Double trouble: Implications and management of twinning in lactating dairy cows

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Abstract
Over the past 4 decades, twinning increased concurrently with milk production in dairy cattle, but recently appears to have plateaued, likely from the adoption of hormonal synchronization protocols. Approximately 95% of Holstein twins are dizygotic, making double ovulations the primary mechanism for twinning. Decreased progesterone (P4) concentrations during preovulatory follicle growth increases double ovulations. Increased hepatic catabolism of P4 because of increased feed intake associated with increased milk production provides a physiological mechanism for decreased P4 concentrations and increased double ovulations. Cows with unilateral twins have more pregnancy losses than cows with bilateral twins which were similar to cows with singletons. Selective embryo reduction followed by P4 treatment did not increase the risk of pregnancy loss for cows with unilateral twins, but increased the risk of loss for cows with bilateral twins. Twinning will likely increase concurrently with milk production over time but can be decreased using 2 approaches: 1) submitting cows to hormonal synchronization protocols that increase P4 during growth of the preovulatory follicle before timed AI; and 2) identifying twins by laterality and allowing cows with bilateral twins to continue through gestation with extra assistance at calving, while selective reduction can be attempted for cows with unilateral twins.

Key words: twinning, double ovulations, dairy cattle

Introduction
Twinning in dairy cattle is associated with many factors, such as cyclicity, breed, parity and milk production, that can negatively impact the health and productivity of cows and calves.[22] Epidemiological data suggests an increase in the prevalence of twinning in U.S. Holsteins over time concurrent with milk production.[40,62] It is estimated that the economic losses due to twinning in dairy cattle are $59 to $151 per twin pregnancy,[9] and the overall negative economic impact of twinning on dairy farm profitability in the United States is $96 million per year.[55] As actual and predicted milk production of U.S. lactating dairy cows continues to increase,[7] twinning in dairy cattle will likely concurrently increase, further perpetuating the problem of twinning on dairy farm profitability.

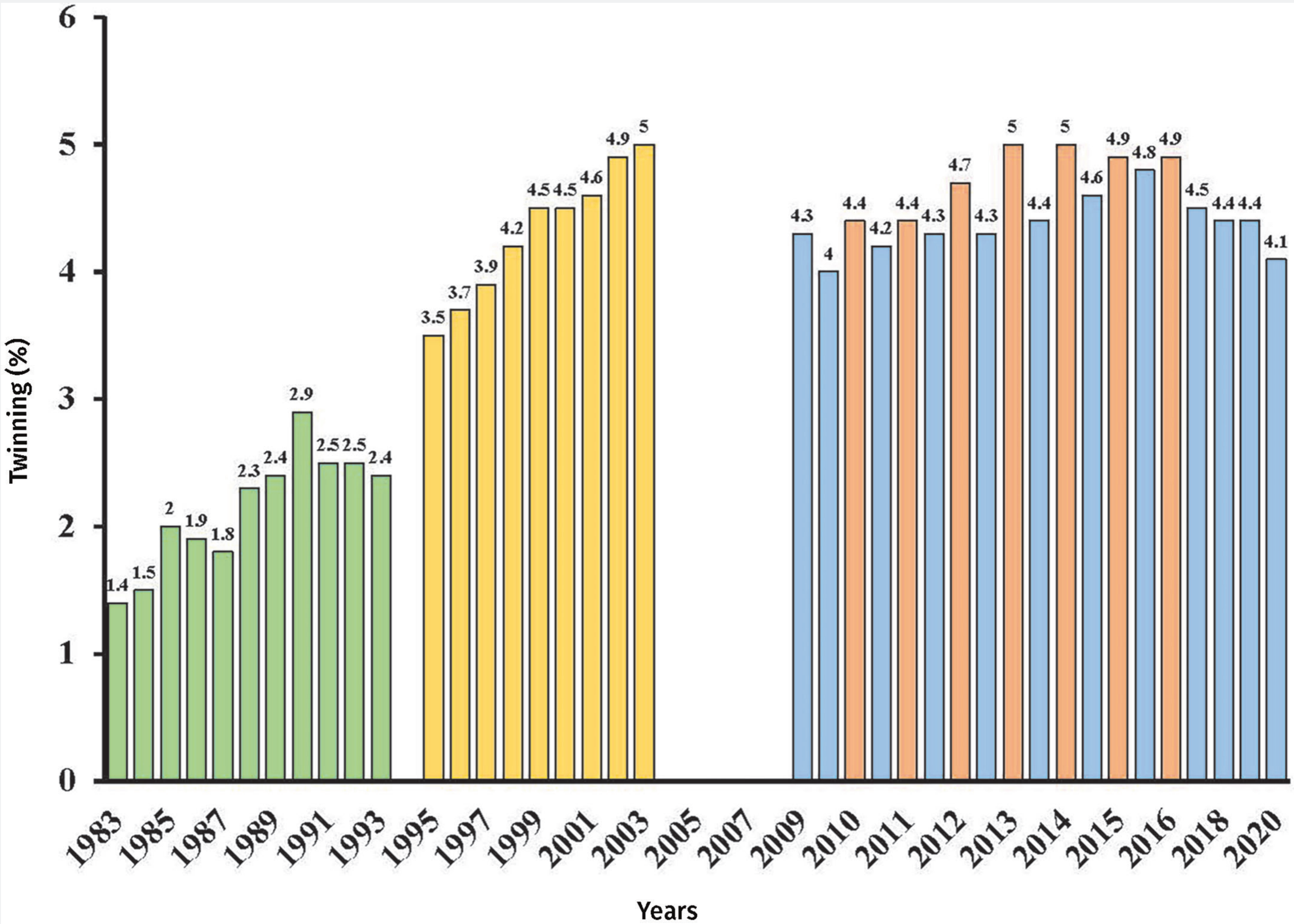
Previously, few controlled experiments examined the endocrinology and physiology behind twinning, making it challenging to provide research-based recommendations to mitigate the negative effects of twinning.[23,21] More epidemiological and randomized-controlled experiments are now being published regarding the incidence and the endocrinology and physiology of twinning in dairy cattle to fill previous knowledge gaps. The objective of this review is to update and expand upon previous reviews[23,21] of twinning in dairy cattle to understand the current incidence, mechanisms, endocrinology and physiology of twinning and the research-based management strategies that can be used to identify and minimize the negative impact of twinning on dairy cows.

Incidence of twinning
Initial epidemiological studies of twinning reported a linear increase in the incidence of twinning in U.S. dairy cattle from 1983 to 1993 (1.4 to 2.4%)[40] and from 1996 to 2004 (3.4 to 4.8%).[66] In recent epidemiological studies, the incidence of twinning reported in Holsteins was 4.7 to 4.8%.[43,62] Cows that calved with twins in the previous lactation were at 1.86 times greater risk for calving with twins at their subsequent calving than cows that previously calved with singletons.[62]. By accumulating these several extensive epidemiological studies, we can characterize the prevalence of twinning in U.S. dairy cattle over the past nearly 4 decades. Figure 1 depicts the incidence of twinning in U.S. dairy cattle based on data from 1983 to 1993,[40] 1996 to 2004,[66] 2010 to 2016[43] and 2009 to 2020.[62]

Over this time, annual milk production per cow in the United States has increased and is predicted to increase over the next 50 years because of improvements in genetics and management.[7] Kinsel et al. (1998) identified increased peak milk production concurrent with increased twinning as the significant factor associated with increased twinning.[40] An observational analysis of Holstein dairy cattle in the Upper Midwest from our laboratory reported a parity by time interaction with the prevalence of twinning increasing at an increased rate in primiparous (4.0 to 5.9%) and multiparous (5.2 to 7.3%) cows during the first and last year of the study than in nonlactating heifers (1.1 to 1.3%).[66] Schambow et al. (2021) reported a positive association with previous lactation 305-d milk production of multiparous Holstein cows with twinning rate (Figure 2).[62] While not causal, a positive association exists between milk production and twinning, suggesting that twinning will continue to increase over time. Interestingly, twinning increased linearly from 1983 to 2016, but then plateaued from 2017 to 2020 (Figure 1). It is likely that the incidence of twinning from 2017 to 2020 plateaued because of the increased adoption of hormonal synchronization protocols that expose the preovulatory follicle to a high progesterone (P4) environment, decreasing double ovulations and twinning.

Mechanisms for twinning
As a monotocous species, dairy cattle typically produce only 1 calf per pregnancy, but occasionally can produce twins, or even rarer, triplets. Twins are classified as either monozygotic or dizygotic due to the different production mechanisms. Monozygotic (identical) twins arise from spontaneous in vivo cleavage of a fertilized oocyte during early embryonic development, producing 2 genetically identical embryos. By contrast, dizygotic (fraternal) twins occur after the ovulation of two follicles and fertilization of 2 oocytes by two individual sperm during the same estrous cycle. The proportion of monozygotic twins in

Figure 1: Incidence of twinning in U.S. dairy cattle from 1983 to 2020. Data includes nonlactating and lactating dairy cattle except Schambow et al., 2021 which only includes lactating dairy cows.



Green bars: data adapted from Kinsel et al., 1998; yellow bars: data adapted from Silva del Río et al., 2007; blue bars: data adapted from Schambow et al., 2021; orange bars: data adapted from Lett and Kirkpatrick, 2018.

dairy cattle was estimated using mathematical models such as Weinberg's Differential Method[72] and Bonnier's Equation[6] focusing on the skewing of the sex ratio from mixed-sex twins to same-sex twins. Genomic testing is now used to determine the proportion of monozygotic twins empirically and accurately because these mathematical models tend to overestimate the observed proportion of monozygotic twins.[65] In an observational experiment in our laboratory, the reported frequency of monozygotic twins in Holstein cows was determined by genotyping all same-sex twins with 7.5% of same-sex and 4.7% of all twins identified as monozygotic twins.[65] Thus, with 95.3% of all twins being dizygotic in Holstein cows, double ovulations are the primary mechanism for twinning in lactating dairy cows.

Twinning and replacement heifers

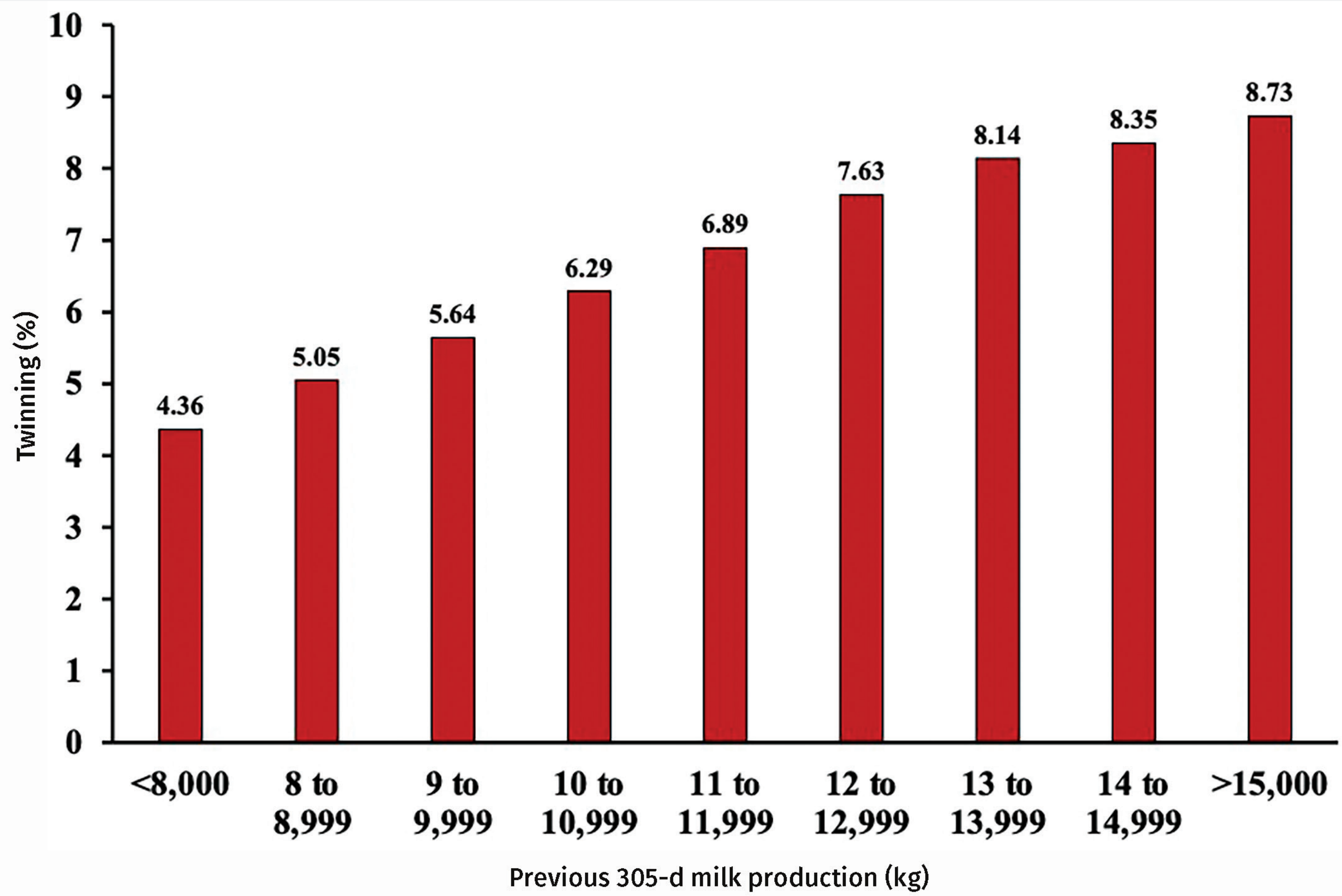
Twinning in dairy cattle is associated with fewer replacement heifers per calving.[56,14, 66] Freemartinism occurs in heifers when gestated co-twin with a bull because of anastomoses of chorionic blood vessels, allowing for Anti-Mullerian hormone to regress the Mullerian ducts in the heifer, causing abnormal reproductive development and infertility. These reproductive abnormalities of freemartins occur at 49 to 52 days after fertilization[39] with approximately 92% of heifers born co-twin to a bull being freemartins.[8] A common misconception is that freemartinism is the primary reason for fewer replacement heifers from twinning, but across 100 singleton or twin calvings, approximately 50 replacement heifers are born. The fewer replacement heifers associated with twinning are because of the increased abortions,[2] stillbirths,[2] and neonatal calf mortality.[66] In a large observational analysis of Holstein dairy cattle, we reported neonatal calf mortality of 7.2% and 28.2% for singleton and twin calvings, respectively. Figure 3 depicts neonatal calf mortality for singleton and twin calvings by parity of U.S. Holstein cows from 1996 to 2004.[66]

Endocrinology and physiology of twinning

Progesterone

In cattle, P4 is the most biologically active progestogen and is primarily produced and secreted by the corpus luteum during the estrous cycle and the placenta during pregnancy. During growth of the preovulatory follicle, P4 concentrations are associated with the incidence of double ovulations. Decreased P4

Figure 2: Association between previous 305-d milk production and observed twinning rate in Holstein cows (adapted from Schambow et al., 2021).



concentrations during growth of the preovulatory follicle are associated with increased double ovulations.[73] When a preovulatory follicle develops without a corpus luteum producing P4, cows have a greater incidence of co-dominant follicles resulting in double ovulations.[35] Valdés-Arciniega et al. (under review) reported a negative association between P4 concentrations at the time of $PGF_{2\alpha}$ treatment in the Breeding-Ovsynch portion of a Double-Ovsynch protocol and double ovulations to the last GnRH treatment (Figure 4).[69]

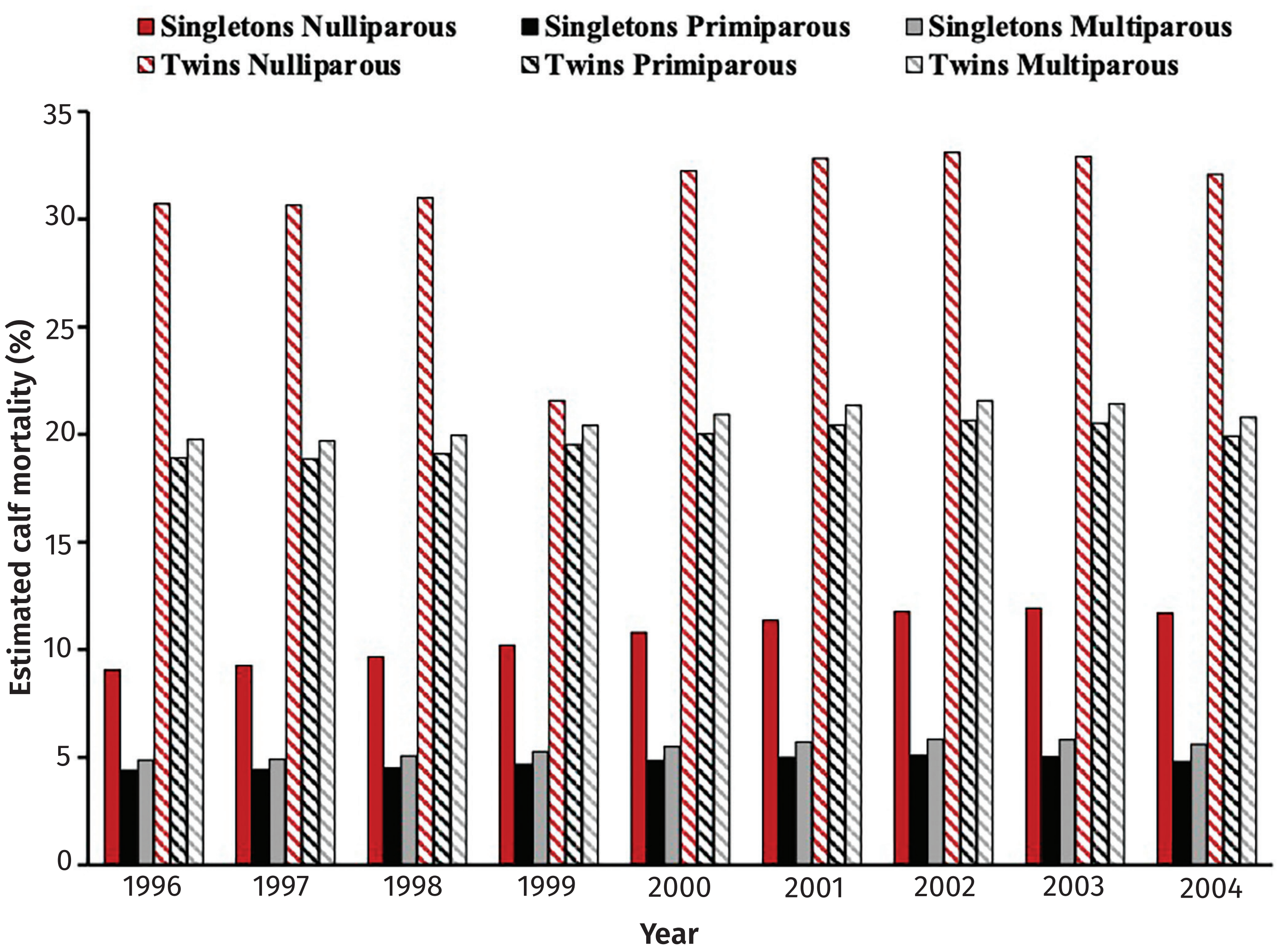
From calving to first ovulation, all lactating dairy cows undergo low P4 concentrations during an anovulatory period postpartum. Anovular cows after a spontaneous estrus had a greater (46.3%) double ovulation rate for their first ovulation than ovular cows at second and greater services (22.4%).[44] Gümen et al. (2003) identified cows as ovular or anovular and were submitted to an Ovsynch protocol for timed AI. Anovular cows had a greater double ovulation rate to the first GnRH treatment of the Ovsynch protocol compared with ovular cows (41 vs. 4%, respectively), but the incidence of double ovulation to the second GnRH treatment did not differ (13 vs. 12%, respectively).[33]

An association exists between lactating dairy cows with cystic ovaries and the incidence of double ovulation[61,51,42] and twinning.[5] For cows identified with ovarian cysts with the absence of a corpus luteum submitted to an Ovsynch protocol, the odds of double ovulation were 3.3 times greater than cows inseminated after a detected estrus or non-cystic cows submitted to an Ovsynch protocol.[63] With an ovarian cyst in the absence of a corpus luteum, the underlying association between ovarian cysts and twinning is likely due to the absence of P4 rather than the ovarian cystic structure.[63] Thus, exposure of the preovulatory follicle to P4 decreases the incidence of double ovulations.

Milk production

In most studies, milk production around the time of ovulation is positively associated with the incidence of double ovulations. Cows with above-average milk production (40.7 kg/d) had a greater incidence of double ovulations after submission to an Ovsynch protocol than cows with below-average production regardless of parity (20 vs. 7%, respectively).[26] Lopez et al. (2005a) reported a positive association of milk production 14 d before a spontaneous estrus with the incidence of double ovulations (Figure 5A).[45] Cows with co-dominant follicles during the first follicular wave produced more milk, had decreased progesterone concentrations, and increased FSH and LH concentrations around the expected deviation of the dominant follicle.[46] Further, a positive association exists between milk production during the 7 d between the first GnRH and $PGF_{2\alpha}$ treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol and the incidence of double ovulations to the last GnRH treatment before timed AI (Figure 5B).[69]

Figure 3: Estimated neonatal calf mortality of singleton and twin calvings based on parity of U.S. Holstein cows (adapted from Silva del Río et al., 2007).



Physiology of twinning

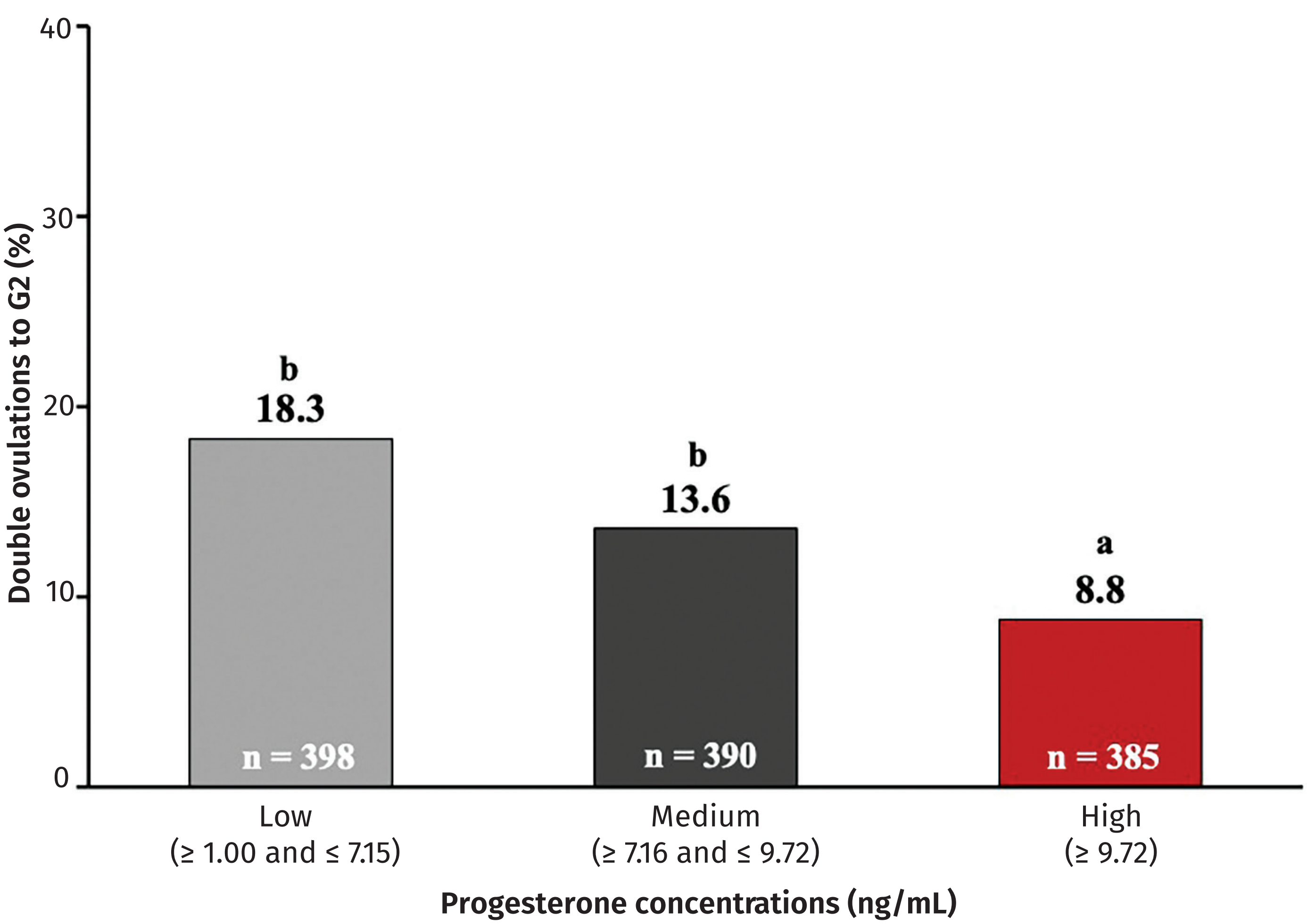
Wiltbank et al. (2000) first proposed a physiological mechanism linking milk production, P4 concentrations, and double ovulations.[73] The steady-state P4 concentration in the circulation of lactating dairy cows is a balance between P4 produced by the corpus luteum and catabolized by the liver.[74] Milk production is highly correlated (r = 0.88) with feed intake[34] which then increases hepatic blood flow as milk production and feed intake increase. With the increased feed intake of a high-producing dairy cow, there is increased hepatic metabolism of steroid hormones such as P4, thereby decreasing circulating P4 concentrations in high-producing dairy cows.[59] Around the time of deviation, decreased P4 concentrations likely cause a delay in the FSH nadir and increase LH pulses, allowing for the selection of 2 or more follicles to be co-dominant during a follicular wave.[74] As milk production has and continues to increase in U.S. lactating dairy cows,[7] circulating P4 concentrations will continue to decrease and increase the incidence of double ovulations and dizygotic twinning. Thus, it is critical to implement management strategies to minimize double ovulations and dizygotic twinning, such as hormonal synchronization before AI, which will be described later in these proceedings.

Identification of cows carrying twins

Transrectal ultrasonography

Management of lactating dairy cows gestating twins requires early and accurate identification. Over the past two decades, as predicted by Fricke (2002), bovine practitioners readily adopted transrectal ultrasonography as a reproductive management tool.[22] In earlier studies, cows gestating twins could be accurately identified using transrectal ultrasonography by 40 to 55 d after insemination.[17,13,15] With most twins being dizygotic[65] and the advancements made in the resolution of ultrasound scanners, cows gestating twins can be accurately identified at an early pregnancy diagnosis conducted 32 to 39 d after AI by the presence of two or more corpora lutea on the ovaries.[22] In addition to evaluating corpora lutea, it is critical to examine the entire length of each uterine horn during a pregnancy diagnosis to accurately diagnose twins using transrectal ultrasonography.[22] With 5% of all twins being monozygotic,[65] cows gestating monozygotic twins would be misidentified as carrying singletons because these cows will have only one corpus luteum. Further, some cows will double ovulate to form two corpora lutea but may only carry a singleton.

Figure 4: Incidence of double ovulations for multiparous Holstein cows to the last GnRH treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol (G2) based on tertiles of serum progesterone concentration at the first $PGF_{2\alpha}$ treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol. Different letters (a-b) denote statistical differences ($P < 0.05$; adapted from Valdés-Arciniega et al., Under Review).



Pregnancy loss

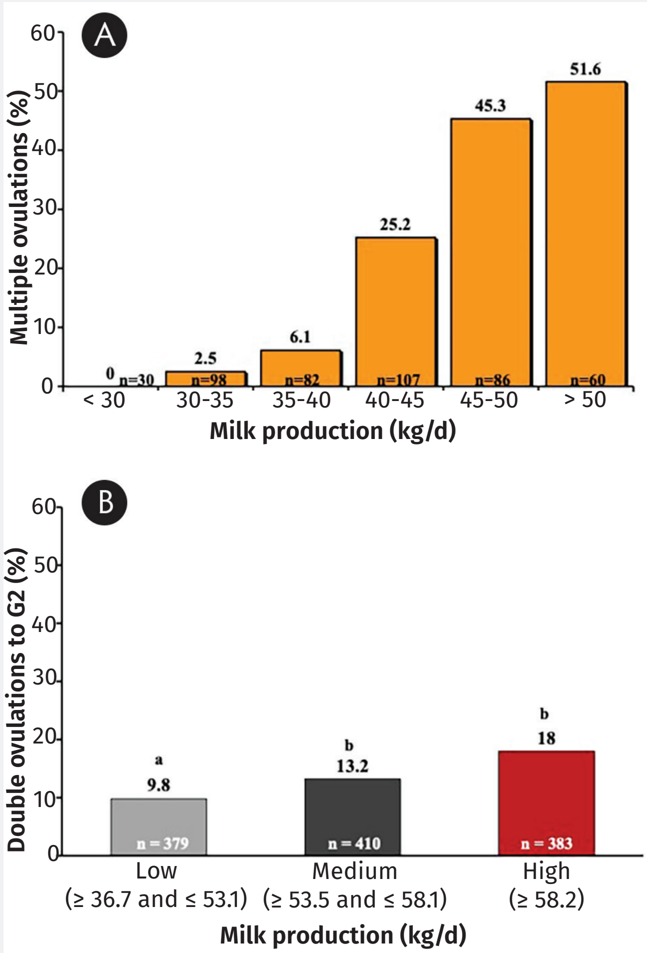
Pregnancy loss confounds the accuracy of the prevalence of twinning in dairy cattle because cows with twins have a greater incidence of pregnancy loss than cows with singletons. Cows with double ovulations tend to have more pregnancies per AI than cows with single ovulations,[26,53,69] but have more pregnancy losses. The pregnancy loss from an initial pregnancy diagnosis to a pregnancy reconfirmation based on transrectal ultrasonography for cows diagnosed with twins was 3 times greater compared with cows diagnosed with singletons, resulting in an embryo survival rate of 76 and 92%, respectively.[63] Cows that double-ovulated to the last GnRH treatment of a Double-Ovsynch protocol had greater pregnancy loss from 32 d after AI to calving than cows that single-ovulated (23.5 vs. 14.1%, respectively).[69]

The laterality of twin pregnancies (unilateral = 2 fetuses in one uterine horn; bilateral = 2 fetuses, one in each uterine horn) affects pregnancy loss. López Gaitius et al. (2020) reported 54.4% of cows gestating multiple fetuses to be unilateral and 45.6% to be bilateral.[49] Pregnancy loss before 90 d in gestation was greater for cows with unilateral (35%) than bilateral twins (8%).[50] Cows with unilateral double ovulations have greater pregnancy losses from 35 to 56 d after AI (16.9 vs. 8.8 vs. 4.2%) and from 23 d after AI to calving (39.3 vs. 21.4 vs. 25.9%) than cows with bilateral double or single ovulations.[53] By contrast, Valdés-Arciniega et al. (under review) reported no differences in pregnancy losses based on laterality from 32 d after AI until calving.[69] Further, the laterality of twin pregnancies affects the incidence of dystocia and calf survival. Cattle were genetically selected for multiple ovulations creating a "Twinner population" for a long-term experiment at the U.S. Meat Animal Research Center in Clay Center, NE.[32] Echternkamp et al. (2007) analyzed calvings and calves from 1996 to 2004 from this population of genetically selected cattle.[18] Bilateral dizygotic twins had increased survival and body weight at birth, longer gestation length, and less dystocia than unilateral dizygotic twins (Table 1).[18] As expected, cows calving twins or triplets had a greater incidence of dystocia than cows calving singletons.[18]

Pregnancy-associated glycoproteins

While the function remains unknown, pregnancy-associated glycoproteins (PAG) are produced by giant binucleate cells of the trophectoderm and are secreted into the maternal circulation serving as an indirect marker for pregnancy establishment,[60,54] placental function[30] and pregnancy loss[29,16] in dairy

Figure 5: Incidence of double ovulations for Holstein cows based on milk production during the 14 d before a spontaneous estrus (A: adapted from Lopez et al., 2005) or during the 7 d between the first GnRH and $PGF_{2\alpha}$ treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol (B: adapted from Valdés-Arciniega et al., Under Review). Different letters (a-b) denote statistical differences ($P < 0.05$).



cattle. Concentrations of PAG in maternal circulation via blood or milk can accurately diagnose pregnancy in dairy cattle and are associated with parity, stage of gestation, milk production and pregnancy loss.[58] Serum PAG concentrations were greater for cows with twin fetuses compared with cows with singleton fetuses and this difference increased as gestation progressed from 35 to 65 d after AI.[48] Giordano et al. (2012) reported differences in serum PAG concentrations between cows gestating singleton vs. twin fetuses by 27 to 29 d after AI.[29] While concentrations of PAG in maternal circulation can accurately diagnose pregnancy, the use of PAG concentrations to accurately identify cows gestating twins is likely limited. Greater pregnancy loss is associated with twin pregnancies[63,69] with some cows undergoing spontaneous embryo reduction and calving with singletons.[49,63] In circulation, PAG concentrations have a relatively long half-life, with PAG concentrations not decreasing to nonpregnant cow concentrations until 9.5 d after induction of pregnancy loss at 39 d in gestation.[29] Further, with most commercial PAG test being qualitative rather than quantitative, using PAG concentrations as diagnostic indicator of twins in dairy cattle is not feasible.

Management strategies to minimize twinning

Pregnancy termination

Spontaneous pregnancy loss in dairy cattle from d 20 to 33 after AI is initiated approximately 50% by conceptus failure or 50% by luteal regression.[16] A drastic method to eliminate twinning in dairy herds is to identify cows gestating twins and inducing pregnancy loss by treatment with the luteolytic agent of $PGF_{2\alpha}$ for luteal regression. Treatment with $PGF_{2\alpha}$ at 39 d of gestation of singleton pregnancies decreased P4 within 24 h with expulsion of the conceptus in all cows within 48 h.[29] Several arguments exist against using proactive pregnancy termination for eliminating twin pregnancies identified early in gestation. First, estimated economic losses incurred due to pregnancy loss range from $46[19] to $300[28] with cows diagnosed with twins often being the highest-producing cows in the herd incurring the greatest economic loss associated with pregnancy loss. Second, the heritability and repeatability estimates for twinning in dairy cattle are low,[71,31,43] but a prior incidence of twinning is a risk factor for subsequent twin calvings.[5,56,62] Third, pregnancy loss before 90 d of gestation for cows with unilateral twins did not differ between control cows and cows receiving manual amnion rupture followed by progesterone supplementation for 21 d[2] and the pregnancy loss of cows gestating bilateral twins was similar to cows gestating singletons.[53] Further, bilateral dizygotic twins have an increased survival and birth weight, longer gestation length, and decreased dystocia than unilateral dizygotic twins.[18] Thus, a possible strategy is to allow cows identified with bilateral twins to continue gestation with extra assistance provided at calving and selective reduction could be attempted for cows identified with unilateral twins.

Selective reduction

In early gestation, selective embryo reduction has been used to mitigate the potentially dangerous maternal effects of multiple births in women[37] and mares.[52] Two controlled experiments using lactating dairy cows reported the efficacy of manually crushing the amnion of one of the embryos in a twin pregnancy to maintain a viable singleton pregnancy.[47,2] Lopez-Gaitus (2005) randomized 33 cows identified with unilateral twins on d 34 of gestation to serve as untreated controls, receive manual amnion rupture, or receive manual amnion rupture with intravaginal P4 supplementation for 28 d.[47] Selective embryo reduction was attempted between 35 to 40 d in gestation because spontaneous reduction of twins for many cows occurs during this period.[50] Pregnancy loss for control cows (i.e., spontaneous loss of both twins) was 27% (3/11), whereas pregnancy loss for cows undergoing manual amnion rupture was 100% (11/11).[47] Interestingly, pregnancy loss for cows supplemented with P4 for 28 d after amnion rupture was 55% (6/11).[47] Of cows supplemented with intravaginal P4 for 28 d after manual amnion rupture, 1 cow calved with twins because an embryo survived the rupture while the remaining 4 calved singletons.[47]

In a follow-up experiment by the same research group, they evaluated the effect of embryo reduction in Holstein cows based on laterality of twin pregnancies on pregnancy maintenance (Table 2).[2] Andreu-Vázquez et al. (2011) blocked by laterality and randomized 55 cows with live twins at 35 to 41 d of gestation to receive manual embryo reduction with intravaginal P4 supplementation for 21 d or serve as untreated controls.[2] Pregnancy loss before 90 d in gestation did not differ between

Table 1: Effect of twinning and laterality on gestation length, calf survival, and birth weight (lb) in cattle genetically selected for multiple ovulations. Different superscript letters (a-c) denote statistical differences (*P* < 0.05; adapted from Echternkamp et al., 2007).

Pregnancy type	Cows (n)	Gestation length (d)	Calves (n)	Calf survival (%) Birth	Calf survival (%) Weaning	Birth Weight (lb)
1 Left	300	284.5 ± 0.2[a]	711	97.3 ± 1.1[a]	87.6 ± 1.5[a]	105.6 ± 0.44[a]
1 Right	360	284.2 ± 0.2[a]	876	97.0 ± 1.0[a]	88.3 ± 1.3[a]	105.4 ± 0.44[a]
2 Left	96	277.2 ± 0.2[b]	446	83.6 ± 1.4[c]	70.7 ± 1.9[b]	81.0 ± 0.66[b]
2 Right	167	277.0 ± 0.1[b]	838	82.7 ± 1.0[c]	73.2 ± 1.4[b]	80.5 ± 0.44[b]
2 Bilateral	259	278.2 ± 0.1[c]	1,158	94.0 ± 0.9[b]	85.4 ± 1.2[a]	85.8 ± 0.44[c]

Table 2: Effect of laterality of twin pregnancy on pregnancy loss before Day 90 for control cows (no manipulation) and cows subjected to manual twin reduction followed by progesterone treatment for 21 d (adapted from Andrieu-Vázquez et al., 2011).

Item	n	Loss rate before 90 d % (n/n)
Unilateral twin pregnancy	27	
Control	14	64 (9/14)
Manual twin reduction	13	54 (7/13)
Bilateral twin pregnancy	28	
Control	14	0 (0/14)
Manual twin reduction	14	29 (4/14)

treatments and occurred in 32% of untreated control cows and 41% of cows after manual amnion rupture with intravaginal P4 supplementation for 21 d.[2] The risk of pregnancy loss, independent of treatment, was 8.7 times greater for unilateral than bilateral twin pregnancies and did not differ between control cows with unilateral twins and unilateral twin reduction cows (62% vs 54%, respectively).[2] By contrast, the pregnancy loss for cows with bilateral twin pregnancies that received embryo reduction was 29% whereas no losses occurred in untreated control cows with bilateral twin pregnancies.[2] Of the cows that received manual amnion rupture, 44% (12/27) of cows calved with singletons compared to 54% (15/28) of control cows calved with twins.[2] The authors concluded that embryo reduction by manual amnion rupture with intravaginal P4 supplementation did not increase the risk of pregnancy loss for unilateral twin pregnancies, but did increase the risk of pregnancy loss in bilateral twin pregnancies.[2]

Transition period nutritional management

Cows gestating twins have 50 to 70% greater energy demands during gestation,[41,57] but decreased prepartum dry matter intake[70] compared with cows gestating singletons. Further, with a decreased gestation length, most cows carrying twins are less likely to experience a full 3-week close-up diet during the dry period.[56] Thus, an opportunity to minimize the negative effects of twinning in dairy cattle could be through feeding management strategies.[3] Previously in our laboratory, an experiment was conducted to determine the effect of dry period feeding management on the metabolic status and lactation performance of Holstein cows gestating singleton and twin pregnancies.[64] Silva del Río et al. (2010) hypothesized that increasing the duration of feeding a close-up diet during the dry period would improve metabolic status and lactation performance for cows gestating twins but not singletons.[64] Interestingly, the metabolic response to the dry period feeding strategy was independent of whether cows gestated singletons or twins indicating that differential management of cows gestating twins during the dry period did not improve metabolic status.[64]

Hormonal manipulation before AI to reduce double ovulation

Adoption of hormonal synchronization protocols by dairy herds for the reproductive management of dairy cows has increased[10] and has been instrumental in increasing reproductive performance.[27] In women, twinning is also increasing over time, but this increase in twinning has been attributed to fertility treatments with exogenous hormonal treatments and in vitro fertilization.[38] Despite the different endocrine physiology in these situations compared with the reproductive management of dairy cattle, a common misconception by dairy farmers is that increased use of hormonal synchronization protocols increases twinning. A common reproductive management strategy for first insemination is to submit lactating dairy cows to a Presynch-Ovsynch protocol with detection of estrus and AI after the Presynch portion of the protocol with the remainder of cows failing to be detected in estrus submitted to an Ovsynch protocol for timed AI.[25] Under this reproductive management, cows that are submitted to an Ovsynch protocol differ from cows detected in estrus and AI because these cows are likely to be the highest-producing cows having a shorter duration of estrus[46] or are anovular cows making them at a greater risk for double ovulations and twinning as previously discussed.

In contrast to the common misconception that hormonal synchronization protocols increase twinning, hormonal manipulations to increase P4 concentrations during ovulatory follicle development decreased the incidence of double ovulations.[68,12] In a Double Ovsynch protocol[67,36] cows are effectively presynchronized to maximize P4 concentrations during the growth of the ovulatory follicle to combat the effects of hepatic steroid catabolism to decrease double ovulation and dizygotic twinning.[74,27] Carvalho et al. (2019) randomized cows to have either a low or high P4 concentrations during the Breeding-Ovsynch portion of the Double-Ovsynch protocol to determine the effect of P4 concentration on reproductive and endocrine outcomes.[11] Cows with low P4 concentrations during the Breeding-Ovsynch portion of the Double-Ovsynch protocol had a 3-fold greater incidence of double ovulation (33 vs. 10%, respectively) and thereby more twin pregnancies at 32 d after timed AI (29 vs. 0%) than cows with high P4 concentrations.[11] Martins et al. (2018) submitted cows to a G7G protocol (Bello et al., 2006) and randomized cows to receive high or low P4 concentrations during the pre-dominance (d 0 to 4) or dominance (d 5 to 7) phase of the second follicular wave creating four groups: high/high, high/low, low/high, and low/low.[53] Figure 6 depicts the effect of high or low P4 concentrations on double ovulations during the pre-dominance and dominance phases.[53] Cows with low P4 concentrations during the pre-dominance (d 0 to 4) and dominance (d 5 to 7) phases of a second follicular wave had increased double ovulations compared with cows with high P4 concentrations (49 vs. 12%, respectively).[53]

Fricke et al. (2016) observed that in Irish Holsteins-Friesian, manipulating P4 concentrations during growth of the ovulatory follicle did not affect double ovulations and speculated this was likely because of feed intake and milk production differences in Irish vs. U.S. production systems.[24] Valdés-Archiniega et al. (under review) reported independent relationships between milk production and P4 concentrations of multiparous Holsteins on double ovulations to the last GnRH treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol (Figure 7).[69] The authors speculate that hepatic steroid metabolism is a major contributor to increased double ovulations but that other mechanisms likely are involved, such as IGF-I, which need to be elucidated.[69] Thus, increasing P4 during growth of the ovulatory follicle decreases double ovulations and mitigates some of the

Figure 6: Effect of treatment (high or low progesterone) during the predominance (d 0 to 4) and/or dominance (d 5 to 7) phase on the incidence of double ovulation to the last GnRH treatment. Different letters (a-c) denote statistical differences (*P* < 0.05; adapted from Martins et al., 2018).

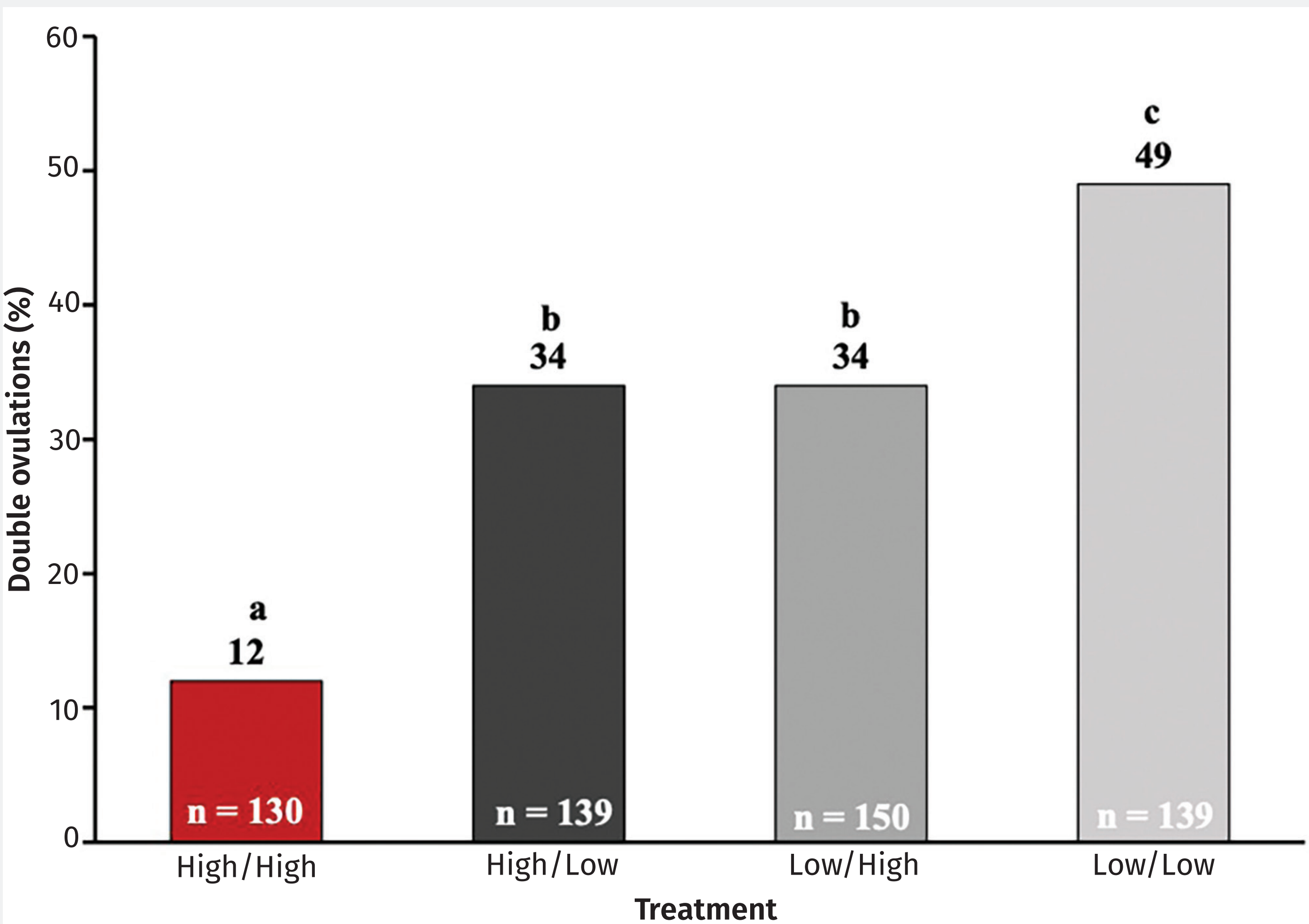
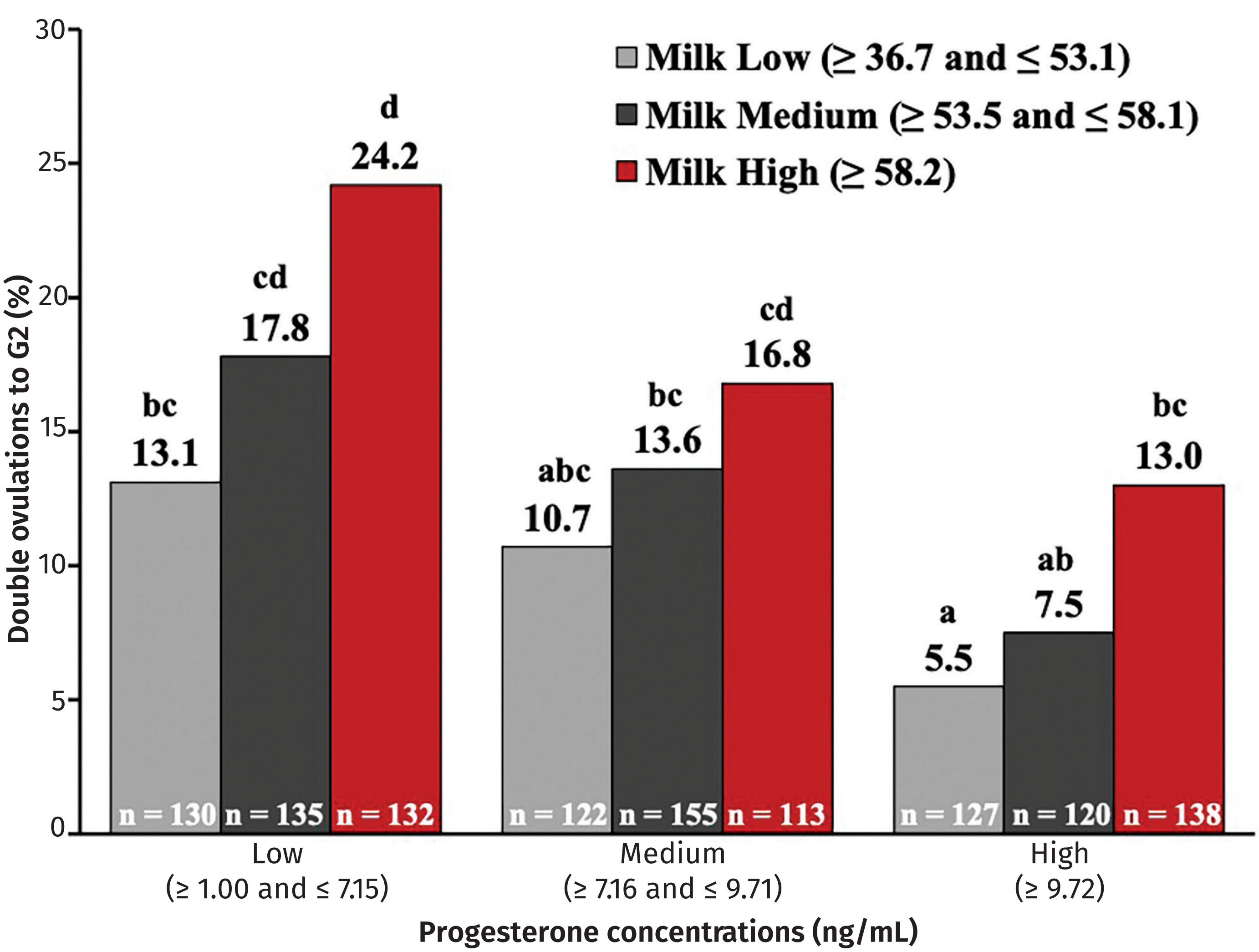


Figure 7: Incidence of double ovulations for multiparous Holstein cows to the last GnRH treatment of the Breeding-Ovsynch portion of the Double-Ovsynch protocol (G2) based on milk production and serum progesterone tertiles. Different letters (a-d) denote statistical differences (*P* < 0.05; adapted from Valdés-Arciniega et al., Under Review).



negative effects of increased milk production associated with increased hepatic steroid metabolism. Further research is needed to understand other physiological mechanisms to decrease double ovulations and dizygotic twinning. Currently, hormonal synchronization using a protocol such as a Double Ovsynch protocol may be the best strategy to decrease double ovulations thereby dizygotic twinning in high-producing dairy cows.

Conclusion

Based on the data presented in this review, dairy herds can implement two approaches in tandem to decrease twinning and the negative impacts of twinning in their herds. The first approach is to submit high-producing dairy cows to hormonal synchronization protocols to increase P4 during the growth of the preovulatory follicle before AI which appears to have plateaued recent twinning rates in dairy herds. Currently, the best reproductive management strategy to accomplish this is to submit cows for first timed AI to a Double-Ovsynch protocol. The second approach is to identify cows with twins based on laterality using transrectal ultrasonography. Cows pregnant with bilateral dizygotic twins should be allowed to continue with gestation because bilateral dizygotic twins have increased survival and birth weight, a longer gestation length, and less dystocia than unilateral dizygotic twins, but should be provided extra assistance at calving. Selective embryo reduction can be attempted in unilateral twins because the pregnancy loss compared to cows with unilateral twins not submitted to selective embryo reduction is similar, but be cognizant that the overall pregnancy losses of unilateral twins are over 50%. In combination, these two strategies should decrease the incidence of twinning in a dairy herd and minimize the negative impacts of twinning on cows that do become pregnant with twins.

References

1. Andreu-Vázquez C, Garcia-Ispierto I, Ganau S, Fricke PM, López-Gatius F. Effects of twinning on the subsequent reproductive performance and productive lifespan of high-producing dairy cows. *Theriogenology* 2012; 78:2061-2070.

2. Andreu-Vázquez C, Garcia-Ispierto I, López-Béjar M, de Sousa NM, Beckers JF, López-Gatius F. Clinical implications of induced twin reduction in dairy cattle. *Theriogenology* 2011; 76:512-521.

3. Bell MJ, Roberts DJ. Effect of twinning on the feed intake, performance and health of dairy cows. *Livest Prod Sci* 2007; 107:274-281.

4. Bello NM, Steibel JP, Pursley JR. Optimizing ovulation to first GnRH improved outcomes to each hormonal injection of Ovsynch in lactating dairy cows. *J Dairy Sci* 2006; 89:3413-3424.

5. Bendixen PH, Oltenacu PA, Andersson L. Case-referent study of cystic ovaries as a risk indicator for twin calvings in dairy cows. *Theriogenology* 1989, 31:1059-1066.

6. Bonnier G. Studies on monozygotic cattle twins. II. Frequency of monozygotic twins. *Acta Agr Suecana* 1946; 1:147-51.

7. Britt JH, Cushman RA, Dechow CD, Dobson H, Humblot P, Hutjens MF, Jones GA, Ruegg PS, Sheldon IM, Stevenson JS. Invited review: Learning from the future—A vision for dairy farms and cows in 2067. *J Dairy Sci* 2018; 101:3722-3741.

8. Buoen LC, Zhang TQ, Weber AF, Ruth GR. Non-Freemartin Rate in Holstein Heterosexual Twins, *Proc Am Assoc Bov Pract Conf* 1992; 300-303.

9. Cabrera VE, Fricke PM. Economics of Twin Pregnancies in Dairy Cattle. *Animals* 2021; 11:552.

10. Caraviello DZ, Weigel KA, Fricke PM, Wiltbank MC, Florent MJ, Cook NB, Nordlund KV, Zwald NR, Rawson CL. Survey of management practices on reproductive performance of dairy cattle on large US commercial farms. *J Dairy Sci* 2006; 89:4723-4735.

11. Carvalho PD, Santos VG, Fricke HP, Hernandez LL, Fricke PM. Effect of manipulating progesterone before timed artificial insemination on reproductive and endocrine outcomes in high-producing multiparous Holstein cows. *J Dairy Sci* 2019; 102:7509-7521.

12. Cerri RLA, Chebel RC, Rivera F, Narciso CD, Oliveira RA, Thatcher WW, Santos JEP. Concentration of progesterone during the development of the ovulatory follicle: I. Ovarian and embryonic responses. *J Dairy Sci* 2011; 94:3342-3351.

13. Davis ME, Heibel GK. Use of real-time ultrasound to identify multiple fetuses in beef cattle. *Theriogenology* 1993; 40:373-382.

14. Day JD, Weaver LD, Franti CE. Twin pregnancy diagnosis in Holstein cows-discriminatory powers and accuracy of diagnosis by transrectal palpation and outcome of twin pregnancies. *CVJ* 1995; 36:93-97.

15. Dobson H, Rowan TG, Kippax IS, Humblot P. Assessment of fetal number, and fetal and placental viability throughout pregnancy in cattle. *Theriogenology* 1993; 40:411-425.

16. Domingues RR, Andrade JPN, Cunha TO, Madureira G, Moallem U, Gomez Leon V, Martins JPN, Wiltbank MC. Is pregnancy loss initiated by embryonic death or luteal regression? Profiles of pregnancy-associated glycoproteins during elevated progesterone and pregnancy loss. *JDS Communications* 2023; 4: 149-154.

17. Echternkamp SE, Gregory KE. Identification of twin pregnancies in cattle by ultrasonography. *J Anim Sci* 1991; 69(Suppl.1):220. (Abstr).

18. Echternkamp SE, Thallman RM, Cushman RA, Allan MF, Gregory KE. Increased calf production in cattle selected for twin ovulations. *J Anim Sci* 2007; 85:3239-3248.

19. Ferguson JD, Galligan DT. The value of pregnancy diagnosis—A revisit to an old art. *Theriogenology Annu Conf Symp*, Milwaukee, WI. Society of Theriogenology, Montgomery, AL, 2011.

20. Frazer GS. Twins. In: Robinson NE, editor. *Current Therapy in Equine Medicine*, 5th edition. Elsevier Science, 2003; 245-248.

21. Fricke PM. Double vision: management of twinning in dairy cows, in *Proc Am Assoc Bov Pract Conf* 2015; 116-124.

22. Fricke PM. Scanning the future – ultrasonography as a reproductive management tool for dairy cattle. *J Dairy Sci* 2002; 85:1918-1926.

23. Fricke PM. Review: Twinning in dairy cattle. *Prof Anim Sci* 2001; 17:61-67.

24. Fricke PM, Carvalho PD, Lucy MC, Curran F, Herlihy MM, Waters SM, Larkin JA, Crowe MA, Butler ST. Effect of manipulating progesterone before timed artificial insemination on reproductive and endocrine parameters in seasonal-calving, pasture-based Holstein-Friesian cows. *J Dairy Sci* 2016; 99:6780-6792.

25. Fricke PM, Giordano JO, Valenza A, Lopes G, Amundson MC, Carvalho PD. Reproductive performance of lactating dairy cows managed for first service using timed artificial insemination with or without detection of estrus using an activity-monitoring system. *J Dairy Sci* 2014; 97:2771-2781.

26. Fricke PM, Wiltbank MC. Effect of milk production on the incidence of double ovulation in dairy cows. *Theriogenology* 1999; 52:1133-1143.

27. Fricke PM, Wiltbank MC. Symposium review: The implications of spontaneous versus synchronized ovulations on the reproductive performance of lactating dairy cows. *J Dairy Sci* 2022; 105:4679-4689.

28. Galligan DT, Ferguson J, Munson R, Remsburg D, Skidmore A. Economic concepts regarding early pregnancy testing, in *Proc Am Assoc Bov Pract Conf* 2009; 48-53.

29. Giordano JO, Guenther JN , Lopes G, Fricke PM. Changes in serum pregnancy-associated glycoprotein, pregnancy-specific protein B, and progesterone concentrations before and after induction of pregnancy loss in lactating dairy cows. *J Dairy Sci* 2012; 95:683-697.

30. Green JA, Parks TE, Avalle MP, Telugu BP, McLain AL, Peterson AJ, McMillan W, Mathialagan N, Hook RR, Xie SC, Roberts RM. The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. *Theriogenology* 2005; 63:1481-1503.

31. Gregory KE, Bennett GL, Van Vleck LD, Echternkamp SE, Cundiff LV. Genetic and environmental parameters for ovulation rate, twinning rate, and weight traits in a cattle population selected for twinning. *J Anim Sci* 1997; 75:1213-1222.

32. Gregory KE, Echternkamp SE, Dickerson GE, Cundiff LV, Koch, RM, Van Vleck LD. Twinning in cattle: I. Foundation animals and genetic and environmental effects on twinning rate. *J Anim Sci* 1990; 68:1867-1876.

33. Gümen A, Guenther JN, Wiltbank MC. Follicular size and response to Ovsynch versus detection of estrus in anovular and ovular lactating dairy cows. *J Dairy Sci* 2003; 86:3184-3194.

34. Harrison RO, Ford SP, Young JW, Conley AJ, Freeman AE. Increased milk-production versus reproductive and energy status of high-producing dairy cows. *J Dairy Sci* 1990; 73:2749-2758.

35. Hayashi KG, Matsui M., Shimizu T, Sudo N, Sato A, Shirasuna K, Tetsuka M, Kida K, Schams D, Miyamoto A. The absence of corpus luteum formation alters the endocrine profile and affects follicular development during the first follicular wave in cattle. *Reprod* 2008; 136:787-797.

36. Herlihy MM, Giordano JO, Souza AH, Ayres H, Ferreira RM, Keskin A, Nascimento AB, Guenther JN, Gaska JM, Kacuba SJ, Crowe MA, Butler ST, Wiltbank MC. Presynchronization with Double-Ovsynch improves fertility at first postpartum artificial insemination in lactating dairy cows. *J Dairy Sci* 2012; 95:7003-7014.

37. Iberico G, Mavarro J, Blasco L, Simon C, Pellicer A, Remohi J. Embryo reduction of multifetal pregnancies following assisted reproduction treatment: A modification of the transvaginal ultrasound-guided technique. *Hum Reprod* 2000; 15:2228-2233.

38. Imaizumi Y. A comparative study of zygotic twinning and triplet rates in eight countries. *J Biosoc Sci* 2003; 35:287-302.

39. Jost A, Prepin J, Vigier B. Freemartins in cattle – first steps of sexual organogenesis. *J Reprod Fertil* 1972; 29:349-+.

40. Kinsel ML, Marsh WE, Ruegg PL, Etherington WG. Risk factors for twinning in dairy cows. *J Dairy Sci* 1998; 81:989-993.

41. Koong LJ, Anderson GB, Garrett WN. Maternal energy status of beef cattle during single and twin pregnancy. *J Anim Sci* 1982; 54:480-484.

42. Labhsetwar AP, Casida LE, Tyler WJ. Analysis of variation in some factors affecting multiple ovulations in Holstein cattle. *J Dairy Sci* 1963; 46(8):840-+.

43. Lett BM, Kirkpatrick BW. Short communication: Heritability of twinning rate in Holstein cattle. *J Dairy Sci* 2018; 101:4307-4311.

44. Lopez H, Caraviello DZ, Satter LD, Fricke PM, Wiltbank MC. Relationship between level of milk production and multiple ovulations in lactating dairy cows. *J Dairy Sci* 2005a; 88:2783-2793.

45. Lopez H, Sartori R, Wiltbank MC. Reproductive hormones and follicular growth during development of one or multiple dominant follicles in cattle. *Biol Reprod* 2005b; 72:788-795.

46. Lopez H, Satter LD, Wiltbank MC. Relationship between level of milk production and estrous behavior of lactating dairy cows. *Anim Reprod Sci* 2004; 81:209-223.

47. López-Gatius F: The effect on pregnancy rate of progesterone administration after manual reduction of twin embryos in dairy cattle. *J Vet Med Assoc* 2005; 52:199-201, 2005.

48. López-Gatius F, Garbayo JM, Santolaria P, Yániz J, Ayad A, de Sousa NM, Beckers JF. Milk production correlates negatively with plasma levels of pregnancy-associated glycoprotein (PAG) during the early fetal period in high producing dairy cows with live fetuses. *Domest Anim Endocrinol* 2007; 32:29-42.

49. López-Gatius F, Garcia-Ispierto I, Hunter RHF. Twin Pregnancies in Dairy Cattle: Observations in a Large Herd of Holstein-Friesian Dairy Cows. *Animals* 2020; 10:9.

50. López-Gatius F, Hunter RHF. Spontaneous reduction of advanced twin embryos: its occurrence and clinical relevance in dairy cattle. *Theriogenology* 2005; 63:118-125.

51. López-Gatius F, López-Béjar M, Fenech M, Hunter RHF. Ovulation failure and double ovulation in dairy cattle: risk factors and effects. *Theriogenology* 2005; 63:1298-1307.

52. Macpherson ML, Reimer JM. Twin reduction in the mare: Current options. *Anim Reprod Sci* 2000; 60-61:233-244.

53. Martins JPN, Wang D, Mu N, Rossi GF, Martini AP, Martins VR, Pursley JR. Level of circulating concentrations of progesterone during ovulatory follicle development affects timing of pregnancy loss in lactating dairy cows. *J Dairy Sci* 2018; 101:10505-10525.

54. Middleton EL , Pursley JR. Short communication: Blood samples before and after embryonic attachment accurately determine non-pregnant lactating dairy cows at 24 d post-artificial insemination using a commercially available assay for pregnancy-specific protein B. *J Dairy Sci* 2019; 102:7570-7575.

55. Mur-Novales R, López-Gatius F, Fricke PM, Cabrera VE. An economic evaluation of management strategies to mitigate the negative effect of twinning in dairy herds. *J Dairy Sci* 2018; 101:8335-8349.

56. Nielen M, Schukken YH, Scholl DT, Wilbrink HJ, Brand A. Twinning in dairy-cattle – a study of risk-factors and effects. *Theriogenology* 1989; 32(5):845-862.

57. Nishida T, Kurihara M, Terada F, Shibata M. Energy requirements of pregnant Holstein dairy cows carrying single or twin Japanese black fetuses in late pregnancy. *Anim Sci Technol* 1997; 68:572-578.

58. Ricci A, Carvalho PD, Amundson MC, Fourdraine RH, Vincenti L, Fricke PM. Factors associated with pregnancy-associated glycoprotein (PAG) levels in plasma and milk of Holstein cows during early pregnancy and their effect on the accuracy of pregnancy diagnosis. *J Dairy Sci* 2015; 98:2502-2514.

59. Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17 beta in dairy cattle. *J Dairy Sci* 2002; 85:2831-2842.

60. Sasser RG, Ruder CA, Ivani KA, Butler JE, Hamilton WC. Detection of pregnancy by radioimmunoassay of a novel pregnancy-specific protein in serum of cows and a profile of serum concentrations during gestation. *Biol Reprod* 1986; 35(4):936-942.

61. Savio JD, Boland MP, Hynes N, Roche JF. Resumption of follicular activity in the early postpartum period of dairy-cows. *J Reprod Fertil* 1990; 88:569-579.

62. Schambow RA, Bennett TB, Dopfer D, Martins JPN. A retrospective study investigating the association of parity, breed, calving month and year, and previous parity milk yield and calving interval with twin births in US dairy cows. *J Dairy Sci* 2021; 104:5047-5055.

63. Silva del Río N, Colloton JD, Fricke PM. Factors affecting pregnancy loss for single and twin pregnancies in a high-producing dairy herd. *Theriogenology* 2009; 71:1462-1471.

64. Silva del Río N, Fricke PM, Grummer RR. Effects of twin pregnancy and dry period feeding strategy on milk production, energy balance, and metabolic profiles in dairy cows. *J Anim Sci* 2010; 88:1048-1060.

65. Silva del Río N, Kirkpatrick BW, Fricke PM. Observed frequency of monozygotic twinning in Holstein dairy cattle. *Theriogenology* 2006; 66:1292-1299.

66. Silva del Río N, Stewart S, Rapnicki P, Chang YM, Fricke PM. An observational analysis of twin births, calf sex ratio, and calf mortality in Holstein dairy cattle. *J Dairy Sci* 2007; 90:1255-1264.

67. Souza AH, Ayres H, Ferreira RM, Wiltbank MC. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. *Theriogenology* 2008; 70:208-215.

68. Stevenson JS, Portaluppi MA, Tenhouse DE. Factors influencing upfront single- and multiple-ovulation incidence, progesterone, and luteolysis before a timed insemination resynchronization protocol. *J Dairy Sci* 2007; 90:5542-5551.

69. Valdés-Arciniega TJ, Anta-Galván E, Leão IMR, Cunha TO, El Azzi MS, Cook NB, Martins JPN. Milk yield was positively associated with double ovulation independently of circulating progesterone concentrations in multiparous high-producing Holstein cows submitted to Double Ovsynch. *J Dairy Sci* (Under Review).

70. Van Saun RJ. Effects of undegradable protein fed prepartum on subsequent lactation, reproduction, and health in Holstein dairy cattle. PhD Thesis. Cornell Univ., Ithaca, NY, 1993.

71. Van Vleck LD, Gregory KE, Echternkamp SE. Ovulation rate and twinning rate in cattle: Heritabilities and genetic correlation. *J Anim Sci* 1991; 69:3213-3219.

72. Weinberg W. Beiträge zur Physiologie und Pathologie der Mehrlingsgeburten beim Menschen Pflügers. *Arch Ges Physiol* 1902; 88:346-430.

73. Wiltbank MC, Fricke PM, Sangsritavong S, Sartori R, Ginther OJ. Mechanisms that prevent and produce double ovulations in dairy cattle. *J Dairy Sci* 2000; 83(12):2998-3007.

74. Wiltbank MC, Souza AH, Carvalho PD, Cunha AP, Giordano JO, Fricke PM, Baez GM, Diskin MG. Physiological and practical effects of progesterone on reproduction in dairy cattle. *Animal* 2014; 8:70-81