**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

Ovarian response and embryo production of cows superstimulated with different FSH regimens and inseminated with conventional or sex-sorted spermatozoa

Natália Picoli Folchini

Dissertation presented to obtain the degree of Master in
Science. Area: Animal Science and Pastures

**Piracicaba
2021**

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Veterinarian

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FSH regimens and inseminated with conventional or sex-sorted spermatozoa**

versão revisada de acordo com a resolução CoPGr 6018 de 2011

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DEDICATION

To my parents, for all their effort, love and support.

“A line is a curve that does not dream” –Manoel de Barros

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SUMMARY

RESUMO	9
ABSTRACT	10
1. INTRODUCTION	11
References	12
2. OVARIAN RESPONSE AND EMBRYO PRODUCTION OF COWS SUPERSTIMULATED WITH DIFFERENT FSH REGIMENS AND INSEMINATED WITH CONVENTIONAL OR SEX-SORTED SPERMATOZOA	17
Abstract	17
2.1 Introduction.....	18
2.2 Material and methods.....	20
2.2.1 Locations and donors	20
2.2.2 Experiment 1	20
2.2.3 Experiment 2	21
2.2.4 Experiment 3	21
2.2.5 Experiment 4.....	21
2.2.6 Combined data from the experiments	22
2.2.7 Superovulation protocol.....	22
2.2.8 Sires, semen evaluation and insemination	23
2.2.9 Evaluation of the response to the protocol.....	24
2.2.10 Embryo collection and evaluation.....	24
2.2.11 Statistical analysis	25
2.3 Results.....	27
2.3.1 Experiment 1	27
2.3.2 Experiment 2	29
2.3.3 Experiment 3	32
2.3.4 Experiment 4.....	35
2.3.5 Combined data from the experiments	38
2.4 Discussion	50
References	57
3. FINAL CONSIDERATIONS	65
APPENDICES	66

RESUMO

Resposta ovariana e produção embrionária de vacas superestimuladas com diferentes regimes de FSH e inseminadas com espermatozoides convencionais ou sexados

Uma sequência de estudos foi realizada para comparar regimes de FSH na superestimulação folicular e produção de embriões *in vivo* de vacas Holandesas e avaliar o efeito do sêmen SexedULTRA 4M de touros com fertilidades distintas a campo na produção *in vivo* de embriões. O protocolo de superovulação consistiu em uma pré-sincronização, seguida do período de superestimulação. No grupo D-FSH, 300 mg de FSH foram distribuídos em 10 doses decrescentes, a cada 12 h. No grupo C-FSH, 300 mg de FSH foram distribuídos em 10 doses constantes. A inseminação ocorreu 12 e 24 h após o GnRH, com sêmen convencional ou SexedULTRA 4M, de acordo com o experimento. As coletas de embrião foram realizadas nos dias 14 ou 15. No Exp. 1, vacas não-lactantes foram superovuladas com crossover de doses de FSH e foram inseminadas com sêmen convencional. No Exp. 2, realizado em arranjo fatorial 2x2, vacas não-lactantes foram superovuladas com crossover de doses de FSH e, parte delas, com crossover de sêmen de touros SexedULTRA, classificados como UF (reconhecidos por maior fertilidade) ou NUF (não-UF). Nos Exp. 3 (não-lactantes) e 4 (lactantes), em arranjo fatorial 2x2, vacas foram superovuladas com os mesmos tratamentos anteriores de FSH e com crossover de touros SexedULTRA 4M. Nos Exp. 1, 2, e 3, D-FSH promoveu maior superestimulação folicular, quando comparado ao C-FSH. No entanto, no Exp. 4 os regimes foram similares. Apenas no Exp. 2, D-FSH teve tendência a menor resposta ovulatória. O número de corpos lúteos e de estruturas totais tenderam ou foram maiores para D-FSH, de acordo com os experimentos. As características embrionárias não foram influenciadas pelas doses, exceto no Exp. 1, onde D-FSH produziu mais estruturas degeneradas. De forma combinada, D-FSH induziu maior superestimulação e produção de embriões degenerados, tendeu a diminuir a resposta ovulatória, contudo, a expressão de cio e demais variáveis foram similares ao C-FSH. Para a avaliação do sêmen SexedULTRA 4M, no Exp. 2, touros UF foram superiores na produção de embriões transferíveis e congeláveis, e produziram menos oócitos não fecundados, comparados a touros NUF. Nos Exp. 3 e 4, touros NUF e UF demonstraram desempenho semelhante nas características embrionárias. Os resultados combinados indicaram superioridade de touros UF na produção de embriões transferíveis, congeláveis e menor produção de estruturas não fecundadas. Além disso, a motilidade espermática de touros UF foi superior em comparação aos NUF. A avaliação combinada de expressão de estro indicou que vacas que demonstraram estro tiveram maior resposta ovulatória, mais corpos lúteos, estruturas totais e embriões transferíveis e congeláveis (apenas doadoras com ≥ 2 estruturas recuperadas). Finalmente, ao se avaliar o efeito da pré-sincronização, não se observou influência do número de corpos lúteos durante a superestimulação, assim como da ovulação no dia zero do protocolo sobre a resposta ovariana e produção embrionária. Em conclusão, embora doses decrescentes de FSH tenham sido superiores na superestimulação folicular, a eficiência de produção embrionária foi similar à dose constante. Finalmente, de forma geral, touros classificados como UF tiveram maior produção embrionária comparados a touros NUF, no entanto, demonstraram ampla variabilidade de resposta.

Palavras-chave: Vaca leiteira, FSH, Superovulação, Sêmen sexado

ABSTRACT

Ovarian response and embryo production of cows superstimulated with different FSH regimens and inseminated with conventional or sex-sorted spermatozoa

A series of studies were performed to compare effects of FSH regimens on follicular superstimulation and *in vivo* embryo production of Holstein cows, and to evaluate the effect of SexedULTRA 4M semen from sires with distinct field fertility on *in vivo* embryo production. The superovulation protocol consisted of a presynchronization, followed by a superstimulation period. In D-FSH group, 300 mg of FSH were distributed over 10 decreasing doses, whereas in C-FSH group, the same amount was distributed over 10 constant doses. Insemination occurred 12 and 24 h post-GnRH, with conventional or SexedULTRA 4M semen, according to the experiments. Embryo collections were performed either on days 14 or 15. In Exp. 1, non-lactating cows underwent superovulations with crossover of FSH doses and were inseminated with conventional semen. In Exp. 2, in a 2x2 factorial arrangement, non-lactating cows underwent superovulations with crossover of FSH doses and, part of them, with crossover of SexedULTRA 4M semen, from sires classified as UF (recognized for higher fertility) or NUF (non-UF). In Exp. 3 (non-lactating) and 4 (lactating), in 2x2 factorial arrangements, cows underwent superovulations with the same FSH dose and with crossover of SexedULTRA 4M sires. In Exp. 1, 2, and 3, D-FSH provided greater follicular superstimulation, compared to C-FSH. However, in Exp. 4, the regimens were similar. Only in Exp. 2, D-FSH tended to have lower ovulatory response. Number of CL and total ova and embryo had a tendency or were greater for D-FSH, according to each experiment. Embryo characteristics were not influenced by doses, except in Exp. 1, where D-FSH produced more degenerate embryos. When data from all experiments were combined, D-FSH induced greater superstimulation and production of degenerate embryos, tended to reduce the ovulatory response, nonetheless, estrus expression and other variables were similar to C-FSH. Regarding SexedULTRA evaluation, in Exp. 2, UF sires were superior in transferable and freezable embryo production, and yielded less unfertilized oocytes, compared to NUF sires. In Exp. 3 and 4, NUF and UF sires showed similar results on embryo characteristics. Combined results indicated that UF were superior in transferable and freezable embryo production, and produced less unfertilized ova. Furthermore, progressive motility of UF sires was superior, compared to NUF. Cows that displayed estrus had greater ovulatory response, more corpora lutea, total ova and embryos, and transferable and freezable embryos (only for donors ≥ 2 structures recovered). Finally, there was no influence of number of corpora lutea during the superovulation protocol, as well as of ovulation on day zero, on ovarian response and embryo production. In conclusion, although decreasing doses of FSH were superior in follicular superstimulation, the embryo production efficiency was similar to constant doses. Finally, overall combined results indicated that UF sires were superior in embryo production, nevertheless, wide variability among bulls still exists.

Keywords: Dairy cow, FSH, Superovulation, Sex-sorted semen

1. INTRODUCTION

Superovulation (SOV) protocols and the surgical embryo collection were developed at the end of the 40s, nevertheless, it was not until the 70s that the embryo transfer (ET) industry spread. Since those years, the utilization of these biotechnological tools provided several benefits, such as reductions in generation intervals, enhanced genetic gain, disease control, the possibility of commercialization of embryos, as well as their maintenance in freezing media for an undetermined time (Mapletoft, 2013; Bó and Mapletoft, 2014; Hasler, 2014).

Many years of research on SOV programs have demonstrated the influence of several factors, such as age, breed, season of the year, heat stress, parity, antral follicle count, quality and purity of the exogenous hormones, nutritional state (under or overnutrition), environment and milk production (Kafi and McGowan, 1997; Hasler, 2010). Additionally, there is wide variation in the response among the donors (Baruselli et al., 2006; Sartori et al., 2010), with a small number of them producing the greatest amount of the embryos (Looney, 1986). Recently, the annual report made by the International Embryo Technology Society (IETS) showed that embryo production in 2019 reached almost 1.5 million. Of this total, 72.7% were produced *in vitro* (IVP) and 27.3% were *in vivo* derived (IVD). Among IVD embryos, 60.8% of them were cryopreserved and the use of sex-sorted sperm represented 18.6% of the flushes, predominantly in dairy breeds (84.8%; IETS, 2020). Even though decades have passed since the beginning of the SOV programs, the number of embryos produced per donor has not shown remarkable improvements (Mapletoft, 2013). Even still, the results for *Bos taurus* dairy cows are better when compared to IVP, which presents low efficiency in this genetic group, the opposite for *Bos indicus* donors (Pontes et al., 2009, 2010). By utilizing *in vivo* production, with conventional sperm, the number of embryos per donor is widely variable (4 to 6, on average), with pregnancy per embryo transfer (P/ET) of approximately 60% (Holm and Callesen, 1998; Pontes et al., 2010). In parallel, IVP technique provides P/ET around 40% (Pontes et al., 2010) and a greater potential for pregnancy loss when these embryos are transferred (Sartori et al., 2016; Ealy et al., 2019; Hansen, 2020). Aiming to optimize the results, SOV protocols were submitted to many adjustments over the years, related to duration and dose of follicle-stimulating hormone (FSH), presence or not of a progesterone (P4) intravaginal implant, time of the prostaglandin F2 α (PGF) treatment and P4 implant withdrawal (Bó and Mapletoft, 2014). Still, the regimen of FSH has shown variations among protocols. The majority of the studies superstimulate the follicles with decreasing doses or compare different total doses of FSH, regarding breed and parity (Lerner et al., 1986; Gonzalez et al., 1990; Ali et al., 2011). Others have used the same dose of FSH in all

treatments with good efficiency (García Guerra et al., 2012, 2015). Thus, there is still no agreement which regimen is the most effective.

Besides, in SOV protocols, artificial insemination (AI) is performed more than once, to provide sufficient number of sperm, able to fertilize at the time when the oocytes are released. With the great appeal of dairy herds in the production of females with a high genetic merit, sex-sorted sperm has gained visibility. Nonetheless, despite the advantages, the conventional sorting technology provides lower pregnancy per AI (P/AI), in approximately 10 to 20 percentage points, when compared to conventional semen (Seidel, 2014). This reduced fertility affects its use and the consistent results in a commercial scale. Regarding superovulated cows, results are also suboptimal, with reduced fertilization rate, greater percentage of degenerate embryos, less transferable embryos and greater number of unfertilized oocytes (Peippo et al., 2009; Sartori et al., 2004, 2010; Kaimio et al., 2013; Mikkola and Taponen, 2017). Recently, to overcome this scenario, a new methodology of sperm sorting was developed. According to Vishwanath and Moreno (2018), this technology, denominated SexedULTRA, consists in a modified protocol with improved steps prior to freezing, which aims better control and maintenance of the integrity and viability of sperm cells. In fact, it has been shown that, *in vitro*, sperm sorted by this method were superior to the previous sorting method in some characteristics, such as total motile sperm, progressive motile sperm and percentage of intact acrosome (Gonzalez-Marin et al., 2017).

Therefore, aiming to maximize SOV results using sex-sorted sperm, it is essential the establishment of an optimized protocol. In this sense, the present study performed a sequence of four experiments to evaluate the effect of the FSH regimen on ovarian response and embryo production, as well as the influence of the SexedULTRA 4M sperm on embryo production outcomes of superstimulated Holstein cows.

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2. OVARIAN RESPONSE AND EMBRYO PRODUCTION OF COWS SUPERSTIMULATED WITH DIFFERENT FSH REGIMENS AND INSEMINATED WITH CONVENTIONAL OR SEX-SORTED SPERMATOZOA

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ABSTRACT

The aim was to evaluate the ovarian response and embryo production of Holstein cows superstimulated either with constant or decreasing doses of FSH, and the effect of SexedULTRA 4M field fertility sires on embryo outcomes. Four experiments were performed with non-lactating (n = 44) and lactating Holstein cows (n = 11). The superovulation protocol, denominated Super Double-Ovsynch, consisted of a presynchronization, followed by a superstimulation period. In D-FSH group, 300 mg of FSH were distributed over 10 decreasing doses, 12 h apart, whereas in C-FSH group, the same amount was distributed over 10 constant doses. Insemination occurred 12 and 24 h post-GnRH, with conventional or SexedULTRA 4M semen, according to the experiments. Embryo collections were performed either on days 14 or 15. In Experiment 1, non-lactating cows underwent superovulations with crossover of FSH doses and were inseminated with conventional semen. In Exp. 2, in a 2x2 factorial arrangement, non-lactating cows underwent superovulations with crossover of FSH doses and, part of them, with crossover of SexedULTRA 4M semen, from sires classified as UF (recognized for higher fertility) or NUF (non-UF). In Exp. 3 (non-lactating) and 4 (lactating), in 2x2 factorial arrangements, cows underwent superovulations with the same FSH dose and with crossover of SexedULTRA 4M sires. No relevant interactions between FSH regimens and field fertility sires were found in all experiments. In Exp. 1, 2, and 3, D-FSH provided greater follicular superstimulation, compared to C-FSH. However, in Exp. 4, the regimens were similar. Only in Exp. 2, D-FSH tended to lower ovulatory response. Number of CL and total ova and embryo had a tendency or were greater for D-FSH, according to each experiment. Embryo characteristics were not influenced by doses, except in Exp. 1, where D-FSH produced more degenerate embryos. Combining the results for all donors (n = 131), D-FSH induced greater superstimulation than C-FSH, respectively (18.6 ± 1.1 vs. 13.2 ± 0.8 , $P < 0.01$), number of CL (13.1 ± 0.9 vs. 10.4 ± 0.6 , $P = 0.01$), tended to reduce the ovulatory response ($70.4 \pm 3.0\%$ vs. $78.3 \pm 2.6\%$, $P = 0.08$) and to produce more total number of ova and embryo (6.7 ± 0.7 vs. 4.9 ± 0.5 , $P = 0.08$). For donors with ≥ 2 structures recovered (n = 107), D-FSH produced more CL and total number of ova and embryos ($P \leq 0.01$), as well as greater number of degenerate embryos, compared to C-FSH, respectively (2.1 ± 0.5 vs. 1.1 ± 0.2 , $P = 0.03$). Number of transferable embryos (4.1 ± 0.5 vs. 3.2 ± 0.3 , $P = 0.40$) and other variables were similar between the regimens. Regarding SexedULTRA 4M evaluation, in Exp. 2, UF sires were superior in transferable and freezable embryo production, and yielded less unfertilized oocytes, compared to NUF sires. In Exp. 3 and 4, NUF and UF sires had similar results on embryo characteristics. Combined results for all donors (n = 96) had no effect of field fertility sires. For donors with ≥ 2 structures recovered (n = 76), UF sires were superior, compared to NUF, respectively, in number of transferable embryos (4.4 ± 0.5 vs. 2.6

± 0.4 , $P = 0.05$), number of freezable embryos (4.0 ± 0.5 vs. 2.1 ± 0.4 , $P = 0.01$), as well as in percentage of those variables. Moreover, UF sires produced less unfertilized ova (1.2 ± 0.3 vs. 3.1 ± 0.6 , $P = 0.02$) and presented greater progressive motility ($55.3 \pm 1.6\%$ vs. $46.4 \pm 1.9\%$, $P = 0.02$) with no differences for sperm vigor (3.5 ± 0.1 vs. 3.2 ± 0.1 , $P = 0.36$). Cows that displayed estrus had greater ovulatory response, more corpora lutea, total ova and embryos, and transferable and freezable embryos (only for donors ≥ 2 structures recovered). Finally, there was no influence of number of corpora lutea during the superovulation protocol, as well as of ovulation on day zero, on ovarian response and embryo production. In conclusion, although decreasing doses of FSH were superior in follicular superstimulation, the embryo production efficiency was similar to constant doses. Finally, overall combined results indicated that UF sires were superior in embryo production, nevertheless, wide variability among bulls still exists.

Keywords: Dairy cow; FSH; Superovulation; Sex-sorted spermatozoa.

2.1 Introduction

Since the beginning of superovulation protocols for cattle in the 40s, the technique has shown the possibility of producing high quality *in vivo* derived embryos (Bó and Mapletoft, 2014; Hasler, 2014). Despite all the physiological abnormalities, which can occur during SOV (Kafi and McGowan, 1997), noteworthy results in pregnancy per embryo transfer (P/ET) and lower pregnancy loss are achieved when compared to *in vitro* produced embryos (Pontes et al., 2009; Hasler, 2010; 2014; Sartori et al., 2016; Ealy et al., 2019). Although the utilization of IVP embryos has been increasing and surpassing IVD embryos during the last few years, the latter still presents satisfactory fertility, even when embryos are transferred frozen, which represents 60.8% of IVD embryos (IETS, 2020). Besides the results in fertility, the efficiency of embryo production by SOV in Holstein cows has also been greater than IVP embryos from ovum pick up (OPU). Pontes et al. (2010) obtained inferior results, such as total oocytes/OPU, viable oocytes/OPU and embryos/OPU-IVP, totaling only 0.7 of pregnancy/OPU-IVP in Holstein cows, when compared to *Bos indicus* and crossbred cows.

To induce superstimulation, it is important that the follicle-stimulating hormone (FSH) surpasses the minimum concentration required for follicular recruitment, known as FSH threshold (Brown, 1978). This rise allows the entrance of sensitive antral follicles in a wave-like pattern. Additionally, this concentration has to be maintained beyond the threshold and beyond the FSH window that occurs physiologically, to overcome the deviation phase (Schipper et al., 1998). Finally, at the end of the protocol, induction of a synchronized ovulation of these superstimulated follicles is performed. Based on these physiological events, many adjustments and protocols have been developed to maximize SOV efficiency (Bó and Mapletoft, 2014). Regarding hormone adjustments, based on physiological changes, decreasing doses of FSH are conventionally employed to superstimulate follicles, over 4 to 7

d (Baruselli et al., 2006; Cirit et al., 2019). Other studies have used constant doses and obtained adequate results, as well (García Guerra et al., 2012, 2015). Although there are studies that use both FSH regimens for follicle superstimulation and embryo production, robust data comparing the efficiency among doses are scarce and the results are still controversial. Some experiments showed that treatment with decreasing doses of FSH provided better embryo production results (Chupin and Procureur, 1983; Takahashi and Kanagawa, 1985), whereas others had the opposite pattern (Sirard et al., 1999, in cows; Berlinguer et al., 2004, in ewes) or, yet, had similar results (Blondin et al., 1997). Thus, there is still no agreement which regimen is more efficient for superovulation.

Moreover, cows submitted to SOV protocols can be inseminated with conventional or sex-sorted spermatozoa. Sex-sorted sperm provides the advantage of generating a greater number of female embryos, which are more valuable for dairy herds. Nonetheless, lower production and quality are associated with embryos produced with this technology (Schenk et al., 2006; Peippo et al., 2009). The sorting process is associated with damage to the plasma membrane, lower acrosome integrity, and less sperm viability (Carvalho et al., 2018). Thus, oocytes fertilized by sex-sorted sperm produce less transferable embryos, more degenerate embryos (DG) and more unfertilized oocytes (UFO) when compared to non-sorted semen (Seidel et al., 1999; Sartori et al., 2004; Peippo et al., 2009). Recently, an improved method for sex-sorting sperm cells was developed, denominated “SexedULTRA”. This new technology produces a straw containing 2 or, more recently, 4 million sex-sorted sperm cells, using an improved sorting process, with control of temperature and pH stabilization, enhanced methods for staining and improved freezing media to retain sperm integrity (Vishwanath and Moreno, 2018). These modifications are implicated in better sperm parameters (Gonzalez-Marin et al., 2017) and fertility (Lenz et al., 2017; Vishwanath and Moreno, 2018) compared to the older sorting technology. Even though there are some studies showing the results achieved following fixed-time artificial insemination (FTAI; Thomas et al., 2019) or AI after estrus detection (Maicas et al., 2020), apparently only one recent study has described the use of SexedULTRA for embryo production of superstimulated cows (Dell’Eva et al., 2019). Besides, considering variability among bulls, SexedULTRA technology categorizes sires identified for providing greater fertility as ULTRA Fertility (UF). This category is based on results of 3% above the average of P/AI at 60 d, of more than 500 inseminations, with reliability to exceed 75%. However, previous studies with SexedULTRA 4M have not evaluated this field fertility category in cows undergoing SOV protocols.

Therefore, the experiments conducted in this study aimed to evaluate the ovarian response and embryo production of Holstein cows superstimulated either with constant or decreasing doses of FSH, and the effect of SexedULTRA4M field fertility sires on embryo outcomes. The specific hypotheses of this study were: 1) both FSH regimens used for superstimulation would provide similar ovarian response and embryo production; 2) semen of sires classified as UF would produce more viable embryos per flush than non-ULTRA Fertility (NUF) sires.

2.2 Material and methods

For this study, four experiments were conducted with lactating and non-lactating Holstein cows, from October 2019 to April 2021. The procedures of all experiments were performed in accordance with the Animal Research Ethics Committee of Luiz de Queiroz College of Agriculture/University of São Paulo (Protocols CEUA #5992300720 and #2018-17).

2.2.1 Locations and donors

Experiments 1, 2 and 3 were conducted with non-lactating multiparous Holstein cows, at the facilities of the Department of Animal Science at Luiz de Queiroz College of Agriculture (ESALQ), University of São Paulo, located in Piracicaba, São Paulo, Brazil. The cows were kept on pasture (*Cynodon ssp.*), received a daily supplementation with haylage (*Cynodon dactylon*), corn and soybean meal-based concentrate with minerals and vitamins once a day, and there was free access to water and mineral salt. The diet was balanced to meet or exceed the nutritional requirements for non-lactating dairy cows (NRC, 2001).

Experiment 4 was carried out in a commercial dairy farm located in São Pedro, São Paulo, Brazil, with eleven lactating Holstein cows. Cows were housed in a cross ventilation barn, were milked by a robotic milking parlor and were fed with a TMR-based diet of corn silage and corn and soybean meal-based concentrate with minerals and vitamins. The diet was balanced to meet or exceed the nutritional requirements of lactating dairy cows (NRC, 2001).

2.2.2 Experiment 1

Non-lactating multiparous Holstein cows ($n = 18$), with BCS 3.1 ± 0.3 (1-5, in a 1 [emaciated] to 5 [obese] scale [Edmonson et al., 1989]) on D0, underwent between one and three superstimulations and embryo collections. Initially, cows were randomly assigned to receive one of two FSH regimens (decreasing [D-FSH] or constant [C-FSH] doses) and there

was a crossover of the FSH regimen in the following SOV. Each cow was inseminated with conventional semen from the same sire in all SOV.

2.2.3 Experiment 2

The experiment was performed as a 2x2 factorial arrangement, with four groups: C-FSH/UF (superstimulated with constant doses and inseminated with UF sires), C-FSH/NUF (superstimulated with constant doses and inseminated with NUF sires), D-FSH/UF (superstimulated with decreasing doses and inseminated with UF sires) and D-FSH/NUF (superstimulated with decreasing doses and inseminated with NUF sires).

Non-lactating multiparous Holstein cows ($n = 14$; BCS 3.1 ± 0.3) underwent between two and four superstimulations and embryo collections. Initially, cows were randomly assigned to receive one of two FSH regimens (D-FSH or C-FSH) and within one of two SexedULTRA 4M field fertility sires (UF or NUF). Each cow was submitted to SOV with both FSH regimens, but only part of them (20/50) underwent a crossover for field fertility sires.

2.2.4 Experiment 3

Non-lactating multiparous Holstein cows ($n = 12$; BCS 2.9 ± 0.4) underwent two superstimulations and embryo collections. Initially, cows were randomly assigned to be inseminated with one of two SexedULTRA 4M field fertility sires (UF or NUF) and, afterward, there was a crossover of the field fertility. Each cow received the same FSH regimen (only D-FSH or C-FSH) during the two SOV protocols. This experiment was performed as a 2x2 factorial arrangement and had the same four groups cited in Experiment 2.

2.2.5 Experiment 4

A total of 11 lactating Holstein cows (six primiparous and five multiparous, BCS 3.1 ± 0.3) underwent two superstimulations and embryo collections. The average DIM and the average daily milk yield at the onset of the first SOV protocol was, respectively, 55.9 ± 0.8 d and 42.9 ± 3.3 kg/d, and at the second, was 97.9 ± 0.8 d and 40.5 ± 2.7 kg/d. The parity ranged from one to four, for both protocols. Initially, the cows were randomly assigned to be inseminated with one of two SexedULTRA 4M field fertility sires (UF or NUF) and, in the second SOV, there was a crossover of the field fertility sires. Each cow received the same FSH regimen (only D-FSH or C-FSH) during the two SOV protocols. This experiment was

performed as a 2x2 factorial arrangement and had the same four groups cited in Experiments 2 and 3.

2.2.6 Combined data from the experiments

Data of experiments were combined, in order to provide more consistent information about the variables evaluated. Data regarding ovulation, number and percentage of CL during SOV for FSH regimens were calculated based on the results of Experiments 1 and 2. Number of follicles on D7, ovulatory response, estrus expression and embryo outcomes for the FSH regimens were calculated based on all experiments. Sperm traits and embryo production for SexedULTRA 4M field fertility sires were estimated based on Experiments 2, 3 and 4.

2.2.7 Superovulation protocol

The same superovulation protocol was used for the four experiments (Figure 1). All cows were synchronized with a modified Double-Ovsynch (DO), termed as “Super Double-Ovsynch” (SDO). Initially, all cows received a pre-synchronization starting with 50 µg lecinrelin (GnRH, TEC-Relin, União Química, Embu-Guaçu, Brazil) on D-16, 0.53 mg cloprostenol sodium (PGF, Estron, União Química) on D-9 and 25 µg GnRH on D-6. On D0 of the superstimulation protocol, cows received 50 µg GnRH. Thirty-six h after D0, treatments with FSH (NIH-FSH-P1 Folltropin-V, Vetoquinol, Mairiporã, Brazil) began (from D1.5 to D6). In groups D-FSH, cows received 300 mg of FSH distributed over 10 decreasing doses 12 h apart (50, 50, 40, 40, 30, 30, 20, 20, 10 and 10 mg), whereas cows in groups C-FSH received 10 constant doses (30 mg each). The PGF (0.53 mg) was given on D5, 5.5 and 6. On D5, an adhesive patch was applied to the tail-head for estrus detection (BOViFLAG, Bovitime Animal Products LTD, Stellenbosch, South Africa or Estrotect, Rockway Inc, Spring Valley, USA) and it was maintained and evaluated twice a day, until FTAI. Finally, on D7, 50 µg GnRH were used to induce ovulation and FTAI was performed twice, at D7.5 and D8. Embryo collections occurred on D14 or D15.

Figure 1. Schematic representation of the superstimulation protocols utilized for the four experiments. On D-16, all cows received GnRH (50 µg lecinirelin), followed by PGF (0.53 mg cloprostenol sodium) on D-9 and GnRH (25 µg) on D-6. On D0, the superstimulation period was started with GnRH (50 µg) and 36 h after (D1.5), the donors received the first FSH. In groups D-FSH, cows received 300 mg of FSH distributed over 10 decreasing doses, 12 h apart and groups C-FSH received 10 constant doses. PGF (0.53 mg) was given on D5, 5.5 and 6. On D5, the cows received an estrus detector adhesive. GnRH (50 µg) was given on D7 and FTAI was performed 12 and 24 h later, with conventional or sex-sorted semen, depending on the experiment. Embryo collections occurred either on D14 or on D15. The minimum interval between embryo collections was 42 d.

2.2.8 Sires, semen evaluation and insemination

All batches of semen were evaluated using a phase-contrast microscope, with subjective visual estimates of three or more random fields of view, for sperm progressive motility (estimated percentage of cells with progressive movement; 0 to 100%) and vigor (estimated velocity of progressive movement; 0 to 5 scale) before AI, with, at least, one straw per AI analyzed. When both AI straws were analyzed, the average between them was considered. In Experiment 1, AI was performed 12 and 24 h after the last GnRH treatment, in the uterine body, with commercial frozen/thawed conventional semen ($> 10 \times 10^6$ sperm/dose), from four Gyr sires (CRV Lagoa, Sertãozinho, Brazil).

In Experiments 2, 3 and 4, AI was done 12 and 24 h after the last GnRH treatment, in the uterine body, with frozen/thawed SexedULTRA 4M (Sexing Technologies, Navasota, TX) semen (4×10^6 sperm/dose), from Holstein sires. Sires classified as UF were those with a minimum of 500 inseminations evaluated, presenting 3% above the average of P/AI at 60 d post-AI, with reliability that exceeded 75%. Sires designated as NUF were bulls classified in other categories of SexedULTRA 4M. In Experiment 2, three sires were classified as UF and three as NUF. In Experiments 3 and 4, three other sires classified as UF and three sires as NUF were used, totaling 12 sires for Experiments 2, 3 and 4 (six UF and six NUF).

2.2.9 Evaluation of the response to the protocol

Transrectal ultrasound examinations in B-mode with a 7.5 MHz linear transducer (E2v, SonoScape, Shenzhen, China) were conducted to evaluate the ovarian response during the protocol. In Experiments 1 and 2, evaluations were performed on D-16 for the ovarian status; on D-9 to confirm ovulation to the first GnRH (by the presence of newly formed corpus luteum; CL) and on D-6, to evaluate luteolysis (by decreasing CL size) and the presence of a pre-ovulatory follicle. On D0, ovulation to the previous GnRH was confirmed by the presence of a newly formed CL. On D5.5, evaluations were performed for tracking follicle growth and ovulation to D0 (by the presence of a newly formed CL). When cows had ovulated after D0 in the same ovary ipsilateral to the CL of D-6, the CL were distinguished by the presence of a cavity, by the size or by the position in the ovary, using the map to record the ovarian structures. On D7, the number of preovulatory follicles (follicles ≥ 8.5 mm) was counted. Finally, on D14 or D15, the number of CL was estimated, before the uterine flushing.

In Experiments 3 and 4, ultrasound evaluations were performed on D2, to count the total number of follicles ≥ 3 mm present in both ovaries, at the onset of superstimulation, and on D7 and on D14, with the same objectives of Experiments 1 and 2.

In addition, the number of CL during SOV considered the total number of CL on D5.5 (0, 1 or 2; Experiments 1 and 2). Ovulatory response was calculated considering the number of CL on the day of embryo collection divided by the number of follicles ≥ 8.5 mm on D7 (all experiments). Estrus expression was evaluated by the color change between the initial application of the patch and the day of FTAI. Estrus was defined to have occurred when the change was $\geq 50\%$ (All experiments). Recovery rate considered the number of total ova and embryos divided by the total number of CL within cow on the day of embryo collection (all experiments).

2.2.10 Embryo collection and evaluation

Embryo collections were performed either on 7 (D14) or 8 (D15) d post-GnRH, utilizing the nonsurgical uterine flushing technique. The donors received an epidural injection (Lidocaine hydrochloride 2%, Anestésico L, Eurofarma, Campo Belo, Brazil) and were submitted to a double uterine flushing, as described by Neto et al. (2005). Initially, each uterine horn was flushed separately and, afterward, the uterine body and the horns were flushed simultaneously, utilizing Dulbecco's modified phosphate-buffered saline flushing solution (dmPBS, Reprodex, Campinas, Brazil).

The recovered ova and embryos were evaluated and classified according to the IETS guidelines (Robertson and Nelson, 1998): grades 1 (excellent/good) and 2 (fair) embryos were considered transferable/viable, whereas grade 1 was defined as freezable. The minimum interval between embryo recoveries was 42 d.

2.2.11 Statistical analyses

Statistical analyses were performed using SAS 9.4 (SAS, Version 9.4 for Windows, SAS Institute Inc., Cary, NC). All data were checked for normality of studentized residuals with the UNIVARIATE procedure and the Shapiro-Wilk method. When necessary, data were transformed to logarithm, square root or exponential, and outliers were removed. The Kenward-Rogers method was used to calculate the degrees of freedom for variance. Continuous and binomial variables were analyzed by GLIMMIX procedure, using Gaussian and Binomial distribution, respectively.

For evaluation of variables such as response to presynchronization (ovulation, number of CL during SOV, number of follicles on D7, ovulatory response), estrus expression, CL, recovery rate, total number of ova and embryos, number and percentage of transferable embryos, percentage of cows with ≥ 2 transferable structures and sperm motility and vigor, results are shown based on embryo collections from all donors, aiming to evaluate the overall response to treatments. For variables such as CL, recovery rate, total number of ova and embryos, number and percentage of transferable and freezable embryos, number and percentage of unfertilized and degenerate structures and individual sire effect, results are also shown based on embryo collections from donors with ≥ 2 structures recovered, aiming to evaluate the effect of treatments on fertilization rate and embryo quality.

In Experiment 1, for analysis of the following variables: presynchronization results, number of follicles on D7, ovulatory response, estrus expression, number of CL, recovery rate and total number of ova and embryos, the final model included FSH regimens (decreasing or constant) as fixed effect and replicate as random effect. For evaluation of embryo characteristics, the final model included FSH regimen, sire, and interaction between these factors as fixed effect and replicate was considered as random effect.

Experiments 2, 3, and 4 were analyzed as a 2x2 factorial arrangement and, for all variables evaluated, the final model included the main effects of treatments (FSH regimens and field fertility sires [NUF and UF]) and their interaction as fixed effect. Additionally, for all variables, replicate and cow were considered as random effect. Although Experiments 2, 3, and 4 were analyzed as a factorial design, no relevant interaction was found between FSH

regimen and field fertility sires. Hence, only the main effects are presented in the results session. In Experiments 3 and 4, the number of follicles on D2 (prior to the beginning of FSH treatments) was tested as a covariate for all the variables, and maintained in the final model when $P < 0.20$. Likewise, in Experiment 4, milk yield, DIM and parity were tested as covariates and maintained in the final model when $P < 0.20$. Further analyses were performed to evaluate the sire effect, regardless of field fertility. In Experiment 2, evaluation of the sire effect considered FSH regimen, sire and interaction between them as fixed effect, whereas in Experiments 3 and 4, the combined evaluation of sires considered FSH regimens, sires, experiments and interaction between sires and experiments.

For the analysis of the presynchronization responses effect, data from Experiments 1 and 2 were combined. To evaluate the effect of ovulation after D0, the final model for all variables included FSH regimens, ovulation, interaction between them, and experiment as fixed effect. To evaluate the effect of the number of CL (< 2 or 2) during the superstimulatory period, the final model for all variables included FSH regimens, number of CL class, interaction between them, and experiment as fixed effect. For both analyses, replicate and cow were considered as random effect.

To evaluate the overall ovarian responses and embryo production according to the FSH regimens, and according to expression of estrus, regardless of treatments, data from all experiments were combined. The final model for the FSH regimen effect included FSH regimen, experiment, and interaction between them, as fixed effect. The final model for expression of estrus effect considered expression of estrus, experiment, and interaction between them as fixed effect. For both analyses, replicate and cow were included as random effect. To evaluate the overall effect of SexedULTRA 4M field fertility on embryo responses, data from Experiments 2, 3, and 4 were combined. All the variables, including sperm parameters of field fertility sires were evaluated using a final model with field fertility sires, experiment, and interaction between these factors as fixed effect, and replicate and cow as random effect. Furthermore, a regression analysis was performed by GLIMMIX procedure, selecting the solution option, to evaluate the correlation between sperm progressive motility of SexedULTRA 4M field fertility sires and number of transferable embryos.

The Tukey adjustment was used to determine differences among classification variables. Significant differences were considered when $P \leq 0.05$ and a tendency was defined when $0.05 < P \leq 0.10$. Values are presented as means $\pm$ standard error of the mean (SEM).

2.3 Results

2.3.1 Experiment 1

The response to the pre-synchronization was similar between C-FSH and D-FSH groups, respectively, considering ovulation on D-16 (72.2% vs. 61.1%; $P = 0.42$), ovulation on D-6 (94.4% vs. 72.2%; $P = 0.13$), ovulation on D0 (83.3% vs. 88.9%; $P = 0.96$) and number of CL during SOV (1.8 vs. 1.6; $P = 0.21$). Number of follicles ≥ 8.5 mm on D7 was greater ($P = 0.01$) for cows receiving D-FSH (19.0 ± 2.1) than for C-FSH (12.1 ± 1.4). However, there was no difference in ovulatory response for cows in C-FSH and D-FSH, respectively ($79.6 \pm 4.5\%$ vs. $72.6 \pm 3.9\%$; $P = 0.25$), as well as in estrus expression (72.2% vs. 83.3%; $P = 0.57$).

Considering all donors, D-FSH resulted in a greater number ($P = 0.02$) of CL compared to C-FSH, resulting in a tendency for D-FSH to have greater number of total ova and embryos compared to C-FSH (Table 1). Recovery rate, number and percentage of transferable embryos, and percentage of cows that produced ≥ 2 transferable structures were similar (Table 2). Considering only the donors with ≥ 2 structures recovered, there was still a tendency for a greater number of CL for D-FSH than for C-FSH (Table 2). There was no difference in recovery rate, number of total structures, transferable and freezable embryos, as well as in UFO between groups. Group D-FSH tended to produce a greater number of degenerate embryos (DG) and produced a greater percentage of DG (Table 2). In addition, there was a tendency among sires in the percentage of sperm with progressive motility ($P = 0.06$; data not shown) and a difference in vigor evaluation ($P = 0.03$; data not shown). Even though, sires had no effect on embryonic outcomes ($P > 0.10$), as well as no effect was found for sperm progressive motility and vigor for C-FSH and D-FSH groups, respectively ($59.3 \pm 2.5\%$ vs. $50.2 \pm 2.2\%$, $P = 0.84$; 3.4 ± 0.1 vs. 3.4 ± 0.1 , $P = 0.98$). Finally, no interaction was found between FSH regimens and sires ($P > 0.20$).

Table 1. Ovarian response, recovery rate, and mean total and transferable structures per cow, according to the FSH regimens, considering all donors (Experiment 1).

Item	C-FSH (n = 18)	D-FSH (n = 18)	<i>P-value</i>
Corpora lutea, n	9.3 ± 1.0	13.8 ± 1.5	0.02
Recovery rate, %	46.0 ± 7.7	49.8 ± 6.4	0.70
Oocytes and embryos, n	4.4 ± 0.9	6.2 ± 1.0	0.10
Transferable embryos, n	2.6 ± 0.6	3.9 ± 0.9	0.25
Transferable embryos, %	61.0 ± 10.3	66.9 ± 8.4	0.36
Cows with ≥ 2 transf. embryos, % (n/n)	55.6 (10/18)	72.2 (13/18)	0.47

Values presented as mean ± SEM.

Abbreviations: C-FSH = constant doses of FSH; D-FSH = decreasing doses of FSH.

Table 2. Ovarian response, recovery rate and mean embryo characteristics per cow, according to the FSH regimens, considering only donors with ≥ 2 structures recovered (Experiment 1).

Item	C-FSH (n = 13)	D-FSH (n = 18)	<i>P-value</i>
Corpora lutea, n	10.1 ± 1.3	13.8 ± 1.5	0.09
Recovery rate, %	61.0 ± 6.8	49.8 ± 6.4	0.25
Oocytes and embryos, n	5.9 ± 1.0	6.2 ± 1.0	0.36
Transferable embryos, n	3.5 ± 0.7	3.9 ± 0.9	0.65
Transferable embryos, %	69.0 ± 10.5	66.9 ± 8.4	0.93
Freezable embryos, n	2.9 ± 0.7	3.5 ± 0.9	0.62
Freezable embryos, %	56.9 ± 10.5	62.1 ± 8.3	0.77
Unfertilized oocytes, n	1.7 ± 0.7	1.5 ± 0.6	0.53
Unfertilized oocytes, %	21.9 ± 8.0	22.6 ± 7.2	0.79
Degenerate embryos, n ¹	0.2 ± 0.2	0.8 ± 0.2	0.06
Degenerate embryos, % ¹	2.7 ± 1.8	9.8 ± 2.8	0.04

Values presented as mean ± SEM.

Abbreviations: C-FSH = constant doses of FSH; D-FSH = decreasing doses of FSH.

Five embryo collections from C-FSH group with ≤ 1 structure recovered were excluded from the analyses.

¹One datum from C-FSH was considered outlier and was excluded from the analyses.

2.3.2 Experiment 2

Response to the pre-synchronization was similar between C-FSH and D-FSH groups, respectively, considering ovulation on D-16 (84.0% vs. 72.0%; $P = 0.34$), ovulation on D-6 (92.0% vs. 92.0%; $P = 0.98$), ovulation on D0 (88.0% vs. 72.0%; $P = 0.16$) and number of CL during SOV (1.8 vs. 1.7; $P = 0.43$). Number of follicles ≥ 8.5 mm on D7 was greater ($P < 0.01$) for cows receiving D-FSH (18.0 ± 1.2), than for C-FSH (13.5 ± 1.4). However, ovulatory response was lower for D-FSH than C-FSH, respectively (62.0 ± 5.8 vs. 75.7 ± 4.9 ; $P < 0.01$). Despite that, estrus expression was similar between groups (64.0% for C-FSH vs. 72.0% for D-FSH; $P = 0.52$).

Considering all donors (Table 3), no difference was found regarding CL, recovery rate and embryo outcomes. Considering only the donors with ≥ 2 structures recovered (Table 4), D-FSH had a greater number of CL than C-FSH, respectively (14.8 ± 0.9 vs. 11.7 ± 1.1 ; $P = 0.05$), resulting in a tendency for a greater number of total ova and embryos for D-FSH (9.8 ± 1.0), compared to C-FSH (7.1 ± 0.8). Recovery rate, number and percentage of transferable and freezable embryos, UFO and DG were similar between groups.

Table 3. Ovarian response, recovery rate, and mean total and transferable structures per cow, according to the FSH regimens, considering all donors (Experiment 2).

Item	C-FSH (n = 25)	D-FSH (n = 25)	<i>P-value</i>
Corpora lutea, n	10.6 ± 1.1	11.8 ± 1.3	0.37
Recovery rate, %	53.7 ± 5.9	53.4 ± 7.1	0.94
Oocytes and embryos, n	6.0 ± 0.8	7.6 ± 1.1	0.19
Transferable embryos, n	2.8 ± 0.6	3.4 ± 0.7	0.60
Transferable embryos, %	41.3 ± 7.1	37.4 ± 7.5	0.77
Cows with ≥ 2 transf. embryos, % (n/n)	52.0 (13/25)	60.0 (15/25)	0.36

Values presented as mean $\pm$ SEM.

Abbreviations: C-FSH = constant doses of FSH; D-FSH = decreasing doses of FSH.

Table 4. Ovarian response, recovery rate and mean embryo characteristics per cow, according to the FSH regimens, considering only donors with ≥ 2 structures recovered (Experiment 2).

Item	C-FSH (n = 21)	D-FSH (n = 19)	<i>P-value</i>
Corpora lutea, n	11.7 $\pm$ 1.1	14.8 $\pm$ 0.9	0.05
Recovery rate, %	63.5 $\pm$ 4.4	67.7 $\pm$ 6.2	0.52
Oocytes and embryos, n	7.1 $\pm$ 0.8	9.8 $\pm$ 1.0	0.08
Transferable embryos, n	3.4 $\pm$ 0.6	4.4 $\pm$ 0.8	0.59
Transferable embryos, %	49.2 $\pm$ 8.1	49.2 $\pm$ 7.2	0.73
Freezable embryos, n	2.9 $\pm$ 0.6	4.0 $\pm$ 0.7	0.41
Freezable embryos, %	41.9 $\pm$ 7.2	46.7 $\pm$ 8.0	0.98
Unfertilized oocytes, n	2.4 $\pm$ 0.7	3.4 $\pm$ 0.9	0.22
Unfertilized oocytes, %	34.2 $\pm$ 7.5	34.6 $\pm$ 8.7	0.68
Degenerate embryos, n	1.3 $\pm$ 0.3	2.0 $\pm$ 0.5	0.60
Degenerate embryos, %	16.6 $\pm$ 3.4	16.2 $\pm$ 3.6	0.72

Values presented as mean $\pm$ SEM.

Abbreviations: C-FSH = constant doses of FSH; D-FSH = decreasing doses of FSH.

Four embryo collections from C-FSH and six from D-FSH with ≤ 1 structure recovered were excluded from the analyses.

Considering groups NUF and UF for all donors (Table 5), differences were greater for the UF group, regarding number and percentage of transferable embryos, as well as percentage of cows that produced ≥ 2 transferable embryos. Considering donors with ≥ 2 structures recovered (Table 6), UF sires had greater number and percentage of transferable and freezable embryos, and lower number and percentage of UFO. An individual effect of sire ($P \leq 0.02$, Appendix A) was found in number and percentage of transferable, freezable embryos and UFO, but not in DG ($P = 0.32$). Individual evaluation of sires utilized in this experiment differed regarding percentage of sperm with progressive motility ($P < 0.01$) and tended to differ in vigor ($P = 0.06$). Moreover, when UF sires were evaluated together, they had superior sperm progressive motility and vigor than NUF sires, respectively ($55.3 \pm 1.7\%$ vs. $42.2 \pm 1.9\%$, $P < 0.01$; 3.4 ± 0.1 vs. 2.9 ± 0.1 , $P = 0.03$).

Table 5. Ovarian response, recovery rate, and mean total and transferable structures per cow, according to the SexedULTRA 4M field fertility sires, considering all donors (Experiment 2).

Item	NUF (n = 25)	UF (n = 25)	<i>P-value</i>
Corpora lutea, n	11.5 ± 1.2	10.9 ± 1.2	0.79
Recovery rate, %	53.4 ± 6.3	53.7 ± 6.8	0.52
Oocytes and embryos, n	6.7 ± 1.0	6.9 ± 1.0	0.92
Transferable embryos, n	1.8 ± 0.5	4.4 ± 0.7	< 0.01
Transferable embryos, %	26.4 ± 6.1	52.3 ± 7.4	0.01
Cows with ≥ 2 transf. embryos, % (n/n)	40.0 (10/25)	72.0 (18/25)	0.02

Values presented as mean ± SEM.

Abbreviations: NUF = non-ULTRA Fertility sires; UF = ULTRA Fertility sires.

Table 6. Ovarian response, recovery rate and mean embryo characteristics per cow, according to the SexedULTRA 4M field fertility sires, considering only donors with ≥ 2 structures recovered (Experiment 2).

Item	NUF (n = 21)	UF (n = 19)	<i>P-value</i>
Corpora lutea, n	13.3 ± 1.1	13.0 ± 1.0	0.83
Recovery rate, %	62.0 ± 5.6	69.3 ± 4.8	0.95
Oocytes and embryos, n	7.9 ± 0.9	8.9 ± 1.0	0.52
Transferable embryos, n	2.2 ± 0.5	5.7 ± 0.6	< 0.01
Transferable embryos, %	31.4 ± 6.7	68.9 ± 5.8	< 0.01
Freezable embryos, n	1.9 ± 0.5	5.2 ± 0.5	< 0.01
Freezable embryos, %	26.8 ± 6.8	63.4 ± 5.8	< 0.01
Unfertilized oocytes, n	4.4 ± 0.9	1.2 ± 0.4	< 0.01
Unfertilized oocytes, %	53.8 ± 7.8	13.0 ± 4.6	< 0.01
Degenerate embryos, n	1.3 ± 0.3	2.0 ± 0.5	0.31
Degenerate embryos, %	14.8 ± 3.2	18.1 ± 3.8	0.56

Values presented as mean ± SEM.

Abbreviations: NUF = non-ULTRA Fertility sires; UF = ULTRA Fertility sires.

Four embryo collections from NUF and six from UF with ≤ 1 structure recovered were excluded from the analyses.

2.3.3 Experiment 3

There was a tendency ($P = 0.07$) for D-FSH to produce more follicles ≥ 8.5 mm on D7 (20.4 ± 3.7), than C-FSH (17.6 ± 2.2). No difference was found in ovulatory response between C-FSH and D-FSH ($76.8 \pm 5.7\%$ vs. $73.7 \pm 7.2\%$, $P = 0.65$) and estrus expression (83.3% vs. 45.5% , $P = 0.14$), respectively.

Considering all donors (Table 7), D-FSH tended to produce more CL (16.3 ± 3.7) than C-FSH (13.1 ± 1.7). The other variables were similar between groups. Considering only the donors with ≥ 2 structures recovered (Table 8), only a greater number of total structures was found for D-FSH. All other variables were similar between groups.

Table 7. Ovarian response, recovery rate, and mean total and transferable structures per cow, according to the FSH regimens, considering all donors (Experiment 3).

Item	C-FSH (n = 12)	D-FSH (n = 11)	<i>P-value</i>
Corpora lutea, n	13.1 ± 1.7	16.3 ± 3.7	0.10
Recovery rate, %	37.3 ± 5.4	35.5 ± 8.2	0.91
Oocytes and embryos, n	4.4 ± 0.9	6.5 ± 1.9	0.27
Transferable embryos, n	2.4 ± 0.7	1.7 ± 1.1	0.42
Transferable embryos, %	45.4 ± 10.0	22.7 ± 10.5	0.28
Cows with ≥ 2 transf. embryos, % (n/n)	58.3 (7/12)	27.3 (3/11)	0.16

Values presented as mean $\pm$ SEM.

Abbreviations: C-FSH = constant doses of FSH; D-FSH = decreasing doses of FSH.

Table 8. Ovarian response, recovery rate and mean embryo characteristics per cow, according to the FSH regimens, considering only donors with ≥ 2 structures recovered (Experiment 3).

Item	C-FSH (n = 11)	D-FSH (n = 7)	<i>P-value</i>
Corpora lutea, n	11.9 $\pm$ 1.3	21.9 $\pm$ 4.4	0.14
Recovery rate, %	40.7 $\pm$ 4.6	46.1 $\pm$ 9.4	0.84
Oocytes and embryos, n	4.8 $\pm$ 0.9	9.7 $\pm$ 2.2	0.04
Transferable embryos, n	2.6 $\pm$ 0.7	2.7 $\pm$ 1.6	0.56
Transferable embryos, %	49.5 $\pm$ 10.0	35.7 $\pm$ 14.6	0.46
Freezable embryos, n	1.6 $\pm$ 0.4	2.7 $\pm$ 1.6	0.55
Freezable embryos, %	34.4 $\pm$ 8.9	35.7 $\pm$ 14.6	0.98
Unfertilized oocytes, n ¹	1.0 $\pm$ 0.4	3.2 $\pm$ 1.3	0.29
Unfertilized oocytes, % ¹	17.1 $\pm$ 5.2	32.5 $\pm$ 13.8	0.55
Degenerate embryos, n	1.6 $\pm$ 0.5	3.7 $\pm$ 1.8	0.75
Degenerate embryos, %	37.9 $\pm$ 10.2	30.5 $\pm$ 12.6	0.91

Values presented as mean $\pm$ SEM.

Abbreviations: C-FSH = constant doses of FSH; D-FSH = decreasing doses of FSH.

One embryo collection from C-FSH and four from D-FSH with ≤ 1 structure recovered were excluded from the analyses.

¹One datum from D-FSH was considered outlier and was excluded from the analyses.

Considering groups NUF and UF for all donors (Table 9) and for donors with ≥ 2 structures recovered (Table 10), no differences were found for any of the variables evaluated. In individual evaluation, sires differed regarding percentage of progressive motility ($P < 0.01$, data not shown), but not in vigor ($P = 0.42$, data not shown). Despite that, when analyzing groups, NUF and UF had similar sperm progressive motility and vigor, respectively ($48.3 \pm 3.8\%$ vs. $51.7 \pm 4.0\%$, $P = 0.44$; 3.5 ± 0.2 vs. 3.6 ± 0.2 , $P = 0.51$). Due to absence of interactions between sires and experiments (3 and 4), individual results of sires for embryo characteristics were combined (Appendix B).

Table 9. Ovarian response, recovery rate, and mean total and transferable structures per cow, according to the SexedULTRA 4M field fertility sires, considering all donors (Experiment 3).

Item	NUF (n = 11)	UF (n = 12)	<i>P-value</i>
Corpora lutea, n	15.6 ± 2.5	13.7 ± 3.0	0.73
Recovery rate, %	39.1 ± 8.2	34.1 ± 5.4	0.50
Oocytes and embryos, n	5.8 ± 1.7	5.0 ± 1.3	0.58
Transferable embryos, n	1.8 ± 0.7	2.3 ± 1.0	0.57
Transferable embryos, %	34.5 ± 11.4	34.6 ± 10.3	0.83
Cows with ≥ 2 transf. embryos, % (n/n)	45.5 (5/11)	41.7 (5/12)	0.94

Values presented as mean ± SEM.

Abbreviations: NUF = non-ULTRA Fertility sires; UF = ULTRA Fertility sires.

Table 10. Ovarian response, recovery rate and mean embryo characteristics per cow, according to the SexedULTRA 4M field fertility sires, considering only donors with ≥ 2 structures recovered (Experiment 3).

Item	NUF (n = 8)	UF (n = 10)	<i>P-value</i>
Corpora lutea, n	16.6 ± 2.6	15.1 ± 3.4	0.77
Recovery rate, %	46.3 ± 8.8	40.0 ± 4.3	0.53
Oocytes and embryos, n	7.7 ± 2.0	5.9 ± 1.3	0.41
Transferable embryos, n	2.5 ± 0.8	2.8 ± 1.2	0.87
Transferable embryos, %	47.5 ± 13.0	41.5 ± 11.1	0.79
Freezable embryos, n	1.5 ± 0.5	2.5 ± 1.1	0.53
Freezable embryos, %	32.9 ± 11.6	36.5 ± 10.6	0.66
Unfertilized oocytes, n	2.1 ± 1.0	1.5 ± 0.7	0.52
Unfertilized oocytes, %	22.0 ± 7.3	22.9 ± 9.0	0.64
Degenerate embryos, n	3.6 ± 1.6	1.5 ± 0.6	0.16
Degenerate embryos, %	35.2 ± 11.0	34.9 ± 11.3	0.95

Values presented as mean ± SEM.

Abbreviations: NUF = non-ULTRA Fertility sires; UF = ULTRA Fertility sires.

Three embryo collections from NUF and two from UF with ≤ 1 structure recovered were excluded from the analyses.

2.3.4 Experiment 4

Values were similar between groups regarding DIM, milk yield, parity, and BCS on both D-16 and D0 ($P > 0.10$). No differences were found between C-FSH and D-FSH, respectively, in the number of follicles on D7 (9.9 ± 1.1 vs. 15.7 ± 3.4 , $P = 0.18$), in ovulatory response ($83.0 \pm 5.9\%$ vs. $82.2 \pm 4.2\%$, $P = 0.96$) and in estrus expression (58.3% vs. 66.7% , $P = 0.96$). All other variables analyzed, considering all donors, were similar between the FSH regimens (Table 11). Considering cows with ≥ 2 structures recovered, the number of CL and the total number of ova and embryos tended to be greater for D-FSH (Table 12), whereas the remaining variables were similar.

Table 11. Ovarian response, recovery rate, and mean total and transferable structures per cow, according to FSH regimens, considering all donors (Experiment 4).

Item	C-FSH (n = 12)	D-FSH (n = 9)	<i>P-value</i>
Corpora lutea, n	8.9 ± 1.4	12.2 ± 2.1	0.36
Recovery rate, % ¹	46.2 ± 10.1	43.4 ± 8.2	0.47
Oocytes and embryos, n	3.6 ± 1.0	5.7 ± 1.3	0.19
Transferable embryos, n ²	2.2 ± 0.6	4.0 ± 1.4	0.23
Transferable embryos, %	46.9 ± 11.2	57.7 ± 12.4	0.78
Cows with ≥ 2 transf. embryos, % (n/n)	58.3 (7/12)	77.8 (7/9)	0.49

Values presented as mean $\pm$ SEM.

Abbreviations: C-FSH = constant doses of FSH; D-FSH = decreasing doses of FSH.

¹One datum from C-FSH and ²one datum from D-FSH were considered outliers and were excluded from the analyses.

Table 12. Ovarian response, recovery rate and mean embryo characteristics per cow, according to the FSH regimens, considering only donors with ≥ 2 structures recovered (Experiment 4).

Item	C-FSH (n = 9)	D-FSH (n = 8)	<i>P</i> -value
Corpora lutea, n	9.44 $\pm$ 1.6	13.3 $\pm$ 2.1	0.06
Recovery rate, %	56.5 $\pm$ 9.1	48.8 $\pm$ 6.9	0.62
Oocytes and embryos, n	4.8 $\pm$ 1.1	6.4 $\pm$ 1.3	0.10
Transferable embryos, n	3.0 $\pm$ 0.7	4.5 $\pm$ 1.3	0.12
Transferable embryos, %	62.5 $\pm$ 10.4	64.9 $\pm$ 11.5	0.93
Freezable embryos, n	2.7 $\pm$ 0.8	3.6 $\pm$ 1.3	0.44
Freezable embryos, %	48.6 $\pm$ 10.9	53.0 $\pm$ 12.9	0.87
Unfertilized oocytes, n ¹	0.7 $\pm$ 0.2	0.4 $\pm$ 0.3	0.23
Unfertilized oocytes, % ¹	20.5 $\pm$ 6.7	6.7 $\pm$ 4.4	0.11
Degenerate embryos, n	0.6 $\pm$ 0.2	1.4 $\pm$ 0.5	0.17
Degenerate embryos, %	14.2 $\pm$ 6.5	27.1 $\pm$ 12.1	0.36

Values presented as mean $\pm$ SEM.

Abbreviations: C-FSH = constant doses of FSH; D-FSH = decreasing doses of FSH.

Three embryo collections from C-FSH and one from D-FSH with ≤ 1 structure recovered were excluded from the analyses.

¹One datum from C-FSH was considered outlier and was excluded from the analyses.

Regarding SexedULTRA 4M fertility, only the total number of structures was superior for NUF, considering all donors (Table 13), and tended to be greater in the same analysis and group, in donors with two or more structures recovered (Table 14). All other evaluations were similar. Sperm progressive motility and vigor did not differ between NUF and UF, respectively (56.3 $\pm$ 6.0% vs. 60.0 $\pm$ 4.4%, P = 0.71; 3.8 $\pm$ 0.2 vs. 3.7 $\pm$ 0.2, P = 0.97). Due to absence of interactions between sires and experiments (3 and 4), individual results of sires for embryo characteristics were combined (Appendix B).

Table 13. Ovarian response, recovery rate, and mean total and transferable structures per cow, according to the SexedULTRA 4M field fertility sires, considering all donors (Experiment 4).

Item	NUF (n = 10)	UF (n = 11)	<i>P-value</i>
Corpora lutea, n	10.8 ± 2.2	9.9 ± 1.2	0.32
Recovery rate, % ¹	51.7 ± 9.8	38.2 ± 8.5	0.13
Oocytes and embryos, n	5.4 ± 1.2	3.6 ± 0.9	0.04
Transferable embryos, n ¹	3.6 ± 1.2	2.3 ± 0.7	0.19
Transferable embryos, %	56.2 ± 11.9	47.3 ± 11.7	0.67
Cows with ≥ 2 transf. embryos, % (n/n)	70.0 (7/10)	63.6 (7/11)	0.76

Values presented as mean ± SEM.

Abbreviations: NUF = non-ULTRA Fertility sires; UF = ULTRA Fertility sires.

¹One datum from UF was considered outlier and was excluded from the analyses.

Table 14. Ovarian response, recovery rate and mean embryo characteristics per cow, according to the SexedULTRA 4M field fertility sires, considering only donors with ≥ 2 structures recovered (Experiment 4).

Item	NUF (n = 9)	UF (n = 8)	<i>P-value</i>
Corpora lutea, n	11.6 ± 2.4	10.9 ± 1.1	0.33
Recovery rate, %	57.4 ± 8.8	47.7 ± 7.2	0.42
Oocytes and embryos, n	6.0 ± 1.4	5.0 ± 0.8	0.08
Transferable embryos, n	4.0 ± 1.3	3.4 ± 0.6	0.20
Transferable embryos, %	62.4 ± 11.3	65.1 ± 10.3	0.91
Freezable embryos, n	3.1 ± 1.3	3.1 ± 0.6	0.41
Freezable embryos, %	43.5 ± 12.4	58.8 ± 10.2	0.21
Unfertilized oocytes, n ¹	0.4 ± 0.2	0.8 ± 0.3	0.23
Unfertilized oocytes, % ¹	14.6 ± 7.3	12.6 ± 4.8	0.87
Degenerate embryos, n	1.0 ± 0.5	0.9 ± 0.3	0.38
Degenerate embryos, %	18.5 ± 7.4	22.3 ± 11.8	0.28

Values presented as mean ± SEM.

Abbreviations: NUF = non-ULTRA Fertility sires; UF = ULTRA Fertility sires.

One embryo collection from NUF and three from UF with ≤ 1 structure recovered were excluded from the analyses.

¹One datum from NUF was considered outlier and was excluded from the analyses.

2.3.5 Combined data from the experiments

2.3.5.1 Response to presynchronization (Experiments 1 and 2)

There were no differences in the response to the presynchronization treatments between C-FSH and D-FSH ($P > 0.10$). Considering all cows, the total mean percentage of ovulation on D-16 was 73.2% (63/86), 88.4% for ovulation on D-6 (76/86) and 83.7% for ovulation on D0 (72/86). The mean number of CL during SOV was 1.7 ± 0.1 . Considering all cows, 72.1% (62/86) had two CL. Considering cows with ≥ 2 structures recovered, 81.7% (58/71) ovulated to D0 and 67.6% (48/71) had two CL during SOV.

When only ovulation on D0 and the number of CL during SOV were evaluated, regardless of treatments, no differences were found in the analyzed variables. Considering all donors, the number of follicles on D7 for cows that did not ovulate ($n = 14$) and cows that ovulated ($n = 72$) on D0 was, respectively, 17.5 ± 2.3 vs. 15.0 ± 0.8 ($P = 0.68$), the percentage of ovulatory response was $78.0 \pm 4.2\%$ vs. $73.9 \pm 2.5\%$ ($P = 0.49$) and the number of CL was 13.7 ± 1.6 vs. 10.9 ± 0.7 ($P = 0.65$). Moreover, the number of follicles on D7 for cows that had < 2 ($n = 26$) or 2 CL ($n = 60$) was, respectively, 17.8 ± 1.5 vs. 14.7 ± 0.9 ($P = 0.56$), the ovulatory response was $75.2 \pm 3.3\%$ vs. $74.3 \pm 2.8\%$ ($P = 0.54$) and the number of CL was 13.4 ± 1.1 vs. 10.5 ± 0.8 ($P = 0.16$). Considering donors with ≥ 2 structures recovered, the number of ova and embryos for cows that did not ovulate ($n = 13$) and cows that had ovulated ($n = 58$) on D0 was, respectively, 8.1 ± 1.4 vs. 7.2 ± 0.5 ($P = 0.66$), the number of transferable embryos was 3.8 ± 0.9 vs. 3.8 ± 0.4 ($P = 0.85$) and the percentage of transferable embryos was $54.9 \pm 9.9\%$ vs. $57.8 \pm 4.7\%$. Finally, for cows that had < 2 ($n = 23$) or 2 CL ($n = 48$) the average number of ova and embryos per cow was, respectively, 8.1 ± 0.9 vs. 7.0 ± 0.6 ($P = 0.31$), the number of transferable embryos was 3.6 ± 0.7 vs. 3.9 ± 0.5 ($P = 0.70$) and the percentage of transferable embryos was $46.3 \pm 7.1\%$ vs. $62.6 \pm 5.1\%$ ($P = 0.11$).

2.3.5.2 Effect of estrus expression on ovarian response and embryo outcomes (all experiments)

Considering all donors (Table 15), cows that exhibited estrus had greater ovulatory response, number of CL and total structures, number and percentage of transferable embryos, as well as percentage of ≥ 2 viable embryos, compared to cows that were not detected in estrus. Considering cows with ≥ 2 structures recovered, the number and percentage of transferable embryos, as well as the number of freezable structures were greater for cows that showed estrus (Table 16).

Table 15. Ovarian response, recovery rate and mean embryo characteristics per cow, according to estrus expression, considering all donors (all experiments).

Item	Estrus expression		<i>P-value</i>
	No (n = 40)	Yes (n = 91)	
Ovulatory response, % ¹	66.9 ± 4.6	77.6 ± 1.9	0.05
Corpora lutea, n	9.6 ± 1.2	13.0 ± 0.7	< 0.01
Recovery rate, %	41.3 ± 5.5	50.4 ± 2.9	0.14
Oocytes and embryos, n	4.3 ± 0.8	6.4 ± 0.4	< 0.01
Transferable embryos, n	1.5 ± 0.4	3.7 ± 0.3	< 0.01
Transferable embryos, %	30.1 ± 6.2	54.6 ± 3.8	< 0.01
Cows with ≥ 2 transf. embryos, % (n/n)	30.0 (12/40)	70.3 (64/91)	< 0.01

Values presented as mean ± SEM.

¹One datum from Yes group was considered outlier and was excluded from the analyses.

Table 16. Ovarian response, recovery rate and mean embryo characteristics per cow, according to estrus expression, considering only donors with ≥ 2 structures recovered (all experiments).

Item	Estrus expression		<i>P</i> -value
	No (n = 26)	Yes (n = 81)	
Ovulatory response, %	76.9 $\pm$ 3.6	79.0 $\pm$ 1.8	0.21
Corpora lutea, n	11.7 $\pm$ 1.4	13.7 $\pm$ 0.7	0.20
Recovery rate, %	59.2 $\pm$ 5.6	56.0 $\pm$ 2.6	0.66
Oocytes and embryos, n ¹	6.4 $\pm$ 1.0	7.2 $\pm$ 0.4	0.33
Transferable embryos, n	2.3 $\pm$ 0.6	4.1 $\pm$ 0.3	< 0.01
Transferable embryos, %	42.5 $\pm$ 7.8	60.2 $\pm$ 3.6	0.04
Freezable embryos, n	2.0 $\pm$ 0.5	3.6 $\pm$ 0.3	0.01
Freezable embryos, %	37.5 $\pm$ 7.7	51.5 $\pm$ 3.6	0.16
Unfertilized oocytes, n	2.6 $\pm$ 0.7	1.8 $\pm$ 0.3	0.18
Unfertilized oocytes, %	34.5 $\pm$ 6.1	22.9 $\pm$ 3.3	0.11
Degenerate embryos, n ²	1.3 $\pm$ 0.3	1.4 $\pm$ 0.2	0.87
Degenerate embryos, % ¹	20.5 $\pm$ 5.7	17.2 $\pm$ 2.3	0.93

Values presented as mean $\pm$ SEM.

Fourteen embryo collections from No group and 10 from Yes group with ≤ 1 structure recovered were excluded from the analyses.

¹One datum and ²two data from Yes group were considered outliers and were excluded from the analyses.

2.3.5.3 Effect of FSH regimens on ovarian response, estrus expression and embryo outcomes (all experiments)

The percentage of cows that did not respond to SOV treatments, yielding ≤ 1 structure, was 18.3% (24/131). Considering all cows, D-FSH stimulated greater number of follicles on D7 (18.6 $\pm$ 1.1, $P < 0.01$), compared to C-FSH (13.2 $\pm$ 0.8). In addition, there was tendency for D-FSH to induce a lower ovulatory response, compared to C-FSH, respectively (70.4 $\pm$ 3.0% vs. 78.3 $\pm$ 2.6%, $P = 0.08$). Estrus expression was similar ($P = 0.73$), totaling 68.7% (44/64) for D-FSH and 68.7% (46/67) for C-FSH. D-FSH group produced more CL per donor, as well as tended to have a greater number of ova and embryos than C-FSH (Table 17). However, no other variables were affected by the regimens.

Considering only cows with ≥ 2 structures recovered (Table 18), the number of CL was greater for D-FSH group, as well as the number of oocytes and embryos and the number of DG. Nevertheless, the number and percentage of transferable and freezable embryos, the number and percentage of UFO and the percentage of DG were not influenced by the regimens.

Table 17. Ovarian response, recovery rate, and mean total and transferable structures per cow, according to the regimen of FSH, considering all donors (all experiments).

Item	C-FSH (n = 67)	D-FSH (n = 64)	<i>P-value</i>
Corpora lutea, n ¹	10.4 $\pm$ 0.6	13.1 $\pm$ 0.9	0.01
Recovery rate, %	46.7 $\pm$ 3.6	48.6 $\pm$ 3.9	0.84
Oocytes and embryos, n ¹	4.9 $\pm$ 0.5	6.7 $\pm$ 0.7	0.08
Transferable embryos, n ²	2.5 $\pm$ 0.3	3.0 $\pm$ 0.4	0.43
Transferable embryos, %	48.3 $\pm$ 4.7	45.9 $\pm$ 4.9	0.72
Cows with ≥ 2 transf. embryos, % (n/n)	55.2 (37/67)	60.9 (39/64)	0.63

Values presented as mean $\pm$ SEM.

Abbreviations: C-FSH = constant doses of FSH; D-FSH = decreasing doses of FSH.

¹One datum from D-FSH, ²two observations from C-FSH and three from D-FSH were considered outliers and were excluded from the analyses.

Table 18. Ovarian response, recovery rate and mean embryo characteristics per cow, according to the regimen of FSH, considering only donors with ≥ 2 structures recovered (all experiments).

Item	C-FSH (n = 54)	D-FSH (n = 53)	<i>P-value</i>
Corpora lutea, n ¹	11.0 $\pm$ 0.6	14.7 $\pm$ 0.8	< 0.01
Recovery rate, %	57.1 $\pm$ 3.1	56.5 $\pm$ 3.7	0.92
Oocytes and embryos, n ²	6.0 $\pm$ 0.5	8.0 $\pm$ 0.7	0.01
Transferable embryos, n	3.2 $\pm$ 0.3	4.1 $\pm$ 0.5	0.40
Transferable embryos, %	56.3 $\pm$ 4.7	55.5 $\pm$ 4.9	0.62
Freezable embryos, n ¹	2.6 $\pm$ 0.3	3.4 $\pm$ 0.4	0.19
Freezable embryos, %	45.1 $\pm$ 4.6	51.1 $\pm$ 4.9	0.56
Unfertilized oocytes, n ¹	1.7 $\pm$ 0.3	2.2 $\pm$ 0.4	0.76
Unfertilized oocytes, % ³	24.7 $\pm$ 3.9	25.5 $\pm$ 4.4	0.78
Degenerate embryos, n	1.1 $\pm$ 0.2	2.1 $\pm$ 0.5	0.03
Degenerate embryos, % ⁴	15.8 $\pm$ 2.7	17.0 $\pm$ 2.8	0.33

Values presented as mean $\pm$ SEM.

Abbreviations: C-FSH = constant doses of FSH; D-FSH = decreasing doses of FSH.

Thirteen embryo collections from C-FSH and 11 from D-FSH with ≤ 1 structure recovered were excluded from the analyses.

¹Two data from D-FSH, ²one datum from D-FSH, ³two data from C-FSH and ⁴two data from C-FSH and one datum from D-FSH were considered outliers and were excluded from the analyses.

In the descriptive analyses, FSH regimens showed distinct patterns of the frequency distribution of CL, ova and or embryos, and number of transferable embryos, either for all donors or for donors with ≥ 2 structures recovered (Figures 2 to 7). Values of standard deviation, variance and coefficient of variance indicated great variability for D-FSH (no statistical analyses performed).

Figure 2. Frequency distribution of number of corpora lutea (CL), according to the FSH regimen, considering all donors (all experiments). SD = standard deviation; Var. = variance; CV = coefficient of variation.

Figure 3. Frequency distribution of number of ova/embryos, according to the FSH regimen, considering all donors (all experiments). SD = standard deviation; Var. = variance; CV = coefficient of variation.

Figure 4. Frequency distribution of number of transferable embryos, according to the FSH regimen, considering all donors (all experiments). SD = standard deviation; Var. = variance; CV = coefficient of variation.

Figure 5. Frequency distribution of number of corpora lutea (CL), according to the FSH regimen, considering donors with ≥ 2 structures recovered (all experiments). SD = standard deviation; Var. = variance; CV = coefficient of variation.

Figure 6. Frequency distribution of number of ova/embryos, according to the FSH regimen, considering donors with ≥ 2 structures recovered (all experiments). SD = standard deviation; Var. = variance; CV = coefficient of variation.

Figure 7. Frequency distribution of number of transferable embryos, according to the FSH regimen, considering donors with ≥ 2 structures recovered (all experiments). SD = standard deviation; Var. = variance; CV = coefficient of variation.

2.3.5.4 Effect of fertility categories of SexedULTRA 4M on embryo outcomes and sperm traits (Experiments 2, 3, and 4)

The fertility categories did not affect the number of CL, recovery rate and embryo outcomes of all donors (Table 19). Nonetheless, for cows with ≥ 2 structures recovered (Table 20), UF provided greater number of transferable embryos, number and percentage of freezable embryos, as well as a tendency for greater percentage of transferable embryos. The number and percentage of unfertilized oocytes were lower for UF sires; however, no differences were found in DG between groups.

Table 19. Ovarian response, recovery rate, and mean total and transferable structures per cow, according to the SexedULTRA 4M field fertility sires, considering all donors (Experiments 2, 3, and 4).

Item	NUF (n = 48)	UF (n = 48)	<i>P-value</i>
Corpora lutea, n ¹	12.5 ± 1.1	10.7 ± 0.8	0.14
Recovery rate, %	50.7 ± 4.5	44.4 ± 4.4	0.17
Oocytes and embryos, n ²	6.2 ± 0.7	5.7 ± 0.7	0.28
Transferable embryos, n	2.4 ± 0.4	3.4 ± 0.5	0.40
Transferable embryos, % ²	33.3 ± 5.0	46.7 ± 5.4	0.29
Cows with ≥ 2 transf. embryos, % (n/n)	47.9 (23/48)	62.5 (30/48)	0.29

Values presented as mean ± SEM.

Abbreviations: NUF = non-ULTRA Fertility sires; UF = ULTRA Fertility sires.

¹One datum from UF and ²one from NUF were considered outliers and were excluded from the analyses.

Table 20. Ovarian response, recovery rate, and mean embryo characteristics per cow, according to the SexedULTRA 4M field fertility sires, considering only donors that had ≥ 2 structures recovered (Experiments 2, 3 and 4).

Item	NUF (n = 39)	UF (n = 37)	<i>P</i> -value
Corpora lutea, n	14.1 $\pm$ 1.1	13.1 $\pm$ 1.1	0.34
Recovery rate, %	58.7 $\pm$ 4.2	56.7 $\pm$ 3.8	0.33
Oocytes and embryos, n ¹	7.4 $\pm$ 0.7	7.3 $\pm$ 0.7	0.26
Transferable embryos, n ¹	2.6 $\pm$ 0.4	4.4 $\pm$ 0.5	0.05
Transferable embryos, %	41.8 $\pm$ 5.5	60.6 $\pm$ 5.0	0.08
Freezable embryos, n ¹	2.1 $\pm$ 0.4	4.0 $\pm$ 0.5	0.01
Freezable embryos, %	32.0 $\pm$ 5.2	55.1 $\pm$ 4.9	0.01
Unfertilized oocytes, n	3.1 $\pm$ 0.6	1.2 $\pm$ 0.3	0.02
Unfertilized oocytes, %	38.1 $\pm$ 5.4	15.6 $\pm$ 3.5	0.01
Degenerate embryos, n	2.3 $\pm$ 0.7	1.6 $\pm$ 0.3	0.38
Degenerate embryos, %	21.2 $\pm$ 3.7	23.6 $\pm$ 4.4	0.73

Values presented as mean $\pm$ SEM.

Abbreviations: NUF = non-ULTRA Fertility sires; UF = ULTRA Fertility sires.

Nine embryo collections from NUF and 11 from UF with ≤ 1 structure recovered were excluded from the analyses.

¹One datum from NUF was considered outlier and was excluded from the analyses.

Finally, UF bulls (n = 48) had a greater percentage of sperm with progressive motility compared to NUF (n = 48), with no differences found for vigor, respectively (55.3 $\pm$ 1.6% vs. 46.4 $\pm$ 1.9, P = 0.02; 3.5 $\pm$ 0.1 vs. 3.2 $\pm$ 0.1, P = 0.36). The linear regression analysis (n = 71) indicated a discrete effect of sperm progressive motility on the number of transferable embryos (P = 0.01) for cows with ≥ 2 structures recovered, however the relationship is low (R^2 = 0.06).

2.4 Discussion

This study investigated the effects of either constant or decreasing doses of FSH in follicular superstimulation and embryo production, showing that the latter stimulated more follicles to grow, however both provided similar embryo yield and viability. Moreover, when we evaluated SexedULTRA 4M field fertility categories for *in vivo* embryo production, there was substantial variability among sires. To our knowledge, this is the study that directly

compared decreasing vs. constant FSH doses for SOV with a larger number of experimental units (131 embryo collections). In addition, this seems to be the first study that evaluated sire effect based on field fertility for *in vivo* embryo production, using this new sorting technology.

Based on the data from Experiments 1 and 2, the “Super Double-Ovsynch” protocol was effective in synchronizing and superovulating Holstein cows for *in vivo* embryo production. It is important to highlight that a double dose of GnRH (50 µg leirelin) was applied at the beginning of the presynchronization (D-16, at a random day of the estrous cycle) and, again, at the beginning of the superovulation period (D0). Considering a possible elevated circulating P4 concentration during these periods, this adjustment aimed to increase the probability of ovulation, possibly through a greater LH release (Giordano et al., 2012). However, the real need of such high dose for this specific GnRH analogue requires further investigation.

In our study, the number of CL during SOV (< 2 or 2) did not affect the ovarian response and embryo viability. Although no hormonal assays for P4 concentration were performed in this research, it is possible to infer that, based on the number of CL during SOV and their ages, the majority of follicles developed under high P4 levels. More than 70% of the donors had two corpora lutea. The remaining cows that ovulated only on D-6 (16.3%; 14/86) had one 7 d old CL at the beginning of FSH treatments, whereas only 11.6% (10/86) ovulated only after the D0 GnRH treatment and started the superstimulation under low P4. In parallel, the insertion of a P4 device proved not to be necessary, since only two donors had premature estrus and ovulation during SOV. In Holstein cows, P4 has an important role during follicular growth. Higher concentrations of P4 reduce LH pulse frequency, preventing premature oocyte maturation (Savio et al., 1993), and may be associated with better quality oocytes and embryos (Ahmad et al., 1995). Rivera et al. (2011) reinforced P4 importance, superstimulating follicles either during the first follicular wave (FFW), FFW with the insertion of two intravaginal devices (FFWP) or during the second follicular wave (SFW), to produce follicle growth under variable P4 concentrations. A greater percentage of embryos classified as transferable ($P = 0.02$) was found for the FFWP and SFW, compared with FFW (lower circulating P4).

Even though this study did not aim to test the response to presynchronization, an interesting finding was that donors that either ovulated or did not ovulate after the GnRH treatment on D0 had a similar ovarian response and embryo yield. Some classical studies demonstrated that initiating FSH treatments near the time of wave emergence improved the

superstimulatory response (Nasser et al., 1993; Adams, 1994), and that presence of a dominant follicle could impair development and ovulation of multiple follicles after FSH treatment (Guilbault et al., 1991; Kim et al., 2001). On the other hand, other studies (Donaldson, 1984; Gray et al., 1992) have demonstrated a similar efficiency in SOV programs, either when treatment with FSH started at the onset of a new follicular wave or after the establishment of follicle dominance, corroborating our results. Despite the lack of effect of ovulation on D0, it is important to be cautious and consider that only 16.3% of the cows (14/86) in our study did not ovulate on D0. In addition, there was no evaluation regarding the exact time of the follicular phase the cows were when superstimulation was initiated. Thus, more experiments designed to test whether it is really necessary to synchronize follicle wave emergence prior to SOV are still needed.

The first hypothesis of the present study, suggesting similar responses regardless of FSH regimens, was partially confirmed. Firstly, it is worth mentioning that two approaches of analyses were considered in this study. Results of embryo collections from all donors were essential to evaluate the overall ovarian response related to treatments, whereas, results of embryo collections from donors with at least two structures recovered were important to evaluate the effect of treatments on embryo quality, as well as on fertilization rates. Decreasing doses of FSH promoted superior superstimulatory effect in all experiments, as evidenced by the greater number of medium to large follicles on the day of ovulation induction. Berlinguer et al. (2004) superstimulated ewes either with four constant ($n = 6$) or decreasing doses ($n = 6$) for OPU and found superior results on the number of follicles $> 5\text{mm}$ for the latter regimen ($P < 0.01$). However, the blastocyst rate and other quality traits were inferior ($P < 0.01$), compared to constant FSH. Similar to Berlinguer et al. (2004) findings, it is possible that, in our study, the first higher doses of FSH given in D-FSH regimen could have stimulated the growth of a greater number of follicles in the superstimulated wave. Thus, at the end of the protocol, there were more follicles $\geq 8.5\text{ mm}$ for D-FSH. However, our approach is not able to explain if this result was caused by a greater follicular growth rate. Also, the D-FSH group had a lower ovulatory response in Experiment 2. However, the combined results only showed a tendency for this variable. Since D-FSH regimen produced more follicles on the day of GnRH, it is possible that circulating concentrations of estradiol were higher in this group and could have, somehow, impaired the ovulatory response, even though there was no hormone assay performed to confirm this hypothesis. Similar to our results, lactating Holstein cows receiving 400 mg FSH had superior superstimulatory response and inferior ovulation rate, compared to cows receiving 200 mg FSH, with similar embryo

yield (Alexandre Souza, personal communication). Despite the tendency for lower ovulatory response, the D-FSH regimen still produced a greater number of CL on the day of embryo collections, and a tendency (all cows) or greater number (cows with ≥ 2 structures recovered) of ova and embryos recovered. Similarly, Chupin and Procureur (1983) showed overstimulation (more than 20 CL) of 23 vs. 7% ($P < 0.10$), being higher for decreasing doses ($n = 26$) than for constant doses ($n = 28$), respectively, in lactating cows. Independent of the greater number of follicles and CL for D-FSH, the number and percentage of transferable or freezable embryos were similar for both regimens. Therefore, either D-FSH or C-FSH showed similar potential to produce good quality embryos. The percentage of cows producing two or more transferable embryos was also similar. Contrasting with our results, some studies showed detrimental effects of D-FSH on embryo traits (Sirard et al., 1999, in cattle; Berlinguer et al., 2004, in sheep), whereas others demonstrated superior results (Chupin and Procureur, 1983; Takahashi and Kanagawa, 1985, in cattle), or, yet, similar outcomes (Blondin et al., 1997, in cattle). Related to embryo quality, the only difference found between groups was the number of DG, being greater for D-FSH. However, the greater number of ova and/or embryos recovered in this group can explain this difference. An interesting finding regarding doses was the pattern of transferable embryos obtained with the FSH regimens. Based only on descriptive results, treatment with D-FSH seemed to have more variability, since embryo collections that yield numerically greater number of transferable embryos are more spread around the mean. Whereas, the C-FSH regimen seemed to produce a more constant pattern of transferable embryos, with great part of them around the mean (no statistical analysis performed).

Relative to estrus, although fixed-time protocols for AI or ET no longer require its detection, its expression (related to physiological estradiol concentrations) is linked to improved results. Cows that display estrus produce embryos of better quality (Larimore et al., 2015) and have greater P/AI in FTAI protocols (Pereira et al., 2016), as well as greater P/ET in FTET programs (Jinks et al., 2013; Pereira et al., 2016). Moreover, greater intensity of estrus was also associated with greater embryo viability in superovulated heifers (Madureira et al., 2020). In the present study, donors that displayed estrus produced more viable embryos. We may infer that oocytes were exposed to adequate concentrations of estradiol during their development, important for follicle and ova competence (Liu et al., 2017). This hormone also optimizes the transport of sperm and oocyte, potentially by altering uterine pH (reviewed by Pohler et al., 2012). DeJarnette et al. (1992) associated estrus display with a greater number of accessory sperm, and enhanced fertilization and embryo quality in AI cows. Even though this

evaluation was not performed in our study, another possible explanation for donors in estrus producing more embryos of high quality could be due to increased accessory sperm cells. All these previous observations are especially important for sex-sorted spermatozoa, that present lower viability (Carvalho et al., 2018), as well as for the altered uterine environment in SOV protocols (Kafi and McGowan, 1997). Finally, estradiol impacts physiological functions in oviduct and uterine epithelium and modulates the environment for embryo development (González-Bulnes et al., 2003). Regarding the protocol used in our experiments, it is important to consider that the newly formed CL from the ovulation to D0 perhaps did not fully regress, even though we applied prostaglandin three times at the end of the protocol (48 h from the first PGF to ovulation induction with GnRH). Thus, progesterone concentrations could have not decreased to lower levels and the residual could have potentially impaired estrus expression.

The hypothesis related to SexedULTRA 4M was partially supported. Considering the combined results of Experiments 2, 3, and 4, it is possible to confirm that SexedULTRA 4M field fertility sires significantly improved embryo production and viability. Moreover, SexedULTRA 4M produced almost four embryos per flush, regardless of fertility groups. Sex-sorted spermatozoa have been studied for production of the desired gender since the 80s (Seidel, 2014) and has a great impact for female production in dairy herds. The studies using this technology show wide variation in embryo production and, due to their different methodologies, is not possible to compare them directly. Regardless of methodologies, the more significant embryo results were obtained by Soares et al. (2011) producing, on average, 6.4 transferable embryos, by Mikkola and Taponen (2017) achieving 5.4 and by Kaimio et al. (2013) producing 4.9. The similar and consistent findings among all these studies were the lower embryo viability and the greater production of DG and UFO, compared to non-sorted spermatozoa (Sartori et al., 2004; Peippo et al., 2009). The standard sex-sorting process involved many steps and manipulations consisting in dilutions, staining, laser exposure, sorting with a sheath fluid under high speed, holding cells, centrifugation and, finally, cryopreservation at low concentrations (Gonzalez-Marin et al., 2017). These processes are related to damages in sperm cells, such as plasma membrane and acrosome alterations (Carvalho et al., 2013, 2018), as well as early capacitation (Bucci et al., 2012), leading to reduced viability and diminishing embryo production and quality. Considering these implications, SexedULTRA was developed through adjustments in the sorting process and it was successful in improving *in vitro* sperm parameters, compared to the older process (Gonzalez-Marin et al., 2017) or in maintaining similar characteristics, compared to

conventional spermatozoa (González-Marín et al., 2018). This new technology has already been used for *in vitro* embryo production (Gonzalez-Marin et al., 2017), for AI following estrus (Maicas et al., 2020) or FTAI in cattle (Lenz et al., 2017; Thomas et al., 2017, 2019), as well as in small ruminants (González-Marín et al., 2021). In SOV, as far as we know, the only published study (Dell'Eva et al., 2019) inseminated lactating Holstein cows three times with straws of two million spermatozoa each, from three bulls, and produced 3.6 transferable embryos (49.6%), on average. Furthermore, the mean percentage of UFO was 33.9%, independent of treatments. In the present study, the number and percentage of transferable embryos were similar to Schenk et al. (2006) findings, using semen from standard sorting, and to Dell'Eva et al. (2019) results, using SexedULTRA 2M. Our study was not able to detect any effect of the sires with greater field fertility in embryo production considering all donors. Nevertheless, considering the donors that yielded ≥ 2 embryos, there was a remarkably effect on embryo yield. The mean number (2.1 ± 0.3) and percentage ($27.1 \pm 3.5\%$) of UFO, regardless field fertility sires, can be considered low compared to rates from other studies using sex-sorted spermatozoa. Furthermore, especially the UF sires had much lower percentage of UFO compared to unselected sires (NUF). Apparently, based on these results, SexedULTRA technology was able to present promising effects in preserving sperm viability.

An interesting finding was the effect of sire fertility within individual experiments. A superior fertility of UF was found especially in Experiment 2. In Experiments 3 and 4, field fertility categories had no influence on embryo outcomes. The sires used in Experiment 2 maintained the pattern of the field fertility on embryo production results, whereas those used in Experiments 3 and 4 had variable patterns. There were bulls classified as NUF that produced good embryo results. In contrast, there were bulls classified as UF that had low embryo yield. It is important to recognize two factors that could have influenced these contrasting results: First, recovery rate in Experiments 3 and 4 was lower than recovery in Experiment 2. Second, we were not able to know whether NUF sires used in experiments 3 and 4 had their evaluation of field fertility completed at the time of this research. That is, some sires may have the potential to enter the UF category subsequently. Therefore, it is not possible to clearly define which factors influenced the results of Experiments 3 and 4. Finally, combining all results, UF still provided superior embryo traits than NUF.

When undergoing SOV protocols, the donor has an important effect on embryo production; however, sires also exert great individual influence, as can be seen in our data. Several other studies also reported this variability among sires not only for SOV. In an elegant

study, DeJarnette et al. (2011) reported a wide range in fertility of Holstein heifers inseminated following estrus with sex-sorted or conventional semen, produced by the same ejaculates. Using SexedULTRA 4M, Thomas et al. (2019) inseminated beef cows at fixed-time with conventional or sex-sorted sperm, from contemporaneous ejaculates, and showed wide variability among bulls. Maicas et al. (2020) have also shown this high variability on fertility results among sires, inseminating dairy cows and heifers following estrus, using the same ejaculate for production of both types of semen. Some sires have similar P/AI regardless of type of semen, whereas others have lower results using sex-sorted semen compared to conventional semen. In SOV protocols, Ortega et al. (2018) inseminated dairy heifers with non-sorted semen from a small number of low (LF) or high fertility bulls (HF). Although the overall percentage of UFO was lower and DG was fewer for HF, there was substantial variation among bulls and, overall, LF sires had similar results as HF. In contrast, O'Callaghan et al. (2021) inseminated superovulated beef heifers with semen from HF or LF bulls and found no difference in fertilization rate, but more embryos in advanced stage of development, as well as greater mean cell number and accessory sperm for HF bulls. Therefore, similar to these previous studies, SexedULTRA 4M sires used in the current study, also demonstrated fertility variations in SOV protocols. Knowing this variation among bulls, efforts have been made to understand the basis behind it and to develop tests for the identification of high fertility sires (Fair and Lonergan, 2018; Butler et al., 2020). However, standard evaluations have not been successful, suggesting that, possibly, a multifactorial approach is required.

In conclusion, even though decreasing doses of FSH induced a greater superstimulatory effect, both regimens were able to produce transferable and freezable embryos with similar efficiency. However, the pattern of embryo production was distinct between doses. Moreover, although overall results showed that UF sires were superior in embryo traits, the conclusion regarding their use in SOV protocols is not yet definitively shown and this gap requires further investigation.

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3. FINAL CONSIDERATIONS

Findings from the present study provided valuable information regarding the pattern of embryo production in Holstein cows superstimulated with distinct FSH regimens, and demonstrated the similar potential to produce *in vivo* viable embryos either with constant or decreasing doses. Furthermore, distinct patterns of embryo production were also found for SexedULTRA 4M sires used in this study, with remarkably superior embryo yield for sires classified as higher fertility sires.

Finally, the Master's period was a growing and extremely valuable challenge. The understanding of reproductive physiology required a deep and constant effort, which led to an analysis of personal and professional strengths and weaknesses on a daily basis. This period was essential to work on critical thinking, to develop important studies as a team and to open brand new opportunities.

APPENDICES

APPENDIX A

Ovarian response and embryo characteristics, according to individual sires of SexedULTRA 4M field fertility, considering donors with ≥ 2 structures recovered (Experiment 2).

Item	NUF sires				UF sires		<i>P-value</i>
	A (n = 7)	B (n = 8)	C (n = 6)	D (n = 7)	E (n = 7)	F (n = 5)	
Corpora lutea, n	15.0 $\pm$ 1.5	13.9 $\pm$ 2.0	10.5 $\pm$ 2.1	13.4 $\pm$ 1.8	11.7 $\pm$ 1.5	14.2 $\pm$ 2.3	0.62
Recovery rate, %	56.2 $\pm$ 11.0	53.9 $\pm$ 8.3	79.6 $\pm$ 6.9	68.1 $\pm$ 9.0	79.5 $\pm$ 7.5	56.7 $\pm$ 5.5	0.34
Oocytes and embryos, n	8.0 $\pm$ 1.7	7.4 $\pm$ 1.4	8.5 $\pm$ 2.0	8.7 $\pm$ 1.6	9.6 $\pm$ 1.7	8.4 $\pm$ 2.1	0.95
Transferable embryos, n	1.1 $\pm$ 0.5 ^b	3.0 $\pm$ 1.0 ^{ab}	2.3 $\pm$ 1.1 ^{ab}	5.0 $\pm$ 0.6 ^{ab}	6.3 $\pm$ 1.2 ^a	6.0 $\pm$ 1.6 ^{ab}	0.02
Transferable embryos, %	18.9 $\pm$ 8.3 ^b	42.5 $\pm$ 12.4 ^{ab}	31.3 $\pm$ 13.4 ^{ab}	62.5 $\pm$ 6.9 ^{ab}	73.2 $\pm$ 10.6 ^a	71.8 $\pm$ 14.6 ^a	< 0.01
Freezable embryos, n	1.1 $\pm$ 0.5 ^b	2.5 $\pm$ 0.9 ^{ab}	2.2 $\pm$ 1.2 ^{ab}	5.0 $\pm$ 0.6 ^a	5.1 $\pm$ 1.0 ^a	5.4 $\pm$ 1.5 ^a	0.02
Freezable embryos, %	18.9 $\pm$ 8.3 ^b	34.7 $\pm$ 12.9 ^{ab}	25.7 $\pm$ 14.3 ^{ab}	62.5 $\pm$ 6.9 ^a	64.0 $\pm$ 12.2 ^a	63.8 $\pm$ 12.8 ^a	0.01
Unfertilized oocytes, n	6.1 $\pm$ 1.7 ^a	3.1 $\pm$ 1.1 ^{ab}	4.0 $\pm$ 1.9 ^{ab}	1.6 $\pm$ 0.6 ^b	0.7 $\pm$ 0.6 ^b	1.4 $\pm$ 0.9 ^b	< 0.01
Unfertilized oocytes, %	71.6 $\pm$ 11.0 ^a	43.7 $\pm$ 14.5 ^{ab}	46.4 $\pm$ 13.4 ^{ab}	15.8 $\pm$ 5.1 ^b	5.4 $\pm$ 4.1 ^b	19.8 $\pm$ 15.5 ^b	< 0.01
Degenerate embryos, n	0.7 $\pm$ 0.3	1.3 $\pm$ 0.5	2.2 $\pm$ 0.7	2.1 $\pm$ 0.7	2.6 $\pm$ 1.1	1.0 $\pm$ 0.6	0.32
Degenerate embryos, %	9.5 $\pm$ 3.9	13.9 $\pm$ 6.0	22.3 $\pm$ 6.4	21.8 $\pm$ 6.0	21.4 $\pm$ 6.9	8.5 $\pm$ 5.9	0.32

Values presented as mean $\pm$ SEM.

Abbreviations: NUF = non-ULTRA Fertility sires (bulls A, B, and C); UF = ULTRA Fertility sires (bulls D, E, and F).

APPENDIX B

Ovarian response and embryo characteristics, according to individual sires of SexedULTRA 4M field fertility, considering donors with ≥ 2 structures recovered (Experiments 3 and 4).

Item	NUF sires				UF sires		<i>P-value</i>
	A (n = 7)	B (n = 6)	C (n = 5)	D (n = 6)	E (n = 6)	F (n = 6)	
Corpora lutea, n	14.4 $\pm$ 3.3	19.0 $\pm$ 4.0	11.0 $\pm$ 2.4	11.5 $\pm$ 1.9	17.2 $\pm$ 5.4	11.0 $\pm$ 1.5	0.14
Recovery rate, %	53.5 $\pm$ 9.5	53.5 $\pm$ 11.6	58.3 $\pm$ 15.4	34.7 $\pm$ 6.7	50.8 $\pm$ 5.8	44.8 $\pm$ 7.6	0.26
Oocytes and embryos, n ¹	5.3 $\pm$ 1.2	9.5 $\pm$ 2.7	5.4 $\pm$ 1.6	4.0 $\pm$ 1.2	7.8 $\pm$ 1.7	4.7 $\pm$ 0.9	0.15
Transferable embryos, n ²	3.7 $\pm$ 1.2	1.4 $\pm$ 0.8	4.0 $\pm$ 1.1	1.3 $\pm$ 0.6	5.5 $\pm$ 1.5	2.3 $\pm$ 0.7	0.15
Transferable embryos, %	45.8 $\pm$ 5.2	41.1 $\pm$ 19.4	80.9 $\pm$ 11.7	39.2 $\pm$ 17.3	67.3 $\pm$ 4.8	49.4 $\pm$ 15.7	0.28
Freezable embryos, n ²	2.9 $\pm$ 1.2 ^{ab}	2.6 $\pm$ 2.4 ^{ab}	2.6 $\pm$ 1.0 ^{ab}	1.2 $\pm$ 0.5 ^b	4.8 $\pm$ 1.6 ^a	2.3 $\pm$ 0.7 ^{ab}	0.01
Freezable embryos, % ³	34.5 $\pm$ 6.0	23.1 $\pm$ 15.0	60.9 $\pm$ 18.6	35.0 $\pm$ 16.1	54.8 $\pm$ 7.6	59.3 $\pm$ 15.0	0.11
Unfertilized oocytes, n	0.4 $\pm$ 0.2	2.7 $\pm$ 1.2	1.6 $\pm$ 0.9	1.5 $\pm$ 1.1	1.5 $\pm$ 0.2	0.5 $\pm$ 0.5	0.38
Unfertilized oocytes, %	11.0 $\pm$ 6.0	27.2 $\pm$ 8.8	21.9 $\pm$ 10.9	21.4 $\pm$ 11.4	21.1 $\pm$ 2.7	12.5 $\pm$ 12.5	0.27
Degenerate embryos, n ¹	1.7 $\pm$ 0.6	3.7 $\pm$ 2.0	1.2 $\pm$ 1.2	1.2 $\pm$ 0.4	0.7 $\pm$ 0.2	1.8 $\pm$ 0.9	0.34
Degenerate embryos, %	27.7 $\pm$ 6.7	32.6 $\pm$ 11.6	17.1 $\pm$ 17.1	39.4 $\pm$ 16.3	10.5 $\pm$ 3.9	38.1 $\pm$ 16.8	0.42

Values presented as mean $\pm$ SEM.

Abbreviations: NUF = non-ULTRA Fertility sires (bulls A, B, and C); UF = ULTRA Fertility sires (bulls D, E, and F).

¹One datum from sire A, ²one datum from sire B and ³one datum from sire F were considered outliers and were excluded from the analyses.