Effect of three different fixed-time AI protocols on follicular dynamics and pregnancy rates in suckled, dual-purpose cows in the Ecuadorian Amazon

Efecto de tres protocolos diferentes de IA a tiempo fijo sobre la dinámica folicular y las tasas de preñez en vacas de doble propósito amamantando en la Amazonía Ecuatoriana

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Originales: Recepción: 13/10/2023 - Aceptación: 09/05/2024

Abstract

Reproductive performance is crucial for profitability of dual-purpose cow-calf production in the Ecuadorian Amazon. To evaluate three different fixed-time artificial insemination (FTAI) protocols in suckled, dual-purpose cows in the Ecuadorian Amazon. Lactating, Brown Swiss cows (n=301) received 2 mg estradiol benzoate (EB) and an intravaginal device containing 0.5 g of progesterone (P4) on Day 0. They were allocated randomly into the following three treatment groups: Cows in the EB group received 500 µg cloprostenol (PGF2α), and P4 devices were removed on Day 7, and 1 mg EB was administered on Day 8. Cows in the ECP group were treated as those in the EB group, except that they received 0.5 mg estradiol cypionate (ECP) at the time of P4 device removal on Day 7 instead of EB on Day 8. Cows in the J-Synch group had the P4 device removed and PGF2α administered on Day 6. All cows were FTAI on Day 9; cows in the J-Synch group also received 100 µg gonadorelin at that time. Although the diameter of the dominant follicle and the resulting CL were greater (P<0.05) in cows in the J-Synch group, pregnancies per AI did not differ (P>0.2) among groups (EB: 51.0%, ECP: 53.0% and J-Synch: 59.4%). The three protocols tested were applied successfully in suckled, dual-purpose cows with no differences in pregnancies per AI.

Keywords
prolonged proestrus • estradiol/progesterone • P4 device • FTAI • pregnancy per AI • lactating dual-purpose cows • tropical

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Resumen

El desempeño reproductivo es crucial para la rentabilidad de la producción doble propósito en la Amazonía Ecuatoriana. Evaluar tres protocolos para Inseminación Artificial a tiempo fijo (IATF) en vacas en lactancia de doble propósito. Vacas Pardo Suizo en lactancia (n=301) recibieron 2 mg de benzoato de estradiol (EB) y un dispositivo intravaginal con 0,5 g de progesterona (P4) el Día 0 y fueron asignadas aleatoriamente en tres grupos. Las vacas del grupo EB recibieron 500 µg de cloprostenol (PGF2α) y se les retiró el dispositivo el Día 7, y recibieron 1 mg de EB el Día 8. Las vacas del grupo ECP fueron tratadas como las del grupo EB, excepto que recibieron 0,5 mg de cipionato de estradiol (ECP) en el día del retiro del dispositivo (Día 7) en lugar de EB el Día 8. En las vacas del grupo J-Synch, el retiro del dispositivo y la administración de PGF2α se realizaron el Día 6. Todas las vacas fueron IATF el Día 9 y las del grupo J-Synch recibieron 100 µg de gonadorelina en ese momento. Aunque el diámetro del folículo dominante y del CL fueron mayores (P<0,05) en las vacas del grupo J-Synch, las tasas de preñez a la IATF no difirieron entre los grupos (EB: 51,0%, ECP: 53,0% y J-Synch: 59,4%). Los tres protocolos probados se pueden aplicar con éxito en vacas de doble propósito amamantando.

Palabras clave

proestro prolongado • estradiol/progesterona • dispositivo de P4 • IATF • preñez por IA • vacas doble propósito en lactancia • tropical

Introduction

Dual-purpose cattle production (*i.e.*, milk and meat) represents a main source of income for small farms in tropical areas around the world, such as the Ecuadorian Amazon. However, there is a need for improvement in terms of reproductive performance and genetics in dual-purpose livestock (31). Less-than-optimal efficiency is due to multiple factors, including the environment (high temperature and humidity), nutrition, health, management, and poor animal welfare, among others (31). One of the technologies that has had the greatest impact on reproductive performance in beef and dairy cattle has been the systematic application of artificial insemination (AI), without estrus detection, known as fixed-time AI (FTAI) (4). Currently, there is a wide range of FTAI protocols used in beef and dairy cattle (4, 15, 47) which requires testing in dual-purpose cattle in the Ecuadorian Amazon. FTAI protocols are classified according to the main hormones used. Ovsynch-type protocols are based on the use of GnRH and prostaglandin F2α (PGF2α; 35, 47) and may be combined with the use of an intravaginal progesterone (P4) releasing device in beef (26, 29) and dairy cattle (41). Estradiol and P4-based protocols (2, 30) have also been commonly used for FTAI in beef and dairy cattle, especially in South America (3, 4, 48). This treatment has been simplified by the administration of estradiol cypionate (ECP) as an ovulation-inducing agent, given at the time of P4 device removal (14) replacing the use of estradiol benzoate (EB) 24 h after P4 device removal in beef (14, 44) and dairy cattle (40, 42). In general, both FTAI protocols (GnRH-based and estradiol/P4-based) result in pregnancies per AI (P/AI) between 40 to 60% in beef (1, 5) and 30 to 50% in dairy cattle (15, 43). In 2012, a new estradiol/P4-based protocol, called J-Synch, was developed (19). In this treatment protocol, the P4-releasing device is inserted for a shorter period of time (6 days) and the administration of ECP at the time of P4 device removal was replaced by the administration of GnRH at the time of AI (72 h after P4 device removal), promoting a longer proestrous period. Subsequent studies have shown that the J-Synch protocol is an efficient treatment to synchronize ovulation in beef (19, 32) and dairy (36) heifers, resulting in greater pregnancy rates than those achieved with the conventional estradiol/P4-based protocols which used ECP or EB to induce ovulation (5, 20, 36). The higher pregnancy rates obtained with the J-Synch protocol were associated with longer exposure to elevated endogenous estradiol concentrations during the proestrus period, which resulted in a more appropriate environment for early embryo development (20).

The current experiment was designed to evaluate three different estradiol/P4-based FTAI protocols, which differ mainly in the duration of the proestrus period, in suckled dual-purpose cows of the Ecuadorian Amazon. The hypothesis to be tested was that the protocol with the prolonged proestrus and utilizing GnRH to induce ovulation, named J-Synch, would result in greater P/AI than conventional estradiol/P4 protocols, which utilized EB or ECP to induce ovulation.

Materials and methods

The study was performed in the Ecuadorian Amazon, at the Center of Postgraduate Research and Conservation of the Amazon (CIPCA) of the Amazon State University in the province of Napo, which is located in the Northeast of Ecuador (Latitude: 01°14'32" South, Longitude: 53°77'13" West), from October 2015 to April 2016. The climate is tropical, with 4000 mm of precipitation per year, average relative humidity of 80% and temperatures ranging between 25 and 30°C. The altitude varies from 580 to 990 m above sea level, and although the soils have a very heterogeneous composition, most originated in fluvial sediments from the Andean plateaus.

Animals and feeding

Lactating, Brown Swiss cows (n = 301) with suckling calves were used. The study was performed in three replicates of 100 animals each. Cows were grazing a mixed pasture based on *Brachiaria decumbens* (17.585 kg DM/ha/year), *Brachiaria brizantha* (26.970 kg DM/ha/year), *Arachis pinto* (6.212 kg DM/ha/year), *Desmodium ovalifolium* (5.890 kg DM/ha/year) and *Stylosanthes guianensis* (15.237 kg DM/ha/year). They received the standard vaccination and health protocols applied commonly to cattle at the CIPCA. This included the application of vitamins and minerals, deworming, insecticide dips against ticks and flies, vaccinations for Foot and Mouth Disease, Bovine Rabies and Vesicular Stomatitis.

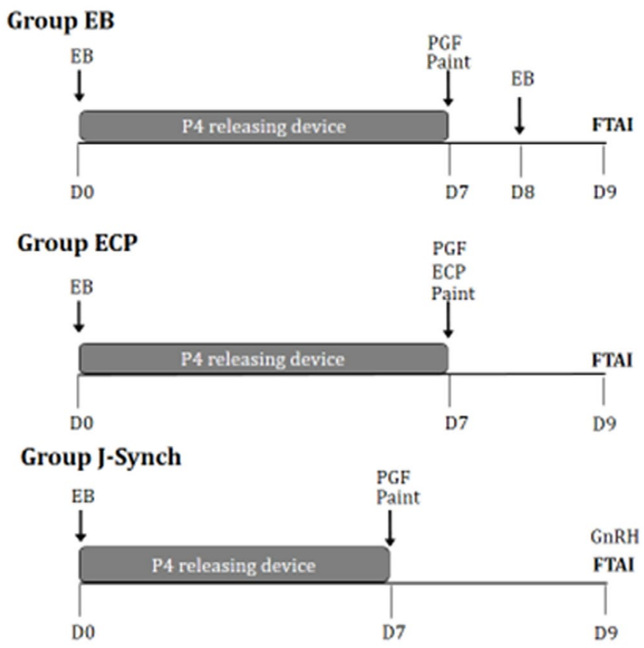
The cows were multiparous (*i.e.*, 2-5 lactations), 112.0±1.0 (mean±SEM) days postpartum, with a mean body condition score (BCS) of 2.4±0.3 (1 to 5 scale; 22), and they were producing 6.7±0.6 Kg of milk per day. Cows were selected for treatment by the presence of a CL, or at least one follicle >10 mm in diameter in their ovaries through an ultrasound examination and without any abnormalities of the reproductive tract. All cows were handled in the same milking facility and were inseminated with frozen-thawed semen from one bull of proven fertility. Animal procedures were approved and supervised by the Animal Care and Use Committee of the CIPCA and the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL) of the University of Villa Maria (UNVM), Argentina.

Treatments

Cows were randomly allocated to one of three FTAI protocols. The EB protocol (n = 100): On Day 0, cows received a P4-releasing intravaginal device (DIB 0.5 g, Zoetis, Ecuador) plus 2 mg EB (Gonadiol, Zoetis) by intramuscular (i.m.) injection. On Day 7, the P4 device was removed, and cows received 500 μg cloprostenol (PGF2α, Ciclase DL, Zoetis) i.m. On Day 8, all cows received 1 mg EB i.m. and the tail heads were painted (Divasa - Farmavic, Spain) for the determination of estrus. On Day 9, (48 to 54 h after P4 device removal) cows were FTAI (figure 1, page 141).

The ECP protocol (n = 100): On Day 0, cows received a P4 intravaginal device plus 2 mg of EB i.m. On Day 7, the P4 device was removed, and cows received PGF2α and 0.5 mg ECP (Cipiosyn, Zoetis) i.m. and the tail heads were painted as in the EB protocol. On Day 9, (48 to 54 h after P4 device removal), cows were FTAI (figure 1, page 141).

The J-Synch protocol (n = 101): On Day 0, cows received a P4 device plus 2 mg of EB i. m. On Day 6, the P4 device was removed, cows received PGF2α i.m. and the tail heads were painted as in the previous groups. On Day 9, (72 h after P4 device removal), cows received 100 μg gonadorelin acetate (GnRH, Gonasyn gdr, Zoetis) and were FTAI concurrently (figure 1, page 141).



EB group: Day 0, P4 releasing device (0.5 g of P4) plus 2 mg EB; Day 7 device removal plus 500 µg cloprostenol (PGF); Day 8, 1 mg of EB; Day 9, FTAI (48 to 54 h after P4 device removal). ECP group: Day 0, P4 releasing device (0.5 g of P4) plus 2 mg EB; Day 7 device removal plus 500 µg cloprostenol (PGF) and 0.5 mg ECP; Day 9, FTAI (48 to 54 h after P4 device removal). J-Synch group: Day 0, P4 releasing device (0.5 g of P4) plus 2 mg EB; Day 6 device removal plus 500 µg cloprostenol (PGF); Day 9, GnRH and FTAI (72 h after device removal).

EB: Día 0, dispositivo liberador de P4 (0,5 g de P4) más 2 mg de EB; Día 7, retiro del dispositivo más 500 µg de cloprostenol (PGF); Día 8, 1 mg de EB; Día 9, IATF (48 a 54 h después de retirado el dispositivo de P4). ECP: Día 0, dispositivo liberador de P4 (0,5 g de P4) más 2 mg de EB; Día 7, retiro del dispositivo más 500 µg de cloprostenol (PGF) y 0,5 mg de ECP; Día 9, IATF (48 a 54 h después de retirado el dispositivo de P4). J-Synch: Día 0, dispositivo liberador de P4 (0,5 g de P4) más 2 mg de EB; Día 6, retiro del dispositivo más 500 µg de cloprostenol (PGF); Día 9, GnRH y IATF (72 h después de retirado el dispositivo de P4).

Figure 1. Treatment Groups.

Figura 1. Grupos de tratamiento.

Ultrasonography

All cows were examined by real-time ultrasonography (Ibex Pro and Lyte ®, USA), with a linear probe of 5.0 MHzon Days 7, 9 and 10. A map was made to measure and record all follicles >5 mm present in both ovaries, and to identify the dominant follicle which was defined as the follicle with the largest diameter at the time of device removal. To determine the timing of ovulation (disappearance of the follicle of greatest diameter) animals were examined at the time of FTAI (Day 9) and then every 12 h for the next 24 h. If the cows ovulated after Day 10, the ovulation time was defined as an ovulation occurring > 24 h after FTAI. The ovulation was reconfirmed by the presence of a CL 7 days after FTAI. Two measurements of the CL were taken using the equipment's software which included the vertical and horizontal diameter (width and height) of each CL as described previously (25).

Estrus detection

Estrus was recorded visually and by the disappearance of the tail-paint. The animals with >30% of the paint removed were considered to be in estrus (13). The visual observations 3 times a day: morning, noon and afternoon of Days 7, 8 and 9 were initiated after the removal of the P4 device.

Blood sampling and progesterone analysis

Seven days after FTAI a blood sample was taken for P4 analysis. After cleaning and disinfecting the base of the tail, samples were taken from the coccygeal vein using 10 mL Vacutainer tubes. Samples were stored at the laboratory at 4°C for 4 to 6 h after extraction and then centrifuged at 3000 RPM for 20 minutes to separate the serum. The serum was extracted and then stored at -20°C until its subsequent analysis (18). The serum P4 concentrations were determined together, in duplicates, using a progesterone ELISA kit (Progesterone Elisa, DiaMetra S.R.L., Italy). The minimum detectable P4 concentration of the kit was 0.05 ng/mL and the low and high intra-assay coefficients of variation were 4.4% and 19.5%, respectively.

Pregnancy diagnosis

The diagnosis of pregnancy was determined by ultrasonography at 35 to 40 days after FTAI.

Statistical analysis

For each continuous variable studied, the arithmetic mean (x) and the standard error (SEM) were estimated. The data were analyzed using ANOVA and means were compared by the Tukey-Kramer HSD test. Differences were considered significant with a P value of <0.05. The statistical analysis was carried out using the JMP program (JMP ®, 2003) in its version 5.0 for Windows. Estrus, ovulation and P/AI rates were compared among groups using logistic regression for binary data (*i.e.*, 1 = success, 0 = failure) and a logit link, using the Infostat program (21).

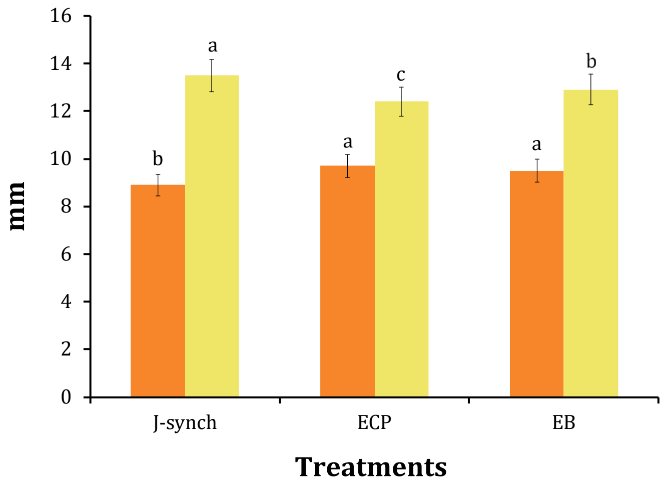
Results

Estrus expression

Overall, 90.4% (272/301) of the cows showed estrus. The percentage of cows in estrus was lower (P < 0.01) in the J-Synch treatment group (78.2%, 79/101), than in the ECP (93%, 93/100) or EB (100%, 100/100) treatment groups.

Follicular characteristics

The diameters of the dominant follicle at the time of P4 device removal and at the time of FTAI are shown in figure 2. Cows in the J-Synch group had the smallest mean diameter follicle at the time of P4 device removal and the largest diameter follicle at the time of FTAI (P < 0.05).



abc Different superscripts indicate significant differences between groups (P<0.05).

abc indica diferencias significativas entre grupos (P<0,05).

Figure 2. Diameter of dominant follicle (means ± SEM) at the time of P4 device removal (orange columns) and at the time of FTAI (yellow columns) in suckled, dual-purpose cows subjected to three different estradiol/P4-based protocols.

Figura 2. Diámetro del folículo dominante (media ± SEM) en el momento del retiro del dispositivo P4 (columnas naranjas) y en la IATF (columnas amarillas) en vacas de doble propósito sometidas a tres protocolos que utilizan estradiol/P4.

Time of ovulation

The mean interval from P4 device removal to ovulation was longer ($P < 0.01$) in the J-Synch group (87.7 ± 0.6 h) than in the ECP (73.7 ± 0.6 h) or EB (75.7 ± 0,6 h) groups. Overall, the percentage of cows that ovulated by 12 h after FTAI was 41.9% (126/301) and it was greater ($P < 0.01$) in the J-Synch group than in the other two groups (table 1). Conversely, the percentage of cows that ovulated between 12.1 and 24 h after FTAI was less in the J-Synch group than in the EB and ECP treatment groups ($P < 0.01$). The percentage of cows that ovulated more than 24 h after P4 device removal was 3% (9/301) and all were in the EB treatment group (9%; $P > 0.1$).

Table 1. Ovulatory response up to 12 h and between 12.1 and 24 h after FTAI in suckled dual-purpose cows subjected to three different estradiol/P4-based protocols.

Tabla 1. Respuesta ovulatoria hasta 12 y entre 12.1 y 24 h después de la IATF en vacas de doble propósito sometidas a diferentes protocolos que utilizan estradiol/P4.

Percentages with different superscripts differ (abP <0.01).

*Ovulations occurring within 24 h after FTAI.

Los porcentajes con diferentes superíndices indican diferencias significativas (abP <0,01).

*Ovulaciones que ocurren dentro de las 24 h posteriores a la IATF.

Treatments	n	Ovulation up to 12 h after FTAI (%)	Ovulation between 12.1 and 24 h after FTAI (%)	Acumulated ovulation* (%)
J-Synch	101	68 (67.3)[a]	31 (30.7)[b]	99 (98.0)
ECP	100	35 (35.0)[b]	65 (65.0)[a]	100 (100.0)
EB	100	23 (23.0)[b]	68 (68.0)[a]	91 (93.0)
Total	301	126 (41.9)	164 (54.5)	290 (97.0)

CL diameter and serum P4 concentration 7 days after FTAI

The CL diameter was greater *($P < 0.05$)* in cows in the J-Synch group compared to the other two groups, which did not differ from one another (table 2). Similarly, serum P4 concentrations were higher *($P < 0.05$)* in the J-Synch group than in the EB group but were intermediate in the ECP group which did not differ from either of the other treatment groups (table 2).

Table 2. CL diameter and serum progesterone concentrations 7 days after FTAI (mean ± SEM) in suckled, dual-purpose cows subjected to three different estradiol/P4-based protocols.

Tabla 2. Diámetro del CL y concentración sérica de progesterona a los siete días de la IATF (media ± SEM) en vacas de doble propósito sometidas a diferentes protocolos que utilizan estradiol/P4.

Means within columns with different superscritps differ ([ab] $P < 0.05$).

Las medias con diferentes superíndices indican diferencias significativas ([ab] P < 0,05).

Treatments	n	CL diameter (mm)	Progesterone concentration (ng/ml)
J-Synch	101	25.4 ± 0.2[a]	11.4 ± 0.3[a]
ECP	100	24.2 ± 0.2[b]	10.6 ± 0.3[ab]
EB	100	24.5 ± 0.2[b]	9.9 ± 0.3[b]

Pregnancies per FTAI (P/AI)

Overall, 164/301 (54.4%) of the cows were pregnant following FTAI. P/AI were numerically, but not significantly higher ($P<0.2$) in cows in the J-Synch group (59.4%, 60/101), compared to the EB (51.0%, 51/100) and ECP (53.0%, 53/100) treatment groups.

Another analysis investigated the effects of the diameter of the dominant follicle at the time of FTAI and the diameter of the ensuing CL on P/AI (table 3). Dominant follicle diameter at the time of FTAI was greater (P < 0.01) in cows that became pregnant than in those that did not become pregnant. Furthermore, the diameter of the CL tended (P=0.0861) to be greater in cows that became pregnant than in those that did not become pregnant to FTAI.

Table 3. Comparison of the follicular and luteal characteristics in suckled, dual-purpose cows that became pregnant or did not become pregnant following treatment with three different estradiol/P4-based FTAI protocols (Mean ± SEM).

Tabla 3. Comparación de las características foliculares y luteales entre vacas que quedaron preñadas o no, en vacas de doble propósito sometidas a tres protocolos diferentes que utilizan estradiol/P4 (media ± SEM).

Variable	Pregnancy Status*	n	Diameter (mm)	P value
DF at FTAI	P	164	13.4 ± 0.2	<0.0001
	O	137	12.3±0.3	
CL 7 days after FTAI	P	164	24.9±0.8	0.0861
	O	137	24.5±0.9	

*Pregnancy Status: (P) pregnant; (O) non-pregnant.

*Estado de gestación: (P) preñadas; (O) no preñadas.

Discussion

The environment, genetics, nutrition, health and management have been reported to be determinants of the reproductive performance in cattle. Although the prolonged proestrus protocol (J-Synch) resulted in the largest dominant follicle at the time of FTAI and the largest CL 7 days after FTAI, the hypothesis that this treatment results in greater P/AI than those in the conventional estradiol/P4 based protocols currently used in South America was not supported. Nevertheless, the results of this study have important implications because they demonstrate that other estradiol/P4-based FTAI alternatives can be applied successfully in suckled dual-purpose cattle in tropical environments. The P/AI exceeded 50% in the three treatment groups, which agrees with other studies performed on beef cattle in tropical regions (1, 5, 27, 38), but superior to those reported with dairy cattle in the same region (40, 46).

Another interesting factor related to tropical regions is the low estrus expression rate and a tendency to show estrus during the night, greatly affecting the efficiency of AI programs (4). However, in the present study, the overall estrus expression was 90.4%, which is higher than those reported for beef and dairy cattle in tropical regions (15, 33, 38, 39). Several studies have shown that the estrus expression at the time of FTAI, detected by tail-paint or estrus detection patches is associated with greater P/AI (13, 33, 38, 39, 42, 43) suggesting that tail-paint or patches are useful aids to determine that cows are indeed showing estrus at the time of FTAI, which is likely to result in improved pregnancy outcomes following FTAI.

The duration of the proestrus period tended to have an influence on P/AI in this study and has been reported to be positively related to an improved uterine environment and fertility (7, 8, 9, 11). Furthermore, the difference in dominant follicle diameter at P4 removal and at the time of FTAI in this study agrees with a similar study reported by de la Mata *et al.* (2018), where cows receiving the J-Synch treatment had the smallest follicles at P4 device removal, but had follicles of the greatest diameter at the time of FTAI compared with two other conventional (short proestrus) protocols. Although P/AI was only numerically greater in cows in the J-Synch group, the cows that became pregnant, regardless of treatment groups, had a greater follicle diameter at the time of FTAI than those cows that did not become pregnant. This is also consistent with the results reported by Yañez *et al.* (2016) on dairy cows in Ecuador. It has also been reported that *Bos taurus* beef heifers have the maximum probability of pregnancy when the follicular diameter at the time of FTAI is >12.8 mm (34). Furthermore, *Bos indicus* cows with follicles > 15 mm in diameter had the greatest probability of ovulating and becoming pregnant in estradiol/P4 based FTAI programs (38, 39). Thus, efforts to increase ovulatory follicle size would appear to be a worthy endeavor.

When the time of ovulation was evaluated in relation to the time of FTAI, the percentage of cows ovulating up to 12 h after FTAI in J-Synch treatment group was the highest (67.3%) compared to the ECP (35.0%) or EB (23.0%) treatment groups; while the percentage of cows that ovulated between 12.1 and 24 h after FTAI was 30.7% for the J-Synch treatment, and 65.0% and 68.0% for the ECP and EB treatment groups, respectively. The determination of the percentage of cows ovulating 12, 24 and after 24 h from FTAI may have important implications for fertility to FTAI programs. It has been estimated that the maximum sperm viability in the female genital tract is 24 to 30 h (23) and after ovulation occurs, the oocyte has an even shorter fertile lifespan (6). Therefore, achieving greater synchrony between ovulation and sperm arrival in the oviduct is very likely to increase P/AI (37). Although pregnancy rates did not differ among groups, inseminating cows nearer to the time of ovulation may have given an advantage to the J-Synch group; however, results did not differ significantly and increasing P/AI in the ECP and EB groups may have been due to later ovulations within 24 h after FTAI, which is still considered appropriate in cattle (16).

In this study CL size 7 days after FTAI was greater in the J-Synch treatment group than in the other groups, which is in agreement with the results reported by de la Mata *et al*. (2018). Nevertheless, it is important to note that when all cows were considered, regardless of their treatment, those that became pregnant had larger dominant follicles and tended to have larger CL than those that did not become pregnant, which is in agreement with other studies (12, 45). Thus, larger ovulatory follicles should result in larger CL and greater P/AI regardless of FTAI treatment protocol, again making this a worthy endeavor.

Endocrine and uterine environments associated with elevated estradiol concentrations before ovulation have been reported to affect the maintenance of the conceptus (9). Furthermore, preovulatory estradiol concentrations have been reported to have a positive impact on subsequent conceptus development (17). Therefore, the positive relationship between the expression of estrus, follicle diameter, plasma P4 concentration and P/AI may be explained by the effects of estradiol and P4 on the endometrial tissue, as has been shown in other studies (10, 20, 24, 28).

Conclusions

Although the prolonged proestrus protocol (J-Synch) resulted in the largest dominant follicle at the time of FTAI and the largest CL 7 days after ovulation, P/AI did not differ from the other estradiol/P4-based treatments in dual-purpose cows. Nevertheless, the present study has shown a positive association between the manifestation of estrus, follicle diameter at the time of FTAI and CL diameter 7 days later in suckled dual-purpose cows synchronized with estradiol/P4-based protocols for FTAI. Finally, it is concluded that the three protocols tested can be successfully applied in lactating dual-purpose cows in the Ecuadorian Amazon.

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Author contributions

GAB: Conceptualization, Writing - review & editing; PRM: Writing - review & editing; JCLP: Supervision, Data curation, Methodology, Writing - original draft. AM: writing-review & editing.

Conflicts of interest

The authors declare no conflicts of interest.

Declaration of Funding and acknowledgments

This research was supported by Fondo Nacional de Ciencia y Tecnología (FONCYT PICT 2017-4550) of Argentina and the Center of Postgraduate Research and Conservation of the Amazon (CIPCA), Ecuador. We also thank our colleagues at the CIPCA for technical assistance and Dr. Reuben J. Mapletoft from the University of Saskatchewan for the critical review of the final version of the manuscript.

A Data Availability Statement

The data that supports this study will be shared upon reasonable request to the corresponding author.