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Use of Applied Reproductive Technologies (FTAI, FTET) to Improve the Reproductive Efficiency in Dairy Cattle

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ABSTRACT

***Background*:** Reproductive inefficiency of dairy cattle, characterized by reduced estrous expression and detection rates, reduced pregnancy per artificial insemination (number of cows pregnant divided by number of cows inseminated), reduced pregnancy rates (number of cows pregnant divided by the number of cows eligible to become pregnant during a time interval), and increased pregnancy losses, has a large financial impact on dairy operations across the world. Although the most important component of reduced reproductive efficiency in dairy cattle is unquestionably poor management and diseases that result from it, the genetic selection and the resulting increased milk yield have caused physiological changes in lactating dairy cows that also affect fertility. The most important of these changes is the increased feed intake and the consequent increased mesenteric and liver blood flow to supply the nutrients necessary for milk yield. This causes significant decreases in concentrations of progesterone and estradiol that affect estrous expression, follicular growth, oocyte quality, and embryo development and survival. This review will discuss reproductive technologies used in large dairy herds to mitigate the effects of these physiological changes on reproductive performance.

***Review*:** The use of ovulation/estrous synchronization protocols (OSP), pre and post-ovulation hormonal treatments, and embryo transfer (ET) in the reproductive management of lactating dairy cows was reviewed. Several OSP have been developed in the past 20 years. To achieve acceptable pregnancy per artificial insemination (P/AI) OSP should result in synchronized recruitment of a new follicular wave, growth of follicles under P4 concentration > 2 ng/mL, synchronized luteolysis, and synchronized ovulation at the end of the protocol. When embryo recipient cows are submitted to OSP, these protocols must aim to tightly synchronize luteolysis and ovulation at the end of the protocol. The use of ET in lactating dairy cows in the U.S. has been limited to herds of registered animals, to mitigate the negative effects of exposure to heat stress, to improve genetics of expanding herds, and in a few herds to salvage repeat-breeders. Lactating dairy cows are sensitive to heat stress because of the high metabolic rate resulting from the increased feed intake necessary to supply nutrients for milk production. Several studies have demonstrated that lactating dairy cows exposed to heat stress that receive ET have improved reproductive performance compared with cows receiving AI. Finally, the use of hormonal treatments to increase P4 concentration during early diestrus was reviewed because several studies have demonstrated a strong association among P4 concentration and embryo development and pregnancy establishment. The effects of hormonal treatments during ovulation synchronization protocols, after AI or at ET on P4 concentration and reproductive outcomes are controversial and likely dependent on management, milk yield, and diet of the lactating dairy cows.

***Conclusion*:** The use of reproductive technologies in lactating dairy cows, particularly AI, is extremely well disseminated and has resulted in significant improvements in milk yield in the past 50 years. Recent developments in the understanding of reproductive physiology of lactating dairy cows have resulted in ovulation synchronization protocols that optimize fertility after AI or ET.

Keywords: Lactating dairy cow, synchronization, artificial insemination, embryo transfer.

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I. INTRODUCTION

Fertility in lactating dairy cows has been decreasing in the past 50 years. This decrease in fertility has been associated with a steady increase in milk yield, which is a consequence of genetic selection for milk yield and improvements in nutritional management. According to the National Animal Health Monitoring System [49], reproductive failure is the most important cause of involuntary culling. The costs of reproductive inefficiency to the cattle industry are extremely important and have been recognized as such for decades. Senger *et al.* [71] suggested that the dairy industry loses approximately $ 300 million per year because of poor estrous detection rate and accuracy. The estimated average value of a pregnancy is $ 275 [20] and that of an abortion is between $ 555 and $ 640 [20,80]. These values are dependent on lactation number, milk yield, days in milk (DIM), price of milk, and cost of replacement animals.

In technical terms, compromised reproductive inefficiency involves fertilization failure - observed from the day of AI to 5-6 d after AI, early embryonic loss - observed from 5-6 d after AI to 17-24 d after AI, late embryonic loss - observed from 17-24 d after AI to 42 d after AI, and fetal loss - observed from 42 d after AI to term [64]. Reduced fertilization and increased early embryonic loss are usually observed as increased return to estrus after AI. Increased late embryonic loss is observed as altered inter-estrus interval or increased abortions if the first pregnancy diagnosis takes place before 42 d after AI. Increased fetal losses are observed as increased number of abortions after 42 d after AI (Figure 1). Ultimately, in large dairy herds, reproductive failure is observed as reduced pregnancy per AI (P/AI; number of pregnancies divided by the number of cows inseminated), increased number of abortions, and decreased pregnancy rates (number of cows pregnant within a time period divided by the number of cows eligible to become pregnant during the same period).

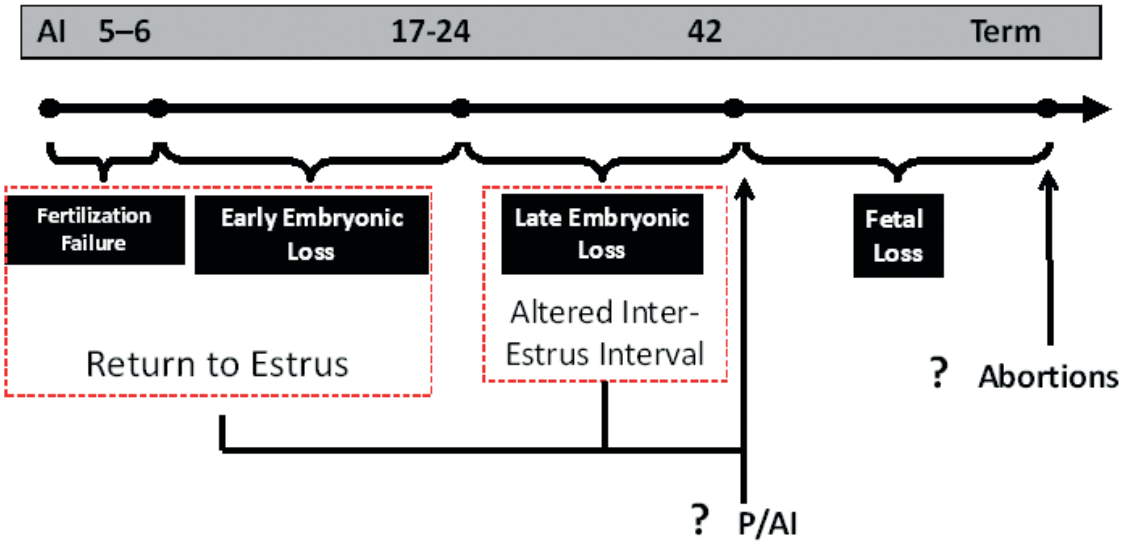

Figure 1. Characterization of reproductive failure in cattle.

Different studies have evaluated the fertilization of oocytes and quality of embryos from lactating dairy cows, heifers, and non-lactating dairy cows [10,12,17,21,67]. Although not all studies compared production and quality of embryos by lactating dairy cows directly with that of heifers and non-lactating dairy cows, it is estimated that non-superovulated lactating dairy cows have approximately 76% of recovered structures fertilized, whereas non-lactating cows and heifers have 78% and 100%, respectively, of recovered structures fertilized. Furthermore, approximately 66% of fertilized structures recovered from lactating dairy cows are classified as excellent/good quality embryos but approximately 74% and 72% of fertilized structures recovered from non-lactating dairy cows and heifers, respectively, are classified as excellent/good quality embryos. Consequently, among all oocyte-embryo recovered from lactating dairy cows, 50% are classified as excellent/good embryos, whereas 58% and 72% of all oocyte-embryo recovered from non-lactating cows and heifers, respectively, are classified as excellent/good embryos. In recent studies conducted by our group in several herds across the U.S.A. we observed P/AI of lactating dairy cows to be between 35 and 40% at 30 to 38 d after first postpartum AI [13,14,62]. Therefore, it is expected that 25% of all excellent/good quality embryos will be lost between 6 and 35 d after first postpartum AI of lactating dairy cows, representing 1.78% of embryonic losses per day.

Summarization of data from 15 different studies conducted in the U.S.A. that reported late embryonic loss demonstrates that pregnancy losses from 27-31 to 38-50 d after AI is approximately 13% with a range of 3 to 43%. This represents pregnancy losses of approximately 0.85% per day during this period. Furthermore, incidence of late embryonic/fetal losses from approximately 40 to 120 d after AI has been reported to range from 8.3 to 10.7%, which represents daily losses of approximately 0.11% of pregnancies diagnosed at 40 d after AI. On the other hand, according to data from six published manuscripts (total of 7,426 AI) P/AI at 38 d after first AI in virgin heifers ranges from 55 to 70% and only approximately 3% of heifers lose pregnancy from 38 to 120 d of gestation, resulting in daily pregnancy loss of approximately 0.05%.

From these data it is obvious that the stages of greatest risk for reproductive failure are fertilization, embryo development, maternal recognition of pregnancy, and placentation. Furthermore, it is clear that lactating dairy cows are less likely to conceive and to carry out the pregnancy to term than virgin heifers. Although the factors associated with reduced fertility in lactating dairy cows are multiple and multi-faceted, they all originate from the ability or lack thereof of lactating dairy cows to cope with the nutritional demands associated with the extremely elevated milk yield. With the onset of colostrum/milk production lactating dairy cows face severe nutritional demands that are usually not fully met by feed intake and result in negative energy balance, metabolic diseases, immune suppression, and increase incidence of diseases. In this manuscript we will discuss the effects of increased milk yield on physiological alterations that affect reproductive efficiency, and we will discuss reproductive technologies used to mitigate the effects of these physiological alterations on reproductive performance.

II. DISCUSSION

2.1 Physiological Changes Associated with Reduced Fertility

There are several hormones that are extremely important to the reproductive function of ruminants [e.g. progesterone (P4), estradiol, GnRH, LH, FSH, and prostaglandin (PG) $F_{2\alpha}$]. In this section, we will briefly discuss the importance of P4 and estradiol, their concentrations, and metabolism in lactating dairy cows.

Estradiol is produced by antral ovarian follicles. Under reduced concentrations of P4 (< 1 ng/mL) estradiol is responsible for signs of estrus and a positive feed-back on the hypothalamus, which stimulates secretion of GnRH that causes the pituitary gland to produce an ovulatory LH-peak. Furthermore, priming of the uterus with estradiol during the proestrus is expected to reduce the binding capacity of oxytocin to its endometrial receptors [43], reducing the positive feed-back of oxytocin on endometrial production of $PGF_{2\alpha}$. For example, ovariectomized cows treated with exogenous estradiol, mimicking concentrations of estradiol during the proestrus, had smaller concentrations of $PGF_{2\alpha}$ metabolite after oxytocin challenge compared with oxytocin-challen-

ged ovariectomized cows not treated with estradiol [43].

Several studies have found a link between metestrus and early diestrus P4 concentrations and embryo development and elongation and the subsequent establishment of pregnancy. Mann and Lamming [42] demonstrated that cows that had the largest embryos at 16 d after AI were also the cows that had the greatest P4 concentrations starting at approximately d 5 after AI and that larger embryos produced greater quantities of interferon-τ. This indicates that increased P4 concentrations during early diestrus should result in hastened development of embryos and improved signaling from the embryo for maternal recognition of pregnancy [42]. Despite the fact that mRNA for P4 receptors can be identified in nuclei of cells of early bovine embryos, *in vitro* exposure of cleaved embryos to elevated P4 concentrations did not affect subsequent development to the blastocyst stage, nor recovery rates of 14 d old *in vitro* produced (IVP) embryos 7 d after transfer [16]. On the other hand, supplementation with P4 between d 3 and 7 of pregnancy did not alter the morphology of embryos recovered in the morula to blastocyst stage, but conceptus from heifers supplemented with P4 were significantly larger at d 13 and 16 after AI [9]. Similarly, when multiple embryos were transferred into superstimulated recipient heifers it was observed that embryos transferred into these heifers were significantly larger at d 13 than embryos transferred into non-superstimulated heifers, indicating a strong association between P4 concentration and embryo development after the blastocyst stage [40]. The establishment of a uterine environment conducive to embryo growth and elongation appears to be P4 dependent, because alterations in uterine gene expression are induced by increased P4 concentrations. For example, messenger RNA expression for transport and secretory proteins (e.g. lipoprotein lipase and connective tissue growth factor) present in the bovine endometrium, thought to contribute to uterine histotroph and thus conceptus elongation, were expressed earlier during diestrus and at higher levels in cows with elevated P4 concentrations [26].

Studies have compared the concentrations of estradiol and P4, the diameter of ovulatory follicles, and corpora lutea (CL) volume between lactating dairy cows and non-lactating cows or heifers. Lopez *et al*. [41] demonstrated that high producing lactating dairy cows (47 kg/day) had smaller peak concentration of estradiol during estrus (6.8 pg/mL) compared with low producers (32 kg/day - 8.6 pg/ml) and heifers (11.3 pg/mL), despite having larger ovulatory follicles (high producers = 18.6 mm, low producers = 17.4 mm, and heifers = 15 mm). Consequently, high producing dairy cows had shorter duration of estrus (high producers = 7 h, low producers = 11.9 h, and heifers = 11.3 h) and had fewer standing events during estrus (high producers = 6.5, low producers = 9.8, and heifers = 16.8 mounts). Furthermore, Sartori *et al*. [66] demonstrated that lactating dairy cows had smaller concentration of estradiol during estrus than non-lactating dairy cows (7.9 vs. 11.3 pg/mL). Besides affecting estrus detection rates, this reduced estradiol concentration is expected to result in prolonged interval from luteolysis to ovulation (lactating cows = 5.2 ± 0.2 d, non-lactating cows = 4.6 ± 0.1 d) [66] because the estradiol threshold necessary to stimulate an LH surge would take longer to be reached in lactating dairy cows. Extended interval from luteolysis to ovulation compromises oocyte quality because pre-ovulatory follicles are exposed to reducing P4 concentrations and increasing LH pulsatility for longer periods resulting in premature oocyte maturation.

Lactating dairy cows also have reduced P4 concentrations compared with heifers starting as early as d 5 of the estrous cycle [66]. The reduced P4 concentrations to which cows are exposed during metestrus and diestrus may result in exposure of follicles to increasing pulsatile release of LH, which causes premature oocyte maturation and reduced embryo quality [8,38]. Oocytes collected on d 8 of the estrous cycle from cows with P4 concentration declining from 1.7 to 0.6 ng/mL from estrous cycle d 6 to 9 were more likely to be in stage II of meiosis compared with oocytes from cows that had P4 concentration increasing from 1.4 to 3.1 ng/mL during the same period [38]. Furthermore, cows exposed to P4 < 1 ng/mL before ovulation are at higher risk for short luteal phase, because the lack of P4 priming results in premature increase in estradiol receptors in the endometrium following ovulation and consequently premature expression of oxytocin receptors in the endometrium, which leads to premature secretion of $PGF_{2\alpha}$ and luteolysis [31,88]. Exposure of cows to

reduced P4 concentration after AI may affect embryo growth and consequently production of IFN-τ, compromising maternal recognition of pregnancy and pregnancy establishment as described previously.

It is not clear whether reduced production of estradiol and P4 or increased metabolism of estradiol and P4 or both are the cause for reduced estradiol and P4 concentrations in lactating dairy cows, but the latter is more likely. Studies conducted in Wisconsin have demonstrated that the rate of metabolism of steroidal hormones in lactating dairy cows is greater than that of non-lactating dairy cows [61]. This seems to be directly correlated with the increased feed intake of lactating dairy cows and the consequent hyperthrophy and hyperplasia of the liver and organs of the gastrointestinal tract. This results in increased blood flow through the liver and greater metabolism of steroidal hormones. Sangsritavong *et al*. [61] demonstrated that unfed cows have reduced blood flow through the liver compared with cows fed 7.8 lb/d, 23.4 lb/d, and 33.4 lb/d (Figure 2), and that faster decreases in P4 and estradiol concentrations are observed after feeding [61]. Similarly, cows receiving 100 and 50% of NRC (2001) recommendations had significantly reduced P4 concentrations compared with cows receiving 25% of NRC recommendations or unfed cows (Figure 3) [85].

This is clear evidence that high yield lactating dairy cows have reduced estradiol and P4 concentrations as a consequence of increased feed intake,

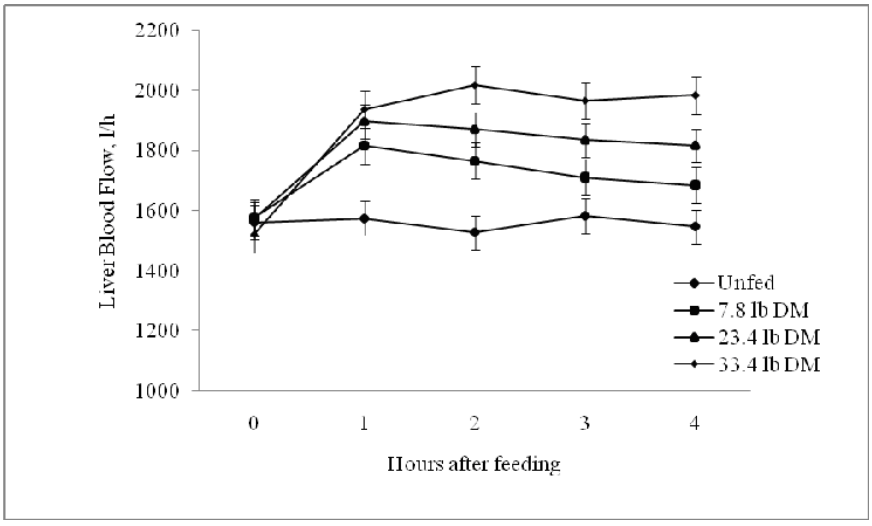

Figure 2. Effect of feed intake on liver blood flow. Adapted from Sangsritavong *et al*. [27].

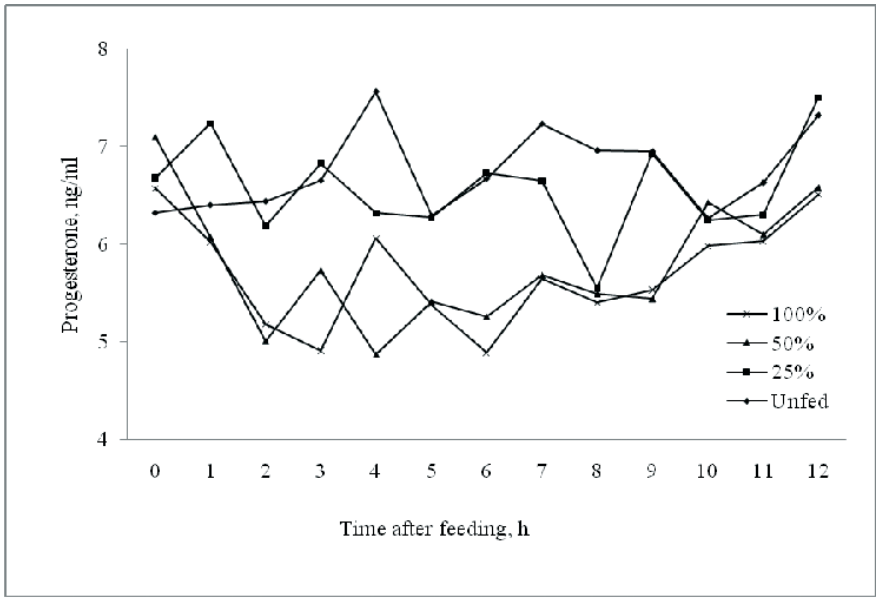

Figure 3. Effect of feed intake on progesterone concentration. Adapted from Vasconcelos *et al*. [28].

which is a consequence of increased milk yield. This poses significant challenges to the reproductive efficiency of these animals because of the importance of estradiol and P4 for reproductive efficiency, explaining in part the significant decreases in reproductive efficiency observed in the past decades.

2.2 Ovulation synchronization protocols

Fixed time AI (TAI) protocols were developed in 1995 with the goal of synchronizing follicular growth, luteolysis, and ovulation. The first protocol developed was the Ovsynch, which consists of one injection of GnRH on d 0, one injection of $PGF_{2\alpha}$ on d 7, a second GnRH injection approximately 56 h after the $PGF_{2\alpha}$ injection and TAI at 12-16 h later [54]. The first GnRH injection synchronizes a new follicular wave, whereas the $PGF_{2\alpha}$ injection synchronizes luteolysis, and the last GnRH injection synchronizes ovulation. Subsequent studies demonstrated that the ideal time to initiate the Ovsynch protocol is between d 5 and 9 of the estrous cycle, because at this stage of the estrous cycle more lactating dairy cows ovulate to the first GnRH injection of the protocol [86]. Later, it was demonstrated that the ovulation to the first GnRH injection of the Ovsynch protocol is critical for embryo quality [9] and P/AI [15] of lactating dairy cows, because cows that do not ovulate to the first GnRH injection have prolonged dominance period of the ovulatory follicle [9] and ovulate aged oocytes [45]. Thus, presynchronization protocols were developed in an attempt to maximize the number of cows that start the timed AI protocol between d 5 and 9 of the estrous cycle.

2.2.1 Presynchronization.

The first presynchronization protocol developed at the University of Florida was based on two injections of $PGF_{2\alpha}$ given 14 d apart (Presynch) [47]. In this study, cows submitted to the Ovsynch protocol 12 d after receiving the Presynch had P/AI approximately 12 percentage points greater than those not presynchronized [47]. By giving 2 injections of $PGF_{2\alpha}$ 14 d apart the percentage of cows that display estrus from 2 to 6 d after the second injection is expected to be 65% [15], depending on compliance to the protocol and the percentage of anovular cows in the herd. Therefore, it is expected that by starting the Ovsynch protocol 10 to 12 d after the last $PGF_{2\alpha}$ injection the majority of cows would be between d 4 and 10 of the estrous cycle.

In an attempt to simplify the Presynch-Ovsynch protocol by giving most injections on the same of the week, Navanukraw *et al.* [50] compared the fertility of cows submitted to the Ovsynch protocol alone with the fertility of cows submitted to a Presynch-Ovsynch with the last $PGF_{2á}$ injection given14 d before the start of the Ovsynch (14-14 Presynch-Ovsynch). In this study, cows receiving the Presynch-Ovsynch (14-14) had greater P/AI than cows receiving the Ovsynch alone.

Galvão *et al.* [29] compared the fertility of cows submitted to the 14-14 Presynch-Ovsynch to that of cows submitted to a 14-11 Presynch-Ovsynch (interval between the last $PGF_{2\alpha}$ injection of the Presynch and the start of the Ovsynch = 11 d). In this study, cows receiving the 14-11 Presynch-Ovsynch had P/AI 6 percentage points higher than cows receiving the 14-14 Presynch-Ovsynch [29]. This improvement in fertility seems to result from the increased percentage of cows that ovulated to the first GnRH injection of the Ovsynch protocol when submitted to the 14-11 Presynch-Ovsynch compared with the 14-14 Presynch-Ovsynch [29].

More recently, presynchronization protocols based on GnRH and $PGF_{2\alpha}$ injections have been developed. Double-Ovsynch is the most know of these protocols as more peer-reviewed data exists [74]. As the name suggests, cows are submitted to a 'presynchronizing-Ovsynch' and 7 d after its end cows are submitted to a 'breeding-Ovsynch'. This protocol has the following potential benefits: improved synchrony of the estrous cycle, anovular cows are more responsive to it than to the Presynch-Ovsynch, and more cows are likely to have growth of the ovulatory follicle under P4 concentrations > 2 ng/ml. The studies published recently comparing the Double-Ovsynch and the Presynch-Ovsynch, however, reported improvements in P/AI only in primiparous cows submitted to Double-Ovsynch, but not in multiparous cows [74]. It is unclear why only primiparous cows benefited from the Double-Ovsynch, but one could speculate that because greater percentage of primiparous cows are expected to be anovular early in lactation compared with multiparous cows, the former would benefit the most from the additional GnRH injections given during the

Double-Ovsynch. It is important to point out, however, that cows submitted to the Double-Ovsynch are less likely to be observed in estrus because of the multiple GnRH injections that they receive and this would affect estrous detection rates.

Therefore, the recommended protocol for first postpartum AI for herds with good estrous detection rate is the Presynch-Ovsynch, with the interval between the last $PGF_{2\alpha}$ injection of the Presynch and the start of the Ovsynch of 10 to 12 d.

2.2.2 Resynchronization.

Most researchers agree that resynchronization protocols to which cows diagnosed non-pregnant are submitted to have to be optimized. Resynchronization protocols used are dependent on herd size, estrous detection rate, and diagnosis of pregnancy by ultrasonography or manual palpation per rectum. Most published research on resynchronization protocols evaluated the effect of timing of initiation of the resynchronization protocol (usually the Ovsynch protocol or an adaptation thereof) on P/AI. It is clear from these published manuscripts that starting the resynchronization protocols before d 28 post-AI will result in reduced P/AI (Figure 4). This is likely because a large percentage of cows that start the resynchronization protocol before d 28 post-AI would be in proestrus, estrus, or metestrus at the start of the resynchronization protocol, affecting ovulation to the first GnRH injection, P4 concentration during ovulatory follicle growth, and synchrony of luteolysis.

Theoretically, if the length of the estrous cycle of lactating dairy cows is 23 d [66], the ideal interval from AI to start the resynchronization protocol would be between 28 and 32 d post-AI (d 5 to 9 of the new estrous cycle). It is interesting to note, however, that recent studies from our laboratory in collaboration with other researchers demonstrated that starting the resynchronization protocol at different intervals after d 27 post-AI does not affect P/AI [6]. In light of the fact that only 52% of cows non-pregnant to a previous AI are observed in estrus between 20 and 24 d post-AI, this finding is not surprising (Chebel personal communication, 2010). Several factors are likely to affect the pattern of return to estrus: 1. approximately 15% of cows submitted to ovulation synchronization protocols do not have the estrous cycle properly synchronized and 10 to 15% of cows inseminated based on signs of estrus are not truly in estrus; 15% of postpartum cows are anovular cows that have shorter luteal phase after first postpartum AI; and, 3. approximately 18% of inseminated cows are expected to have early/late embryonic death. Thus, it is not surprising that we can poorly predict the stage of the estrous cycle that cows are in at the start of resynchronization protocols, making necessary strategies to presynchronize the estrous cycle of non-pregnant cows before the start of resynchronization. A limiting factor to controlling the estrous cycle of inseminated cows before the start of resynchronization protocols is the fact that $PGF_{2á}$ cannot be used until cows are diagnosed non-pregnant.

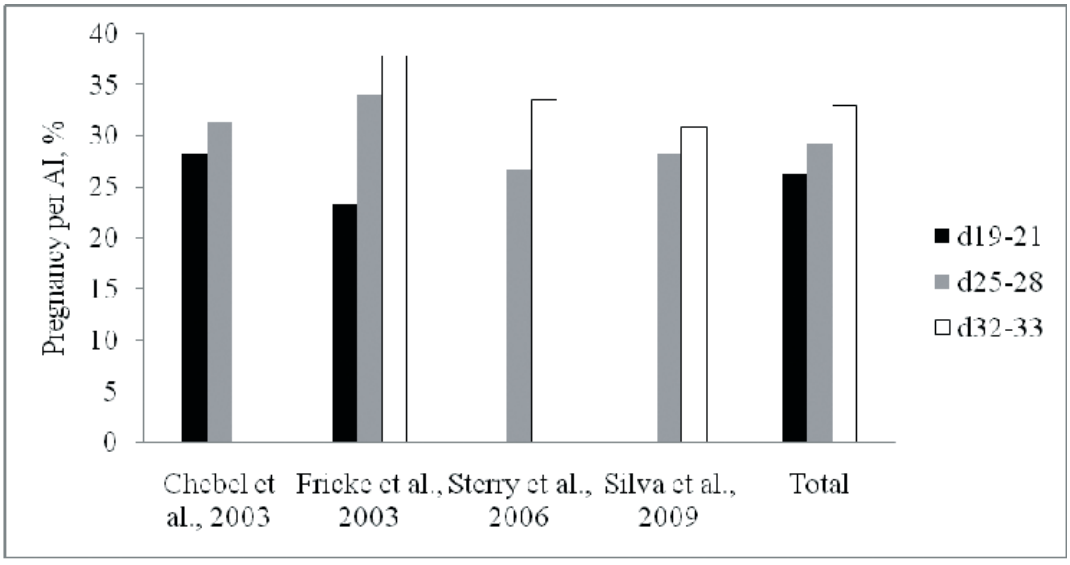

Figure 4. Effects of interval between AI and initiation of the resynchronization protocol (Ovsynch or a variation thereof) on pregnancy per AI.

Although the indicated label use of CIDR inserts is to improve return to estrus when used from 14 to 21 d after initial AI, the use of CIDR inserts according to label recommendations has proven to be inefficient, as the interval to re-insemination and proportion of cows re-inseminated prior to pregnancy diagnosis is not improved [15,29].

Recently, presynchronization protocols for non-pregnant cows before the start of the resynchronization have been explored. In one study, non-pregnant cows were either resynchronized with the Ovsynch protocol starting at 33 d post-AI or with a presynchronizing injection of $PGF_{2\alpha}$ at 34 d post-AI and the Ovsynch protocol at 46 d post-AI [72]. Cows presynchronized with $PGF_{2\alpha}$ had greater P/AI than cows resynchronized with the Ovsynch alone (35.2 vs. 25.6%) [72]. Similarly, cows resynchronized with the Double-Ovsynch (start of the 'presynchronization-Ovsynch' 22 d post-AI, non-pregnancy diagnosis 29 d post-AI by ultrasonography, and start of the 'breeding-Ovsynch' 39 d post-AI) had greater P/AI than cows resynchronized with the Ovsynch alone starting at 32 d post-AI (38.5 vs. 30%) [32]. Although these experiments clearly demonstrate that improvements in P/AI to resynchronized AI could be obtained from presynchronizing the resynchronization protocol, these protocols resulted in inter-AI interval 7 to 13 d longer compared with resynchronizing with the Ovsynch alone, which could offset the improvements in P/AI.

Our laboratory has recently conducted several experiments evaluating different resynchronization protocols. In the most recent study [24], cows at 31 ± 3 d post-AI were selected to receive one of three resynchronization protocols: Cosynch72 starting at non-pregnancy diagnosis; CIDRsynch = Cosynch72+CIDR starting at non-pregnancy diagnosis; or G7G = GnRH injection at enrollment and start the Ovsynch at non-pregnancy diagnosis. All cows were examined for pregnancy 7 d after enrollment, at 38 ± 3 d post-AI. Throughout this study, cows observed in estrus were re-inseminated on the same day. Among cows re-inseminated at fixed (after the completion of the resynchronization protocol), cows in the G7G and CIDRsynch treatments had the greatest P/AI (Cosynch72 = 22.1%, G7G = 31.2%, CIDRsynch = 29.5%) [24]. When we evaluated the data from all cows, including those re-inseminated in estrus, the differences in overall P/AI after re-insemination were smaller (Cosynch72 = 28%, G7G = 32%, CIDRsynch = 31%) [46]. That was mainly because the presynchronizing GnRH injection given to G7G cows and the treatment with CIDR during the resynchronization protocol reduced the percentage of G7G cows and CIDR cows that were re-inseminated in estrus (Figure 5) [46]. Among cows submitted to the Cosynch72 treatment the P/AI of those re-inseminated in estrus was significantly better than that of cows re-inseminated at fixed time, which increased their overall P/AI (Figure 6) [46].

In a subsequent study, we evaluated the effects of a presynchronizing GnRH injection given at different intervals post-AI. In this study, cows received a presynchronizing GnRH injection at 17 or 24 d post-AI and started the Ovsynch 7 d later [7, 46]. Thus, there were 4 treatments: EGGPG – d 17

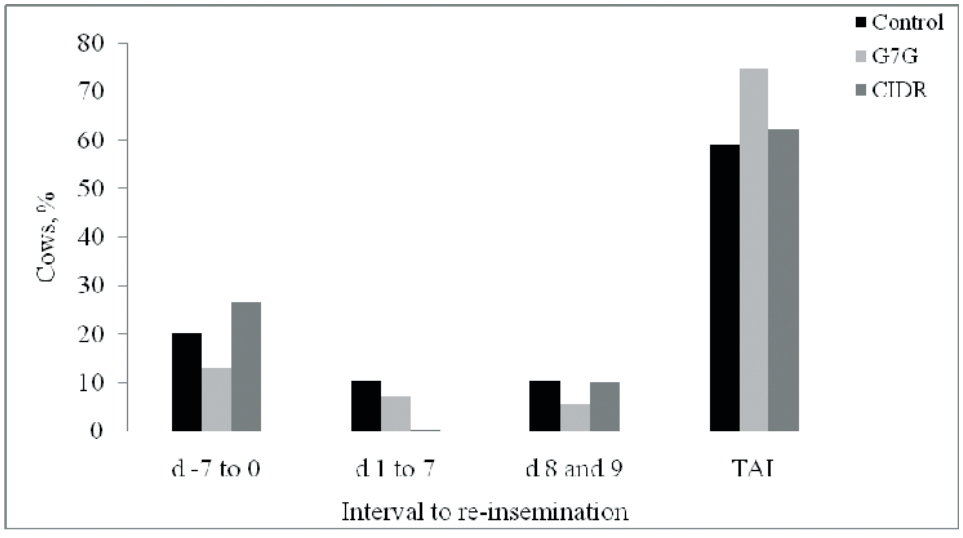

Figure 5. Percentage of cows re-inseminated in estrus at different intervals in relation to the start of the resynchronization protocol (Day 0) and at fixed time. Adapted from Mendonça *et al*. [42].

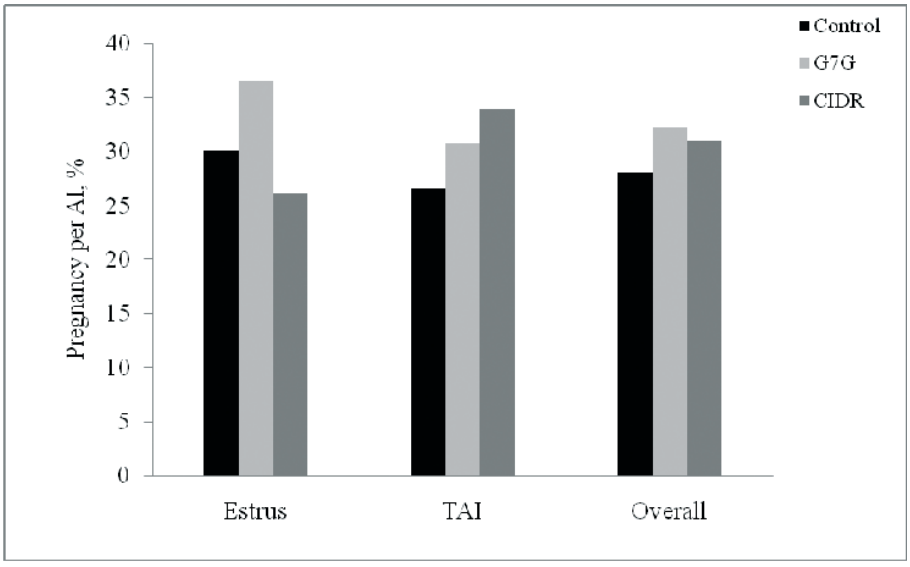

Figure 6. Pregnancy per AI according to resynchronization protocol and re-insemination procedure (AI in estrus or at fixed time (TAI)). Adapted from Mendonça *et al*. [42].

GnRH, d 24 GnRH, d 31 $PGF_{2\alpha}$ (if diagnosed non-pregnant), d 33 GnRH, and d 34 TAI; EOVS – same as EGGPG without the presynchronizing GnRH on d 17 post-AI; LGGPG – d 24 GnRH, d 31 GnRH (if diagnosed non-pregnant), d 38 $PGF_{2\alpha}$, d 40 GnRH, and d 41 TAI; and, LOVS – same as LGGPG without the presynchronizing GnRH on d 24 post-AI [7,23]. Cows were re-inseminated at any time if observed in estrus. Percentage of cows re-inseminated in estrus was smallest for EGGPG treatment and greatest for LOVS treatment (EGGPG=23.7, EOVS = 41.6, LGGPG = 49.0, LOVS = 57.6%) and the interval to re-insemination was slightly shorter for EGGPG and EOVS cows (EGGPG = 13.7 ± 0.2, EOVS = 11.6 ± 0.2, LGGPG = 15.4 ± 0.3, LOVS = 14.6 ± 0.3 d) [7,23]. Overall P/AI [including cows re-inseminated in estrus or at fixed time (TAI) upon completion of the resynchronization protocol] was not different among treatments (EGGPG = 26.2, EOV = 29.1, LGGPG = 30.5, LOV = 30.5%) [7,23]. Regardless of treatment or farm, cows re-inseminated in estrus had greater P/AI at 66 d post-AI than cows that received TAI (36.0 vs. 23.9%). Among cows receiving TAI upon completion of the resynchronization protocol treatment did not affect P/AI (EGGPG = 26.1, EOV = 19.4, LGGPG = 25.3, LOV = 23.8%) [7,23].

The use of CIDR within the resynchronization protocol has also been evaluated by our group in collaboration with other researchers [6]. In this study non-pregnant cows were initiated in the resynchronization protocol at 32 or 39 d post-AI and receive or did not receive a CIDR insert during the resynchronization protocol [6]. In this study, we observed that the interaction between time of initiation of the resynchronization protocol and CIDR treatment affected P/AI, because CIDR treatment tended to increase P/AI of cows starting the resynchronization protocol at 39 d post-AI (28 vs. 23.7%) but had no effect on P/AI of cows that started the resynchronization protocol at 32 d post-AI (no CIDR = 26.9 and CIDR = 24.2%) [6].

In summary, resynchronization protocols should start after 28 d post-AI. Up to date, no presynchronization protocols or additional hormonal treatments (e.g. CIDR during the resynchronization) have been able to increase P/AI of cows that start the resynchronization protocol between 28-34 d post-AI. On the other hand, cows that start the resynchronization protocol at 35-41 d post-AI should be presynchronized (e.g. $PGF_{2\alpha}$ or GnRH or Ovsynch) or treated with CIDR during the resynchronization protocol to increase P/AI to resynchronized TAI. Regardless of the resynchronization protocol chosen, herds that have good estrous detection accuracy and rate should not use 100% timed AI for re-insemination of non-pregnant cows because more than likely P/AI of cows re-inseminated in estrus will be higher than that of cows re-inseminated upon completion of resynchronization protocols.

2.2.3 Reducing the period of dominance of the ovulatory follicle (5d-Cosynch).

Cows submitted to the Ovsynch protocol are expected to have an interval from ovulatory follicle emergence to ovulation of 8.5 d approximately.

According to Cerri *et al.* [10] reducing the interval from emergence to ovulation in 2.3 d (from 8.1 to 5.8 d) results in significant improvements in embryo quality. Thus, we have tested the hypothesis that reducing the interval from the first GnRH injection of the timed AI protocol to insemination would improve fertility.

Because to achieve this reduction in interval from the first GnRH injection to AI we would have to treat cows with $PGF_{2\alpha}$ 5 d after the GnRH injection, which could result in suboptimal luteolysis, in a pilot study, we compared the percentage of cows that had luteolysis when $PGF_{2\alpha}$ was given on d 7 (COS72; GnRH on d 0, PGF2α on d 7, and GnRH+TAI on d 10), on d 5 (COS5d1; GnRH on d 0, PGF2α on d 5, and GnRH+TAI on d 8), or on d 5 and 6 (COS5d2; GnRH on d 0, PGF2a, on d 5 and 6, and GnRH+TAI on d 8) after the first GnRH injection [62]. As expected the percentage of cows that had luteolysis was smallest for those receiving one injection of $PGF_{2\alpha}$ on d 5 after the GnRH (COS72 = 79.0, COS5d1 = 59.1, COS5d2 = 95.7%) [62].

In a subsequent study, 933 cows were submitted to the Presynch and 12 d later to either the COS72 or the COS5d2 described previously [62]. Cows receiving the COS5d2 had smaller ovulatory follicles (18.4 ± 0.3 and 16.8 ± 0.3 mm), reflecting the shorter interval from follicle recruitment to ovulation, and were more likely to have luteolysis (96.3%) compared with COS72 cows (91.5%) [62]. Because greater luteolysis in COS5d2 cows could confound the effect of treatment on P/AI, we analyzed P/AI including only cows that had luteolysis and observed that COS5d2 cows had higher P/AI than COS72 cows at 38 (39.3 and 33.9%) and 66 (36.7 and 32.5%) d after AI [62]. Thus, COS5d2 treatment increased P/AI by reducing the dominance period of the ovulatory follicle [62].

2.2.4 Low P4 concentration and fertility.

The start of the timed AI protocols at 5 to 9 d of the estrous cycle is not only important to maximize the percentage of cows that ovulate to the first GnRH injection of the protocol and to assure that synchronized luteolysis will occur at the end of the protocol, but also to assure that ovulatory follicles grow under elevated P4 concentrations. As mentioned above, reduced concentrations of P4 before ovulation may result in increased exposure of follicles to pulsatile release of LH, which causes premature oocyte maturation and reduced embryo quality [8,38]. In recent studies conducted by my laboratory [23,56] we evaluated whether the reduced P/AI observed in anovular cows and cows induced to ovulate follicles of the first follicular wave was caused by the exposure of ovulatory follicles to reduced concentrations of P4. In these two studies we demonstrated that reduced P/AI of anovular cows and cows induced to ovulate the dominant follicle of the first follicular wave is a consequence of compromised embryo quality because of exposure to P4 concentration < 2 ng/mL during follicle growth (Figure 7 and 8). Interestingly, there was no effect of P4 concentration during growth of the ovulatory follicle on percentage of cows with short luteal phase after AI [46], indicating that the benefits of P4 concentration were likely associated with health of oocyte.

2.3 Embryo transfer

Embryo transfer (ET) is not a new technology as in 1891 Heape [36] reported the transfer of two 4-cell Angora embryos into inseminated Belgium rabbits and the production of four Belgium and two Angora young from the same dam. Only six decades later successful ET pregnancy [80] and birth of an ET calf [90] were reported. Since then, the growth of commercial application of ET in the cattle industry has been significant and, in 2007, 823,160 embryos were transferred [78].

According to the National Animal Health Monitoring System [49], approximately 11.5% of U.S.A. dairy herds have transferred at least one embryo into a lactating dairy cow or heifer in 2006. Interestingly, a similar percentage of operations transferred embryos into only heifers or cows (8.9 and 8.6, respectively) and slightly more operations transferred fresh than frozen embryos (8.2 vs. 7.7%). Although ET is primarily seem as a technique to improve the genetic composition of the herd, in recent years, ET has been used in dairy operations in an attempt to improve reproductive performance of lactating dairy cows.

2.3.1 Mitigation of effects of heat stress on reproductive efficiency.

High production dairy cows (daily milk yield > 35 kg) often consume 20 to 30 kg of dry matter per day. The increased dry matter intake (DMI) results in significant increases in metabolic rates and heat production, such that the daily heat production by

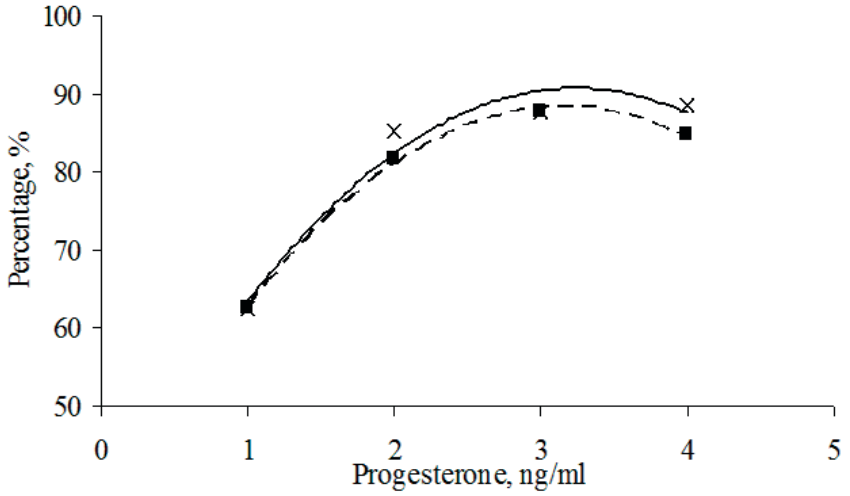

Figure 7. Correlation among P4 concentration during the superstimulation protocol (P4) and percentage of cows producing at least one transferable (solid line; percentage of cows producing at least one transferable embryo = 33.7 + (35.2 x P4) – (5.4 x P4^2); r^2 (Adj.) = 0.96) or one freezable (dashed line; percentage of cows producing at least one freezable embryo = 33.6 + (34.6 x P4) – (5.5 x P4^2); r^2 (Adj.) = 1.0) embryo. Adapted from Rivera *et al*. [47].

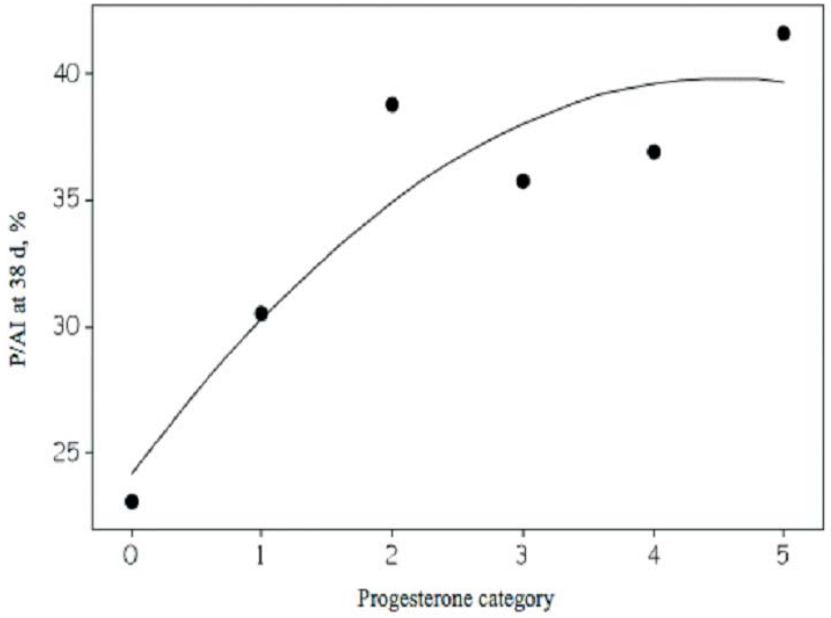

Figure 8. Percentage of cows conceiving after first postpartum AI according to progesterone concentration during ovulatory follicle growth. Progesterone category: 0 = 0 to 0.99 ng/ml; 1 = 1 to 1.99 ng/ml; 2 = 2 to 2.99 ng/ml; 3 = 3 to 3.99 ng/ml; 4 = 4 to 4.99 ng/ml; and, 5 > 5 ng/ml. Pregnancy per AI (P/AI) = 24.2 + (6.9 x P4) – (0.76 x P4^2); r^2 (Adj.) = 75.8%. Adapted from Denicol *et al*. [46].

lactating dairy cows increased 30 MJ since the 50s [4]. This has prompted researchers to re-evaluate characterization of heat stress in lactating dairy cows and to determine that increases in milk yield from 34 kg/d to 46 kg/d result in decreased threshold temperature of 9°F [5]. Consequently, heat stress affects high producing dairy cows more dramatically than lower producing dairy cows and non-lactating animals [1,66].

Oocytes and embryos of lactating dairy cows are damaged by heat stress. Several reports have demonstrated that exposure to heat stress degenerates thecal and granulosa cells, and reduces percentage of fertilized oocytes and percentage of recovered structures classified as excellent-good quality embryos in lactating dairy cows, and *in vitro* and *in vivo* embryonic development [28,34,44,47,50]. Embryos older than 3 to 4 d of age produced *in vivo* from

donors not exposed to heat stress or produced *in vitro*, however, are more resistant to heat stress because they are capable to produce heat-shock protein 70 and because they have greater number of cells [69].

Several studies have demonstrated that, during heat stress, reproductive performance of lactating dairy cows receiving embryos is improved compared with lactating dairy cows receiving AI [28,30,51,72]. In a recent study conducted in Brazil [82] lactating dairy cows were assigned to receive fixed time AI or ET after having their ovulation synchronized with one of two protocols. Although no differences in percentage of cows pregnant were observed between cows receiving AI or ET during the mild weather months, during the summer months a significant decrease in percentage of cows pregnant after AI was observed, whereas season did not affect percentage of cows pregnant at ET (Table 1).

In a study conducted in two commercial dairy farms in TX, cows were randomly selected to receive AI or IVP embryos, fresh or vitrified, during heat stress season [77]. *In vitro* produced embryos were inseminated with sex-sorted semen. Because embryo recipient cows only received embryos if they had a CL on the day of ET, whereas all cows assigned to the AI treatment received AI, the researchers calculated two reproductive outcomes: pregnancy rates (including all cows enrolled in each treatment, regardless if recipient cows received an embryo or not), and P/AI or pregnancy per ET (P/ET; including only cows that received AI or ET) [77]. Cows receiving fresh IVP embryos had the best reproductive performance (P/ET, pregnancy rates, and percentage of cows producing a live calf) followed by cows receiving vitrified IVP embryos and cows receiving AI, respectively (Table 2). Furthermore, 80 to 85% of offspring of cows receiving IVP embryos were female calves (Table 2). Even though the cost of a dose of semen was $ 20 and the cost of IVP embryos was $ 60, the significant improvements in reproductive performance during heat stress and the increased number of live heifers produced from transfer of IVP embryos compared with cows receiving AI, transfer of IVP embryos resulted in an estimated return over investment of $ 22-42/lactating cow/year [19].

Table 1. Reproductive performance of lactating dairy cows from two TX dairy herds exposed to heat stress and submitted to fixed time AI (TAI), *in vitro* produced (IVP) fresh and vitrified embryos. Adapted from Vasconcelos *et al*. [63].

Items	Winter	Spring	Summer	Fall	*P* - value
Pregnancy rate 28 d, %					
TAI	37.1[a]	32.9[a]	21.6[b]	27.0[ab]	0.08
ET	45.1	44.7	41.2	45.1	0.48
Pregnancy rate 60 d, %					
TAI	33.7[a]	28.4[ab]	18.2[b]	25.8[ab]	0.02
ET	38.3	37.1	35.9	39.1	0.50

[a,b] Within a row, means without a common superscript differed ($P < 0.05$).

Table 2. Reproductive performance of lactating dairy cows from two TX dairy herds exposed to heat stress and submitted to fixed time AI (TAI), *in vitro* produced (IVP) fresh and vitrified embryos. Adapted from Bilby *et al.* [64].

Items	AI	IVP fresh embryo	IVP vitrified embryo	*P* - value
Number of cows receiving AI or ET	219	134	188	
Pregnancy per AI or ET[1],%	22.9[a,A]	45.5[b]	30.9[B]	< 0.01
Pregnancy rates, %	18.3[a]	42.1[b]	29.3[c]	< 0.01
Cows delivering a life calf, %	14.6[a]	27.5[b]	17.1[a]	< 0.01
Percentage of live calves of female sex[2], %	50[a,A]	79.1[b]	72.5[B]	< 0.01

[1]Data regarding cows with properly synchronized estrous cycle. [2]Deliveries referent to the services performed during the study.

Unquestionably, improved *in vivo* and *in vitro* production of embryos has favored the use ET-based reproductive strategies to improve reproductive performance of lactating dairy cows, particularly during seasons of heat stress. The more disseminated use of IVP embryos for the reproductive management of lactating dairy cows, however, depends on the creation of cryopreservation procedures that result in higher and more consistent P/ET.

2.3.2 Reproductive performance of repeat-breeder cows.

Dairy cows are classified as repeat breeders once they have received more than 3 AI and do not conceive. Considering that the average pregnancy rate of dairy herds in the U.S.A. is approximately 16% and P/AI approximately 30%, it is not surprising that nearly 10 to 20% of lactating dairy cows in the U.S.A. may be considered as repeat-breeders. The causes of the reproductive failure of repeat-breeder cows are not completely elucidated. Even though the definition of repeat-breeder cows is sub-fertility of animals that do not present anatomical or infectious abnormalities at the time of diagnosis, it is likely that dystocia, occurrence of postpartum diseases (e.g. mastitis, metritis, endometritis, displacement of abomasum), and exposure to heat stress, among other things, are predisposing factors to this condition. Therefore, it is obvious that correction of predisposing factors that cause repeat-breeders is the best solution to this problem. Nonetheless, in situations in which oocyte quality and/or uterine environment are compromised, the use of ET to improve reproductive performance seems to be a good alternative. Recently, researchers have demonstrated in a large retrospective study (n = 9,551) that pregnancy outcomes of repeat-breeder cows was significantly improved when they received ET (41.7%) compared with repeat-breeder cows that received AI (17.9%) [58].

2.3.3 Effects of P4 concentration on embryo survival.

Although the role of P4 on the growth of embryos is well known, conflicting results have been reported regarding the effects of P4 concentration on establishment of pregnancy after ET. Although a few studies with *Bos indicus* influenced beef heifers and lactating dairy cows in tropical environment indicated that P4 concentration at the time of transfer affected P/ET [17,28,39] studies with *Bos taurus* influenced beef and lactating dairy cows demonstrated no correlation between P4 concentration at the time of transfer and P/ET [40,44,60,64,84]. Similarly, P4 concentrations 5 to 7 d after transfer did not affect P/ET in lactating dairy cows according to a few studies [30,81] but increased P/ET in beef and lactating dairy cows according to others [39,51].

An important consideration that must be made when evaluating published data regarding the effect of P4 concentration at the time of ET or 7 d later on P/ET is whether the synchrony of the estrous cycle of the recipient cows was determined and controlled for. In a recent study conducted by our group [39], we determined synchrony of the estrous cycle of recipient lactating Holstein cows based on P4 concentrations at each injection of the OVP and at the time of ET. Cows that did not have a properly synchronized estrous cycle had greater P4 concentration on the day of ET (3.9 ± 0.2 vs. 2.2 ± 0.2 ng/ml), but had lower P4 concentration 7 d after ET (4.5 ± 0.3 vs. 5.6 ± 0.2 ng/ml). That was mainly because among cows with properly synchronized estrous cycle only 3.6% had luteolysis from the day of ET to 7 d later, whereas among cows that did not have a properly synchronized estrous cycle 20% had luteolysis from the day of ET to 7 d later [39]. Consequently, P/ET 60 d after ET of cows that did not have a properly synchronized estrous cycle was lower than P/ET of cows that had a properly synchronized estrous cycle (17.1 vs. 27.2%). In this study, when we excluded from the analysis cows that did not have the estrous cycle properly synchronized and evaluated the effects of P4 concentration 7 d after ET on P/ET, we observed a significant correlation between them (Figure 9) [39].

2.4 Hormonal treatments to increase P4 concentration during diestrus and pregnancy after AI or ET.

Because of the importance of P4 concentration on embryo development, attempts have been made to increase P4 concentrations during metestrus and diestrus of cows receiving AI or ET through different hormonal treatments during the OVP or after presumptive ovulation.

Several research groups have attempted to increase P4 concentration during metestrus and diestrus by treating cows with human chorionic gonadotropin (hCG) or GnRH at different intervals after presumptive ovulation. This is expected to cause ovulation of large/dominant follicles present in the ovary at the time of treatment, formation of an accessory CL, and increase P4 concentrations.

Treatment with 1,500 to 3,300 IU of hCG at 5 to 7 d after presumptive ovulation results in greater percentage of cows forming accessory CL and, in most studies, significant increases in P4 concentrations approximately 7 d later [12,24,50,52]. The effects of hCG given 5 to 7 d after AI or presumptive ovulation on pregnancy outcomes, however, are a little more conflicting with some studies reporting higher pregnancy for hCG treated cows (51,53,60,65] and other studies reporting no effect of hCG treatment on pregnancy outcomes [41,51,78] (Figure 10).

The effects of GnRH treatment after presumptive ovulation on P4 concentration and pregnancy outcomes also are conflicting. Lactating dairy cows [14,17,35] receiving GnRH 5 to 7 d after presumptive ovulation and lactating dairy cows receiving GnRH 11 d after presumptive ovulation [89] had slightly higher P4 concentrations 5 to 7 d after treatment than

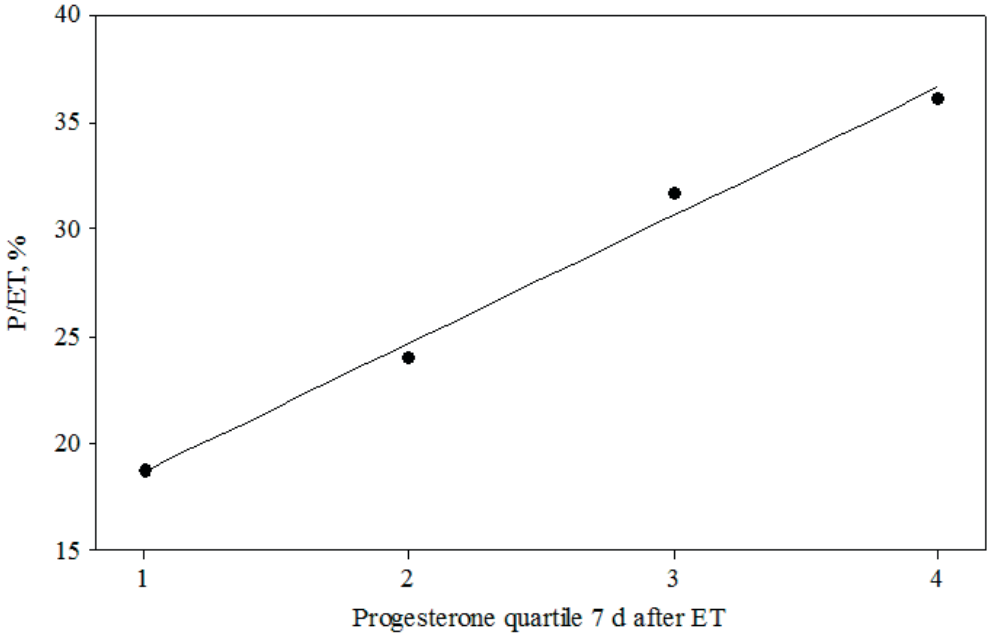

Figure 9. Correlation between P4 concentration 7 d after ET and pregnancy per ET (P/ET) 53 d after ET. Average (± SEM) P4 concentrations on 7 d after ET according to quartiles were: quartile 1 = 2.7 ± 0.2, quartile 2 = 4.7 ± 0.1, quartile 3 = 5.9 ± 0.1, and quartile 4 = 8.4 ± 0.3 ng/ml. P/ET = 12.7 + (6.0 x P4); r^2 (Adj.) = 98.5%. Adapted from Kenyon *et al*. [74].

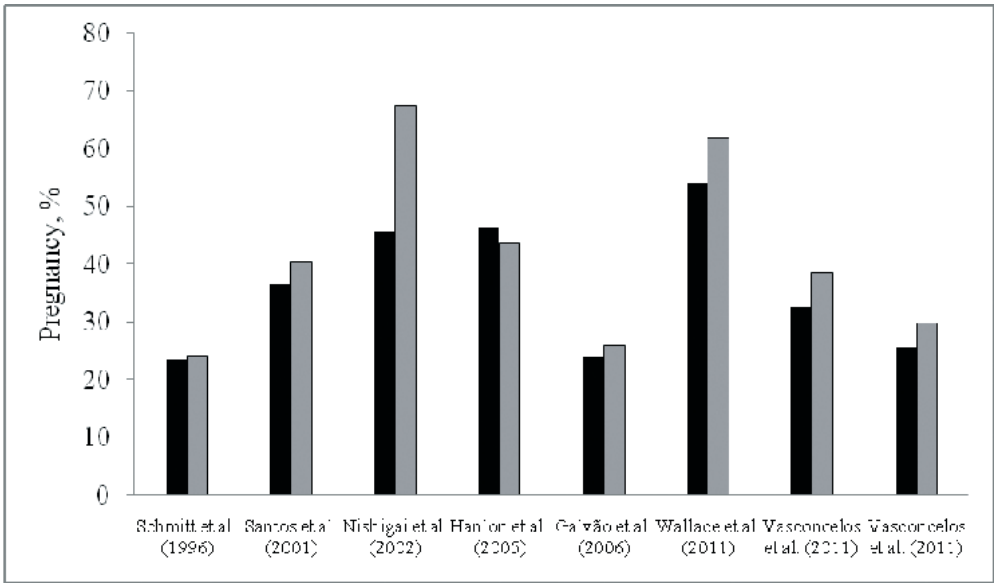

Figure 10. Effect of hCG treatment 5 to 7 d after presumptive ovulation on pregnancy outcomes after AI or ET in dairy heifers [Schmitt *et al*. (69)], beef cows [Nishigai *et al*. (51);Wallace *et al*. (89)], and dairy cows [Santos *et al*. (64); Hanlon *et al*. (69); Galvão *et al*. (30); Vasconcelos *et al*. (81)].

non-treated cows. The increases in P4 concentration after GnRH treatment are smaller and less consistent compared with those observed after hCG treatment because hCG has a longer half-life than GnRH (30 vs. 5 h) and, consequently, a more potent LH-like activity that not only causes new ovulations but extends the functional life of CL already present in the ovaries [18]. Lactating dairy cows treated with GnRH had higher pregnancy outcomes according to some [30,81] but not all studies [24,46]. In a meta-analysis conducted by Peters et al. [53], the effects of GnRH treatment 11 to 14 d after presumptive ovulation on pregnancy outcomes was evaluated based on 16 studies and 8,535 inseminations. According to this meta-analysis, the interaction between study and treatment affected the pregnancy outcomes, which deems this meta-analysis quite inconclusive [86]. When the authors included in the logistic regression important independent variables that could affect the pregnancy outcomes (e.g. OVP to which cows were submitted, cattle breed), however, GnRH treatment did not affect pregnancy outcomes [86]. In recent studies we conducted in dairies in CA, MN, and TX, we demonstrated that GnRH treatment at 17 or 31 d after presumptive ovulation did not reduce pregnancy losses from 31 to 66 d after AI [87], but cows treated with GnRH at 17 and 24 d after AI had greater P4 concentration at 31 d after AI and reduced pregnancy losses from 31 to 66 d after AI (7.5 vs. 11.9%) [88]. It is interesting to point out that according to this later study [88] there was a strong correlation between P4 concentration at 31 d after AI and incidence of pregnancy loss from 31 to 66 d after AI (Figure 11).

Treatment of cows and heifers with equine chorionic gonadotropin (eCG) during OVP has garnered interest for its potential to increase P4 concentration after ovulation and increase pregnancy per AI or ET. In general, the studies that evaluated the effects of eCG treatment on P4 concentration and pregnancy outcomes attempted to promote hastened growth and ovulation of dominant follicles and,

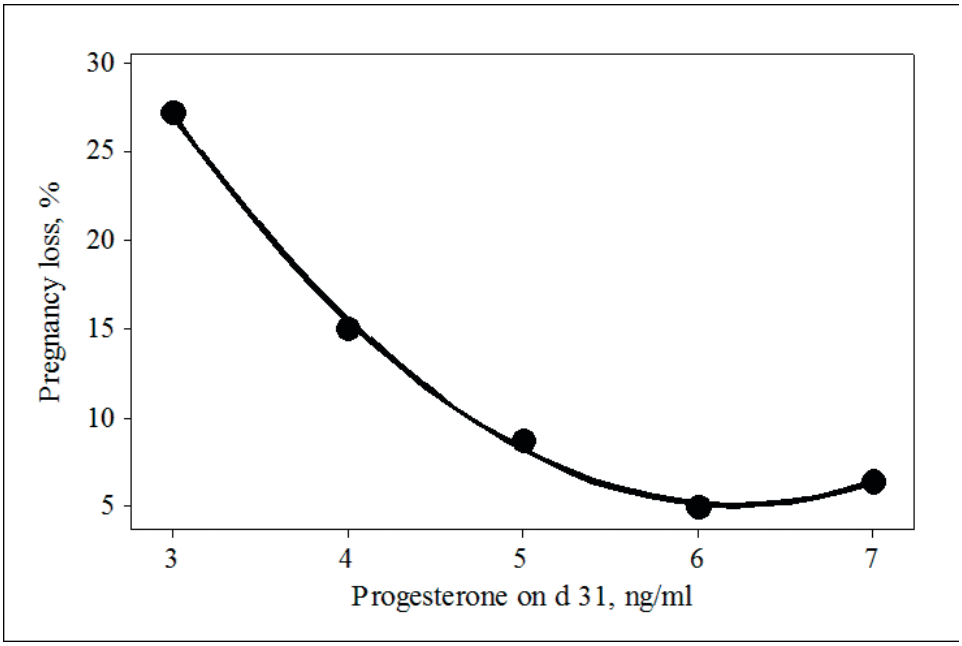

Figure 11. Correlation between progesterone concentration 31 d after AI (P4) and pregnancy loss from 31 to 66 d after AI. Pregnancy loss = 87.41 – (26.5 x P4) + (2.1 x P4^2); r^2 (Adj.) = 99.7%. Adapted from Scanavez *et al*. [88].

consequently, increased P4 concentration after AI or ET by treating animals with eCG close to the expected time of ovulation [32,36,47,56,90]. Although these studies do not promote consensus regarding the effects of eCG treatment on pregnancy outcomes, it appears that anovular cows and cows with low body condition score would benefit the most from eCG treatment. On the other hand, treatment of embryo recipient lactating dairy cows with eCG around the time of follicular wave emergence to promote superstimulation and higher P4 concentration at the time of ET does not seem to improve P/ET (control =

32.8 vs. eCG = 37.3%) [39]. According to this study, treatment of cows with 800 IU of eCG around the time of follicular wave emergence resulted in reduced percentage of cows with synchronous estrous cycle (61.0 vs. 71.7%) and reduced the percentage of cows selected to receive ET (79.1 vs. 87.5%) [39]. It is not clear how eCG affects synchrony of the estrous cycle in lactating dairy cows, but the eCG's long half-life is likely to be involved.

Important factors that may affect the effects of hormonal treatments during the OVP and after presumptive ovulation on pregnancy outcomes are body condition score, lactation status, environmental conditions, and cattle breed.

III. CONCLUSIONS

The constant pressure for more efficient milk production demands that high producing dairy cows be used by dairy operations. Because of the dependency of milk yield on dry matter intake, it is unavoidable that lactating dairy cows will continue to demonstrate physiological changes (e.g. reduced steroidal hormone concentration and increase sensitivity to heat stress) that predispose them to compromised reproductive performance. The use of fixed time AI protocols and ET in the reproductive management of lactating dairy cows will continue to be important, particularly in situations of heat stress.

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