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Ovarian luteal category at the time of exogenous progesterone treatment alters pre-ovulatory follicle size and pregnancy outcome but not initial GnRH treatment in repeat-breeder crossbred dairy heifers submitted to the 7-day fixed-time AI protocol

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ABSTRACT

Repeat breeding is a substantial problem in heifer and cow breeding leading to greater infertility for female dairy herds. The aim of present study was to investigate the impact of corpus luteum (CL) presence and category and the first gonadotropin-releasing hormone (GnRH) administration concurrent with exogenous progesterone (P4) treatment on the largest follicle (LF) size and pregnancy rate (PR) in repeat-breeder crossbred dairy heifers submitted to the fixed-time artificial insemination (AI) protocol. Heifers (n= 243) were synchronised with (+GnRH) or without (–GnRH) first GnRH in the 7-day P4-GnRH-prostaglandin F2α-based programme. Each GnRH group was divided on presence of CL into two groups (+CL and –CL) in a 2 × 2 factorial arrangement. The PR was similar among –GnRH–CL (20.7%), –GnRH+CL (68.8%), +GnRH–CL (30.4%), and +GnRH+CL (68.3%) groups. However, presence of CL in heifers produced a 43.6% increase in PR compared to PR of heifers without CL (odds ratio = 6.550). Heifers bearing large-sized CL had greater large-sized LF on the day of fixed-time AI and PR. Plasma P4 concentration was positively related with CL diameter (r= 0.845; $p < 0.001$). The diameter of ovarian LF on the day of fixed-time AI was positively associated with P4 concentrations (r= 0.512; $p < 0.001$). We highlight that ovarian CL presence and category at the time of exogenous P4 treatment alters pre-ovulatory follicle size and PR but not initial GnRH treatment in repeat-breeder crossbred dairy heifers submitted to service with the 7-day fixed-time AI programme.

1. Introduction

In dairy cattle, repeat breeding have been defined as normal oestrous cycling females with no clinically detectable reproductive disorders which failed to conceive after at least three successive inseminations (Puglisi et al., 2013). The high incidence of repeat breeders has been found in 24.4–45.4% of female dairy herds (lactating and non- lactating cows and heifers) (Deka et al., 2021). The phenomenon of the repeat breeding leads to infertility in heifers (Singh et al., 2005) and cows (Yusuf et al., 2010). This phenomenon challenges the normal reproductive output of the dairy farm (Nebel & Jobst, 1998) and repeat breeding heifers has considerable impact on the economy of dairy

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farmers (Båge et al., 2002). Reproductive performance of heifers is crucial for the economics of the heifer enterprise because decreasing time to conception decreases the management and opportunity costs of herd replacement (Masello et al., 2021). Therefore, effective breeding programmes to manage the reproduction of repeat-breeder dairy heifers may have a pronounced effect on the overall reproductive ability of dairy herds (Yaginuma et al., 2019). To manage reproduction in the replacement heifer herds, the 7-day progesterone (P4)-gonadotropin releasing hormone (GnRH)-prostaglandin F2α (PGF2α)-based protocol has been applied to induce the ovulation of dominant follicle (DF) and inseminate with fixed-time artificial insemination (AI) in heifers (Mellieon et al., 2012; Stevenson et al., 2008). This protocol assumes that the first GnRH treatment induces the growing DF to ovulate, which leads to emergence of an ovulatory wave (new ovarian follicular wave) with a new DF (Colazo & Mapletoft, 2014). To control follicular growth and prevent early ovulation before the second GnRH treatment, P4 device is added to this programme for 7 days. Seven days after first GnRH, administration with PGF2α results in degeneration of the corpus luteum (CL) (Colazo & Mapletoft, 2014). In turn, on day 9 of the programme, a new DF will be induced to ovulate by the second injection of GnRH (Colazo & Mapletoft, 2014). The appearance of a new ovarian follicular wave is induced only when first GnRH administration causes ovulation (Mellieon et al., 2012). If the initial GnRH does not induce the emergence of an ovarian follicular wave, ovulation following the second GnRH injection may be poorly synchronised (Colazo & Mapletoft, 2014; Martinez et al., 1999). Unfortunately, according to Mellieon et al. (2012), Stevenson et al. (2008), and Sahu et al. (2014), treatment of first GnRH irrespective of oestrous cycle stage resulted in an ovulation rate of only 31–49% and depends on the maturation stage of the follicles (Colazo & Mapletoft, 2014) and ovarian structure (Urzúa Gonzáleza et al., 2017) at the time of programme initiation. Because of the importance of ovarian structure, dairy cows with a functional CL at the time of first GnRH tretment had more pregnancy outcome than cows lacking a functional CL at first GnRH injection regardless of their ovulatory response to the first GnRH treatment (Carvalho et al., 2018). This implies that, although ovulatory response to first GnRH tretment of the GnRH-based protocol can affect pregnancy to timed AI, a functional CL at the time of programme initiation has a greater effect on pregnancy than ovulatory response to first GnRH treatment (Carvalho et al., 2018). However, relatively little is known about the importance of ovarian luteal presence at the time of first GnRH treatment in repeat-breeder dairy heifers subjected to the fixed- time AI protocol. On the basis of these observations, there are still two questions to be resolved in the 7-day P4-GnRH-PGF2α-based programme when this protocol is applied irrespective of oestrous cycle stage in repeat-breeder dairy heifers: (1) the impact of CL presence at the time of the P4 treatment on follicle and fertility and its relationship with pre-ovulatory follicle size and pregnancy rate (PR); and (2) the necessity of the initial GnRH administration at the time of the P4 treatment of the fixed-time AI protocol. However, the impact of ovarian luteal category, blood P4 concentrations, and first GnRH treatment at the of exogenous P4 administration on pre-ovulatory follicle size and pregnancy outcome had not yet been investigated in repeat-breeder crossbred dairy heifers with a random stage of the ovarian cyclicity submitted to the 7-day P4-GnRH-PGF2α-based protocol. Moreover, enhanced knowledge of ovarian biology is essential to increasing the reproductive ability of normal and infertile female ruminants (Palmieri et al., 2011; d'Orey Branco et al., 2016; U-krit et al., 2022; Yama et al., 2022).

Collectively, these observations led us to hypothesise that, during a random stage of the ovarian cyclicity, CL presence at the time of exogenous P4 treatment in the 7-day P4-GnRH-PGF2α-based protocol with or without first GnRH would result in enhanced diameter of the largest follicle (LF) on the day of fixed-time AI and PR after fixed-time AI in repeat-breeder crossbred dairy heifers. In addition, the different CL category (diameter) and blood P4 levels at the time of exogenous P4 treatment would associate to the LF size on the day of fixed-time AI and

PR after fixed-time AI. The aim of present study, therefore, was to investigate the impact of CL presence and category and the first GnRH administration concurrent with exogenous P4 treatment on the ovarian LF size and PR in repeat-breeder crossbred dairy heifers with a random stage of the ovarian cyclicity submitted to the fixed-time AI with the 7-day P4-GnRH-PGF2α-based protocol.

2. Materials and methods

2.1. Ethics

The ethical approval for this experiment was obtained from the Animal Care and Use Committee of Maejo University (approval number: MACUC025A/2564).

2.2. Heifers and management

Repeat-breeder crossbred Holstein-Friesian (75.0–87.5%) dairy heifers ($n = 243$) were first selected based on their failure to conceive after three artificial inseminations (Arishima et al., 2017) and no evidence of genital tract pathologies being diagnosed after ultrasonic evaluation (Puglisi et al., 2013). Heifers were raised indoors in a free-stall barn, and fed chopped maize silage and fresh Napier grass (*Pennisetum purpureum*) *ad libitum* supplemented with a commercial concentrate. Clean drinking water and mineralised salt bricks were provided *ad libitum*. Parameters of the dairy heifers, including age (mean ± standard deviation [SD]; 21.8 ± 5.3 mo), body weight (BW, 372.1 ± 63.0 kg), body condition score (BCS; [3.1 ± 0.5]), and insemination number (4.4 ± 0.9) were recorded prior to the initiation of the 7-day P4-GnRH-PGF2α-based protocol.

2.3. Experimental design, treatments, and fixed-time AI

A total of 243 repeat-breeder crossbred dairy heifers was distributed to a 2 × 2 (GnRH × CL) factorial arrangement. On a random stage of the ovarian cyclicity, heifers were synchronised with the 7-day P4-GnRH-PGF2α-based protocol. On the day of initiation of this protocol (day of the P4 treatment; day 0), heifers in the +GnRH group received a 10 μg dose (buserelin) of first GnRH (Receptal, MSD Animal Health, New Zealand) while heifers in the –GnRH group did not receive the first GnRH. Luteal structures were evaluated using an HS-1600V ultrasound machine (Honda Electronics, Japan) with a 7.5 MHz linear-array probe to determine the CL presence. Heifers in both GnRH groups (–GnRH and +GnRH) were divided on presence of CL into two groups (with [+CL] and without [–CL] CL) in a 2 × 2 factorial arrangement: –GnRH–CL ($n = 29$), –GnRH+CL ($n = 109$), +GnRH–CL ($n = 23$), and +GnRH+CL ($n = 82$) groups.

On day 0, heifers in all groups were implanted with a P4-releasing device with 1.38 g of P4 (controlled internal drug release [CIDR], Eazi-Breed; Zoetis Inc., New Zealand) which were withdrawn on day 7. On day 7, all heifers received a 250 μg dose (cloprostenol) of PGF2α (Estrumate; MSD Animal Health, New Zealand). The day of the fixed-time AI was designated as day 9. On the day of the fixed-time AI, all heifers received a 10 μg dose (buserelin) of GnRH. All heifers were inseminated with a dose of frozen-thawed bull semen at the same time as the last injection of GnRH.

2.4. Ovarian ultrasonography, assessment of luteal parameters, and ovarian LF

On day of the initiation of this protocol (day 0), ovarian follicular and luteal structures were evaluated using an HS-1600V ultrasound machine with a 7.5 MHz linear-array probe by a single examinator to evaluate the CL presence and diameter. To assess the ovarian luteal parameters, the location and diameter of CL were sketched on an ovarian map relative to each CL, and ultrasound images of each CL were

saved for further calculation. During ultrasound examination, the CLs were categorised according to size as class 1 ($\leq$ 9.9 mm in diameter), class 2 (10.0–14.9 mm in diameter), class 3 (15.0–19.9 mm in diameter), or class 4 ($\geq$ 20 mm in diameter) (Escalante et al., 2013). We followed the calculation of each CL cross-sectional area based on previously reports (Jitjumnong et al., 2020; Miura et al., 2015), using the following formulation (Eq. (1)):

$$CLcross - sectional area = \pi/4 \times (mean D of CL)^2 \qquad (1)$$

where π is 3.1416 and D is the diameter. If a CL cavity was appeared, each CL cross-sectional area was calculated according to the following formulation (Eq. (2)):

$$CLcross - sectional area = \pi/4 \times (mean D of CL)^2 - \pi/4 \\ \times (mean D of cavity)^2 \qquad (2)$$

where π is 3.1416 and D is the diameter.

We followed the calculation of each CL volume based on previously reported (Tortorella et al., 2013), through the following formulation (Eq. (3)):

$$CLvolume = 4/3\pi r^3 \qquad (3)$$

where π is 3.1416 and r is the radius.

On the day of the fixed-time AI (day 9), a sketch of the location and diameter of ovarian LF was recorded. During ultrasound examination, we followed the classification of the LFs based on previously reported (Escalante et al., 2013), according to diameter: small-sized (2.0–5.0 mm), medium-sized (6.0–9.0 mm), or large-sized ($\geq$ 10.0 mm).

2.5. Blood sampling and hormonal assay

On day 0, all blood samples were taken from the coccygeal vein using venipuncture into plastic tubes containing anticoagulant. Plasma samples were centrifuged at $1200 \times g$ for 15 min and stored at -80 °C until subsequent P4 determination. Plasma P4 concentration assays were performed using the competitive enzyme-linked immunosorbent assay. All plasma samples were pipetted in duplicate for each run, and the sensitivity of the assay was 0.02 ng/mL. The intraassay and interassay coefficients of variation were 9.6% and 11.7%.

2.6. Pregnancy diagnosis and calculations of PR and embryonic loss

On days 32 and 60 after fixed-time AI, all heifers were subjected to pregnancy diagnosis by transrectal ultrasonography. We followed the calculations of PR and embryonic loss based on previous reports (Urzúa Gonzáleza et al., 2017) as follows. The PRs were defined as the proportion of heifers that were diagnosed as pregnant on days 32 or 60 after fixed-time AI, divided by the total number of bred heifers at fixed-time AI. The embryonic loss was presented as the proportion of heifers that lost their pregnancy between days 32 and 60 after fixed-time AI, divided by the total number of pregnant heifers on day 32 after fixed-time AI.

2.7. Statistical analysis

The first GnRH treatment and CL presence were included in the statistical model as class variables, and age, BW, BCS and insemination number were also included in the statistical model as covariates. Data were analysed using the generalized linear mixed model regarding the GnRH factor (with and without), the CL factor (with and without) and its interaction. In heifers with (+CL) and without (–CL) CL, the Duncan's New Multiple Rang Test was used to compare the means of P4 concentrations and LF diameter among the CL categories. Data of P4 concentrations and LF diameter are expressed as the mean $\pm$ standard error of the mean (SEM). Linear regression was applied to evaluate the linear relationship between CL characteristics and ovarian LF sizes. The odds

ratio (OR) and 95% confidence intervals (CI) were used to evaluate the association between the PR and GnRH treatments, CL presence, age, BCS, and BW. A probability of p-value $\leq$ 0.05 indicated a significant difference.

3. Results

3.1. Main effect of the GnRH and CL factors and its interaction on plasma P4 concentrations, LF diameter, and PRs in repeat-breeder crossbred dairy heifers

Regardless of the stage of ovarian cyclicity, there was no interaction (GnRH $\times$ CL) effect on the plasma P4 concentrations on day 0 ($p > 0.05$), LF diameter on the day of the fixed-time AI (day 9) ($p > 0.05$), proportion of heifers with small-sized LF on day 9 ($p > 0.05$), PRs on days 32 and 60 after fixed-time AI ($p > 0.05$), and embryonic loss during 32–60 days after fixed-time AI ($p > 0.05$) (Table 1).

There was no significant effect of GnRH (–GnRH and +GnRH) on the plasma P4 concentrations on day 0 ($p > 0.05$), LF diameter on day 9 ($p > 0.05$), proportion of heifers with small-sized and large-sized LFs on day 9 ($p > 0.05$), PRs on days 32 and 60 after fixed-time AI ($p > 0.05$), and embryonic loss during 32–60 days after fixed-time AI ($p > 0.05$) (Table 1). There was no significant effect of CL (–CL and +CL) on the embryonic loss during 32–60 days after fixed-time AI ($p > 0.05$) (Table 1). However, a significant effect of CL (–CL and +CL) was observed on the plasma P4 concentrations on day 0 ($p < 0.05$), LF diameter on day 9 ($p < 0.05$), proportion of heifers with small-sized, medium-sized and large-sized LFs on day 9 ($p < 0.05$), and PRs on days 32 and 60 after fixed-time AI ($p < 0.05$) of repeat-breeder crossbred dairy heifers (Table 1). There was an GnRH $\times$ CL interaction effect on the proportion of heifers with medium-sized and large-sized LFs on day 9 ($p < 0.05$) (Table 1). The proportion of heifers having medium-sized LF on day 9 was greater ($p < 0.05$) in –GnRH–CL group than –GnRH+CL, +GnRH–CL, and +GnRH+CL groups (Table 1). On the other hand, the percentage of heifers with large-sized LF was less ($p < 0.05$) in –GnRH–CL group than –GnRH+CL, +GnRH–CL, and +GnRH+CL groups (Table 1).

3.2. Influence of different CL categories on plasma P4 concentrations, ovarian LF size, and PRs

In heifers with CL (+CL) on their ovaries, plasma P4 concentrations prior to start the exogenous P4 treatment on day 0 was greatest ($p < 0.05$) in heifers bearing CL class 4 compared with heifers bearing CL classes 3, 2, and 1 (8.6 ± 0.36 ng/mL vs. 6.1 ± 0.15 ng/mL, 3.3 ± 0.30 ng/mL, and 1.4 ± 0.14 ng/mL, respectively; Fig. 1a). In heifers with (+CL) and without (–CL) CL, ovarian LF size on the day of the fixed-time AI (day 9) was greatest ($p < 0.05$) in heifers bearing CL classes 2, 3, and 4 (12.6 ± 0.19 mm, 13.5 ± 0.17 mm, and 13.9 ± 0.18 mm, respectively) compared with heifers bearing CL class 1 and without CL (10.2 ± 0.22 mm and 11.1 ± 0.64 mm; Fig. 1b). On day 9, the percentage of heifers having small-sized LF was fewest ($p < 0.05$) in heifers bearing CL classes 1, 2, and 3 (3.0%, 2.0%, and 0.0%, respectively; Fig. 1c) compared with heifers without CL (11.5%); however, it was not different when compared with heifers bearing CL class 4 (3.7%) on their ovaries (Fig. 1c). The percentage of heifers having medium-sized LF was higher ($p < 0.05$) in CL class 1 (48.5%) and without CL (32.7%) than in CL classes 2, 3, and 4 (14.3%, 11.0%, and 3.7%, respectively; Fig. 1d). On the other hand, the percentage of heifers with large-sized LF was higher ($p < 0.05$) in CL classes 2 (83.7%), 3 (89.0%), and 4 (92.6%) than in CL class 1 (48.5%) and without CL (55.8%; Fig. 1e). The PRs on days 32 (Fig. 1f) and 60 (Fig. 1g) after fixed-time AI were greater ($p < 0.05$) for heifers bearing CL classes 3 and 4 than for heifers bearing CL classes 1 and 2 and without CL on day 0 (87.8% and 81.5% vs. 51.5% and 59.2% and 28.9% for PR on day 32; 82.9% and 77.8% vs. 48.5% and 53.1% and 25.0% for PR on day 60, respectively).

Table 1

Effect of first GnRH administration (–GnRH or +GnRH), CL presence (–CL or +CL), and its interaction on plasma P4 concentrations (mean ± SEM), ovarian LF diameter (mean ± SEM), ovarian LF categories, PR, and embryonic loss of repeat-breeder crossbred dairy heifers receiving the 7-day P4-GnRH-PGF2α-based protocol.

Items [1]	Treatment [2]				Main effect				p-value		
	–GnRH		+GnRH		GnRH		CL				
	–CL	+CL	–CL	+CL	–GnRH	+GnRH	–CL	+CL	GnRH	CL	GnRH × CL [5]
Total heifers (*n*)	29	109	23	82	138	105	52	191			
Plasma P4 concentrations on day 0 (ng/mL) [3]	0.3 ± 0.02	4.7 ± 0.27	0.3 ± 0.01	5.2 ± 0.30	3.7 ± 0.26	4.1 ± 0.31	0.3 ± 0.01[B]	4.9 ± 0.20[A]	0.5026	0.0001	0.4869
LF diameter on day 9 (mm) [4]	10.4 ± 0.85	12.7 ± 0.26	12.0 ± 0.95	12.8 ± 0.32	12.3 ± 0.28	12.6 ± 0.32	11.1 ± 0.64[B]	12.8 ± 0.20[A]	0.1961	0.0080	0.0837
LF category on day 9 (%, *n/n*) [4]											
Small size (2.0–5.0 mm)	10.3 (3/29)	1.8 (2/109)	13.0 (3/23)	2.4 (2/82)	3.6 (5/138)	4.8 (5/105)	11.5 (6/52)[A]	2.1 (4/191)[B]	0.5940	0.0022	0.7353
Medium size (6.0–9.0 mm)	44.8 (13/29)[a]	14.7 (16/109)[b]	17.4 (4/23)[b]	17.1 (14/82)[b]	21.0 (29/138)[A]	17.1 (18/105)[B]	32.7 (17/52)[A]	15.7 (30/191)[B]	0.0410	0.0131	0.0151
Large size (≥10.0 mm)	44.8 (13/29)[b]	83.5 (91/109)[a]	69.6 (16/23)[a]	80.5 (66/82)[a]	75.4 (104/138)	78.1 (82/105)	55.8 (29/52)[B]	82.2 (157/191)[A]	0.0929	0.0002	0.0324
PR (%, *n/n*)											
On day 32 after fixed-time AI	24.1 (7/29)	72.5 (79/109)	34.8 (8/23)	74.4 (61/82)	62.3 (86/138)	65.7 (69/105)	28.9 (15/52)[B]	73.3 (140/191)[A]	0.3743	0.0001	0.5366
On day 60 after fixed-time AI	20.7 (6/29)	68.8 (75/109)	30.4 (7/23)	68.3 (56/82)	58.7 (81/138)	60.0 (63/105)	25.0 (13/52)[B]	68.6 (131/191)[A]	0.5258	0.0001	0.4807
Embryonic loss (%, *n/n*)	14.3 (1/7)	5.1 (4/79)	12.5 (1/8)	8.2 (5/61)	5.8 (5/86)	8.7 (6/69)	13.3 (2/15)	6.4 (9/140)	0.6144	0.7653	0.8170

[1] AI, artificial insemination; LF, largest follicle; PR, pregnancy rate; P4, progesterone. [2] GnRH, gonadotropin releasing hormone; CL, corpus luteum. [3] Plasma P4 concentrations at the time of exogenous P4 treatment (day 0). [4] Ovarian LF diameter and category on day of the fixed-time AI (day 9). [5] Interaction GnRH with CL (GnRH × CL) effects. [A,B] Values with different superscript letters indicate significant differences between the main effects at p-value < 0.05. [a,b] Values with different superscript letters indicate significant differences among treatments at p-value < 0.05.

3.3. Influence of different LF diameter at the time of fixed-time AI on PRs and embryonic loss

Across the treatment, the PRs on days 32 and 60 after fixed-time AI were greater ($p < 0.05$) for heifers bearing LF ≥11.0 mm in diameter than for heifers bearing LF <11.0 mm in diameter on the day of the fixed-time AI (day 9) (84.3% vs. 7.7% for PR on day 32; 78.7% vs. 6.2% for PR on day 60, respectively). There was no difference in the embryonic loss during 32–60 days after fixed-time AI between heifers bearing LF <11.0 mm in diameter and heifers bearing LF ≥11.0 mm in diameter on day 9 (20.0% vs. 6.7%; $p < 0.05$).

3.4. Association among ovarian CL characteristics at the time of exogenous P4 treatment, plasma P4 concentrations, and LF size on the day of the fixed-time AI

Plasma P4 concentration prior to the start of the exogenous P4 treatment on day 0 displays an increasing trend with increase in CL diameter on day 0 ($r = 0.845$ and $p < 0.001$; Fig. 2a). The diameter of ovarian LF on the day of the fixed-time AI (day 9) shows an increasing trend with increase in plasma P4 concentration ($r = 0.512$ and $p < 0.001$; Fig. 2b), CL diameter ($r = 0.459$ and $p < 0.001$; Fig. 2c), CL area ($r = 0.428$ and $p < 0.001$; Fig. 2d), and CL volume ($r = 0.397$ and $p < 0.001$; Fig. 2e) on day 0.

3.5. Factor relating to a positive PR in repeat-breeder crossbred dairy heifers

Based on the OR, the risk of PR increased in heifers bearing CL (+CL) on their ovaries at the time of exogenous P4 treatment (day 0). Compared to heifers without CL (–CL), heifers with CL (+CL) on day 0 were at a 29% increased likelihood of PR (OR = 6.550, 95% CI = 3.416–12.558, and $p < 0.01$; Table 2).

4. Discussion

The purpose of this study was to gain a better understanding of the impact of CL presence and category, and the first GnRH administration concurrent with exogenous P4 treatment on the ovarian LF size and PR in repeat-breeder crossbred dairy heifers with a random stage of the ovarian cyclicity submitted to the fixed-time AI protocol. To the best of the researchers' knowledge, this is the first investigation to provide data on the important factors (hormonal treatment and luteal stage) for the improvement of fertility in crossbred dairy heifers with repeat breeding syndrome.

The present results confirm that eliminating of first GnRH treatment during a random stage of the ovarian cyclicity concurrent with exogenous P4 treatment (day 0) in the 7-day P4-GnRH-PGF2α-based protocol for repeat-breeder crossbred dairy heifers did not reduce optimal size of ovarian LF and did not decrease the appearance of large-sized LF on the day of the fixed-time AI (day 9) as indicted by the success in synchronous emergence of preovulatory follicular wave, similar to a previous report for ovarian follicular dynamics in pasture-based dairy heifers (Sahu et al., 2014). Although no evaluation of ovulatory response to the treatment with first GnRH concurrent with exogenous P4 treatment in the 7-day P4-GnRH-PGF2α-based protocol was attempted in the present experiment, our findings support the results of Mellieon et al. (2012), Stevenson et al. (2008), and Sahu et al. (2014) who indicated that the treatment with first GnRH achieved ovulation in a small percentage (31–49%) of dairy heifers. For these female dairy cattle, the lack of ovulation in response to treatment with first GnRH may have been due to the stage of ovarian follicular development at the time of the first GnRH injection (Wijma et al., 2017) and suppression of ovulation by the negative feedback impact of P4 on GnRH-induced LH release (Giordano et al., 2012; Lima et al., 2013; Stevenson & Pulley, 2016). This implies that the previous GnRH administration in the 7-day P4-GnRH-PGF2α-based protocol was unnecessary for the recruitment of the new follicular wave (preovulatory follicular wave) and was not essential to achieving acceptable PR in crossbred dairy heifers with a random stage of the ovarian cyclicity.

Interestingly, the results strongly imply that mean diameter of LF and the proportion of repeat-breeder crossbred dairy heifers having large-sized LF (≥10.0 mm) on the day of the fixed-time AI, as indicted by the success in synchronous emergence of preovulatory follicular wave,
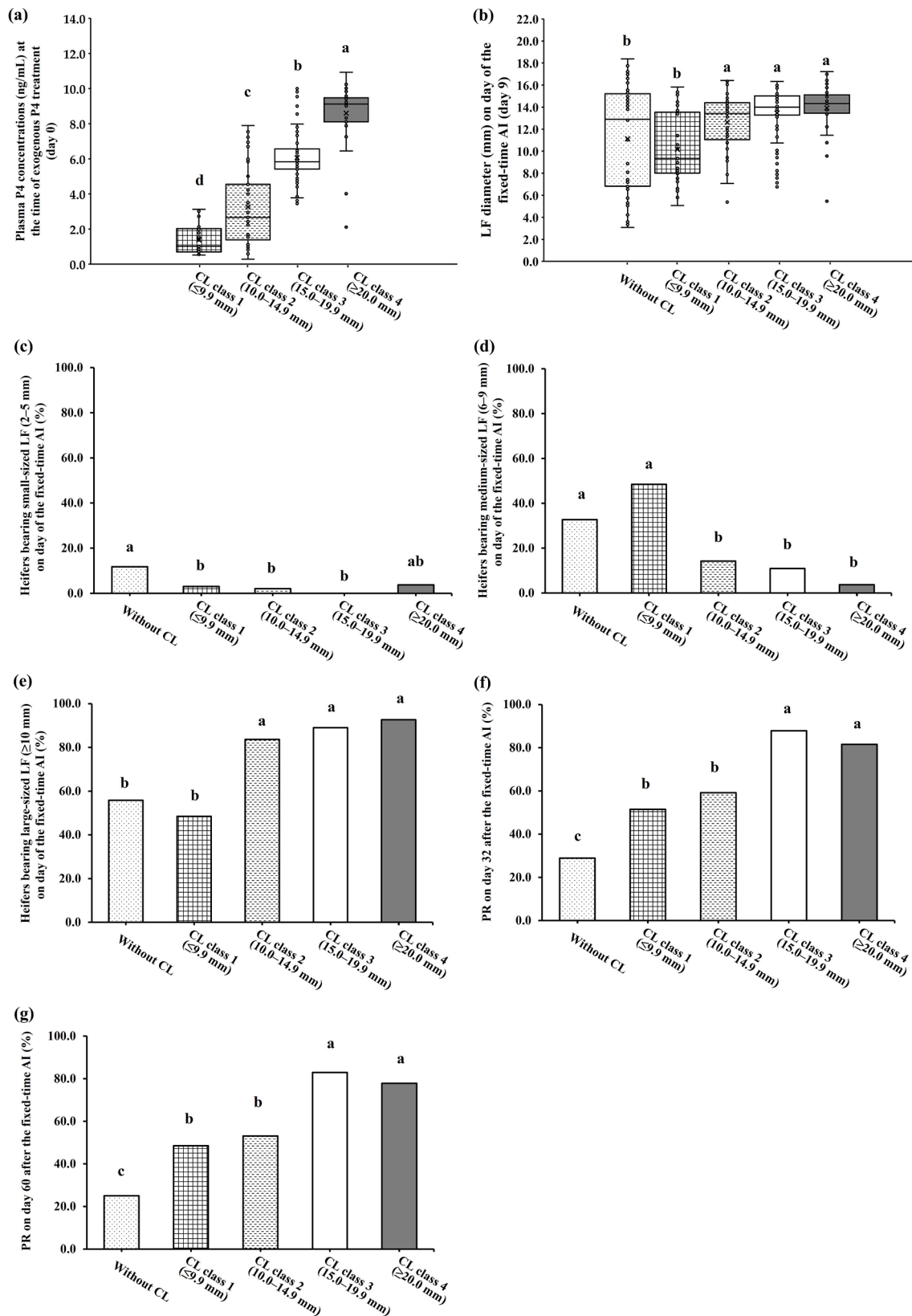
Fig. 1. Box plot showing the distribution and variability of plasma P4 concentrations (a) and LF diameter (b) on the day of the fixed-time AI (day 9) for repeat-breeder crossbred dairy heifers without CL (*n*= 52) and for repeat-breeder crossbred dairy heifers appearing different CL categories (*n*= 191) at the time of exogenous P4 treatment (day 0). The proportions of heifers bearing small-sized LF (c), medium-sized LF (d), and large-sized LF (e) on day 9 with different CL categories on day 0. PRs on days 32 (f) and 60 (g) after fixed-time AI of heifers appearing different CL categories on their ovaries. [a,b,c,d] Values with different superscript letters indicate significant differences among animal groups at *p*-value < 0.05. CL, corpus luteum; AI, artificial insemination; LF, largest follicle; PR, pregnancy rate; P4, progesterone.
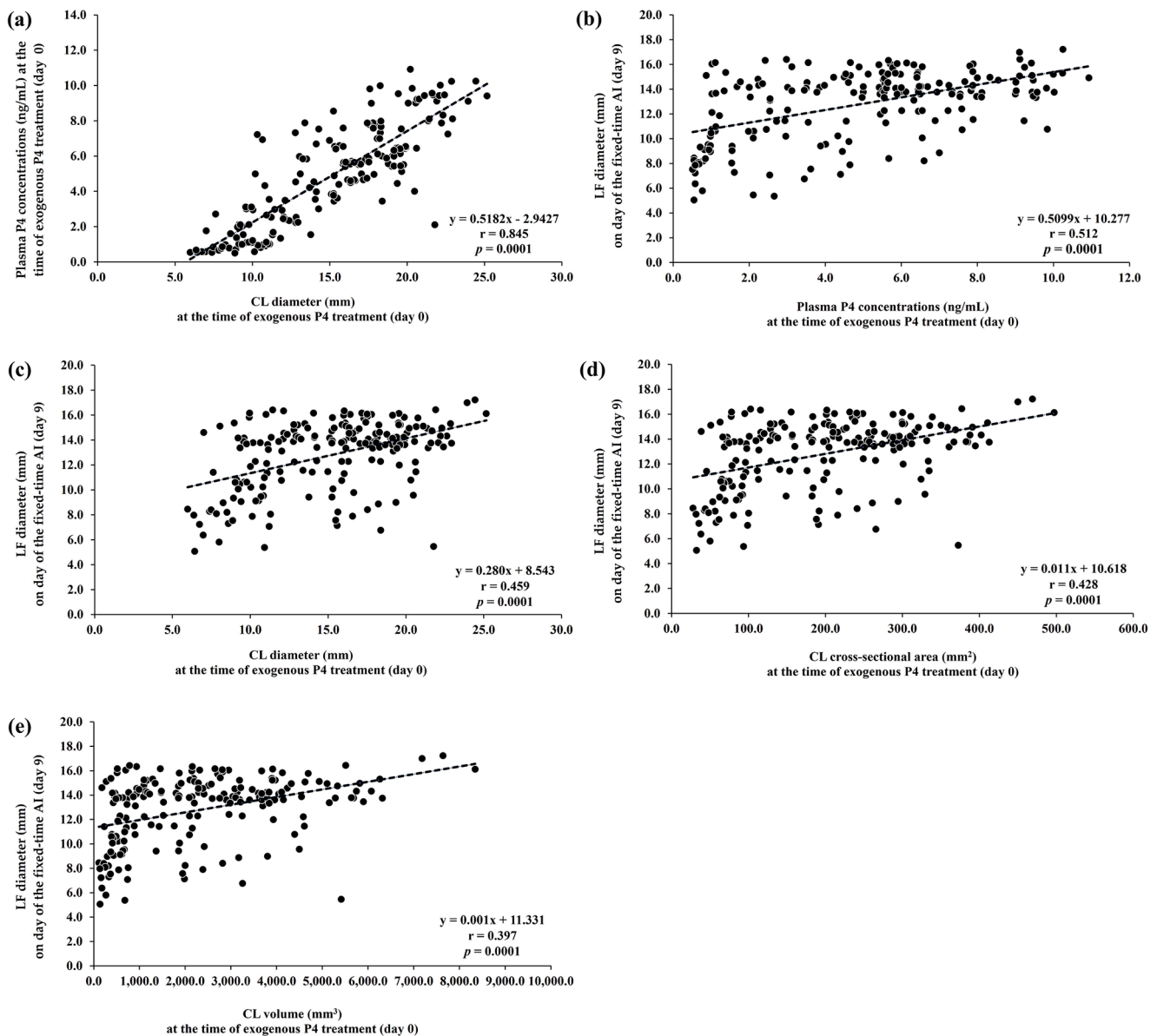
Fig. 2. Relationship between plasma P4 concentrations and CL diameter (a) and relationship between ovarian LF diameter on the day of the fixed-time AI (day 9), plasma P4 concentrations (b) and the characteristics of CL at the time of exogenous P4 treatment (day 0) for diameter (c), cross-sectional area (d), and volume (e) in repeat-breeder crossbred dairy heifers ($n= 191$). A linear relationship ($p < 0.001$) was identified for all analyses. CL, corpus luteum; AI, artificial insemination; LF, largest follicle; P4, progesterone.

were greater in heifers with CL (luteal phase) at the time of exogenous P4 treatment than heifers without CL (follicular phase) on their ovaries. This implies that the appearance of a functional CL (optimal P4 production) on the ovary at the time of exogenous P4 insert leads to an increase in the percentage of dairy heifers with synchronous emergence of preovulatory follicular wave and an increase in fertility (Yusuf et al., 2016). As stated above, although the secretory patterns of LH were not evaluated in the present experiment, we speculate, based on previous data (Kinder et al., 1996), that low circulating P4 levels (sub-luteal concentrations) increased the pulse frequency of LH secretion, supporting the DF in retaining its dominance over other ovarian follicles, thus preventing a new follicular wave (preovulatory follicular wave) from emerging (Atanasov et al., 2015; Bridges & Fortune, 2003; Kinder et al., 1996).

Overall, in the present study, repeat-breeder heifers attained 62–66% of PR in the 7-day P4-GnRH-PGF2α-based programme. This increase in PR was probably due to the fact that a combination of GnRH and PGF2α

in the P4-based fixed-time AI programme results in synchronous follicular wave emergence, timely follicular development, synchronous ovulation following the last injection of GnRH (Kim et al., 2007), and it can overcome the problem of delayed ovulation and anovulation in repeat-breeder dairy cattle (Bhattacharyya & Hafiz, 2009). As stated above, repeat breeder heifers have a smaller preovulatory LH surge than virgin heifers (Gustafsson et al., 1986) and, therefore, an increase in the spontaneous LH surge that results from the treatment of GnRH at onset of the final stage of DF development affects the fertility favourably (Ahmed et al., 2016; Kaim et al., 2003).

5. Conclusion

Taken together, we conclude that initial administration of GnRH concurrent with exogenous P4 treatment in the 7-day P4-GnRH-PGF2α-based protocol is not essential to achieving acceptable fertility in repeat-breeder crossbred dairy heifers at a random stage of the ovarian

Table 2

The OR for the risk factors contributing to the PR of repeat-breeder crossbred dairy heifers with (+CL) or without (–CL) CL on their ovaries that received (+1GnRH) or did not receive (–GnRH) first GnRH (GnRH) in the 7-day P4-GnRH-PGF2α-based protocol (*n*= 243).

Variable [1]	Pregnant heifers (*n*)	Non-pregnant heifers (*n*)	PR (%, *n/n*) [2]	OR [3]	95% CI [4]	*p*-value
1GnRH administration (Ref = +GnRH) [5]						
+GnRH	63	42	60.0 (63/105)			
–GnRH	81	57	58.7 (81/138)	0.947	0.564–1.590	0.838
Luteal presence (Ref = –CL) [6]						
–CL	13	39	25.0 (13/52)			
+CL	131	60	68.6 (131/191)	6.550	3.416–12.558	0.001
Age (mo) (Ref = <21 mo) [7]						
<21	71	52	57.7 (71/123)			
21–23	56	26	68.3 (56/82)	1.577	0.877–2.836	0.128
>23	17	21	44.7 (17/38)	0.593	0.285–1.232	0.161
BCS (Ref = <2.5) [7]						
<2.5	7	10	41.2 (7/17)			
2.5–3.5	116	74	61.1 (116/190)	2.239	0.832–6.031	0.111
>3.5	21	15	58.3 (21/36)	2.000	0.618–6.472	0.247
BW (kg) (Ref = <350 kg) [7]						
<350	62	50	55.4 (62/112)			
350–400	38	29	56.7 (38/67)	1.057	0.573–1.949	0.860
>400	44	20	68.8 (44/64)	1.774	0.931–3.383	0.082
Insemination number (Ref = 4–5) [7]						
4–5	130	82	61.3 (130/212)			
6–7	12	14	46.2 (12/26)	0.541	0.240–1.218	0.138
>7	2	3	40.0 (2/5)	0.421	0.072–2.451	0.335

[1] GnRH, gonadotropin releasing hormone; CL, corpus luteum; BCS, body condition score; BW, body weight. [2] PR, pregnancy rate. [3] OR, Odds ratio. [4] CI, confidence interval. [5] First GnRH treatment concurrent with exogenous P4 treatment (day 0). [6] Luteal presence at the time of exogenous P4 treatment (day 0). [7] Age, BCS, BW and AI service number before the start of the synchronisation programme.

cyclicity. Repeat-breeder crossbred dairy heifers with the luteal stage when subjected to the 7-day P4-GnRH-PGF2α-based programme show improved fertility.

Ethical statement

The study was conducted according to the ethical principles and guidelines for the use of animals of the National Research Council of Thailand and approved by the Animal Care and Use Committee of Maejo University (approval number: MACUC025A/2564).

CRediT authorship contribution statement

Warunya Chaikol: Conceptualization, Methodology, Writing – original draft. **Chayanon Yadmak:** Conceptualization, Funding acquisition. **Punnawut Yama:** Methodology, Writing – original draft. **Jakree Jitjumnong:** Methodology, Writing – original draft. **Molarat Sangkate:** Methodology, Writing – original draft. **Warittha U-krit:** Methodology, Writing – original draft. **Nalinthip Promsao:** Methodology, Investigation. **Assawadet Suriard:** Methodology, Data curation. **Raktham Mektrirat:** Methodology, Writing – review & editing. **Julakorn Panatuk:** Methodology, Conceptualization. **Hien Van Doan:** Methodology, Writing – review & editing. **Chien-Kai Wang:** Methodology, Writing – review & editing. **Pin-Chi Tang:** Methodology, Writing – review & editing. **Tossapol Moonmanee:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

References

Ahmed, N., Kathiresan, D., Ahmed, F. A., Lalrintluanga, K., Mayengbam, P., & Gali, J. M. (2016). Pattern of induced estrus and conception rate following Ovsynch and Ovsynch based gonadotropin-releasing hormone treatments initiated on day 6 of estrous cycle in repeat breeding crossbred cows. *VeterinaryWorld, 9*, 342–345.

Arishima, T., Sasaki, S., Isobe, T., Ikebata, Y., Shimbara, S., Ikeda, S., et al. (2017). Maternal variant in the upstream of FOXP3 gene on the X chromosome is associated with recurrent infertility in Japanese Black cattle. *BMC Genetics, 18*, 103.

Atanasov, B., De Koster, J., Bommelé, L., Dovenski, T., & Opsomer, G. (2015). Pathways of the dominant follicle after exposure to sub-luteal circulating progesterone concentrations are different in lactating dairy cows versus non-lactating heifers. *Animal Reproduction Science, 154*, 8–15.

Båge, R., Gustafsson, H., Larsson, B., Forsberg, M., & Rodríguez-Martínez, H. (2002). Repeat breeding in dairy heifers: Follicular dynamics and estrous cycle characteristics in relation to sexual hormone patterns. *Theriogenology, 57*, 2257–2269.

Bhattacharyya, H. K., & Hafiz, A. (2009). Treatment of delayed ovulation in dairy cattle. *Indian Journal of Animal Research, 43*, 219–210.

Bridges, P. J., & Fortune, J. E. (2003). Characteristics of developing prolonged dominant follicles in cattle. *Domestic Animal Endocrinology, 25*, 199–214.

Carvalho, P. D., Santos, V. G., Giordano, J. O., Wiltbank, M. C., & Fricke, P. M. (2018). Development of fertility programs to achieve high 21-day pregnancy rates in high-producing dairy cows. *Theriogenology, 114*, 165–172.

Colazo, M. G., & Mapletoft, R. J. (2014). A review of current timed-AI (TAI) programs for beef and dairy cattle. *The Canadian Veterinary Journal, 55*, 772–780.

Deka, R. P., Magnusson, U., Grace, D., Randolph, T. F., Shome, R., & Lindahl, J. F. (2021). Estimates of the economic cost caused by five major reproductive problems in dairy animals in Assam and Bihar, India. *Animals, 11*, 3116.

d'Orey Branco, R. A., Neuendorff, D. A., Lewis, A. W., Vann, R. C., Welsh, T. H., Jr., & Randel, R. D. (2016). Comparison of follicular dynamics and subsequent progesterone profiles in Brahman cows with either two or three ovarian follicular waves. *Journal of Animal Science, 94*, 535.

Escalante, R. C., Poock, S. E., & Lucy, M. C. (2013). Follicular populations and luteal function in dairy heifers treated with a controlled internal drug release insert for 14 days as a method to synchronize the estrous cycle before prostaglandin F2α treatment and artificial insemination. *Journal of Dairy Science, 96*, 3806–3816.

Giordano, J. O., Fricke, P. M., Guenther, J. N., Lopes, G., Herlihy, M. M., Nascimento, A. B., et al. (2012). Effect of progesterone on magnitudeof the luteinizing hormone surge induced by two different doses of gonadotropin-releasing hormone in lactating dairy cows. *Journal of Dairy Science*, 3781–3793.

Gustafsson, H., Larsson, K., Kindahl, H., & Madej, A. (1986). Sequential endocrine changes and behaviour during oestrus and metoestrus in repeat breeders and virgin heifers. *Animal Reproduction Science, 10*, 261–273.

Jitjumnong, J., Moonmanee, T., Sudwan, P., Mektrirat, R., Osathanunkul, M., Navanukraw, C., et al. (2020). Associations among thermal biology, preovulatory follicle diameter, follicular and luteal vascularities, and sex steroid hormone concentrations during preovulatory and postovulatory periods in tropical beef cows. *Animal Reproduction Science, 213*, Article 106281.

Kaim, M., Bloch, A., Wolfenson, D., Braw-Tal, R., Rosenberg, M., Voet, H., et al. (2003). Effects of GnRH administered to cows at the onset of estrus on timing of ovulation, endocrine responses, and conception. *Journal of Dairy Science, 86*, 2012–2021.

Kim, U. H., Suh, G. H., Hur, T. Y., Kang, S. J., Kang, H. G., Park, S. B., et al. (2007). Comparison of two types of CIDR-based timed artificial insemination protocols for repeat breeder dairy cows. *Journal of Reproduction and Development, 53*, 639–645.

Kinder, J. E., Kojima, F. N., Bergfeld, E. G., Wehrman, M. E., & Fike, K. E. (1996). Progestin and estrogen regulation of pulsatile LH release and development of persistent ovarian follicles in cattle. *Journal of Animal Science, 74*, 1424–1440.

Lima, F. S., Ribeiro, E. S., Bisinotto, R. S., Greco, L. F., Martinez, N., Amstalden, M., et al. (2013). Hormonal manipulations in the 5-day timed artificial insemination protocol to optimize estrous cycle synchrony and fertility in dairy heifers. *Journal of Dairy Science, 96*, 1–12.

Martinez, M. F., Adams, G. P., Bergfelt, D., Kastelic, J. P., & Mapletoft, R. J. (1999). Effect of LH or GnRH on the dominant follicle of the first follicular wave in heifers. *Animal Reproduction Science, 57*, 23–33.

Masello, M., Perez, M. M., Granados, G. E., Stangaferro, M. L., Ceglowski, B., Thomas, M. J., et al. (2021). Effect of reproductive management programs for first service on replacement dairy heifer economics. *Journal of Dairy Science, 104*, 471–485.

Mellieon, H. I., Pulley, S. L., Lamb, G. C., Larson, J. E., & Stevenson, J. S. (2012). Evaluation of the 5-day versus a modified 7-day CIDR breeding program in dairy heifers. *Theriogenology, 78*, 1997–2006.

Miura, R., Haneda, S., & Matsui, M. (2015). Ovulation of the preovulatory follicle originating from the first-wave dominant follicle leads to formation of an active corpus luteum. *Journal of Reproduction and Development, 61*, 317–323.

Nebel, R. L., & Jobst, S. M. (1998). Evaluation of systematic breeding programs for lactating dairy cows: A review. *Journal of Dairy Science, 81*, 1169–1174.

Palmieri, C., Schiavi, E., & Della Salda, L. (2011). Congenital and acquired pathology of ovary and tubular genital organs in ewes: A review. *Theriogenology, 75*, 393–410.

Puglisi, R., Cambuli, C., Capoferri, R., Giannino, L., Lukaj, A., Duchi, R., et al. (2013). Differential gene expression in cumulus oocyte complexes collected by ovum pick up from repeat breeder and normally fertile Holstein Friesian heifers. *Animal Reproduction Science, 141*, 26–33.

Sahu, S. K., Cockrem, J. F., Parkinson, T. J., & Laven, R. A. (2014). The effects of exclusion of progesterone or day 0 GnRH from a GnRH, prostaglandin, GnRH + progesterone program on synchronization of ovulation in pasture-based dairy heifers. *Theriogenology, 82*, 643–651.

Singh, B., Saravia, F., Båge, R., & Rodríguez-Martínez, H. (2005). Pregnancy rates in repeat-breeder heifers following multiple artificial inseminations during spontaneous oestrus. *Acta Veterinaria Scandinavica, 46*, 1–12.

Stevenson, J. L., Dalton, J. C., Santos, J. E. P., Sartori, R., Ahmadzadeh, A., & Chebel, R. C. (2008). Effect of synchronization protocols on follicular development and estradiol and progesterone concentrations of dairy heifers. *Journal of Dairy Science, 91*, 3045–3056.

Stevenson, J. S., & Pulley, S. L. (2016). Feedback effects of estradiol and progesterone on ovulation and fertility of dairy cows after gonadotropin-releasing hormone-induced release of luteinizing hormone. *Journal of Dairy Science, 99*, 3003–3015.

Tortorella, R. D., Ferreira, R., dos Santos, J. T., de Andrade Neto, O. S., Barreta, M. H., Oliveira, J. F., et al. (2013). The effect of equine chorionic gonadotropin on follicular size, luteal volume, circulating progesterone concentrations, and pregnancy rates in anestrous beef cows treated with a novel fixed-time artificial insemination protocol. *Theriogenology, 79*, 1204–1209.

U-krit, W., Wadsungnoen, S., Yama, P., Jitjumnong, J., Sangkate, M., Promsao, N., et al. (2022). Understanding the ovarian interrelationship with low antral follicle counts (AFC) in the in vivo Bos indicus cow model: Unilateral and bilateral main AFC as possible biomarkers of ovarian response to hormonal synchronisation. *Biology, 11*, 523.

Urzúa González, E., Péreza, L. A. V., Garzab, A., Mapesc, G., Gutiérreza, G. G., & Hernández-Ceróna, J. (2017). Pregnancy rate in dairy cows treated with human chorionic gonadotropin five days after insemination. *Austral Journal of Veterinary Sciences, 49*, 119–122.

Wijma, R., Stangaferro, M. L., Masello, M., Granados, G. E., & Giordano, J. O. (2017). Resynchronization of ovulation protocols for dairy cows including or not including gonadotropin-releasing hormone to induce a new follicular wave: Effects on re-insemination pattern, ovarian responses, and pregnancy outcomes. *Journal of Dairy Science, 100*, 7613–7625.

Yaginuma, H., Funeshima, N., Tanikawa, N., Miyamura, M., Tsuchiya, H., Noguchi, T., et al. (2019). Improvement of fertility in repeat breeder dairy cattle by embryo transfer following artificial insemination: Possibility of interferon tau replenishment effect. *Journal of Reproduction and Development, 65*, 223–229.

Yama, P., Yadmak, C., Sangkate, M., Jitjumnong, J., U-krit, W., Promsao, N., et al. (2022). In vivo follicular and uterine arterial indices as an indicator of successful hormonal stimulation for inactive ovaries in repeat-breeder crossbred dairy cows using a short-term progesterone-based programme. *Animals, 12*, 292.

Yusuf, M., Nakao, T., Long, S. T., & Fujita, S. (2016). Risk factors influencing conception rate in Holstein heifers before artificial insemination or embryo transfer. *Media Peternakan, 39*, 148–154.

Yusuf, M., Nakao, T., Ranasinghe, R. B., Gautam, G., Long, S. T., Yoshida, C., et al. (2010). Reproductive performance of repeat breeders in dairy herds. *Theriogenology, 73*, 1220–1229.