Comparison of estradiol benzoate and cypionate in Girolando cows submitted to a timed artificial insemination

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ABSTRACT
The various studies on the manipulation of the estrous cycle culminated in the development of estrous and ovulation synchronization protocols, to the point of defining an optimum moment for artificial insemination. The induction of ovulation is a primordial part for the determination of the moment of the TAI, so to study which hormones and how they behave allows a better understanding of this physiological process and, to manipulate it efficiently. The aim of the present study was to compare the use of estradiol benzoate (EB) and cypionate (ECP) as ovulation inducer in Girolando cows submitted to Timed Artificial Insemination (TAI). Ovulations of 108 Girolando cows were synchronized and the initial day of treatment, recorded as D0, which was when the animals received an intravaginal device containing 1g of progestogen and an intramuscular application (im) of 2mg of EB. After 8 days (D8), the device was removed and 500μg of cloprostenol (im) was administered to all females. Then, the animals were randomly divided into two treatments: BG Group (n = 52) and CG Group (n = 56). The CG animals received 1mg of ECP (im) as the ovulation inducer at the time of device removal, while BG Group cows received 1mg of EB (im) 24 h later (D9). TAI occurred at D10. After device removal, ultrasound evaluations were performed every 12 h up to ovulation. The following reproductive parameters were evaluated: interval from intravaginal device removal to ovulation - IDO (hours); interval from ovulation to TAI-IOT (hours); diameter of the largest follicle at intravaginal device removal (mm); maximum diameter of the DF (mm) at D10; the dominant follicle growth rate (mm/day); synchronization rate - SR (%); ovulation rate - OR (%) and PR - pregnancy rate (%). Only the ovulation rate presented a statistical difference (p<0.05). In conclusion, despite the ovulation rate difference, both estradiol esters administered were effective and presented similar pregnancy rates in Girolando cows submitted to TAI.

Keywords: Follicular dynamics. Estradiol esters. Induction of ovulation.

RESUMO
Os diversos estudos sobre manipulação do ciclo estral culminaram com o desenvolvimento de protocolos de sincronização do estro e da ovulação, a ponto de se definir um momento ótimo para a inseminação artificial. A indução da ovulação é primordial para a determinação do momento da IATF. Dessa forma, estudar quais hormônios e como eles se comportam permite compreender melhor esse processo fisiológico e, ainda, manipulá-lo de forma eficiente. O objetivo do presente estudo foi comparar o uso de benzoato de estradiol (BE) e cipionato (ECP) em vacas Girolando submetidas à inseminação artificial em tempo fixo (IATF). As ovulações de 108 vacas Girolando foram sincronizadas e no início do tratamento, D0, os animais receberam um dispositivo intravaginal contendo 1g de progestágeno e uma aplicação intramuscular (im) de 2mg de BE. No D8, o dispositivo foi removido e 500μg de cloprostenol (im) foram administrados a todas as fêmeas. Depois disso, os animais foram divididos aleatoriamente em dois tratamentos: Grupo GB (n = 52) e Grupo GC (n = 56). Os animais do Grupo GC receberam 1mg de ECP (im) como indutor de ovulação no momento da retirada do dispositivo, enquanto as vacas do Grupo GB receberam 1mg de BE (im) 24 horas depois (D9). IATF ocorreu em D10. Após a remoção do dispositivo, as avaliações ultrassonográficas foram realizadas a cada 12 horas até a ovulação. Os parâmetros reprodutivos avaliados foram: intervalo entre a retirada do dispositivo intravaginal de P4 e
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Introduction
The average Brazilian productivity in 2017 was 1963 liters/cow/year (Instituto Brasileiro de Geografia e Estatística, 2017), an inexpressive productivity. The challenge to increase productivity of the cows in Brazil is related to numerous factors such as breed, environment and nutrition. In short, the goal to obtain better and more productive cows will undoubtedly include the selection of genetically superior animals.

Artificial insemination is the main tool to promote genetic improvement in Brazilian herds, but the poor identification of the estrus results in low service and conception rates and, consequently, low reproductive efficiency.

The search for alternatives to circumvent these difficulties led to the development of timed artificial insemination (TAI). The need for protocols that synchronize ovulations in a practical, cheap and efficient way led researchers to study the different hormones available on the market. The association of hormones also reduces the number of days of handling the animals, which is a great advantage as it will reduce the impact not only on animal welfare, but also save on labor.

Among the hormones used, estradiol cypionate is already widely used in beef cattle herds, since its protocol synchronizes ovulation with less handling differently from estradiol benzoate. A decrease in management and handling makes the process more practical, cheaper, less stressful to the animals and guarantees efficiency for the producer. There are several forms of estrogen, including 17-β-estradiol, biologically active molecule, estradiol benzoate and estradiol cypionate. Although all are estrogens, each produces distinct 17-β-estradiol profiles in the circulation due to peculiarities in the esterification of the molecule, thus leading to changes in absorption and, consequently, its metabolism (Souza et al., 2005). The administration of 17β-estradiol causes a faster action, since it does not need to be metabolized and transformed in the organism, according to observations by Larson & Ball (1992).

Estradiol cypionate, which is formed by the esterification of propionic acid cyclopentane, results in low solubility in water and, therefore, slower release at the site of administration, prolonging the biological activity when compared to estradiol benzoate and 17β estradiol (Vynckier et al., 1990). According to Pancarci et al. (2002), the administration of 2mg EC (intramuscular) promotes an increase in plasma estradiol concentrations 12 h after administration, peaks at 36 h after administration and remains elevated for more than 96 h. Therefore, studies that compare these different hormones seeking to simplify and reduce the costs of TAI protocols for dairy cows are very important. Such studies can ensure good or even better reproductive efficiency and encourage insemination in Brazil. Consequently, the production costs will reduce and the products will be more competitive and, at the same time, the reproductive efficiency of the national herd will improve. The hypothesis was that EC administration (at device removal) decreases cow handling without affecting conception rate of dairy cows submitted to TAI programs, compared to EB given 24 h after P4 device removal. The objective of this study was to compare the use of benzoate and estradiol cypionate in Girolando cows submitted to Timed Artificial Insemination.

Materials and Methods
The present experiment was approved by the Commission of Ethics in the Use of Animals of the Animal Science Institute of the Federal Rural University of Rio de Janeiro (CEUA/IZ/UFRRJ) under process nº. 23083.024213/2017-14.

The experiment was carried out in Seropédica, RJ. Geographically, the region is located at 22°45’53”S, 43°41’56”W
and is 33m above sea level. A total of 108 Girolando cows that were non-pregnant, multiparous, lactating, cyclic and acyclic with a mean body score of 3.1 ± 0.5 (range 1 to 5) and mean age 7.2 ± 2.2 years were used. All animals were previously submitted to a gynecological examination by rectal palpation with the aid of ultrasound for the detection of cyclicity and abnormalities of the reproductive tract before initiating hormonal protocols and monitoring follicular dynamics.

The hormonal protocols for TAI were initiated on a random day of the estrous cycle, and this day was recorded as day Zero (D0), when all cows received an intravaginal device with 1g of progestogen (DIB®, Coopers, Sao Paulo, Brazil) and an intramuscular (im) application of 2.0 mg of estradiol benzoate (EB-ESTROGIN®, Biofarm, Jaboticabal-SP, Brazil). After 8 days (D8), the intravaginal device was removed and 500μg of cloprostenol (CIOSIN®, Intervet Schering Plow Animal Health, Sao Paulo, Brazil) was given intramuscularly (im) to all animals. At the same time, the animals were randomly divided into two experimental groups: Estradiol Cypionate Group (CG; n = 56) and Estradiol Benzoate Group (BG; n = 52). The cows in the CG received as the ovulation inducer 1.0 mg of estradiol cypionate intramuscular (ECP®, Pfizer, Sao Paulo, Brazil) at the time of removal of the intravaginal device, and the cows in the BG received 1.0 mg of estradiol benzoate the morning of the following day (D9). TAI occurred on D10 and the CG cows were inseminated in the morning (48 h after DIB® removal) and BG in the afternoon (60 h after DIB® removal). All hormonal applications were done at the same time of day (08:00 AM).

Ultrasound examinations were performed using the transrectal technique with a 7.5MHz linear transducer (Mindray DP2200 vet) and started on D8 of the synchronization protocol, which is the same day the intravaginal device was removed. These evaluations were repeated every 12 h until 24 h after TAI. The moment of ovulation was defined as the time of disappearance of a previously identified dominant follicle (DF) from one ultrasound examination to the next, minus 6 h. In each evaluation, the largest follicle with a diameter greater than 4 mm was measured in order to monitor follicular growth and determine maximum diameter of the dominant follicle (FD).

The following reproductive parameters were evaluated: interval from intravaginal device removal to ovulation - IDO (hours); interval from ovulation to TAI-IOT (hours); diameter of the largest follicle at intravaginal device removal (mm); maximum diameter of the DF (mm) at D10; the dominant follicle growth rate (mm/day); synchronization rate - SR (Synchronized animals/treated animals × 100); ovulation rate - OR (ovulated animals/treated animals × 100), and PR - pregnancy rate (pregnant animals/treated animals × 100). The diagnosis of gestation was performed by rectal palpation with the aid of the ultrasound device accompanied by a linear transrectal transducer of 7.5MHz (Mindray DP2200 vet), 30 days after insemination.

The experimental design was completely randomized. All data were tested for normality by the Anderson-Darling test with a significance level of 5%. For the description of the results, standard deviations and means (mean ± standard deviation) were used. The differences between the groups were tested by the general linear model of variance (ANOVA) and the Kruskal-Wallis test. In the analysis of variance, the effects of the classification variables were analyzed using estradiol benzoate (EB) and estradiol cypionate (ECP). The chi-square test, with a significance level of 5%, was used to compare the differences that imply proportions, such as those found in synchronization, ovulation and pregnancy rates.

**Results**

The results found in the present study for follicular growth rate, dominant follicle diameters measured in D10, intervals from progesterone device removal to ovulation and intervals from ovulation to TAI for BG and CG cows are presented in Table 1. None of the parameters evaluated above presented statistical difference.

### Table 1 – Follicular growth rate, dominant follicle (DF) diameter at D10, intervals from progesterone device removal to ovulation and from TAI to ovulation, after estradiol cypionate (CE) and benzoate (BE) treatments in Girolando cows

| Treatments (CE×BE) | Follicular growth rate (mm/day) | DF diameter (mm) | Interval from ovulation to |
|--------------------|---------------------------------|------------------|---------------------------|
|                    |                                 |                  | Progesterone device removal (h) | TAI (h) |
|                    |                                 |                  | (n=40)                     | (n=40)  |
| BG                 | 1.26 ± 1.33ª                   | 14.06 ± 2.26ª    | 67.91 ± 8.68ª              | 8.68 ± 6.94ª |
| (n = 49)           | (n = 49)                       |                  | (n = 47)                   | (n = 47)  |
| CG                 | 1.14 ± 1.27ª                   | 13.11 ± 3.02ª    | 63.90 ± 10.49ª             | 16.05 ± 10.20ª |
| (n = 50)           | (n = 50)                       |                  | (n = 40)                   | (n = 40)  |

Values with different superscripts within the same column are different (p ≤ 0.05). TAI = Timed Artificial Insemination; BG = benzoate; group; CG = cypionate group.
Table 2 presents the pregnancy rates after using estradiol benzoate and cypionate according to the different intervals from TAI and ovulation. There was only difference in conception rate in the 12- to 24 h-interval between TAI and ovulation (p = 0.03).

The mean follicle diameters at D10 for pregnant and non-pregnant cows of the BG and CG are presented in Table 3. No statistical difference was observed between these values.

Synchronization, ovulation and pregnancy rates according to EB or ECP in TAI protocols are presented in Table 4. Non-ovulating animals were not considered for the calculation of the interval from intravaginal progesterone device removal to ovulation and the interval from ovulation to TAI. The ovulation rate of benzoate group was higher than cypionate (p = 0.01). The synchronization and pregnancy rates showed no difference (p>0.05).

Table 2 – Pregnancy rate according to interval from TAI to ovulation in Girolando cows submitted to TAI

| Interval from TAI to Ovulation (h) | Pregnancy rate (%) | P Value |
|-----------------------------------|---------------------|---------|
| Ovulated                          | BG (n = 46)         | 42.86 (3/7) | 0.2     |
| 0-12 hours                        | BG (n = 46)         | 65.38 (17/26) | 0.8    |
| 12-24 hours                       | BG (n = 46)         | 33.33 (4/12) | 0.03   |
| >24 hours                         | BG (n = 46)         | 100 (1/1) | 0.2    |

Table 3 – Mean follicle diameter and standard deviation of the largest follicle evaluated at D10 as a function of the treatments with ECP and EB and the number of pregnant Girolando cows submitted to TAI

| Treatments | Status | Mean follicle diameter and standard deviation |
|------------|--------|---------------------------------------------|
| BG (n = 48)                      | Pregnant (n = 25) | 14.17 ± 1.65       |
| BG (n = 48)                      | Non-Pregnant (n = 23) | 13.98 ± 2.84   |
| CG (n = 52)                      | Pregnant (n = 26) | 13.69 ± 2.33       |
| CG (n = 52)                      | Non-Pregnant (n = 26) | 12.41 ± 3.66   |

Table 4 – Synchronization, ovulation and pregnancy rates according to EB or ECP in TAI protocols

| Reproductive parameters | BG (n = 52) | CG (n = 56) | p value |
|-------------------------|-------------|-------------|---------|
| Synchronization rate (%) | 98.08 (51/52) | 94.43 (54/56) | 0.6     |
| Ovulation rate (%)      | 90.38 (47/52) | 71.43 (40/56) | 0.01    |
| Pregnancy rate (%)      | 48.08 (25/52) | 46.43 (26/56) | 0.8     |

Discussion

To our knowledge, this is the first report studying the effects of distinct ovulation inducers in estradiol and progesterone-based protocols on Girolando females submitted to TAI.

Studies from Borges et al. (2004), using Gir cows, demonstrated follicular growth rates ranging from 1.2 to 1.4 mm/day in animals that were not submitted to TAI protocols, which means that the results obtained in the present study approximate the values of a normal estrous cycle in Gir cows. On the other hand, Ferraz et al. (2013) obtained the highest follicular growth rate when they used GnRH as an ovulation inducer, obtaining a value of 1.07 ± 0.3 mm/day in Nelore cows, and the group that used estradiol benzoate as an inducer had a follicular growth rate of 0.58 ± 0.3 mm/day. Tortorella et al. (2017), working with Bos taurus females (cows and heifers), found a rate of 0.49 ± 0.02 mm/8 h for cows and 0.47 ± 0.06 mm/8 h for heifers. The dominant follicle growth rate appears to be higher in Bos taurus cows (Ginther, 2018) than in Bos indicus cows (Coutinho et al., 2007). The values found in the present study are intermediate to those found in the literature and can be explained since the cows used are Girolando (crossbred from Bos taurus x Bos indicus).

The variation in the values of follicular growth rates can be influenced by the subspecies to which the animals belong (Bos taurus x Bos indicus) or animal category (cows x heifers) (Hadiya et al., 2016). The use of different ovulation inducers did not appear to be a relevant factor in follicular growth rate. Plasma concentrations of progesterone seem to have a more significant influence than the ovulation inducer used, as demonstrated by Savio et al. (1993) who, by monitoring the estrous cycle of cows, demonstrated that high levels of progesterone decrease the follicular growth rate and, consequently, the size of the dominant follicle.

There was no significant difference between the diameter of the dominant follicle at D10 for the BG and CG groups. Andrade et al. (2012), using estradiol esters to induce ovulation in Nelore cattle, observed that the follicle diameter did not differ between the treatments that they used (ECP D8 x EB D9), obtaining, respectively, 13.03 ± 2.24 mm and 12.40 ± 1.34 mm. This result is like Sales et al. (2012), who found equivalent follicular diameters for treatment with estradiol cypionate (13.90 ± 0.40 mm) and estradiol benzoate (13.10 ± 0.40 mm) for the Nelore breed.

The results presented in the present study resemble other studies that have worked with dairy cows such as Franca et al. (2015), who used crossbred Holstein x Gir cows, and showed no difference related to the mean follicular
diameter for animals receiving ECP at D8 (10.71 ± 2.43 mm) and EB at D9 (11.45 ± 2.34 mm). More recently, crossbred cows showed 9.77 ± 1.36 mm and 10.97 ± 1.24 mm (Ramos Sobreira et al., 2017) for EB and ECP, respectively, with no statistical difference. Although the results obtained in the present study demonstrate a numerically superior means to the other studies mentioned, the similarity between the follicular diameters obtained with the use of both ovulation inducers suggests that the two were effective to stimulate the growth and also to obtain follicles greater than 10 mm which, according to Sartori et al. (2001), is a size that already has the capacity to respond to the action of LH and, consequently, to ovulate.

In the present study, EB produced ovulations at a similar time interval compared to ECP as demonstrated by Sales et al. (2012) and Callejas et al. (2016). In 2016, some researchers, who were evaluating the plasma concentrations of 17β-estradiol, reported that the application of ECP at the same time as the device removal and the application of EB one day after the withdrawal of the device generate similar hormonal profile. This characteristic occurs due to the pharmacokinetics of estradiol esters. Estradiol benzoate has a shorter half-life, and it induces an increase in the biologically active molecule (17β-estradiol) more rapidly, which explains why BE is administered 1 day after device removal and not at the same time.

Regarding the interval from TAI to ovulation, it was possible to observe that there was a statistical difference only between the BG and CG groups in the interval of 12-24 h, that is, the animals of the CG group that ovulated in this interval had a higher pregnancy rate when compared to BG.

Dransfield et al. (1998) and Roelofs et al. (2005) reported in their studies that the chance of an animal becoming pregnant increases as AI approaches the time of ovulation. Thus, in order to maximize the contact of the sperm with the oocyte, the AI should be performed close to the time of ovulation so that the egg does not age waiting for the spermatozoon. Based on the studies cited above and from other authors, the best time for AI would probably be between 16 and 26 h before ovulation (Dransfield et al., 1998; Roelofs et al., 2005). Therefore, the highest pregnancy rate in the 12- to 24 h-interval between AI and ovulation for CG is due to the fact that cows ovulate at a more favorable conception period. For example, in CG, 57.5% of the cows ovulated after 12 h in relation to AI, different from BG, where 71.73% of cows ovulated in the first 12 h after AI. This ovulation closest to AI is probably associated with the half-life of estradiol benzoate as explained above.

Success in TAI programs can be influenced by several factors, including the diameter of the dominant follicle at the time of artificial insemination (Pfeifer et al., 2015). The follicle diameter at the time of TAI is an important success factor in pregnancy rates, since larger follicles contain higher concentrations of estradiol and, therefore, a greater possibility of ovulation; consequently, there is an increase in the fertility indexes in synchronized herds (Sá et al., 2010). In addition, larger follicles give rise to a larger corpus luteum, thereby increasing the chances of establishing and maintaining pregnancy (Lonergan et al., 2013). Thus, a high concentration of pre-ovulatory estradiol generated by the greater diameter of the ovulatory follicle may influence the fertilization of females by promoting changes in the uterine environment, improving sperm transport and favoring conception (Sá et al., 2010).

Several researchers have reported the importance of follicular diameter on the day of insemination (Fujita et al., 2013; Ribeiro et al., 2013; Silveira et al., 2016). Silveira et al. (2016), working with the Nelore breed, found larger follicular diameters in the cows that conceived (13.26 mm × 10.99 mm). Pfeifer et al. (2015) proposed to evaluate the size of the follicle on the day of TAI as a way to improve the fertility of the cows that will be inseminated and, depending on the size of the dominant follicle, it might be necessary to wait a few more hours and delay the TAI in order to optimize the pregnancy rate of the herd.

The present study did not observe a statistical difference between the follicular diameter on the day of the TAI and the pregnancy rate, since we did not use any other additional hormone with the objective of optimizing follicular growth, which is different from most of the above mentioned studies. Most of the researchers used eCG, a hormone that acts directly on the final follicle growth, generating a follicle with a larger diameter (Baruselli et al., 2008; Maciel et al., 2017).

Only the ovulation rate presented a difference between the groups. Estradiol benzoate was better than estradiol cypionate to induce ovulation. This may be associated with the fact that EB promotes higher concentrations of 17β-estradiol in the bloodstream earlier than the ECP, thus contributing to better ovulation rates, as discussed by Souza et al. (2005).

Despite the difference in ovulation rates, no statistical significance was observed in the pregnancy rates. This fact is possibly related to the CG cows, where these animals ovulated in a more propitious period for conception. So, despite the fact that the group presented lower ovulation rates, this was not sufficient to affect the pregnancy rates, since ovulations occurred at a more appropriate time than the BE group animals.
Conclusion
Although estradiol benzoate is more efficient in inducing ovulation than estradiol cypionate, both can be used with the same efficiency in TAI programs for Girolando cows because they produce the same pregnancy rates.

Conflict of Interest
The authors have no competing interest.

Ethics Statement
The present investigation attended all of the rules proposed by Brazilian National Council of Animal Experimentation (CONCEA) for controlling the use of animals for teaching and research and was also approved by the Ethic Comission of Animals Usage in Experiments (CEUA) from the Federal University of Rio de Janeiro, Brazil.

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