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Donor category and seasonal climate associated with embryo production and survival in multiple ovulation and embryo transfer programs in Holstein cattle

L.M. Vieira [a], C.A. Rodrigues [b], M.F. Mendanha [a], M.F. Sá Filho [a], J.N.S. Sales [c], A.H. Souza [d], J.E.P. Santos [e], P.S. Baruselli [a,*]

[a] Departamento de Reprodução Animal, FMVZ-USP, São Paulo, Brazil
[b] Clínica Veterinária SAMVET, São Carlos, São Paulo, Brazil
[c] Centro de Ciências Agrárias, UFPB, Areia, Paraiba, Brazil
[d] University of California Cooperative Extension, Tulare, California, USA
[e] Department of Animal Sciences, University of Florida, Gainesville, USA

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ABSTRACT

The present study investigated the effect of Holstein donor category (cows vs. heifers) and climate variation (hot vs. cooler season) on the efficiency of *in vivo* embryo production programs as well as embryo survival after transferred to Holstein recipient cows. A total of 1562 multiple ovulation (MO) procedures (cows: n = 609, and heifers: n = 953) and 4076 embryo transfers (ETs) performed in two dairy herds were evaluated. Donor cows had greater number of CLs (10.6 ± 0.6 vs. 7.5 ± 0.4; P < 0.0001) and ova/embryos recovered (7.6 ± 0.6 vs. 4.6 ± 0.4; P < 0.0001) compared with donor heifers. However, fertilization rate (47.9 vs. 82.4%; P < 0.0001) and proportion of transferable embryos (31.5 vs. 67.4%; P < 0.0001) were lower in donor cows than heifers, respectively. Regardless of donor category, the proportion of freezable embryos was less (P < 0.001) during hot season than in cooler season (21.4 vs. 32.8%). However, greater decline in the proportion of freezable embryos during the hot season was observed in cows (21.7 vs. 10.7%) compared with heifers (46.2 vs. 38.1%; P = 0.01). In contrast, the season on which the embryo was produced (hot or cool) did not affect pregnancy rate on Day 31 (30.5 vs. 31.7%; P = 0.45) and 45 (25.3 vs. 25.1%; P = 0.64) of pregnancy. Regardless of the season in which the embryos were produced, embryonic survival after transferring embryos retrieved from donor cows was greater on Days 31 (36.0 vs. 30.7%; P = 0.001) and 45 (28.3 vs. 23.1%; P = 0.001) of pregnancy when compared with embryos from donor heifers. In conclusion, MO embryo production efficiency decreased during the hot seasons both in cows and heifers; however, the decline was more pronounced in donor cows. Regardless of the embryo source, similar pregnancy rate was observed in the recipient that received embryos produced during the hot and cooler seasons. Curiously, embryos originating from donor cows had higher embryonic survival when transferred to recipient cows than embryos originating from heifers.

1. Introduction

The multiple ovulation (MO) technique for *in vivo* embryo production and embryo transfer (ET) programs has been an important breeding technology applied to accelerate the gain in genetic progress and, consequently,

* Corresponding author. Tel.: +55 11 3091 7674; fax: +55 11 3091 7412.
E-mail address: barusell@usp.br (P.S. Baruselli).

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Table 1
Descriptive statistics of multiple ovulation (MO) response, *in vivo* embryo production, and embryonic survival after embryo transfer (ET) performed between the years of 2007 and 2010.

Items	No. of MO protocols	No. of ET	Items	No. of ET
Total	*1562*	*4076*	Embryo stage development[c]	
Farm			Morula	2224
A	1354	4030	Early blastocyst	983
B	208	46	Blastocyst	760
Year			Expanded blastocyst	109
2007	272	787	Embryo quality[c]	
2008	503	951	Grade 1	662
2009	374	1007	Grade 2	2202
2010	413	1331	Grade 3	1212
Embryo source			Number of CL at ET	
Heifer	953	2314	1	3750
(Warmer/cooler season)[a]	*(354/599)*	*(596/1718)*	≥2	326
Cows	609	1762	CL size at ET[d]	
(Warmer/cooler season)	*(270/339)*	*(576/1186)*	Good	2249
Season of embryo production			Fair	1676
Warmer season	624	1172	Poor	151
(Heifer/cow)[b]	*(354/270)*	(596/576)	Season of TE	
Cooler season	938	2904	Warmer season	2012
(Heifer/cow)	*(599/339)*	*(1718/1186)*	(Heifer/cow)[e]	*(1205/807)*
Embryo status			Cooler season	2064
Fresh	–	2279	(Heifer/cow)	*(1109/955)*
Freeze-thaw	–	1797	Sires	36

[a] Within embryo source (heifer or cow), number of MO processes performed during the warmer and cooler season.
[b] Within season of embryo production (warmer or cooler), number of MO processes performed in heifer or cow donors.
[c] Classification according to International Embryo Transfer Society [26].
[d] Good: CL represents 75% of the ovary volume; fair: 50% of the ovary volume; and poor: 25% of the ovary volume.
[e] Within season of ET (warmer or cooler), number of ET performed with embryos from heifer and cow donors.

improve herd productivity. During the last years, the world production of *in vivo* embryos presented an increase of 4.4% from 2009 to 2011, with a total of 732,862 viable embryos produced in 2011 [1]. Intriguingly, although MO has been used over the past 40 to 50 years [2], the overall efficiency of this method has remained fairly stable, with an average of 6.2 embryos being produced per uterine flush with no signs of recent improvements in embryo yield [1].

The variability in response to MO treatments has been an important commercial limitation [3] due to various multifactorial reasons [2,4–6]. Researchers reported lower fertilization rate [7], lower proportion of viable embryos [8,9], and greater number of unfertilized oocytes [7,9] in lactating cows undergoing MO compared with heifers. In addition, studies with *in vitro* embryo production in heat-stressed donors have reported fewer viable embryos as well as greater proportion of embryos with delayed development [10,11]. Collectively, it appears that embryo production and quality in dairy cattle is affected by donor category and heat stress.

Several studies have shown that ET can be applied to dairy herds to improve fertility compared with artificial insemination (AI), particularly during the hot months of the year [12–19]. In addition, embryo transfer yields satisfactory pregnancy results especially in females classified as repeat breeder recipients [16,20], that is, females with four or more AI. Therefore, pregnancy outcome after ET according to the season and/or recipient category has been extensively studied; however, few researches [13,21,22] have focused on the effect of the embryo donor category (cow or heifer) as a potential factor affecting the recipients' pregnancy maintenance, especially when working strictly with Holstein females.

Therefore, the main objective of this study was to evaluate the effect of the Holstein embryo donor category (cow or heifer) on the recipients pregnancy rate, and, second, the effects of nuisance variables such as climate and donor category that could be associated with embryo production. We hypothesized that the heifer donors would produce embryos with greater quality, and therefore, result in increased pregnancy rate after ET. However, the embryo production of both donor categories would be negatively affected during the warmer period.

2. Materials and methods

2.1. Farms and managements

The study evaluated data from 1562 MO procedures (lactating cows: n = 609, and heifers: n = 953) and 4076 embryos transferred as fresh (n = 2279) or frozen/thawed (n = 1797) into lactating Holstein recipients. The procedures were carried out in two commercial dairy herds in southwestern Brazil (farm A = 22 01′27′S/47 53′19″W, and farm B = 20 32′19″S/47 24′03″W), both located in tropical humid climate. A total of 36 sires were used to breed donors undergoing MO. Multiple ovulation and ET records were collected from 2007 to 2010, and all the techniques were performed by a single veterinarian practitioner (Table 1).

Heifers were kept in pasture and lactating cows were housed in free stall facilities and milked three times daily. The free stall facilities and the milk parlor had fans and sprinklers activated when temperature was over 19 °C; once activated, the fans were put on for 24 hours per day and the sprinklers on/off cycle from 6 am to 10 pm, in a 4

minute on and 4-minute off cycle. The daily milk yield and days in milk during MO protocol and ET procedure were 25.8 ± 7.6 L/day and 346.8 ± 9.2 days for the lactating donors and 23.5 ± 5.8 L and 320.7 ± 2.3 days for the recipients. In addition, all lactating cows received 500 mg of recombinant bovine somatotropin (Boostin, MSD Animal Health, São Paulo, Brazil) every 12 days.

All enrolled cows and heifers received nutritionally balanced rations on the basis of corn silage as the main forage and corn and soybean meal-based concentrates, sufficient to meet or exceed the nutritional requirements for lactating dairy cows and heifers [23]. All techniques were approved by the Animal Welfare committee of the School of Veterinary Medicine and Husbandry of the University of São Paulo, Brazil (protocol number 2198/2011).

2.2. Superstimulatory treatments

Donors were treated with a synchronization protocol and gonadotropins followed by timed AI as previously described by Martins et al. [24]. The superstimulation and synchronization of ovulations (Fig. 1) consisted of the insertion of one (heifers) or two (cows) subcutaneous ear implants containing 3 mg of norgestomet each (Crestar, MSD Animal Health) and the intramuscular administration of 2 mg of estradiol benzoate (Gonadiol, MSD Animal Health). After 4 days, females received six treatments of follicle-stimulating hormone (heifers: 250, and cows: 500 IU porcine FSH equivalent to 25 mg of FSH; Pluset, Hertape Calier, Minas Gerais, Brazil) administered twice daily in decreasing doses (heifers: 55, 55, 42.5, 42.5, 27.5, 27.5 IU, and cows: 110, 110, 85, 85, 55, 55 IU) over a 3-day period. On the third day of FSH treatment, 0.265 mg of cloprostenol sodium ($PGF_{2\alpha}$; Ciosin, MSD Animal Health) was administrated at the same time of the fifth and sixth dose of FSH. In the morning of Day 7, 400 (heifers) and 450 IU (cows) of equine chorionic gonadotropin (Folligon, MSD Animal Health) was administrated, and during the afternoon the norgestomet ear implants were removed according to Mattos et al. [25]. In addition, a dose of 250 μg of gonadorelin (GnRH; Fertagyl, MSD Animal Health) was

A

	Donor heifers	
Days of treatment	**AM**	**PM**
0	One ear implant + EB (2 mg)	
4	FSH (55 IU)	FSH (55 IU)
5	FSH (42.5 IU)	FSH (42.5 IU)
6	FSH (27.5 IU) + Cloprostenol sodium (0.265 mg)	FSH (27.5 IU) + Cloprostenol sodium (0.265 mg)
7	eCG (400 IU)	Ear implant removal
8	Gonadorelin (250 μg)	TAI
9	TAI	
15	Uterine flush	

B

	Donor cows	
Days of treatment	**AM**	**PM**
0	Two ear implants + EB (2 mg)	
4	FSH (110 IU)	FSH (110 IU)
5	FSH (85 IU)	FSH (85 IU)
6	FSH (55 IU) + Cloprostenol sodium (0.265 mg)	FSH (55 IU) + Cloprostenol sodium (0.265 mg)
7	eCG (450 IU)	Ear implants removal
8		Gonadorelin (250 μg)
9	TAI	TAI
15		Uterine flush

Fig. 1. Superovulation protocol with timed artificial insemination (TAI) in Holstein heifer (A) and cow (B) donors.

administered 48 hours (cows) and 36 hours (heifers) after the last $PGF_{2\alpha}$, and cows were time inseminated 12 and 24 hours after GnRH injection. Embryo recovery was performed by nonsurgical uterine flush 7 days after the first timed AI.

On the day of the uterine flush, the number of CL was estimated by transrectal palpation to evaluate the number of CL before uterine flush, MO rate (proportion of donors with two or more CL divided by the total number of donors submitted to MO protocol), and recovery rate (proportion of ova/embryos recovered divided by total number of CL observed before the uterine flush).

2.3. Embryo evaluation, freezing, and transfer

The procedures were performed according to the guidelines of the International Embryo Transfer Society [26]. Recovered embryos (n = 4076) were kept in cell culture dishes containing holding solution TQC (AB Technology, Nutricell, Campinas, Brazil) during embryo evaluation (stage of development and quality) under stereoscope (×50). The embryos were classified according to stage of development (1 = unfertilized embryo, 4 = morula, 5 = early blastocyst, 6 = blastocyst, and 7 = expanded blastocyst) and quality (1 = excellent or good, 2 = fair, 3 = poor, and 4 = degenerated) according to International Embryo Transfer Society [26]. Therefore, embryos graded as 1, 2, and 3 were defined as transferable, and embryos with grades 1 and 2 were classified as freezable.

The total ova/embryos collected per donor was recorded to evaluate the following responses: fertilization (proportion of embryos grade 1 to 4 divided by total ova/embryo recovered), transferable embryos (proportion of embryos grade 1 to 3 divided by total ova/embryo recovered), freezable embryos (proportion of embryos grade 1 and 2 divided by total ova/embryo recovered), and degenerated embryos (proportion of embryos grade 4 divided by total ova/embryo recovered).

After classification, the fresh embryos were loaded into 0.25 mL straws (IMV, France) and kept at 25 °C until ET. Frozen embryos underwent freezing procedure with ethylene glycol TQC (AB Technology, Nutricell) using an automatic freezing machine (TK 2000, program P1-01, BOV/E/O1; TK and Nutricell, Campinas, Brazil). When the procedure was completed, the straws were transferred to liquid nitrogen (−196 °C) and stored until ET. During thawing before ET, the frozen straws were removed from liquid nitrogen, held at room temperature for 10 seconds, and then submerged in water at 25 °C for another 10 seconds. All embryos were transferred nonsurgically into the uterine horn ipsilateral to the CL (Table 1).

2.4. Recipient management

The embryo recipients were lactating Holstein cows with more than 150 days in milk or those considered as “repeat breeders” (three or more previous AI services). The recipient cows received an *in vivo*–produced embryo after a synchronization protocol for fixed-time ET or on detection of estrus, as previously described in detail by Rodrigues et al. [27]. Briefly, the synchronization program for recipients consisted of the insertion of an ear implant containing 3 mg of norgestomet and an intramuscular administration of 2 mg of estradiol benzoate. After 8 days, the ear implant was removed, and the females received 0.265 mg of cloprostenol sodium, 1.0 mg of estradiol cypionate (ECP, Zoetis Animal Health, São Paulo, Brazil), and 400 IU of equine chorionic gonadotropin. All embryos were transferred nonsurgically into the uterine horn ipsilateral to the CL at 9 days after ear implant removal. Alternatively, in recipients detected in natural estrus, embryos were transferred 6 to 8 days after estrus. All recipients received 100 μg of GnRH immediately before the ET.

On the day of ET, recipients were assessed by transrectal palpation for the presence of CL. A subjective estimation of the size and number of CL was performed. Only animals with at least one CL received an embryo [28]. The subjective CL size classification was performed by transrectal palpation [28,29] following the criteria used by the veterinarian (relationship between CL and ovary volume): good = when CL represented 75% of the ovarian volume; fair = 50% of the ovarian volume; and poor = 25% of the ovarian volume (Table 1).

2.5. Assessment of environmental temperature

The maximum daily temperature was recorded by a data logger-thermometer located on the farm. Maximum temperature records were used for retrospective analysis of climate conditions, as proposed by García-Ispierto et al. [30]. Therefore, because of the location and climate parameters of the farm (Table 2), months of the year were classified as warmer season, from November to May, and cooler season, from June to October (Table 1).

2.6. Pregnancy diagnosis and embryonic survival evaluation

Pregnancy diagnosis was performed by transrectal ultrasound at 24.5 ± 6.5 days after ET (31.5 ± 6.5 days of pregnancy; P/ET31). Real-time ultrasound scanner equipment with a 5-MHz linear transducer (Aloka SSD500, Japan) was used. The detection of an embryonic vesicle with a viable embryo (presence of heartbeat) was used as an indicator of pregnancy. The P/ET was calculated as the proportion of females pregnant at 31 days divided by the total number of females that received an embryo. Pregnant

Table 2
Mean monthly temperatures, RH, and monthly cumulative rainfall conditions during the study period (2007–2010).

Month	Minimum T (°C)	Maximum T (°C)	RH (%)	Rainfall (mm)
January	20.0	34.6	70.5	315.5
February	20.2	35.6	62.7	177.6
March	19.3	35.2	60.8	279.5
April	17.2	32.1	62.1	103.6
May	13.5	28.4	61.4	37.7
June	11.7	28.3	63.4	20.2
July	11.6	28.0	54.7	56.4
August	12.4	29.0	49.6	45.1
September	15.4	32.9	49.0	95.5
October	17.4	34.7	52.8	68.0
November	18.4	37.0	56.8	135.1
December	19.6	37.1	62.2	227.6

Abbreviations: RH, relative humidity; T, temperature.

females were submitted to transrectal palpation 38.5 ± 6.5 days after ET (45.5 ± 6.5 days of pregnancy; P/ET45) to confirm pregnancy and to determine whether late embryonic loss had occurred 4 weeks later. Pregnancy loss (PL) was calculated by the number of nonpregnant females on Day 45 divided by the number of pregnant females on Day 31.

2.7. Statistical analysis

Data were analyzed using the GLIMMIX procedure of SAS (Statistical Analysis Software 9.3, SAS Institute Inc., Cary, NC, USA). The response variables for embryo production were MO rate, number of CL before uterine flush, number of ova/embryo recovered, recovery rate, fertilization rate, and number and rate of transferable, freezable, and degenerated embryos. These response variables were assumed binomial for proportions and as Poisson distribution for continuous data.

The statistical model for embryo production-related variables included the fixed effects of donor category (cow vs. heifer), season of embryo production (warmer or cooler), farm, year of embryo production, and interactions. The effects of donor category within the farm and season of embryo production within year were included as random effects for analyses of superovulation rate and number of CL. For embryo production and quality, sire within farm was also included as a random effect.

For P/ET and PL, the variables initially included in the model were donor category (cow vs. heifer), season of embryo production (warmer vs. cooler), stage and embryo quality, type of embryo (fresh or frozen), size and quantity of CL recipient, season of ET, and interactions. Variables were removed from the statistical model by backward elimination on the basis of the Wald statistics criterion when $P > 0.20$. The final model for P/ET31 included donor category, embryo grade quality, type of embryo (fresh or frozen), and CL quality and quantity. The final model for P/ET45 included donor category, embryo quality, type of embryo (fresh or frozen), and CL quality and quantity. For PL, the final model included donor category, season of embryo production, embryo quality, and season of embryo transfer. The effect of donor category within the farm, season of embryo production within year, and sire within farm were included as random effects for models for P/ET and PL.

Differences with $P \leq 0.05$ were considered statistically significant, and those with $0.05 < P \leq 0.10$ were considered as tendency. Data are presented as mean ± SEM or percentage with respective adjusted odds ratio and 95% confidence intervals.

3. Results

3.1. Embryo production

Overall, 88.7% of the donors submitted to MO protocols responded with two or more CL. The average number of estimated CL, recovered ova/embryos, and recovery rate per donor were 9.3 ± 0.2%, 7.1 ± 0.2%, and 68.6%, respectively. Regarding the recovered ova/embryos, 64.4% were fertilized (4.6 ± 0.1 embryos grades 1–4 per uterine flush). However, only 54.1% of the ova/embryos were transferable embryos (3.8 ± 0.1 embryos grades 1–3 per uterine flush) and 10.3% were degenerated embryos (0.7 ± 0.04 embryos grade 4 per uterine flush).

The MO rate did not differ ($P = 0.25$) between donor lactation categories (heifers = 89.7% and cows = 91.9%) and only a tendency ($P = 0.09$) was observed between seasons (warmer = 89.3% and cooler = 92.2%). In addition, donor cows had greater ($P < 0.001$) number of estimated CL (10.6 ± 0.6 vs. 7.5 ± 0.4) than donor heifers, and fewer ($P = 0.03$) CL were observed in donors superovulated during the hot compared with cooler seasons (8.3 ± 0.5 vs. 9.7 ± 0.5). There was no interaction between donor category and season for the variables previously mentioned (Table 3).

Donor cows had greater numbers of ova/embryos (7.6 ± 0.6 vs. 4.6 ± 0.4, $P < 0.001$) and recovery rate (77.6 vs. 58.7%, $P < 0.001$) than donor heifers. Season influenced MO response, and donors superstimulated in the hot season produced fewer ($P = 0.04$) ova/embryos (5.3 ± 0.5 vs. 6.7 ± 0.5) and had lower ($P = 0.006$) recovery rate (65.7 vs. 72.3%) than donors superstimulated in cooler periods of the year. However, there was no category and season interaction for the number of ova/embryos and the recovery rate (Table 3).

The fertilization rate was much lower ($P < 0.001$) in donor cows (47.9%) compared with donor heifers (82.4%). Although the season only tended ($P = 0.07$) to influence fertilization (hot season = 64.9 and cooler season = 70.0%), it was observed a smaller ($P = 0.004$) number of fertilized ova in donor cows during the hot than cooler season (Table 3). The number of transferable embryos did not differ ($P = 0.15$) between donor categories (heifer = 3.8 ± 0.3 and cow = 3.3 ± 0.4,). However, regardless of donor category, the number of transferable embryos was less ($P = 0.002$) during the hot than in cooler seasons (2.8 ± 0.3 vs. 4.4 ± 0.4). Also, an interaction ($P < 0.001$) between donor category and season was observed for the number of freezable embryos, mainly because of the greater decline in number of freezable embryos for donor cows than donor heifers during the hot season (Table 3). The number of degenerated embryos recovered was greater ($P < 0.001$) for donor cows than donor heifers.

The rates of transferable (31.5 vs. 67.4%, $P < 0.001$) and freezable embryos (15.4 vs. 42.1%, $P < 0.001$) were both less in donor cows than donor heifers. Similarly, the rates of transferable (44.6 vs. 54.0%, $P = 0.001$) and freezable embryos (21.4 vs. 32.8%, $P < 0.001$) were less for females superstimulated during the hot period of the year compared with the cooler period. The decline in the rate of freezable embryos during the hot season was more pronounced ($P = 0.01$) in donor cows compared with donor heifers (Table 3).

3.2. Pregnancy establishment after ET

Overall, P/ET31 was 31.0% (n = 4076) and P/ET45 was 25.3% (n = 4076), with PL of 18.3% (n = 1513) after ET in recipients.

The P/ET of recipients was greater when they received embryos from donor cows than donor heifers on Days 31

Table 3
Multiple ovulation and embryo production outcomes according to donor category (heifers or cows) superovulated during warmer or cooler months of the year (2007–2010).

Items	Heifer		Cow		P-value		
	Cooler (n = 599)	Warmer (n = 354)	Cooler (n = 339)	Warmer (n = 270)	Category	Season	Category* Season
MO rate (%)[a]	92.5	86.1	91.9	91.9	0.25	0.09	0.05
CL (n)	8.1 ± 0.5	6.8 ± 0.4	11.3 ± 0.7	9.8 ± 0.7	<0.001	0.03	0.93
Ova/embryo (n)	5.2 ± 0.5	4.1 ± 0.4	8.5 ± 0.7	6.7 ± 0.7	<0.001	0.04	0.44
Recovery rate (%)[b]	64.1	53.1	79.3	76.4	<0.001	0.006	0.17
Fertilized structures (n)	5.1 ± 0.3^{Aa}	3.7 ± 0.4^{Ax}	5.5 ± 0.4^{xa}	3.8 ± 0.5^{Yx}	0.32	0.03	0.004
Fertilization rate (%)[c]	83.1	81.6	52.4	43.2	<0.001	0.07	0.27
Transferable embryos (n)	4.4 ± 0.4	3.2 ± 0.4	4.3 ± 0.5	2.4 ± 0.4	0.15	0.02	0.10
Transferable/total (%)[d]	69.5	65.2	37.8	25.8	<0.001	0.001	0.10
Freezable embryos (n)	1.9 ± 0.2^{Aa}	1.6 ± 0.2^{Bx}	1.6 ± 0.7^{xa}	0.8 ± 0.1^{Yy}	<0.001	0.02	<0.001
Freezable embryos rate (%)[e]	46.2^{Aa}	38.1^{Bx}	21.7^{xb}	10.7 ^{Yy}	<0.001	<0.001	0.01
Degenerated embryos (n)	0.5 ± 0.1	0.4 ± 0.1	0.8 ± 0.2	0.7 ± 0.1	<0.001	0.13	0.67
Degenerated embryos rate (%)[f]	9.6	11.9	8.6	11.1	0.48	0.05	0.88

Different capital letters $^{(A\ e\ B/x\ e\ Y)}$ in the same row differ ($P \leq 0.05$) within the same donor.
Different lowercase letters $^{(a,\ b/x,\ y)}$ in the same row differ ($P \leq 0.05$) within season.
[a] MO rate: proportion of donors with two or more CL divided by the total number of donors submitted to MO protocol.
[b] Recovery rate: proportion of ova/embryos recovered divided by total number of CL previous to uterine flush.
[c] Fertilization rate: proportion of embryos grade 1, 2, 3, and 4 divided by total ova/embryo recovered.
[d] Transferable embryos rate: proportion of embryos grade 1, 2, and 3 divided by total ova/embryo recovered.
[e] Freezable embryos rate: proportion of embryos grade 1 and 2 divided by total ova/embryo recovered.
[f] Degenerated embryos rate: proportion of embryos grade 4 divided by total ova/embryo recovered.

(36.0 vs. 30.7%; P = 0.007) and 45 (28.3 vs. 23.1%, P = 0.001) of gestation. However, donor category did not affect (P = 0.14) PL (donor cow = 16.6 and donor heifer = 18.2%). Although the season on which the embryo was produced (hot or cool) did not affect pregnancy rate on Days 31 (30.5 and 31.7%; P = 0.45) and 45 of gestation (25.3 and 25.1%; P = 0.64), surprisingly, the PL was lower (P = 0.002) for recipients that received embryo during the hot season compared with the cooler (17.4 and 22.1%). No donor category-by-season interaction was observed for P/ET on Days 31 (P = 0.55) and 45 (P = 0.63) and for PL (P = 0.36).

Table 4
Factors associated with pregnancy per embryo transfer (P/ET) in lactating dairy cow recipients.

Item		Pregnancy per embryo transfer				Pregnancy loss	OR (95% CI)
		31 days	OR (95% CI)[a]	45 days	OR (95% CI)		
Donor category							
Cow	2314	36.0	Ref[b]	28.3	Ref	16.6	Ref
Heifer	1762	30.7	0.82 (0.70–0.95)	23.1	0.77 (0.66–0.90)	18.2	1.22 (0.94–1.60)
Flush season							
Cooler	2904	31.7	Ref	25.1	Ref	22.1	Ref
Warmer	1172	30.5	0.94 (0.81–1.10)	25.3	1.06 (0.84–1.33)	17.4	0.57 (0.40–0.82)
Embryo quality							
Excellent or good	662	37.8	Ref	30.7	Ref	15.8	Ref
Fair	2202	34.0	0.84 (0.71–1.01)	25.3	0.76 (0.63–0.92)	21.8	1.51 (1.06–2.16)
Poor	1212	28.4	0.64 (0.52–0.78)	21.3	0.61 (0.49–0.75)	21.8	1.46 (0.97–2.19)
Embryo stage							
Morula	2224	33.3	Ref	26.0	Ref	19.3	Ref
Early blastocyst	983	32.6	0.97 (0.83–1.14)	26.6	1.03 (0.87–1.23)	16.5	0.84 (0.61–1.15)
Blastocyst	760	34.4	1.06 (0.87–1.28)	25.9	1.00 (0.82–1.24)	21.2	1.13 (0.79–1.61)
Expanded blastocyst	109	25.2	0.68 (0.43–1.07)	21.2	0.77 (0.48–1.23)	13.3	0.64 (0.21–1.97)
Embryo status							
Fresh	2279	34.0	Ref	27.2	Ref	17.2	Ref
Freeze-thaw	1797	32.0	0.89 (0.77–1.03)	24.1	0.83 (0.71–0.96)	22.3	1.21 (0.88–1.66)
CL grade[c]							
Good	2056	37.4	Ref	28.8	Ref	18.2	Ref
Fair	1855	34.1	0.86 (0.75–0.99)	27.0	0.91 (0.79–1.06)	16.5	0.89 (0.68–1.16)
Poor	165	28.5	0.65 (0.45–0.94)	21.4	0.66 (0.44–0.99)	19.9	1.10 (0.52–2.31)
Number CL							
1	3710	35.8	Ref	27.8	Ref	18.2	Ref
≥ 2	366	30.8	0.80 (0.62–1.02)	23.5	0.79 (0.60–1.04)	18.1	0.98 (0.60–1.61)

Pregnancy diagnosis was performed on Days 31 and 45 of gestation and pregnancy loss was evaluated between both diagnoses.
[a] OR = odds ratio; CI = confidence interval.
[b] Reference (Ref) point odds ratio = 1.0.
[c] Good: CL represents 75% of the ovary volume; fair: 50% of the ovary volume; and poor: 25% of the ovary volume.

The embryo grade quality was linearly associated ($P < 0.001$) with pregnancy on Days 31 and 45 (Table 4). As grade quality declined, so did pregnancy per ET. However, the PL only tended ($P = 0.07$) to be influenced by embryo quality with the smallest loss for recipients receiving an embryo graded as 1 compared with grades 2 to 3 (Table 4). The stage of embryo development did not affect P/ET31 ($P = 0.25$), P/ET45 ($P = 0.64$) and PL ($P = 0.37$). Yet, when a fresh embryo was transferred, recipients had greater ($P = 0.01$) P/ET45 compared with recipients receiving a frozen-thawed embryo (27.2 vs. 24.1%). Finally, recipients with CL graded as good had greater ($P = 0.01$) P/ET31 than those with CL graded as poor or fair. In addition, the number of CL the recipient had on the Day of ET only tended to affect P/ET31 (1CL = 35.8% and ≥ 2CL = 30.8%, $P = 0.07$) and P/ET45 (1CL = 27.8% vs. ≥2CL = 23.5%, $P = 0.09$), but had no effect ($P = 0.94$) on PL (1CL = 18.2% vs. ≥2CL = 18.1%).

4. Discussion

4.1. Superovulation and embryo production

In the current retrospective nonrandomized study, donor category and environmental temperatures had a profound effect of Holstein embryo production. Obviously, differences in feeding management or even gonadotropin dose used between cows and heifers might account for differences in ovarian responses observed herein, but this is out of scope of this retrospective study. Heifer donors had lower estimated number of CL and less collected structures compared with cows. Despite of that, because cows had lower fertilization rates and embryo quality, the final numbers of transferable embryos were not largely affected by the category of the donor. These findings corroborate with previous reports in the literature, suggesting that embryo quality is reduced during lactation [4,8,9,31]. Nonetheless, the present results indicate that poor fertilization rates rather than embryo degeneration accounted for most losses in embryo quality and number of transferable embryos in donor cows compared with heifers. For example, the proportion of degenerated embryos based on the total numbers of collected structures was similar ($P = 0.48$) between cows (9.2%) and heifers (11.9%); however, the proportion of transferable embryos based on the total numbers of collected structures was much lower for cows (41%) compared with heifers (65%). Poor fertilization results in superovulated lactating cows remain unclear but likely related to disturbances in spermatozoa and/or ova transport and suboptimal oocyte quality [32] and is aggravated as the dose of FSH increase [33], for example, lactating cows compared with heifer donors.

Another important finding of the present study was the association between the environmental temperature with MO and embryo production efficiency. It is widely accepted that heat stress can compromise oocyte and embryo quality in dairy cattle [4,13,34,35]. Exposure of dairy cattle to high environmental temperatures clearly results in disturbances in theca and granulosa cell physiology, impaired oocyte quality, reduced fertilization rate, impaired embryonic development, and, ultimately, reducing pregnancy success [4,10,11,36–43]. As expected, although hot season had an overall detrimental impact on embryo production and quality, its negative effects were more pronounced in donor cows than heifers. For example, donor cows had greater decline in fertilized structures and freezable embryos during the hot season compared with the heifers. Lactating cows are known to generate more body heat and suffer greater hyperthermia when exposed to similar environments compared with growing heifers [29]. In that prospective, although body temperature was not measured in the current experiment, it is plausible to suggest that lactating cows had more problems with thermoregulation, which in turn affected embryo production mainly by limiting fertilization results. Thus, implementation of strategies to mitigate detrimental effects of heat stress on MO need to be in place for improved *in vivo* embryo production, particularly when utilizing lactating cows as donors.

4.2. Embryonic survival

Surprisingly, the season in which embryos were produced did not seem to have major effects in embryo survival after transplantation to recipients. These results are in agreement with reports that observed greater impact of heat stress on early embryonic development [10,36]. Therefore, it appears that embryos that were able to endure heat stress and bypass the initial critical stage of development (first days after fertilization) were able to maintain pregnancy at similar rates to embryos produced under cooler months of the year. However, whether early changes in gene expression in embryos produced under heat stress conditions [44] can alter future offspring health and performance remains undetermined.

Interestingly, recipients receiving embryos originating from donor cows showed improved pregnancy results when compared with embryos derived from heifers. These puzzling results contrast with previous studies that found no difference in embryonic survival between embryos derived from lactating or nonlactating donors [13,45]. In the present study, it is important to highlight that donor cows used to yield the embryos were producing 23.5 ± 5.8 L with average days in milk of 320.7 ± 2.3 days, and perhaps undergoing less metabolic challenges when compared with lactating cows used in other studies. Despite of that, a number of factors could be hypothesized to play a role in these improved pregnancy results from embryos produced in lactating cows. For example, perhaps embryos produced in lactating cows were simply more adjusted to the endocrine milieu (i.e., low circulating progesterone post ovulation) which they encountered after being transferred back into lactating recipients. In this regard, circulating P4, for instance, can alter uterine gene expression and the amount of histiotrophic support for the growing embryo [46]. Possibly, the sudden change in uterine environment and histiotrophic support experienced by embryos derived from heifers might explain, at least partially, the observed drop in pregnancy results in recipients receiving embryos produced in heifers. Alternatively, considering that the commonly technique used to grade embryos in the field is through visual morphology,

ultrastructural alterations between cow and heifers' embryo might be unnoticed and account for these findings. Furthermore, other uncontrolled factors such as differences in diets used for heifers and cows in the current trial might also have an unpredictable impact in embryo viability [9,47–49].

Embryo grade and quality of the CL in the recipient had a major impact in pregnancy results. As mentioned previously, although far from perfect [50], stereoscopic-based embryo grading is still the most standard method used to grade embryos and appears to correlate fairly well to pregnancy outcome in the field. In addition, recipient selection can drastically improve results of embryo transfer. Previous studies have reported that larger CL structures maintain higher circulating P4 in diestrus and result in greater pregnancy rate after ET [18,51]. Controversially, earlier reports failed to indicate positive correlations between CL size and plasma P4 concentration with pregnancy rates in recipients [21,52–54]. In addition, one limitation in the current study is the fact that CL quality was assessed by transrectal palpation, and although recipients classified as having better CL structures had improved fertility, we would expect that selecting recipients with less subjective methods such as ultrasound would improve the selection accuracy and potentially fertility in ET recipients.

In conclusion, the results suggest that MO embryo production efficiency is compromised during the hot seasons both in cows or heifers; however, the failure is more pronounced in donor cows. Therefore, adequacy of environment (e.g., cooling systems) is required to mitigate detrimental effects of heat stress on MO programs. In contrast, similar embryonic survival was observed in recipient cows that received embryos produced during the hot and cooler seasons. Curiously, embryos originating from donor cows were more viable (higher embryonic survival) when transferred to recipient cows than embryos originating from heifers.

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