Short communication: Greater intensity of estrous expression is associated with improved embryo viability from superovulated Holstein heifers

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ABSTRACT

The aim of this study was to determine the association between estrous expression, measured using a breeding indicator and an automated activity monitor (AAM), and the success of embryo collection after superovulation. Holstein heifers ($n = 51$; 10.5 to 14.5 mo, and 325.0 ± 21.1 kg of body weight) were superovulated ($n = 69$ events) for the collection of embryos using a protocol based on sequential administration of FSH for follicle superstimulation and GnRH to induce ovulation. Artificial insemination (AI) was performed twice, once at the moment of GnRH administration and again 12 h later, using thawed, sexed semen. Ovaries were scanned via ultrasonography on the day of the first AI to count the total number of preovulatory follicles and 7 d later for the total number of corpora lutea present. Embryos were collected 7 d post-AI, counted, and assessed for viability. A breeding indicator (Estrotec, Rockway Inc., Spring Valley, WI) and a collar-mounted AAM (CowScout Activity Monitoring System, GEA, Dusseldorf, Germany) were used to measure standing mounts and an algorithmic estimate of estrous expression, respectively. A score for the breeding indicator was given as follows: score 1 = 100% of the indicator was intact; score 2 = 50% of the indicator was rubbed off; score 3 = greater than 50% of the indicator was rubbed off. Estrous expression detected by the AAM was quantified through the relative increase in physical activity and duration of time spent above a set threshold. Data were analyzed by ANOVA using the MIXED procedures of SAS (SAS Institute Inc., Cary, NC). The number of follicles present at AI was not affected by estrous expression. The mean ($\pm$ SD) ovulatory response was $67.5 \pm 26.3\%$. We found an effect of estrous

expression as detected by the breeding indicator on the ovulatory response (42.1 ± 8.0 , vs. 78.2 ± 9.0 , vs. $74.0 \pm 4.9\%$, for scores 1, 2, and 3, respectively) but not from the AAM. Heifers that had a score of 3 (versus those with scores of 1 and 2) on the breeding indicator had a greater number of embryos (4.1 ± 0.5 , vs. 1.2 ± 1.0 , vs. 1.8 ± 1.0 embryos), and a greater percentage of these embryos were viable (43.1 ± 0.05 , vs. 35.5 ± 0.1 , vs. $34.3 \pm 0.1\%$). Similarly, heifers that showed a greater intensity of activity (as measured by the AAM) had a greater number of embryos collected (10.2 ± 1.2 vs. 6.0 ± 1.3 embryos), and a greater percentage of those embryos were viable (53.1 ± 5.0 vs. $23.4 \pm 5.1\%$). Longer-duration estrus episodes were associated with a higher percentage of viable embryos (51.2 ± 5.2 vs. $25.3 \pm 5.3\%$). In conclusion, stronger estrous intensity was associated with a greater number of total embryos collected and a greater percentage of viable embryos. These results suggest that monitoring the intensity of estrus could be used to predict superovulatory response as well as embryo quality in Holstein heifers.

Key words: embryo transfer, estrous expression, automated activity monitor

Short Communication

Superovulation was developed in the 1970s (Hasler, 2014) and can be used to accelerate gains in genetic progress (Jaton et al., 2016). The procedure involves providing a combination of hormones to superstimulate the ovarian follicles, leading to multiple ovulations (Peippo et al., 2011). Successful multiple ovulations are influenced by factors related to the donor (including parity, age, and breed) and to management (including the superovulation protocol, climate, and nutrition; Chebel et al., 2008). Factors that contribute to fertilization rates in superovulated animals include semen fertility, type of semen, and AI technician skills (Sartori et al., 2004; Schenk et al., 2006). Together these factors can influence fertilization rates and embryo production;

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the efficiency of superovulation methods has remained fairly stable in recent years, with an average yield of 6.2 embryos per superovulation event (IETS, 2013).

The intensity of estrous expression is related to cow fertility, both when spontaneous estruses are detected by automated activity monitors (AAM; Madureira et al., 2015) and when following a timed AI program (Pereira et al., 2016; Madureira et al., 2019). Similarly, the occurrence of estrus in recipient cows appears to increase pregnancy rates following embryo transfer in estradiol-based (Pereira et al., 2016) or GnRH-based protocols (Jinks et al., 2013). Furthermore, stronger displays of estrus are associated with decreased pregnancy losses after embryo transfer (Pereira et al., 2016). The reasons for this link between estrous expression and fertility are not well understood but may be associated with the hormonal milieu surrounding estrus, as both progesterone and estradiol play important roles in the final maturation of the oocyte, embryo development, and receptivity of the uterus, as well as being associated with differences in the occurrence and expression of estrus (Denis-Robichaud et al., 2018; Madureira et al., 2018). To date, limited literature exists about the effect of estrous expression on the superovulatory response and viability of embryos (Jinks et al., 2013; Larimore et al., 2015).

The main objective of this study was to evaluate the effect of the intensity of estrous expression on the viability of in vivo-produced embryos. We hypothesized that heifers that displayed greater estrous expression would yield a greater number of embryos, of which a greater proportion would be viable and freezable. This study was conducted at a commercial farm in Minas Gerais State, Brazil (19°18'40"S, 46°02'56"W). The practices outlined in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS, 1999) were used for all procedures as part of the local requirements.

A total of 51 nulliparous Holstein heifers ($n = 69$ events; 10.5 to 14.5 mo of age; 325.0 ± 21.1 kg of BW) were enrolled in the study 1 wk before the superovulation protocol. Heifers were housed in an open-pack compost-bedded barn. Animals were fed a TMR 3 times daily, which was pushed up 3 times daily. Water and TMR were available for ad libitum intake. At the time of enrollment, BCS was recorded (1 to 5 scale at increments of 0.25; Edmonson et al., 1989). Heifers were monitored continuously by a collar-mounted sensor (CowScout Activity Monitoring System, GEA, Dusseldorf, Germany) starting 1 wk before the superovulation protocol until the moment of timed AI, allowing for sufficient time for the AAM to create a baseline before the initiation of the protocol.

Heifers were submitted to a superovulation protocol based on FSH and progesterone, as described in Figure 1. Timed AI was performed twice, first at the moment of GnRH administration (d 9) and then 12 h later (d 10) using thawed, sexed semen delivered by a trained technician. All heifers were bred regardless of estrous expression. At the time of the progesterone insert removal (d 8), all heifers received a breeding indicator for detection of estrus (Estroject, Rockway Inc., Spring Valley, WI) placed on the spine, halfway between the hip and the tail head; all heifers received both the AAM and the breeding indicator. At the day of first AI, a score was given to the breeding indicator based on the proportion of the original color that remained on it (score 1 = 100% of the indicator was intact, and animals were classified as not being in estrus; score 2 = 50% of the indicator was rubbed off, and animals were classified as having displayed a moderately intense estrus; and score 3 = greater than 50% of the indicator was rubbed off, and the animals were classified as having displayed a high intensity of estrous expression). The AAM system was equipped with a wireless antenna-receiver, located in the open-pack barn, that sent data to a processing unit for recording and analysis. The estrus event alarm was triggered when at least 3 consecutive data block-intervals of 2 h were above a set threshold recommended by the manufacturer. Two traits of estrous expression were calculated from the AAM: intensity of activity (maximum activity during an estrus episode) and duration (total time the animal spent with activity above the threshold) to describe the expression of estrus (Madureira et al., 2015). Ovulatory response was calculated via the number of follicles on the day of AI (≥ 10 mm) divided by the number of corpora lutea on the day of collection.

Heifers were flushed 7 d after the first AI by a transcervical procedure using a silicone Foley catheter (number 18; Solidor, Well Lead Medical Instruments, Guangzhou, China). The balloon of the Foley catheter was placed into the uterine horn ipsilateral to the CL. Approximately 500 mL of flushing solution (Dulbecco's modified PBS, Biodux, Campinas, Brazil) was used to recover structures into a sterile filter (WTA, Cravinhos, SP, Brazil). For heifers with CL on both ovaries, both horns were flushed as described previously. Oocytes or embryos collected were evaluated for fertilization and grade quality (G1 = excellent or good, G2 = fair, G3 = poor, and G4 = degenerated) as described by the International Embryo Technology Society (IETS, 2013). Each embryo was also classified by its stage of development, as follows: morula, initial blastocyst, blastocyst, or expanded blastocyst (Robertson and Nelson, 1998). The initial blastocyst and blastocyst groups were later

combined due to low numbers of embryos in these stages, and no embryos were found in the expanded blastocyst category. After classification, embryos were placed into a straw (0.25 mm) with ethylene glycol solution (Holding, Biodux) for a fresh or frozen-thawed transfer to a recipient cow.

Distributions and normality tests were obtained using the Univariate procedure of the SAS software, version 9.4 (SAS Institute Inc., Cary, NC). Class variables used for analyses are as follows. The BCS categories were average (<3.0) or moderate (≥ 3.0). Duration of estrus was calculated as the number of hours above the alert threshold detected by the AAM and was categorized as shorter or longer using the median. Intensity of activity from the AAM was defined as the maximum activity value recorded during the estrus event, and was also categorized using the median, into moderate and greater estrous expression groups. Percentage and number of viable embryos, number of follicles, ovulatory response, and percentage of morula and blastocysts were used as continuous dependent variables and assessed with ANOVA using the MIXED procedure, with AI event as the experimental unit and heifer as a random effect. Age was used as a continuous independent variable. Breeding indicator score, BCS, and intensity and duration of estrous expression (as measured through the AAM) were used as categorical independent variables; breeding indicator score and intensity and duration of estrous expression were considered collinear variables and thus not included within the same models. Only variables with a P -value <0.15 were maintained in the

final model. Differences with $P \leq 0.05$ were considered significant, and those between $0.05 > P \leq 0.10$ were designated as a tendency.

The main objective of our study was to evaluate the association between estrous expression measured by a breeding indicator and AAM, on the viability of embryos produced in vivo. Increased estrous expression was associated with increased embryo production. Heifers with a score of 3 on the breeding indicator had a greater number and percentage of viable embryos compared with those with a score of 1 or 2 (Table 1; $P < 0.05$). Similarly, greater intensity of estrous expression detected by the AAM was also associated with a greater number ($P = 0.01$) and percentage of viable embryos ($P < 0.01$) compared with heifers expressing moderate intensity of activity. Moreover, heifers that had a longer duration of estrus, as measured using the AAM, tended to have more viable embryos ($P = 0.10$) and a greater percentage of viable embryos ($P < 0.01$) than did heifers that had shorter duration of estrus. Although the viability of embryos increased with increased estrous expression, the stage and quality of the embryos were unaffected. The total percentage of embryos at the morula, initial blastocyst, and blastocyst stages in this study were 78.9, 18.6, and 2.5%, respectively.

Estradiol and progesterone are the main hormones acting on the hypothalamus to trigger estrous behavior (Woelders et al., 2014). Standing to be mounted has been the gold standard for estrus detection (Roelofs et al., 2010), but this behavior is well known to be reduced in lactating dairy cows (Rivera et al., 2010). In

Figure 1. The superovulation synchronization protocol used was as follows. An intravaginal progesterone implant of 1.9 g of progesterone previously used for 9 d (controlled internal drug release, CIDR; Zoetis, Sao Paulo, Brazil). A 2.0-mg (i.m.) injection of estradiol benzoate (EB; 2.0 mL of RIC-BE, Agener Uniao, Sao Paulo, Brazil) on p.m. of d 0. On p.m. d 4 a 2.0-mL (i.m.) injection of FSH (Folltropin, Vetoquinol, Mairiporã, Brazil). On a.m. and p.m. d 5, respectively, a 2.0- and 1.5-mL (i.m.) injection of FSH. On a.m. and p.m. d 6, respectively, a 1.5- and 1.0-mL (i.m.) injection of FSH as well as a 25-mg (i.m.) injection of dinoprost tromethamine (PGF; 5.0 mL of Lutalyse, Zoetis) at p.m. d 6. On a.m. and p.m. d 7, respectively, a 1.0- and 0.5-mL (i.m.) injection of FSH, and a PGF injection on a.m. d 7. On a.m. d 8 the last injection of FSH 0.5 mL (i.m.), CIDR removal, and estrus detection. On a.m. d 9 a 4-mg (i.m.) injection of Gonadorelin (GnRH; 4 mL of Fertagyl, MSD Animal Health, São Paulo, Brazil). A first AI at p.m. d 9 and second AI at a.m. d 10. On d 16, oocyte or embryo collection.

Table 1. Number of viable embryos, percentage of viable embryos, number of follicles, and number of viable embryos per follicle according to breeding indicator score and automated activity monitor (AAM) measures of estrous expression

Variable	Number of viable embryos ¹ (no. $\pm$ SE)	Viable embryos ¹ (% $\pm$ SE)	Number of follicles (no. $\pm$ SE)	Viable embryos per follicle ¹ (%)
Breeding indicator				
Score 1	1.2 $\pm$ 1.0 ^a	35.5 $\pm$ 0.1 ^a	7.7 $\pm$ 1.9 ^a	15.6 ^a
Score 2	1.8 $\pm$ 1.0 ^a	34.3 $\pm$ 0.1 ^a	10.3 $\pm$ 1.9 ^a	17.8 ^a
Score 3	4.1 $\pm$ 0.5 ^b	43.1 $\pm$ 0.05 ^b	13.9 $\pm$ 0.9 ^b	29.5 ^b
AAM device: Intensity ²				
Moderate	2.0 $\pm$ 0.6 ^a	23.4 $\pm$ 0.05 ^a	12.1 $\pm$ 1.3	16.5 ^a
Greater	4.6 $\pm$ 0.6 ^b	53.1 $\pm$ 0.05 ^b	13.6 $\pm$ 1.3	33.8 ^b
AAM device: Duration ³				
Longer	4.1 $\pm$ 0.6 ^a	51.2 $\pm$ 0.05 ^a	13.8 $\pm$ 1.5	29.7
Shorter	2.7 $\pm$ 0.6 ^b	25.3 $\pm$ 0.05 ^b	11.8 $\pm$ 1.3	22.8

^{a,b}Different superscript letters indicate differences between variables within columns ($P < 0.05$).

¹Embryos were considered viable if they were fertilized and of excellent or good quality.

²Intensity of estrous expression using the AAM was determined as the maximum activity during an estrus episode and categorized as moderate or greater using the median.

³Duration of estrous expression using the AAM was determined as the total time the heifer spent with activity above the threshold and categorized as longer or shorter using the median.

our study, a total of 34.8% of heifers did not stand to be mounted (score 1 on the breeding indicator) but had an increase in physical activity detected by the AAM. Recent studies have shown that the intensity and duration of the estrus event is associated with ovulation rates and timing, pregnancy per AI, and pregnancy loss (Madureira et al., 2015, 2019; Burnett et al., 2017, 2018; Polsky et al., 2017; Silper et al., 2017), but the physiological mechanisms that explain this link are still unclear.

One possible explanation for decreased estrous behavior close to ovulation may be the abnormal pattern of concentrations of progesterone at and after AI (Madureira et al., 2018). Increased progesterone decreases LH pulses from the anterior pituitary, resulting in lower circulating estradiol concentrations (Stevenson and Pulley, 2016), thus inhibiting or decreasing its effects in the hypothalamic nuclei that trigger modifications in behavior (Lyimo et al., 2000). However, estradiol concentrations do not seem to be highly correlated with increased physical activity as detected by AAM (Lyimo et al., 2000; Madureira et al., 2015). Increased estradiol concentration increases the duration and frequency of standing behavior (Lyimo et al., 2000), but perhaps increased physical activity is dependent on estradiol for its initiation but not for its intensity. Nonetheless, it is remarkable that animals enrolled in and responding to a multiple ovulation protocol do not express estrous behavior, considering the elevated preovulatory plasma concentrations of estradiol (Soumano et al., 1996).

In our study, heifers displaying greater intensity of estrus had a greater proportion of viable embryos. Studies have shown the importance of progesterone in

the first week after conception to support embryo and fetal development (Bisinotto et al., 2010; Spencer et al., 2016). In a recent study, Madureira et al. (2019) showed that cows with greater estrous expression had lower concentrations of progesterone at AI and greater concentrations of progesterone 7 d post-AI. In addition, Pereira et al. (2016) reported that cows that expressed estrus had lower concentrations of progesterone compared with cows that did not express estrus near timed AI. This suggests that the circulating progesterone profile around the time of AI affected both estrous behavior and fertilization and early embryo development.

Total number of follicles was associated with estrous expression as detected by the breeding indicator but not by the AAM. Response to a superovulation protocol is important to optimize the number of fertilized and, ideally, transferable embryos. The total mean ($\pm$ SD) ovulatory response in this study was $67.5 \pm 26.3\%$. Heifers with a breeding indicator score of 1 had a lower ovulatory response ($42.1 \pm 8.0\%$) than did those that had some or all of the indicator rubbed off (i.e., score 3; $74.0 \pm 4.9\%$; $P < 0.01$). Variability in superovulation responses have been reported previously (Keller and Teepker, 1990; Kafi and McGowan, 1997; Bó and Mapletoft, 2014), and this variability may be related to the superovulation protocol itself, as well as to other factors related to the animal and environment (Kakar et al., 2005; Chebel et al., 2008).

In conclusion, greater estrous expression was associated with a greater number of embryos collected and a greater percentage of viable embryos. The strength of estrous expression was not associated with the number of follicles present at AI but was associated with a greater ovulatory response.

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