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Reproductive strategies for dairy heifers based on 5d-Cosynch with or without an intravaginal progesterone device and observed estrus

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HIGHLIGHTS

- Heifers' Reproductive management requires consideration of economic efficiency.
- Intravaginal progesterone device in 5d-Co-synch' appropriateness depends on season.
- 5d Co-synch achieved profitable conception and economic rates in dairy heifers.

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ABSTRACT

The objective of this randomized, controlled study was to evaluate the reproductive and economic performance of dairy Holstein heifers managed for first to third artificial inseminations (AIs) with or without an intravaginal progesterone device (IPD) under different temperature-humidity indexes (THI) and combined with AI after observed estrus. A total of 503 heifers from one rearing commercial farm were randomly assigned for first AI to the 5d Co-synch 72 h protocol (5dCO; n=261) or to the 5d Co-synch protocol plus IPD (5dCOP4; n=242). In a subset of heifers (n = 193) we determined progesterone (p4) and performed an ovarian ultrasound scanner on Days 0, 5, 8 and 15. Animals were considered to be synchronized on Day 5 if p4 > 1 ng/mL and a corpus luteum present; synchronized on Day 8 if p4 < 1 ng/mL, luteolysis and a follicle > 8 mm diameter was observed; and synchronized on Day 15 if p4 > 1 ng/mL and ovulation occurred, defined as the presence of a CL in the ovary where a follicle had been detected on Day 8. Pregnancy diagnosis was performed by ultrasound scanner on Days 28-35 after AI. The diagnosis confirmation was done by ultrasound on Days 50-56 and again on Days 100-113 after AI. Non-pregnant heifers (n=205) were resynchronized with the same protocol for second fixed-time AI (FTAI) and 104 for third FTAI. Pregnancy per AI (P/AI) and pregnancy loss after each AI were calculated. Reproductive costs were calculated at the individual level, based on costs for pregnancy and cost for the open days. Estrus observation was performed by visual inspection for 20 min periods, twice a day and heifers observed in estrus inseminated (OEAI). Observed in estrus inseminated heifers were 10.5% at first, 26.8% at second and 24.0% at third AI (P >0.05). The global P/AI after first AI was 58.6%; the P/AI after FTAI, 58.0%; and the P/AI after first OEAIs, 64.2%. Pregnancy per AI values were better in the IPD group [55.2% for 5dCO vs. 62.4% for 5dCOP4; odds ratio (OR) 0.35, 95% confidence interval (CI) 0.18-0.70; P=0.003], and a significant effect of the temperature humidity index (THI) on P/AI was observed (P = 0.03). The protocol 5dCOP4 led to a significantly better synchronization rate (85.7% vs. 40.0% for 5dCO; P = 0.01) and a numerically higher P/AI (74.5% vs. 49.0% for 5dCO; P = 0.24) when THI values were ≥ 70. During the cold season, there were no differences between the experimental groups for the synchronization rate (P = 0.9) nor for the P/AI (P = 0.6). The P/AI was 52.2% after the second AI and 60.6% after the third AI. Inseminations per pregnancy and open days did not differ

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significantly across experimental groups. However, reproduction costs per heifer were 130.8 ± 116.1 and 152.3 ± 129.5€ for 5dCO and 5dCOP4 groups, respectively, for the whole study ($P = 0.051$) and the average cost per AI was 58.23 ± 27.9 € for 5dCO and 76.3 ± 36.2 € for 5dCOP4 ($P < 0.0001$). During the cold season, protocols were associated with similar costs to the whole study, but they differed notably during the hot season (194.3 ± 137.6 vs. 177.3 ± 134.2 for 5dCO and 5dCOP4, respectively; P=0.49), reflecting the better reproductive performance with an IPD during the summer ($P = 0.003$). Advisors and farmers need to consider farm conditions and characteristics (herd management, staff training, and seasonality, among others) to achieve the best economic and reproductive performance in the dairy herds, when implementing hormonal synchronization protocols. Thus, cost-effectiveness depends on seasonality when implementing reproductive strategies with 5 d Co-synch 72h and combined OEAI at a rearing farm. With high THI-values, the inclusion of IPD in hormonal protocols in heifers is recommended, while it is not required during the cool season.

1. Introduction

In different countries in the world, heifers are frequently raised on so-called rearing farms while dairy farms concentrate on milking cows (Wolf and Harsh, 2001). Rearing farms are highly specialized and their objective is to give the optimal heifer (in terms of health, yield potential and longevity) back to their farm of origin, after having raised them in the most efficient way (Wolf and Harsh, 2001). One goal is to achieve an age at first calving that optimizes rearing costs and productive life; the optimal age is thought to be 22-24 months for dairy heifers (Akins, 2016; Ettema and Santos, 2004; Heinrichs et al., 2017). To achieve this optimal age at first calving, heifers should become pregnant between 13 and 15 months.

Traditionally, the insemination of heifers has been based on observation of estrus (OE), which requires extra work that farms are often unwilling or unable to undertake (Masello et al., 2019). One strategy to overcome this problem is to apply hormonal protocols for fixed-time artificial insemination (FTAI; Stevenson, 2016). The protocols used most widely to inseminate dairy heifers at fixed time are "short" variations (Masello et al., 2019) of the original Ovsynch® (Pursley et al., 1995). The in heifers frequently used protocol "5d Co-synch" shortens the interval between first administration of gonadotropin–releasing hormone (GnRH) and administration of prostaglandin (PGF) from seven days to five days (Colazo and Ambrose, 2015; Santos et al., 2010), requiring two doses of PGF before the last GnRH. The prefix "Co" means that the FTAI is performed at the same time as the last administration of GnRH, shortening the period of animal management. In brief, the 5d-Cosynch protocol consists of GnRH at Day 0 (if the protocol includes an IPD, it is inserted on Day 0), IPD removal and PGF administration on Day 5, second PGF administration on Day 6, and second GnRH administration concurrently with FTAI at 72 h after the first PGF dose (Bridges et al., 2008). Recent studies have consistently reported values of P/AI ranging from 50 to 60 % in dairy heifers subjected to the five-day (5d) FTAI program (Lima et al., 2011; Rabaglino et al., 2010). These values are comparable to those observed in heifers inseminated after OE (Kuhn et al., 2006; Masello et al., 2019). Several protocols have also included the use of an intravaginal progestin device (IPD; Colazo and Mapletoft, 2014). A classical Ovsynch® protocol, and a 7d-Co-synch 72 h + IPD protocol were compared to a double administration of PGF 14 d apart to observe estrus (McDougall et al., 2013). Those authors found that 7d-Co-synch 72 h + IPD resulted in the highest fertility and economic benefit. Other studies suggested improvements to the Co-synch + IPD protocol, such as omitting the initial GnRH administration (Colazo and Ambrose, 2011; Lima et al., 2011; Macmillan et al., 2017), applying one (Colazo and Ambrose, 2011; Rabaglino et al., 2010) or two PGF doses (Say et al., 2016), or removing the IPD after 4 d (Fishman-Holland et al., 2019), 5 d (Colazo and Ambrose, 2011; Macmillan et al., 2017) or 7 d (Colazo and Ambrose, 2011).

Different studies have reported a specifically better ovarian response to hormonal protocols with IPD, under heat stress (Fishman-Holland et al., 2019). Heat stress may reduce the fertility by reducing steroidogenesis, hindering follicle selection, and shifting follicular waves, thereby damaging oocyte quality (Badinga et al., 1993; García-Ispierto et al., 2007; Hansen, 2002; Roth et al., 2001, 2000; ; Wolfenson et al., 1995). It also shortens corpus luteum life span and reduces progesterone production (Wolfenson et al., 1997), with several of these effects being partially ameliorated with the use of an IPD (Kasimanickam et al., 2014; Lima et al., 2011).

Including an IPD in the hormonal synchronization protocol can increase fertility of dairy heifers to 58-63% (Chebel and Cunha, 2020; Kasimanickam et al., 2014), but it also increases the amount of work and therefore human resources costs. Global costs (taking into account: days open, pregnancy per AI, etc.) should be considered to determine if IPD inclusion is profitable. When implementing FTAI in farms, protocol selection should not be based only on the expected P/AI. The associated costs (including drugs, time, extra work, days open, overall pregnancy rates, among others) also need to be taken into consideration and global reproductive strategies need to be evaluated (Gabler et al., 2000). The reproductive and economic efficiency of dairy heifers is critical for the farm because reducing time to pregnancy decreases costs of rearing and opportunity costs for replacements (Ettema and Santos, 2004; Lopes et al., 2013; Silva et al., 2015). However, few studies have compared the reproductive and economic efficiency of reproductive programs implementing hormonal protocols with or without an IPD in the same herd, combined with AI after observed estrus and new approaches which link economics to reproduction in dairy heifers are required to improve financial and reproductive efficiency in rearing farms.

Thus, our objective in the present study was to compare the reproductive and economic efficiency of reproductive strategies based on the implementation of 5d Co-synch 72 h protocol in dairy heifers with or without an IPD, combined with estrus observation. We also evaluated to what extent seasonality, i. e. heat stress and other environmental factors influence the potential reproductive and economic benefits of these strategies.

2. Material and methods

2.1. Animals and farm

Nulliparous heifers from a single commercial rearing farm in northeast Spain (Vilanant, Girona) were enrolled in the study from January 2018 to November 2019. The temperature-humidity index (THI) was monitored daily with a portable device (Benetech GM1365 Digital Humidity & Temperature Data Logger; Shenzhen Jumaoyuan Science and Technology, Shenzhen, China). THI was calculated by combining the maximum temperature in Celsius and minimum relative humidity using a published formula (Bohmanova et al., 2007). Mean daily THI over the cold season of the study was 58.6 (range 44.9 to 76.1) and over the hot season 73.1 (range 71 to 76.1). Heifers were housed in freestall barns with concrete flooring and self-locking head gates in the feeding lane. Surfaces were covered with pack sawdust bed. Heifers were fed a total mixed ration once a day following the National research Council, 2001, with *ad libitum* access to feed and water.

2.2. Experimental design

To be included in the study, heifers had to be healthy, at least 350 days old, and at least 135 cm tall to the withers. They had to have no previous insemination when included for first AI, and diagnosed non-pregnant or observed in estrus when submitted for subsequent AIs. Health monitoring was performed daily by the farm staff, identifying sick heifers based on their general attitude and nutrition behaviour. Heifers detected sick were examined for rectal temperature and veterinarian general exam.

Initially, 521 virgin, non-pregnant Holstein heifers were included in the study at two-week intervals from January 2018 to November 2019. Every two weeks, on Day 0, available heifers (18-22 animals per batch) were randomly assigned to undergo the 5d Co-synch 72 h (Bridges et al., 2008) protocol as previously described (5dCO group), or the 5d Co-synch 72 h protocol with an IPD (5dCOP4 group). A total of 18 heifers were not included into the study (3.4 %; 18/521) due to IPD loss in the first three days after insertion (n = 11), ovarian adherence (n = 1), unicornuate uterus (n = 1), error in AI data entry (n = 1), or death prior to pregnancy diagnosis (n = 4). Thus, 503 first inseminations were included in the analysis: 261 in the 5dCO group, and 242 in the 5dCOP4 group. The same two veterinarians who performed AI diagnosed pregnancy by ultrasound using an iScan system equipped with a 7.5-MHz linear transducer (Draminski, Olsztyn, Poland) on Days 28-35 after AI.

Heifers diagnosed non-pregnant (n = 205) were resynchronized with the same protocol they received before (5dCO or 5dCOP4) at the day of pregnancy diagnosis, and subjected to a second FTAI, immediately at the same moment of pregnancy diagnosis. If they were not pregnant after the second AI, the same procedure was repeated for the third AI (n=104). The average interval between first and second AI was 48d (8-132d) and between the second and third inseminations was 42 (14-108d). Only heifers younger than 18 months at the moment of AI were kept in the study (non-pregnant heifers >18m old were "problem heifers" and differently managed by the farm advisors). The last study day for each heifer was the last pregnancy diagnosis.

Pregnancy was confirmed by ultrasound on Days 50-56 and again on Days 100-113 after AI. If the heifer was non-pregnant after being considered pregnant on one of the previous examinations, the result was recorded as pregnancy loss, and the heifer was resubjected to AI, if younger than 18m old.

Estrus was detected by the farm staff, by visual observation during 20 min twice a day throughout the study, with the heifer standing to be mounted being the accurate sign of estrus (Allrich, 1993). Synchronized heifers included in the study but observed in estrus prior to the scheduled day for FTAI were inseminated after OE, and these AIs were recorded as OEAIs. Three heifers in the 5dCO group that became pregnant at the second AI but subsequently lost the pregnancy were excluded from the study because they were older than 18m old. The experimental design is summarized in Fig. 1.

The factors THI and type of semen were considered to classify heifers in two categories: those having had at least one insemination with THI≥70 or none; the same classification was made for heifers having had at least one AIs with sex-sorted semen or none. For the number of FTAIs vs. OEAIs, we classified heifers having had 0, 1, 2 or 3 FTAIs. These groups were included in the analysis of the probability of pregnancy and AIs per pregnancy (Table 4).

2.3. Hormonal protocols

The protocol 5dCO consisted of intramuscular administration (semitendinosus muscle) of 100 µg GnRH (Cystoreline® CEVA Santé Animale SA, Libourne, France) at Day 0. At Days 5 and 6, 500 µg cloprostenol (analogue of $PGF_{2\alpha}$; Cyclix®, Virbac, Carros Cedex, France) and 100 µg GnRH (Cystoreline® CEVA) were administered intramuscularly at the time of AI (Day 8, Fig. 2). The 5dCOP4 protocol was identical to the 5dCO protocol, except for the insertion of an IPD containing 1.55 g of

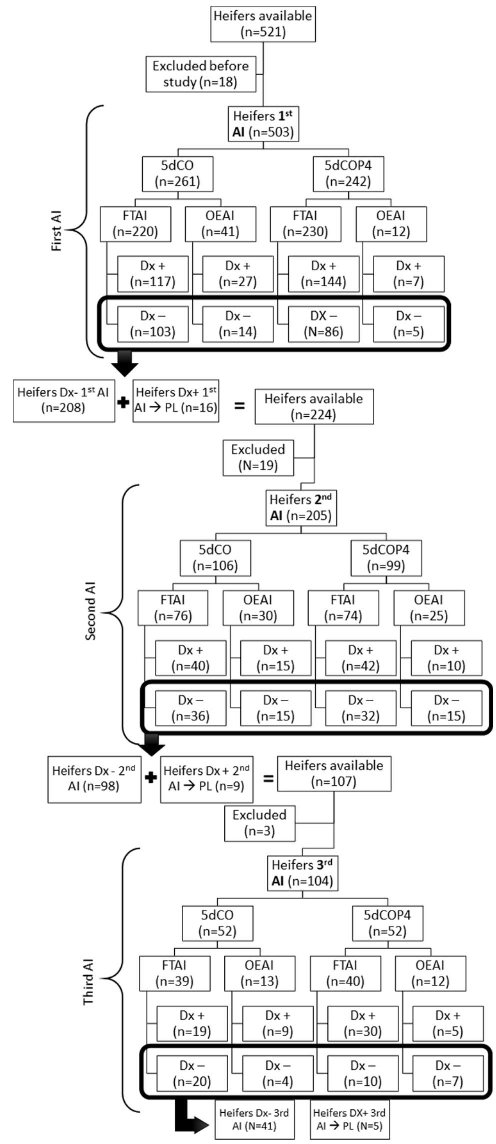

Fig. 1. Experimental design and heifer distribution after AI assignment, protocol, and pregnancy diagnosis.
Abbreviations: 5dCO = 5 day Co-synch 72h; 5dCOP4 = 5 day Co-synch 72h with and intravaginal progesterone device; AI = artificial insemination; FTAI = fixed time AI; OEAI = AI after observed estrus; Dx = pregnancy diagnosis; PL = pregnancy loss.

progesterone (PRID-delta®, CEVA) on Day 0, with subsequent removal on Day 5.

At Day 8, age, height, and body condition score (BCS) on a scale of 1-5 (Edmonson et al., 1989) were recorded by one of the veterinarians who performed FTAI. The distribution of FTAIs between veterinarians was random. Additional information recorded at each insemination were as follows: date, AI-technician, bull, sex-sorted or conventional seminal dose, and temperature-humidity index. Conventional semen from seven bulls and sex-sorted semen from five bulls was used for the inseminations randomly.

2.4. Ultrasound examinations and progesterone evaluation

Transrectal ultrasonography was performed by the same experienced veterinarian on all heifers. In a representative subset of heifers included for first AI (38.4 %; 193/503, comprising 85 from the 5dCO group and 108 from the 5dCOP4 group) or included for second and third AIs (27 %;

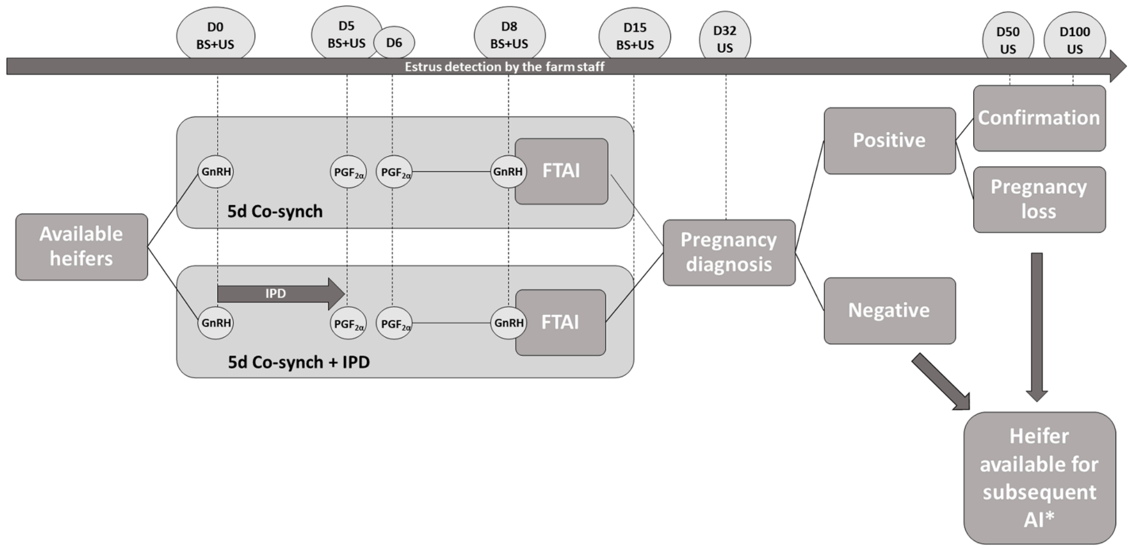

Fig. 2. Experimental design and description of both protocols including hormone administration, ultrasound scanner, and blood sampling schedules. Abbreviations: D = day; US = ultrasound scanner; BS = blood sampling; IPD = intravaginal progesterone device; FTAI = fixed-time artificial insemination. * Heifer available for subsequent AI based on the same Co-synch protocol as before.

83/309, comprising 43 from the 5dCO group and 40 from the 5dCOP4 group), a blood sample was taken and ovaries were examined by ultrasound on Days 0, 5, 8, and 15 of the synchronization (Fig. 2).

Blood samples were taken from the coccygeal vein into 4-mL EDTA K2 vacutainer tubes (Fisher Scientific, Pittsburgh, PA, USA), immediately centrifuged at 4,500 g for 15 min at room temperature (22-25°C), and the plasma was transferred to a fresh tube and stored at -80 ℃ until progesterone determination. Plasma progesterone concentrations were measured in a single analysis using a by enzyme-linked immunosorbent assay (ELISA) (Demeditec Diagnostics, Kiel-Wellsee, Germany). Assay sensitivity was 0.045 ng/mL and the manufacturer-specified intra-assay variation coefficient was 5 %.

The diameter and location of the greatest follicles and corpus luteum (CL) were recorded. Detailed ovarian maps based on the location, number and size of follicles, and CL were obtained to link hormonal results to ovarian structure and function. Ovulation was considered to have occurred after the first GnRH administration if a CL was observed on Day 5 in the ovary where a follicle had been detected on Day 0. Luteolysis was considered to have occurred after the second prostaglandin dose if there was no CL on Day 8. The largest follicle diameter (in mm) was measured on Day 8. Ovulation was considered to have occurred after the second GnRH administration if there was a CL on Day 15 at the ovary where a preovulatory follicle was previously observed on Day 8.

Animals were considered to be synchronized on Day 5 (synchronization rate at Day 5; SD5) if a CL was present and plasma progesterone level > 1 ng/mL; synchronized on Day 8 (SD8) if luteolysis and a follicle with > 8 mm diameter were observed and plasma progesterone < 1 ng/mL; and synchronized on Day 15 (SD15) if the animal had ovulated after the second GnRH (CL in the ovary where a preovulatory follicle had been detected on Day 8) and plasma progesterone > 1 ng/mL. The total synchronization rate (TSR) was calculated based on the number of animals synchronized on Days 5, 8 and 15. To have a positive, complete TSR the heifer should have achieved all synchronization criteria on all three Days 5, 8 and 15.

2.5. Reproductive efficiency of the heifers and economic cost calculation

The economic evaluation was made at the level of heifer, based on the definitive status (pregnant or non-pregnant) of each animal at the end of the study. To calculate the insemination cost at the individual level, the following calculation (cost of the protocol and cost of the insemination work) were based on the costs recorded at the farm, which were similar to those found in the literature (Masello et al., 2021). The cost of the 5dCO protocol was 9.8 € (8.0 € drugs + 1.8 € staff work), while the cost of the 5dCOP4 protocol was 21.5 € (19.0 € drugs + 2.5 € staff work). The insemination work cost was 27.5 € (25 € seminal dose + 2.5 € staff work), and it was considered the same for all AIs.

The cost for first AI implemented after OE included the cost of the protocol, because these heifers received all the hormones before showing estrus except the last GnRH. The cost of inseminations achieved after second and third inseminations implemented after OE (OEAIs) without a hormonal protocol before that AI, included only the insemination costs.

The reproductive cost per heifer was calculated as the sum of the insemination cost, plus the cost of pregnancy diagnoses performed, plus the cost associated to “open days”. Cost of pregnancy diagnosis was estimated in 4€/diagnosis. Pregnancy diagnosis was performed between AIs when not observed in estrus before. The cost for each “open day”, i.e. for each day that a heifer was not pregnant, was estimated for the farm in this study as 2.3 €/heifer. This cost is similar to that recently published for a rearing farm in the UK (£2.8; Boulton et al., 2017). Open days were calculated from the first AI until the heifer got pregnant or until the end of the study for each non-pregnant heifer (last pregnancy diagnosis after last AI). The heifers that exited the study as not pregnant before the third AI (19 after first OEAI and 3 after second AI) had the corresponding open days until pregnancy diagnosis.

Additionally, the costs per AI were calculated for the 812 AIs of the study using the same criteria as described above, and median cost per AI was calculated for both experimental groups.

2.6. Statistical analyses

All data were analyzed using SPSS® version 25 (IBM, Armonk, NY, USA). *P*-values less than or equal to 0.05 were considered significant. Binary outcomes were reported as a mean percentage for categorical variables or as mean ± standard deviation (SD) for continuous variables. Binary outcomes (i.e. P/AI and pregnancy loss) were analyzed using logistic regression, odds ratios (OR) were determined, and a stepwise forward method based on the Wald statistic criterion of $P > 0.10$ was applied. The statistical model included the batch of inclusion as random effect to control for it, and in the model for second and third inseminations, the heifer was included as a random effect because some heifers contributed with data from more than one insemination. Treatment was a fixed effect in all models.

To explore whether progesterone values on Day 0 influenced synchronization efficacy, we categorized the progesterone levels in two categories using the classical cut-off value of 1 ng/mL (Kasimanickam et al., 2014). An additional analysis was performed categorizing progesterone in three levels: low (< 0.6 ng/mL), intermediate (0.6-5 ng/mL), or high (≥ 5 ng/mL) based on a previous study in dairy cattle (Carvalho et al., 2018) that reported higher P/AI with intermediate progesterone levels on Day 0.

Three separate models were built depending on the subset of data involved, and they were analyzed separately using different regression models in order to describe the effects of the different factors on conception and pregnancy loss rates on first inseminations (first subset), second and third inseminations (second subset), and global reproduction performance (third subset).

The following factors were included in the model: experimental group (5dCO vs. 5dCOP4); level of progesterone at Day 0 in three categories (low/intermediate/high); SD5, SD8, SD15, and TSR; THI (high/low) at insemination, for which THI was stratified as ≥ 70 for the hot season or < 70 for the cold season (Mader et al., 2006; Pinto et al., 2020); AI technician; semen used (conventional vs. sex-sorted semen), dominant follicle diameter at Day 8 (in cm); height (in cm), BCS (scale 1-5), and age (in days) at insemination.

Economic efficiencies were compared using the *t*-test and generalized linear models. In the case of heifer costs, the model included two categories by THI at AI: having had at least one insemination with THI≥70 or none; two categories by the use of sex-sorted semen: having had at least one AIs with sex-sorted semen or none; and four categories for the number of FTAIs vs. OEAIs performed: having had 0, 1, 2 or 3 FTAIs.

3. Results

Of the 503 heifers available for the study (261 in the 5dCO group and 242 in the 5dCOP4 group), 53 were inseminated after OE before ending the protocol (Fig. 1). The global P/AI after first AI was 58.7 % [295/503; P/AI after FTAI, 58.0 % (261/450); P/AI after OE, 64.2 % (34/53)]. Detailed conception results are shown in Table 1.

The heifers with OEAI were 10.5 % (53/503) at first AI. Non pregnant heifers in which synchronization protocols were initiated for first FTAI but which were then classified as OEAIs were included for pregnancy diagnosis but not submitted to subsequent FTAIs; this was the case for 19 non-pregnant heifers with first OEAI, comprising 14 from the 5dCO group and 5 from the 5dCOP4 group.

3.1. Results after first AI

The distribution of the possible confounding factors analyzed in the 503 heifers were similar in both experimental groups. The height (140.3 ± 3.3 vs. 140.1 ± 3.1 cm, for 5dCO and 5dCOP4 groups, respectively), BCS (3.4 ± 0.2 vs. 3.4 ± 0.2), age at first AI (395.1 ± 31.0 vs. 394.2 ± 18.8 m), THI at AI (57.7 ± 9.0 vs. 58.3 ± 9.3) and progesterone level at Day 0 (6.3 ± 5.1 vs. 5.9 ± 5.2 ng/mL) did not differ statistically between groups (P>0.05).

Table 2 describes the distribution of the possible confounding categoric factors analyzed in the 503 heifers submitted to first insemination and in the subset of 193 heifers more intensively explored. Both experimental groups were similar in terms of the measured factors. The logistic regression showed significant effects due to the type of semen used for first inseminations and the synchronization protocol. P/AI values were 7 percentage points worse for the protocol without IPD (5dCO; P=0.03) and 25 percentage points worse for procedures using sex-sorted semen (P<0.001; Table 2).

Among the continuous or ordinal factors in the regression model, only the height at insemination differed significantly between pregnant and non-pregnant heifers (139.9 ± 3.1 cm for pregnant vs. 140.7 ± 3.3 cm for non-pregnant heifers; OR 0.93, 95% CI 0.875-0.990; P = 0.02). Age did not significantly influence pregnancy probability (394.9 ± 19.1 for pregnant vs. 394.5 ± 33.2 d for non-pregnant heifers: P = 0.06).

Since the interaction between THI and protocol was significant in the regression model, a separate analysis of the data by THI was performed (Table 3). No significant effects were observed in the hot or cold season for any of the analyzed factors.

The percentages of TSR were 40.0 % (6/15) for 5dCO and 85.7 % (18/21) for 5dCOP4 during the hot season (OR 0.13; 95% CI 0.025-0.63; P = 0.01), while during the cold season the TSR did not differ between the two groups [46/70 (65.7 %) for 5dCO vs. 58/87 (66.7 %) for 5dCOP4); P = 0.90.

Height, BCS, and age at AI did not significantly influence the P/AI, based on regression of data stratified by THI. The same was observed for the diameter of the largest follicle at Day 8 of synchronization.

A total of 16 cases of pregnancy loss were observed after first AIs (16/295; 5.4 %) with no significant difference between experimental groups [3/144 (2.1 %) in the 5dCO group and 13/151 (8.6 %) in the 5dCOP4 group]. A regression model that included the same factors as for P/AI showed a significant increase in pregnancy losses when THI at insemination was high [12/233 (5.2 %) with THI < 70 vs. 4/61 (6.6 %) with THI ≥ 70; OR 1.19, 95% CI 1.00-1.41; P = 0.05]. Additionally, conventional semen showed a protective effect against pregnancy loss after

Table 1
P/AI in dairy heifers synchronized with either of two hormonal protocols, stratified by order and type of artificial insemination.

AI	Group	Total AIs	Global P/AI, n/N (%)	P/AI after FTAI, n/N (%)	P/AI after OEAI, n/N (%)
First	5dCO	261	144/261 (55.2)	117/220 (53.2)	27/41 (65.9)
	5dCOP4	242	151/242 (62.4)	144/230 (62.6)	7/12 (58.3)
	Total	503	295/503 (58.7)	261/450 (58.0)	34/53 (64.2).
Second*	5dCO	106	55/106 (51.9)	40/76 (52.6)	15/30 (50.0)
	5dCOP4	99	52/99 (52.5)	42/74 (56.8)	10/25 (40.0)
	Total	205	107/205 (52.2)	82/150 (54.7)	25/55 (45.5)
Third**	5dCO	52	28/52 (53.8)	19/39 (48.7)	9/13 (69.2)
	5dCOP4	52	35/52 (67.3)	30/40 (75.0)	5/12 (41.7)
	Total	104	63/104 (60.6)	49/79 (62.0)	14/25 (56.0)
All AIs	5dCO	419	227/419 (54.2)	176/335 (52.5)	51/84 (60.7)
	5dCOP4	393	238/393 (60.6)	216/344 (62.8)	22/49 (45.0)
	Total	812	465/812 (57.3)	392/679 (57.7)	73/133 (54.9)

Abbreviations: AI, artificial insemination; FTAI, fixed-time AI; OEAI, AI after observed estrus; P/AI, pregnancy per AI; 5dCO, synchronization protocol 5 days Co-synch 72h; 5dCOP4, synchronization protocol 5 days Co-synch 72 h with intravaginal progesterone device.

*To non-pregnant heifers after first AI (208) 16 heifers which had had pregnancy loss after first pregnancy (3 from group 5dCO and 13 from 5dCOP4) were added resulting in 117+3=120 heifers for 2nd AI in the 5dCO group and 91+13=104 in group 5dCOP4. Finally, 19 OEAI non-pregnant heifers were excluded (14 from group 5dCO and 5 from 5dCOP4) which made a total of 106 second AIs in the 5dCO group (120 – 14=106) and 99 second AIs in the 5dCOP4 group (104-5=99).

** To non-pregnant heifers after second AI (98) 9 heifers which had had pregnancy loss after second pregnancy (4 from group 5dCO and 5 from 5dCOP4) were added resulting in 51+4=55 heifers for 3rd AI in the 5dCO group and 47+5=52 in the group 5dCOP4. Finally, 3 heifers were excluded (all three from group 5dCO) which made a total of 52 third AIs in the 5dCO group (55 – 3=52) and 52 third AIs in the 5dCOP4 group.

Table 2
Categorical factors that were measured at first insemination of dairy heifers synchronized with either of two hormonal protocols and that may affect P/AI and logistic regression of P/AI after first insemination in dairy heifers synchronized with either of two hormonal protocols.

Factor	Category	Total n/N	5dCO n/N (%)	5dCOP4 n/N (%)	P/AI, n/N (%)	OR[1]	95% CI			*P*-Value
P4 (ng/mL)	< 0.6	26/193	11/85 (12.9)	15/108 (13.9)	15/26 (57.7)					0.93
	0.6-5	66/193	29/85 (34.1)	37/108 (34.3)	40/66 (60.6)					0.70
	>5	101/193	45/85 (52.9)	56/108 (51.9)	59/101 (58.4)					0.95
P4-1 (ng/mL)	≤ 1	36/193	14/85 (16.5)	22/108 (20.4)	22/36 (61.1)					0.48
	> 1	157/193	71/85 (83.5)	86/108 (79.6)	92/157 (58.6)					
THI at AI	≥ 70	62/503	212/261 (81.2)	191/242 (79.0)	62/100 (62.0)					0.52
	< 70	403/503	49/261 (18.8)	51/242 (21.1)	233/403 (57.8)					
TSR	No	65/193	33/85 (38.8)	32/108 (29.6)	30/65 (46.2)					0.59
	Yes	128/193	52/85 (61.2)	76/108 (70.4)	84/128 (65.6)					
SD5	No	3/193	3/85 (3.5)	0/108 (0.0)	1/3 (33.3)					0.94
	Yes	190/193	82/85 (96.5)	108/108 (100)	113/190 (59.5)					
SD8	No	60/193	30/85 (35.3)	30/108 (27.8)	29/60 (48.3)					0.92
	Yes	133/193	55/85 (64.7)	78/108 (72.2)	85/133 (63.9)					
AI-tech	Tech-1	334/503	179/261 (68.6)	155/242 (64.0)	199/334 (59.6)					0.59
	Tech-2	169/503	82/261 (31.4)	87/242 (36.0)	96/169 (56.8)					
Semen	Conventional	343/503	178/261 (67.9)	165/242 (68.2)	228/343 (66.5)	3.19	2.07	-	4.92	<0.001
	Sex-sorted	160/503	83/261 (31.8)	77/242 (31.8)	67/160 (41.9)	reference				
Protocol	5dCO	261/503			144/261 (55.2)	0.35	0.18	-	0.70	<0.001
	5dCOP4	242/503			151/242 (62.4)	reference				
Interaction		THI by protocol				0.41	0.18		0.92	0.03

Abbreviations: n/N, number of pregnant heifers/total number of inseminated heifers in each category; P/AI, pregnancy per AI in %; OR, odds ratio; CI, confidence interval; P4, serum progesterone level at Day 0 of the synchronization, classified into three categories; P4-1, P4 classified in two categories; THI, temperature-humidity index; TSR, total synchronization rate at Days 5, 8, and 15; SD5, synchronized at Day 5; SD8, synchronized at Day 8; AI-tech, artificial insemination technician; 5dCO, synchronization protocol 5 days Co-synch 72h; 5dCOP4, synchronization protocol 5 days Co-synch 72h with intravaginal progesterone device.
P-values come from a logistic regression model. [1]OR values only given for significantly affecting factors.

first insemination compared to sex-sorted semen [12/227 (5.3 %) *vs.* 4/67 (6.0 %); OR 0.033, 95% CI 0.002-0.566; $P = 0.02$].

3.2. Results at second and third AIs

A total of 208 non-pregnant heifers were available for second FTAI. Additionally, 16 heifers (three in group 5dCO and 13 in group 5dCOP4) that suffered pregnancy loss after first AI were included. A total of 19 non-pregnant heifers inseminated after OE at first AI (14 in group 5dCO and 5 in group 5dCOP4) were excluded for a further syncrhonization. Therefore, 205 heifers (106 in group 5dCO and 99 in group 5dCOP4) were hormonally resynchronized using the same protocol as for first AI inseminations (Fig. 1). A total of 55 heifers (26.8 %) was inseminated after OE [OEAI; 30/106 (28.3 %) from group 5dCO and 25/99 (25.3 %) from group 5dCOP4]. The global P/AI after second AI was 52.2 % (107/205); P/AI after second FTAI, 54.7 % (82/150); and P/AI after second OEAI, 45.5 % (25/55) (Table 1). Third inseminations were conducted on 98 non-pregnant animals after the second insemination, as well as six heifers that lost pregnancies after the second AI (one in group 5dCO and five in group 5dCOP4). Among the 104 third inseminations (52 in group 5dCO and 52 in group 5dCOP4), 24.0 % (25 heifers) were inseminated after OE [OEAI; 13/52 (25 %) from group 5dCO and 12/52 (23.1 %) from group 5dCOP4]. The global P/AI after third AIs was 60.6 % (63/104); P/AI after third FTAI, 62.0 % (49/79); and P/AI after third OEAI, 56.0 % (14/25). Further conception results are described in Table 1.

The same factors as described for first inseminations were analyzed for second and third inseminations. The distribution of these possible confounding factors analyzed in the heifers submitted to second and third inseminations was similar in both experimental groups (5dCO and 5dCOP4).

Second and third inseminations were considered jointly in the regression analysis. Similarly to that observed after first inseminations, the use of conventional semen was associated with better P/AI after second and third inseminations [60.1 % (143/238) after AI with conventional semen vs. 38.0 % (27/71) after the use of sex-sorted semen; OR 2.22; 95% CI 1.22-4.06; $P = 0.01$]. The P/AI differed between the technicians inseminating, with technician 1 performing better than technician 2 [60.4 % (113/187) vs. 46.7 % for technician 2 (57/122); OR 1.77; 95% CI 1.05-2.98; $P = 0.03$]. No other factor or interaction of factors significantly influenced P/AI or risk of pregnancy loss after these inseminations.

A total of 14 cases of pregnancy loss were observed (8.2 %), nine of them after pregnancy from second AIs (four in group 5dCO and five in group 5dCOP4) and five from the third AIs (three in group 5dCO and two in group 5dCOP4). The pregnancy loss rates were similar between the two experimental groups and were not significantly affected by any of the analyzed factors.

3.3. Reproductive and economic efficiency

By the last day of the study, among the 503 included heifers (261 in group 5dCO and 242 in group 5dCOP4), 68 ended non-pregnant [44/261 (16.8 %) in group 5dCO and 24/242 (9.9 %) in group 5dCOP4] and 435 were pregnant (Table 4). The global P/AI after three AIs was 86.5 % (435/503) across all animals, 217/261 (83.1 %) in group 5dCO and 218/242 (90.0 %) in group 5dCOP4 ($P = 0.098$).

Heifers that were subjected to one or two FTAIs (1 or 2 FTAI out of 3 AIs) were significantly more likely to become pregnant than those that underwent only FTAIs (3 FTAI out of 3 AIs)and only OEAIs (0 FTAI out of 3 AIs). Heifers inseminated at least once with sex-sorted semen became pregnant significantly less often than those inseminated with conventional semen.

The evaluation of costs in each experimental group based on the reproductive efficiency of the heifers is shown in Table 5. The average insemination cost per heifer in the 5dCO group was 58.23 ± 27.9 and 76.3 ± 36.2 € in the 5dCOP4 group ($P < 0.001$).

Although the average of inseminations per pregnancy and number of open days did not differ significantly between experimental groups, the cost of insemination was significantly higher with the IPD, and the reproduction cost per heifer significantly tended to (130.8 ± 116.1 vs. 152.3 ± 129.5€; P=0.051).

Similar results were obtained for the cold season, after stratifying the data by heifers that were inseminated at least once with THI ≥ 70 or always inseminated with cold temperatures. However, for the heifers inseminated at least once during the hot season, the reproductive cost per heifer in the 5dCO group was 17 € more expensive than that for

Table 3
P/AI after first insemination in dairy heifers synchronized with either of two hormonal protocols, stratified by cold or hot season.

Factor	Value	THI < 70		THI ≥ 70	
		n/N	P/AI, n/N (%)	n/N	P/AI, n/N (%)
P4 (ng/mL)	< 0.6	22/157	13/22 (59.1)	4/36	2/4 (50.0)
	0.6-5	56/157	35/56 (62.5)	10/36	5/10 (50.0)
	>5	79/157	43/79 (54.4)	22/36	16/22 (72.7)
P4-1 (ng/mL)	≤ 1	31/157	20/31 (64.5)	5/36	2/5 (40.0)
	> 1	126/157	71/126 (56.2)	31/36	21/31 (67.7)
TSR	No	53/157	25/53 (47.2)	12/36	5/12 (41.7)
	Yes	104/157	66/104 (63.5)	24/36	18/24 (75.0)
SD5	No	2/157	1 /2 (50.0)	1/36	0/1 (0)
	Yes	155/157	90/155 (58.1)	35/36	23/35 (65.7)
SD8	No	49/157	24/49 (49.0)	11/36	5/11 (45.5)
	Yes	108/157	67/108 (62.0)	25/36	18/25 (72.0)
AI-Tech	Tech-1	259/403	153/259 (59.1)	75/100	46/75 (61.3)
	Tech-2	144/403	80/144 (55.6)	25/100	16/25 (64.0)
Semen	Conventional	243/403	166/243 (68.3)	100/100	62/100 (62.0)
	Sex-sorted	160/403	67/160 (31.9)	0/100	0/0 (0)
Protocol	5dCO	212/403	120/212 (56.6)	49/100	24/49 (49.0)
	5dCOP4	191/403	113/191 (59.2)	51/100	38/51 (74.5)

Abbreviations: n/N indicates number of pregnant heifers by the total of inseminated heifers at each category; P/AI = pregnancy per AI in %; P4 = serum progesterone level at Day 0 of the synchronization classified in three categories; P4-1 = P4 classified in two categories; THI = temperature-humidity Index; TSR = totally synchronized at Days 5, 8, and 15; SD5 = synchronized at Day 5; SD8 = synchronized at Day 8; AI-Tech = artificial insemination technician; 5dCO = synchronization protocol 5 days Co-synch 72h; 5dCOP4 = synchronization protocol 5 days Co-synch 72h with intravaginal progesterone device.

heifers with the 5dCOP4 protocol, despite the much higher insemination cost associated to the 5dCOP4 protocol. This reflects the higher reproductive efficiency achieved with the 5dCOP4 protocol, under heat stress (Table 5). Even when numbers were not statistically significant, the numerical differences (17€) may be important for farm economics.

4. Discussion

In this work, we aimed to evaluate and compare the reproductive and economic suitability of two synchronization protocols in dairy heifers. After three consecutive inseminations, despite the improvement in P/AI results due to the 5dCOP4 protocol, the effect on the average AI per pregnancy was only slightly improved with 5dCOP4 protocol. On the other hand, despite the higher costs per insemination in the 5dCOP4 group ($P < 0.001$), global reproduction costs per heifer difference was not significant, showing a statistical trend (130.8 ± 116.1 vs. 152.3 ± 129.5; $P = 0.051$). Reproductive parameters (less open days and AI per pregnancy) and costs (17€ cheaper) were clearly more favourable for the protocol with IPD during the hot season, whereas during the cold season, the two protocols were similarly efficient and 5dCO was less expensive.

Our study revealed that, comparing the two protocols at first insemination, the 5dCOP4 improved fertility (P/AI 55.2 % for 5dCO vs. 62.4 % 5dCOP4; $P < 0.001$). This finding is consistent with previous work (Kasimanickam et al., 2014; McDougall et al., 2013). In the present study, this positive effect of the 5dCOP4 protocol was due to the notable improvement in synchronization rate (> 30 % better than 5dCO) and thereafter a higher P/AI (> 14 % better than 5dCO) during the hot season. In accordance with our results, previous studies demonstrated that progesterone supplementation via IPD between the GnRH and the PGF injections can prevent premature estrus, luteinizing hormone (LH) surge, and ovulation in heifers with no endogenous progesterone (Fishman-Holland et al., 2019; Kasimanickam et al., 2014; Lima et al., 2011). Our observation that these effects were more noticeable during the hot season has also been reported in previous work (Wang et al., 2020), which associated heat stress with lower embryo quality (Putney et al., 1989) and lower progesterone levels (Wolfenson et al., 2002). Heat stress also reduces the duration and intensity of estrus (Gangwar et al., 1965), resulting in silent or weak estrus expression, reducing pregnancy rates (Rensis and Scaramuzzi, 2003). Moreover, heat stress compromises follicular development, steroidogenesis, and oocyte quality, contributing to a reduced P/AI (Howell et al., 1994; Wilson et al., 1998). Hence, IPD enhances ovarian synchronization and oocyte quality for inseminating heifers under heat stress, thereby improving P/AIs (Wang et al., 2020). We observed these results in our animals at first AI, but not at second and third AIs. We also cannot exclude that the lack of a significant difference in TSR and P/AI is an artefact of the relatively small sample of second and third AIs in our study.

Table 4
Global P/AI of dairy heifers after three artificial inseminations, stratified by synchronization protocol.

Factor	Class	P/AI, n/N (%)	OR[1]	95% CI		*P-value*
Type of AI	0/3 FTAI	35/54 (64.8)	reference			
	1/3 FTAI	274/280 (97.9)	22.16	8.10	60.63	<0.0001
	2/3 FTAI	95/110 (86.4)	4.39	1.89	10.20	0.001
	3/3 FTAI	31/59 (52.5)	0.65	0.29	1.45	0.289
Type of Semen	No AI with sex-sorted semen	296/321 (92.2)	reference			
	At least one AI with sex-sorted semen	139/182 (76.4)	0.31	0.16	0.58	<0.0001
Protocol	5dCO	217/261 (83.1)	0.59	0.31	1.10	0.098
	5dCOP4	218/242 (90.0	reference			
Heat stress at AI	No AI with THI ≥70	102/120 (85.0)				
	At least one AI with THI ≥ 70	333/383 (86.9)				0.990

Abbreviations: n/N = number of pregnant heifers by the total of inseminated heifers at each category; P/AI = pregnancy per AI in %; OR = odds ratio; CI = confidence interval; THI = temperature-humidity index; OE = insemination after observed estrus; 5dCO = synchronization protocol 5 days Co-synch 72h; 5dCOP4 = synchronization protocol 5 days Co-synch 72h with intravaginal progesterone device.
P-values from logistic regression model. [1]OR values only given for significantly affecting factors.

The weaker synchronization effectiveness observed in the heifers of group 5dCO is also reflected in the higher percentage of animals being submitted to OEAI before the date for first FTAI (15.7 % for 5dCO vs. 4.9 % for 5dCOP4). Interestingly, the P/AI after first OEAI was better with the 5dCO protocol (65.8 % for 5dCO vs. 58.3 % for 5dCOP4). Similarly to our 5dCO group, Macmillan and colleagues (2017) observed a higher

Table 5
Economic evaluation of the reproductive efficiency of dairy heifers after three AIs, stratified by synchronization protocol.

Variables	All heifers (n=503) 5dCO (n=261) Average ± SD	5dCOP4 (n=242) Average ± SD	*P*-value	Heifers with at least one AI with THI>70 (n=120) 5dCO (n=57) Average ± SD	5dCOP4 (n=63) Average ± SD	*P-value*
Age at conception (d)	416.3 ± 37.0	417.6 ± 40.0	0.79	427.9± 44.3	422.4 ± 46.0	0.55
AI per pregnancy	1.93 ± 0.8	1.82 ± 0.7	0.73	2.1 ± 0.85	1.79 ± 0.85	0.10
Open days/heifer[1]	29.0 ± 37.9	30.3 ± 40.4	0.70	48.6± 45.8	37.8± 42.2	0.18
Cost due to open days/ heifer (€)[2]	66.9 ± 87.6	70.1 ± 93.4	0.70	112.3 ± 105.7	87.4 ± 97.5	0.18
Cost of insemination/heifer (€)[3]	58.23 ± 27.9	76.3 ± 36.2	<0.0001	74.7 ± 31.4	83.5 ± 37.8	0.17
Reproduction costs/heifer (€)	130.8 ± 116.1	152.3 ± 129.5	0.051	194.3 ± 137.6	177.3 ± 134.2	0.49

Abbreviations: 5dCO = synchronization protocol 5 days Co-synch 72h; 5dCOP4 = synchronization protocol 5 days Co-synch 72h with intravaginal progesterone device; SD, standard deviation.
P-values come from the *t*-test.
[1] Open days refer to the interval between the first AI and pregnancy. For heifers pregnant at first AI, the number of open days was zero. For heifers who became pregnant after subsequent AIs, the number of open days was the number of days between first and last AIs. Non-pregnant heifers were assumed to become pregnant at the forth AI (see Methods).
[2] Cost of each open day was estimated as 2.3 € (see Methods).
[3] Cost of insemination was calculated as described in Methods.

P/AI after OEAI (67.6 %) than after FTAI (58.2 %; Macmillan et al., 2017). Traditionally, this higher fertility after OE has been attributed to increased estrogen release from a larger follicle (Bridges et al., 2010; Mellieon et al., 2012). In fact, one study reported no pregnancy losses after OEAI compared to 4% loss after FTAI (Macmillan et al., 2017). However, the lower fertility of heifers inseminated after OE in the 5dCOP4 group than of heifers after OEAI in the 5dCO group may be due to the difference in ovarian stage at Day 0. The 5dCO protocol synchronized fewer heifers. Therefore, a higher percentage of heifers, normally cyclic and at diestrus at Day 0, were not properly synchronized; these animals showed estrus before the FTAI date and ovulated a fertile oocyte. Heifers not properly synchronized after the 5dCOP4 protocol were probably not in diestrus at Day 0, and they likely had a medium follicle that did not respond to the first GnRH administration; they showed estrus after IPD removal and prostaglandin administration, so they ovulated an old, less fertile oocyte. One study (Moreira et al., 2000) revealed that initiating a timed AI protocol at metaestrus (D2, where D0 was considered ovulation) may lead to lower quality of the pre-ovulatory follicle and lower developmental competence of the oocyte than initiating the protocol at diestrus (D5, D10 or D15). In fact, in the 5dCO group, 77 % (17/22) of the first OEAIs had a corpus luteum at D0, compared with only 25 % (3/12) in the 5dCOP4 group, and the largest follicle at Day 8 was smaller in this group (1.95 mm in 5dCOP4 vs. 2.20 mm in 5dCO). Follicles grown with progesterone levels < 1 ng/mL are less likely to induce pregnancy (Ginther et al., 2013), and lower circulating progesterone during follicular growth is associated with greater LH pulsatility. This triggers premature resumption of oocyte meiosis and germinal vesicle breakdown, decreasing oocyte quality and thereby fertility (Ahmad et al., 1995; Cardoso Consentini et al., 2021; Moreira et al., 2000; Revah and Butler, 1996). In one study, high progesterone concentrations near the insemination time were negatively associated with pregnancy risk, and synchronized heifers that showed estrus before FTAI (Days 5 to 8 of the synchronization) lost fertility over time on the protocol (Bruinjé et al., 2017). Unfortunately, we were unable to examine our data for such associations because the estrus detection rate was relatively low on the study farm, and relatively few heifers were inseminated after OEAI. Moreover, OEAI heifers in the two experimental groups showed a similar average number of days at estrus after synchronization.

Our results support the relevance of achieving an adequate estrus observation rate in commercial dairy herds, despite the difficulty involved (Stevenson, 2016). One study (Lopes et al., 2013) showed that heifers receiving FTAI with 5 d Co-synch + IPD had a similar average number of days to first AI, but delayed time to pregnancy, as heifers that underwent OEAI. In fact, maximizing the rate of insemination after OE is key for optimizing efficiency when combined with FTAI programs (Fricke et al., 2014; Giordano et al., 2012; Masello et al., 2019). For example, one study concluded that a combined program (FTAI + OEAI) is more effective than a program with only FTAIs (Masello et al., 2021). In the current study, outcomes with 5dCO improved through OE, even if average 18.6 % of observed estrus was fairly low. Moreover, the heifers more probable to get pregnant were those receiving one or two FTAIs (i. e. one or two OEAIs), when compared to those with all three FTAIs or with those heifers with all three OEAIs.

The differences in fertility with type of semen in our study are consistent with previously published work (Chebel and Cunha, 2020; Macmillan et al., 2017), which reflect the negative effect of process to select sperm cells on their fertility. Additionally, a protective effect was confirmed for conventional semen compared to sex-sorted semen for pregnancy loss after first insemination of heifers [5.3 % (12/227) for 5dCO vs. 6.0 % (4/67) for 5dCOP4; $P = 0.02$]. The bull factor may contribute to pregnancy loss (Lopez-Gatius et al., 2004): specifically, when sex-sorted semen is used (Karakaya et al., 2014; Underwood et al., 2010), the identity of the bull can explain several-fold differences in pregnancy loss rates (Pegorer et al., 2007).

A significant increase of pregnancy loss when the THI at insemination was observed after first inseminations [5.2 % (12/233) with THI < 70 vs. 6.6 % (4/61) with THI ≥ 70; $P = 0.050$]. Pregnancy loss is a complex, multifactorial event (Fernandez-Novo et al., 2020), with heat stress frequently described as a risk factor (Carpenter et al., 2006; García-Ispierto et al., 2006; Souza et al., 2019). High THI reduces follicle quality, progesterone secretion by a smaller corpus luteum, placentation, and embryo viability (López-Gatius and Hunter, 2020; Roth, 2020, 2018).

In our study, the serum level of progesterone at Day 0 of the synchronization protocol did not affect P/AI or synchronization rate. Previous work (Kasimanickam et al., 2014) reported better results in terms of P/AI when heifers at Day 0 had progesterone levels > 1 ng/mL, suggesting that follicles grown under high progesterone levels respond more sensitively to LH, enhance fertility, and induce appropriate luteal phase lengths (Ginther et al., 2013). Another study (Carvalho et al., 2018) found that intermediate progesterone levels (0.5-6 ng/mL) in dairy cows resulted in better P/AI. In fact, that work revealed a 51 % relative decrease in P/AI for cows with progesterone < 1 ng/mL on Day 0 of the protocol. We expected to observe similar results in dairy heifers. However, we found no progesterone effect in first AIs. This may be due to differences between heifers and adult cows. Probably, ovarian functionality is better in younger animals than in parous cows because of higher progesterone secretion ability, lower metabolic stress in the absence of milk production, and stronger immunity due to no post-partum or milk-related diseases (Bruinjé et al., 2017; Cardoso Consentini et al., 2021).

Economic balance is a key issue in any farm. Although the costs associated with reproduction on rearing farms are not so high (occupying seventh place among major costs), they do require human resources, which are the largest expense after feeding costs (Gabler et al., 2000). In one study it was demonstrated that rearing costs are smaller in bigger farms (> 100 milking cows) and when seasonal rearing in autumn and summer is implemented vs. rearing during the whole year (Boulton et al., 2017). Reproduction at an appropriate age is essential for turning the heifer into a productive cow. Therefore, rearing farms strive to achieve calving at 22-23 months in order to ensure efficient reproduction (Kenny et al., 2018; Krpálková et al., 2014; Perry, 2012). In our study, reproduction cost per heifer, despite the higher costs per insemination for the 5dCOP4 protocol (58.23 ± 27.9 vs. 76.3 ± 36.2€, $P < 0.001$), was not significantly different between the two protocols (130.8 ± 116.1 vs.152.3 ± 129.5 € for 5dCOP4, $P = 0.051$), however, this difference can be considered a significantly statistical trend. Obviously, the protocol with IPD is more expensive due to the higher hormone cost and greater human work to insert and remove the device. The economic advantage of using protocols with IPD was previously reported in a comparison of Ovscynch + IPD, 5d Co-synch + IPD and 2 $PGF_{2\alpha}$ (McDougall et al., 2013). In the present study, the most efficient protocol was 5dCOP4, in terms of fertility. Although the 5dCOP4 protocol is more expensive, when analysing economic outputs (open days, number of inseminations per heifer, etc.), the 5dCOP4 protocol is more profitable than 5dCO only under heat stress. One recent work (Masello et al., 2021) reported greater cost-effectiveness for FTAI with IPD than for PGF+OEAI, although the two protocols led to similar percentages of non-pregnant heifers at 100 days. Many studies have reported the reproductive advantage for the use of an IPD, as well as the efficiency of analogous synchronization protocols without IPD (Colazo and Mapletoft, 2014; Stevenson, 2016; Wathes et al., 2014). However, few studies have compared protocols with or without IPD in the same herd. Our study compared the same synchronization protocol with or without IPD on the same farm under real conditions. Moreover, we considered inseminations occurring after OE and followed our heifers for at least three AIs and during cold and hot seasons, in order to obtain a complete picture of reproductive efficiency. In fact, we observed that the protocols affected heifer fertility differently depending on THI: the 5dCOP4 protocol was better than the 5dCO protocol when THI ≥ 70 at AI. This suggested different economic efficiency depending on season. Actually, we observed that the reproductive cost per heifer with the 5dCOP4 protocol was significantly lower during hot weather than cold weather. Our results may be the first direct evidence that the greater cost of the IPD protocol can be compensated by the higher reproductive efficiency during the hot season. The suitability of a given synchronization protocol or reproductive strategy depends on the conditions of the farm and region (Heinrichs et al., 2017; McDougall et al., 2014). On our study farm, OE was performed throughout the study (although the efficiency of estrus detection was far below the optimal), which means that our results, especially the economic analysis, may differ from results on a farm where only FTAI is performed, or where a better estrus observation rate is achieved (Masello et al., 2021).

5. Conclusion

Reproductive results were more favorable for the 5dCOP4 protocol, and its costs were numerically lower to those of the 5dCO protocol during the hot season. However, during the cold months, the two protocols showed similar reproductive efficiency, while the 5dCO was less expensive. Advisors and farmers need to take into account all farm conditions and characteristics to achieve economic and reproductive efficiency of the herds. In farms where OE of heifers is not performed or during long periods of hot weather, the 5CdOP4 protocol may be more effective, in reproductive and economic terms. However, on farms where OE is performed or when heat stress is absent, the 5dCO protocol may be a reasonable choice to manage dairy heifers for AI.

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CRediT authorship contribution statement

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.livsci.2021.104588.

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