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Observed frequency of monozygotic twinning in Holstein dairy cattle

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Abstract

Bonnier's equation is used to mathematically estimate the frequency of monozygotic (MZ) twinning in epidemiologic studies of twinning in dairy cattle; however, no empirical determination of MZ twinning has been reported in the literature. Our objectives were to empirically determine the frequency of MZ twinning in lactating Holstein cows and to compare this result with published estimates predicted using Bonnier's equation. Ear biopsies were collected from 107 sets of Holstein twins from six Wisconsin dairies resulting in 40 opposite-sex twins, 29 same-sex male twins, and 38 same-sex female twins. To empirically determine the frequency of MZ twinning, DNA extracted from ear biopsies collected from the 67 same-sex twins was PCR amplified using primers for a minimum of 5 polymorphic microsatellite DNA markers. Opposite-sex twins were classified as dizygotic (DZ) as well as same-sex twins differing in at least one microsatellite DNA marker. Same-sex twins were classified as MZ when all genotypes for a minimum of five markers were identical. Of the 67 same-sex twins, 62 were classified as DZ and 5 MZ resulting in a MZ twinning frequency of 7.5% of same-sex twins and 4.7% of all twins. The estimated frequency of MZ twinning in this population of twin calves using Bonnier's equation was 39.5% of same-sex twins and 24.7% of all twins. We concluded that MZ twinning occurred infrequently in Holstein cattle and perhaps less frequently than that reported in studies using Bonnier's equation to estimate MZ twinning.

Keywords: Holstein twinning; Monozygotic; Bonnier's equation; Cattle

1. Introduction

Cattle are a monotocous species; under most circumstances, a successful pregnancy results in the birth of a singleton calf. Occasionally, however, the reproductive process in cattle, as with many other monotocous species, results in the birth of twins. In dairy cattle, twinning is an undesirable trait that reduces overall profitability through negative effects on cows calving twins as well as on calves born as twins [1]. That twinning has increased over time in the dairy cattle population [2,3] has brought attention to this phenomenon because of its negative economic impact on dairy farms.

Twinning can be classified into two types: monozygotic (MZ) and dizygotic (DZ). Monozygotic twins, also referred to as identical twins, result from spontaneous cleavage of one fertilized oocyte during embryonic development, whereas DZ twins, also referred to as fraternal twins, result from fertilization of oocytes from two follicles that ovulate during the same estrous cycle. In cattle, the evidence for MZ twinning is based on abbatoir findings of concurrent embryos from reproductive tracts in which the ovaries

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contained only one corpus luteum [4] and by the existence of conjoined twins or double-monster fetuses [5]. In cattle, the study of morphological and physiological characteristics to identify MZ twinning has not been as successful as in humans due to intensive selection and inbreeding. The examination of placental membranes to identify zygosity, as suggested by Benirschke [6] in humans, is tedious and prevents the identification of dichorionic diamniotic identical twins which represent 25–30% of MZ twins in humans. In addition, zygosity studies based on red cell antigens are not reliable in cattle because blood exchanged between conceptuses during pregnancy results in a majority of DZ twins with identical red cell factors. New technologies such as DNA fingerprinting and ultrasonography are better able to identify zygosity in cattle. To the best of our knowledge, no DNA analyses have been conducted to accurately determine zygosity in Holstein twins, whereas extensive data have been published estimating the frequency of MZ twinning based on gender distribution of the twin population [7–9].

Assuming that after a double ovulation each oocyte has the same probability of being fertilized by either an X or a Y bearing sperm, approximately half of DZ twin pairs should be of the same-sex and half of the opposite-sex. Therefore, the expected gender distribution would be 25% same-sex female twins, 50% opposite-sex twins, and 25% same-sex male twins. By contrast, MZ twins would constitute only same-sex pairs. In 1902, Weinberg's Differential Method was used to estimate the frequency of MZ twins based on a skewing of the expected sex distribution for DZ twins towards a greater percentage of same-sex twin pairs within a study population of twins, under the assumption of equal sex ratio for both MZ and DZ twins [10]. In 1946, the Swedish animal geneticist, Gert Bonnier, introduced a modification to Weinberg's Differential Method that used the observed sex ratio in the sample population rather than Weinberg's assumption of equal sex ratio [11]. Since the introduction of this modification to Weinberg's Differential Method, Bonnier's equation has been widely used to estimate MZ twinning frequency in dairy cattle. Two studies published in the 1970s estimated MZ twinning based on Bonnier's equation to be 9.0% of same-sex Holstein twins [7] and 13.5% of same-sex Swedish Friesian twins [8]. Alternatively, a higher occurrence of MZ twinning was estimated in a later study that reported a frequency of 23.8% of same-sex Holstein twins [9].

Reported estimates of MZ twinning based on Bonnier's equation conflict with recent studies using ultrasound which have reported a high incidence of multiple ovulation with rates of 20.4% after spontaneous ovulations [12] and 14.1% after synchronized ovulations [13] in lactating Holstein cows. Holstein cows producing $\geq$45 kg milk/d during the 14 d preceding estrus had a double ovulation rate of 47.9%, whereas the double ovulation rate of herdmates producing 30–40 kg of milk was only 3.9% [14]. A better understanding of the physiology of twinning could aid the development of new strategies to prevent its occurrence.

Our objectives were to empirically determine zygosity in a population of Holstein twins and to compare the results with previously reported estimates of MZ twinning. Our hypothesis was that MZ twinning occurs infrequently in Holstein dairy cattle and that Bonnier's equation may overestimate the frequency of MZ twinning.

2. Materials and methods

2.1. Collaborating farms and ear biopsy collection

Six commercial dairies in Wisconsin comprising 400–1850 lactating cows were invited to participate in this study for a 12 month period beginning in July, 2001. Although all six farms initially agreed to participate for the duration of the study, participation of individual farms ranged from 1 to 14 consecutive month. Visits were made to each farm at the onset of the study to instruct and train herd personnel to collect ear biopsies from each pair of twin calves born during the study period. Each farm was supplied with an ear biopsy tool (Temple Tag Co., Temple, TX, USA), a 500-mL plastic rinse bottle containing 70% EtOH, and a set of color-coded, paired, 10 mm × 75 mm polypropylene tubes with fitted caps. Within 24 h after birth, herd personnel used the ear biopsy tool to punch an 8 mm circular ear biopsy sample from one ear from each calf within the twin pair. The ear biopsy tool was rinsed with 70% EtOH between each sample collection to minimize sample cross-contamination and to clean the ear biopsy tool.

Ear biopsies were immediately placed into polypropylene tubes after collection, the tubes were fitted with caps, and ear biopsies were stored at −20 °C in a freezer located on the farm. Polypropylene tubes were preassembled in the laboratory and delivered to each farm so that they were fastened together in pairs using masking tape colored coded to correspond to each participating farm. For each farm, tubes within each preassembled paired set were individually color-coded to allow recording of the sex of the calves within each twin pair (e.g., two blue tubes for same-sex male twins;

two red tubes for same-sex female twins; one blue and one red tube for opposite-sex twins). This recording system was used to facilitate ease of on-farm data collection, eliminate the need for farm personnel to record information by hand, and minimize inaccuracies in collection of farm and calf sex data. Samples stored at each farm were collected periodically throughout the study and were transported to the laboratory on ice and stored at −20 °C.

2.2. DNA fingerprint to assess zygosity

Monozygotic twins share the same genetic material and consequently are always of the same-sex; however, DZ twins can be of the same or opposite-sex and are no more genetically alike than siblings from the same sire and dam born during separate gestations. Therefore, opposite-sex twin pairs were classified as DZ twins with no further genetic analysis.

2.2.1. Isolation of DNA

DNA from ear biopsies collected from same-sex twin pairs ($n = 67$) was isolated by using a modified salt extraction method. Briefly, ear skin tissue was separated from ear cartilage, minced, and placed in a conical tube with 3 mL of digestion buffer (10 mM Tris–HCl, 400 mM NaCl, 2% SDS, and 50 mM Na_2EDTA, pH 8.0), 20 μL of trypsin (10 mg/mL; Gibco BRL, Grand Island, NY, USA) and 20 μL of proteinase K (10 mg/mL; Sigma, St. Louis, MO, USA). The conical tube containing the digestion solution was incubated at 50 °C while shaking at 120 rpm, until the ear tissue was digested. One-third volume of 5 M NaCl (1 mL) was added to the digested tissue and mixed gently, followed by centrifugation and removal of the supernatant. Two and one-half volumes of 95% EtOH were added to the supernatant, and the DNA pellet was removed and washed in 70% EtOH. The DNA was rehydrated with 300 μL of 10 mM buffered Tris–HCl (pH 8) and its concentration was estimated by spectrophotometry (OD at 260 nm). Working DNA solutions were prepared by diluting samples in buffered Tris–HCl to a concentration of 50 ng/μL.

2.2.2. Microsatellite DNA markers

Six bovine microsatellite DNA markers were initially selected to classify twin pairs as either MZ or DZ based on their proven polymorphic character (Table 1) and diversity in their genomic location [15]. All microsatellite DNA markers were PCR-amplified in 12 μL volumes with 17 ng of template DNA. Reagent concentrations were 1.5 mM $MgCl_2$, 10 × *Taq* buffer, 0.2 mM of each dNTP, and 0.04 units/μL of *Taq* DNA polymerase (Promega, Madison, WI, USA). Reactions were initially denatured at 95 °C for 90 s, then cycled 20 times in the following order: 95 °C for 30 s, 62 °C for 30 s with a decrease of 0.5 °C per cycle, 72 °C for 30 s, followed by 12–18 similar cycles with an annealing temperature of 56 °C, finishing with a final extension step at 72 °C for 20 min. Variations for optimization of particular primers included touchdown ranges of 62–58 °C ending at 60 °C, 62–56 °C ending at 58 °C, and 58–48 °C ending at 52 °C. Polymerase chain reaction products were separated by size using an ABI 310 capillary electrophoresis DNA analyzer. Genotypes were scored using ABI Genescan and ABI Genotyper (Applied Biosystems, Foster City, CA, USA) analysis software.

Same-sex twins were classified as DZ when genotype readings indicated genomic differences in one or more microsatellite DNA marker; whereas to be classified as MZ all the genotypes had to be identical for a minimum of five microsatellite DNA marker readings. All genotype readings were interpreted by two or more trained laboratory personnel, and genotypes for all samples with ambiguous initial results were reanalyzed.

Table 1
Characteristics of the microsatellite DNA markers used to identify zygosity in Holstein twin calves

Microsatellite DNA marker	No. alleles[a]	No. alleles identified[b]	Heterozygosity[a]	Heterozygosity[b]	Chromosome[a]
RM103	12	9	50	74	5
BMS823	10	8	58	68	10
BMS360	11	8	61	81	6
BMS650	16	8	71	67	19
BM4305	10	6	69	73	14
BM6425	13	6	75	63	14
BM9289	12	8	73	13	7
UWCA20	12	3	62	50	7

[a] Based on USDA-MARC genome mapping results.
[b] Based upon results from the current study.

However, several microsatellite DNA markers failed to amplify all genotypes represented by the ear biopsy samples resulting in inconclusive outcomes for one or both of the samples for five twin pairs. To determine zygosity for these paired samples, two additional markers, UWCA20 and BMS6425, were selected and used to obtain a definitive classification.

2.3. Estimation of monozygotic twinning using Bonnier's equation

The frequency of MZ twins in the sample population was estimated using a modification of Weinberg's Differential Method [10] published by Bonnier in 1964 [11]. Assuming equal probability for either an X or a Y bearing sperm to fertilize an oocyte, sex distribution of DZ twins is expected to be 50:50, with half of the twin pairs of the opposite-sex and half of the same-sex. Because MZ twins result from the cleavage of one fertilized oozyte and carry the same genetic material, they must be of the same-sex. Therefore, the number of same-sex twins in a population that includes both MZ and DZ twin pairs would be expected to represent a greater frequency of the population than the number of same-sex twins in a population that includes only DZ twin pairs. Bonnier's equation, assumes the observed sex ratio to be the same for both MZ and DZ twins. The proportion of MZ twins, or m, among same-sex twins in a population is calculated using the following mathematical equation:

$$m = \frac{2npq - n_2}{2pq(n - n_2)}$$

where n represents the total number of twin pairs, n_2 the total opposite-sex twin pairs, p the proportion of male calves, and $q = 1 - p$ is the proportion of female calves among calves from all twin births.

2.4. Effect of season of calving on twinning

Complete data which included information for all calvings that occurred during a consecutive 12 month period (July 2001 to June 2002) were available for two of the six farms representing a total of 1212 calvings. Only data from these two farms were included to evaluate the effect of season on twinning to avoid possible bias introduced by incomplete data from the remaining farms. The seasonal effect on twinning rate was evaluated for these two farms by dividing the 12 month period into four tri-monthly seasons. A combined total of 355 calvings from January to March, 336 calvings from April to June, 215 calvings from July to September, and 306 calvings from October to December. Farm 1 represented a total of 315 calvings and Farm 2 a total of 897 calvings, with an overall twinning rate for the 12 month period of 12.1 and 3.9%, respectively.

2.5. Statistical analyses

The observed sex ratio for the sample population of twins was compared to a 50% male, 50% female sex ratio, and the observed sex combination ratios for twin calves were compared to a 25% same-sex male, 50% opposite-sex, and 25% same-sex female sex ratio using the FREQ procedure of SAS [16]. Differences in proportions were tested using the Cochran–Mantel–Haenszel Chi-square test statistic. Calving data collected from two farms for a 12 consecutive month period were analyzed using the GLIMMIX procedure of SAS to assess the effect of season on the risk of twinning. Seasons were defined as S1 from January to March, S2 from April to June, S3 from July to September and S4 from October to December. The statistical model included season as the explanatory variable with farm included as a random variable. Orthogonal contrasts were constructed to test differences among seasons. The association between MZ twinning frequency as estimated using Bonnier's equation and the observed twinning rate based on a survey of published literature was assessed using the CORR and REG procedures of SAS. Because the observations used in the meta-analysis were from a limited number of studies that varied greatly in sample size, data from each study were weighted by the number of total observations included in each study.

3. Results

3.1. Monozygous twinning identified with DNA fingerprint

Genotypes from the 67 same-sex twins showed that 62 twin pairs were classified as DZ, whereas 5 twin pairs were classified as MZ. This resulted in a MZ twin frequency in the sampled population of 7.5% among same-sex twins and 4.7% among all twin births. Of the 62 DZ twin pairs, 29 differed in one marker and 33 in more than one marker. Of the five MZ twin pairs identified, two were classified after obtaining genotypes for six microsatellite DNA marker loci and three after obtaining genotypes for five microsatellite DNA markers, each pair resulting in identical readings for all markers. Of the five MZ twin pairs identified, four were female pairs and one was a male pair.

Table 2
Frequency of dizygotic (DZ) and monozygotic (MZ) twinning in a population of twin Holstein calves determined empirically or estimated mathematically using Bonnier's equation

Classification	Observed, *n*	Empirical classification[a]		Mathematical estimate[b], MZ, %
		DZ, % (*n*)	MZ, % (*n*)	
Same-sex male twins	29	86.2 (25)	2.6 (1)	–
Same-sex female twins	38	97.4 (37)	13.8 (4)	–
All same-sex twins	67	92.5 (62)	7.5 (5)	39.5
Opposite-sex twins	40	100.0 (40)	–	–
All twins	107	95.3 (102)	4.7 (5)	24.7

[a] DNA extracted from ear biopsies collected from same-sex twins was PCR amplified using primers for eight polymorphic microsattelite DNA markers. Opposite-sex twins were classified as DZ as well as same-sex twins differing in at least one microsatellite DNA marker. Same-sex twins were classified as MZ when all genotypes for a minimum of five microsatellite DNA markers obtained for each twin pair were identical.
[b] Frequency of MZ twinning estimated mathematically using Bonnier's equation [11].

Table 3
Reported estimates of monozygotic (MZ) twinning in twin calves based on Bonnier's equation

Dairy breed	Total calvings (*n*)	Twinning rate (%)	MZ twinning (% of all twins)	MZ twinning (% of all births)	Reference
Holstein Friesians	10,885	2.91	4.47	0.13	[17]
Holstein Friesians	7,387	4.58	6.55	0.30	[31]
Swedish Red & White	495,470	1.45	4.52	0.17	[8]
Swedish Friesians	169,144	1.95	7.23	0.14	[8]
German Schwarzbunt	734,297	1.97	14.26	0.28	[8]
Bavaria	140,054	1.84	9.78	0.18	[8]
German Fleckvieh	683,807	3.16	10.41	0.33	[8]
German Braunvieh	271,283	3.16	13.98	0.44	[8]
Holstein	23,978	4.75	4.74	0.23	[7]
Holstein Friesians	11,951	3.20	7.19	0.23	[28]
Holstein Friesians	24,843	5.40	13.7	0.74	[9]

3.2. Bonnier's estimates of monozygous twinning

The frequency of MZ twinning estimated mathematically using Bonnier's equation for the 107 twin pairs in the sample population was 39.5% among same-sex twins and 24.7% of all twins (Table 2). The distribution of 29 same-sex male twins, 40 opposite-sex twins, and 38 same-sex female twins differed from the expected 1:2:1 ratio if all twins born were DZ ($P < 0.05$). However, the overall twin sex ratio of 45.8% male and 54.2% female did not differ statistically from the expected 50:50 ratio but was greater for females than males, as a result from greater female–female pairs reported in the farm contributing the most observations for 14 month (21 female–female pairs out of 42 twin pairs).

3.3. Monozygotic twins per calving event

For the two farms from which complete calving data were available for a consecutive 12 month period, the frequency of MZ twins was calculated to be 5.5% among all twin births (4/73), and 0.33% per birth (4/1212) based on the empirical assessment of MZ twins. In a summary of the literature, Bonnier's estimates of MZ twinning per calving event in dairy cattle (Table 3) ranged from 0.13% [17] to 0.74% [9]. Although the occurrence of MZ twins

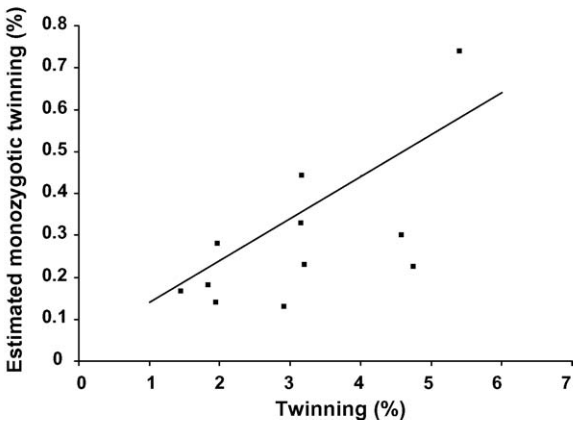

Fig. 1. Relationship between observed twinning rate and estimated monozygotic frequency using Bonnier's equation with data from Table 3. $y = 0.10x + 0.04$, R^2 − adjusted = 0.59, $P < 0.05$; $r = 0.80$.

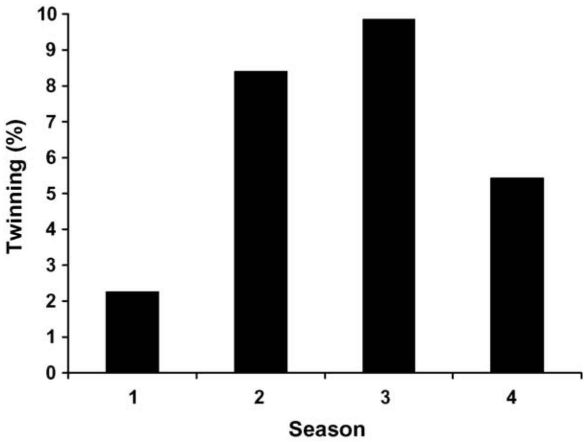

Fig. 2. Effect of season on twinning rate in lactating Holstein cows from two farms in Wisconsin. Seasons were defined as: (1) January to March (355 calvings), (2) April to June (336 calvings), (3) July to September (215 calvings), and (4) October to December (306 calvings). Farm 1 represented a total of 315 calvings and Farm 2 a total of 897 calvings with an overall twinning rate of 12.1 and 3.9%, respectively. The observed twinning rates from April to June and July to September were greater ($P < 0.005$) than that observed from October to December and January to March.

is expected to be roughly constant per calving event and independent of the rate of DZ twinning, twinning rate and the frequency of MZ twinning estimated using Bonnier's equation were positively correlated ($r = 0.8$; $P < 0.05$) suggesting an association between risk factors for MZ and DZ twins (Fig. 1). However, when the present results were extrapolated to the previously mentioned equation, with an overall 6.0% (73/1212) of twinning incidence, we would have expected a higher occurrence of MZ, 0.64% of all calvings, whereas the observed was 0.33%.

3.4. Effect of season of calving on twinning

The effect of season of calving on twinning rate was examined using calving data from the two farms from which ear biopsy samples and calving data were collected for a consecutive 12 month period. The overall twinning rate was 12.1% (38/315) for Farm 1 and 3.9% (35 /897) for Farm 2. Season of calving affected ($P < 0.05$) the risk of twinning (Fig. 2). The observed twinning rate was 8.3% from April to June and 9.7% from July to September, both of which were greater ($P < 0.005$) than the observed twinning rate of 5.2% from October to December and 2.3% from January to March.

4. Discussion

Although MZ twinning has been known to occur in cattle, to the best of our knowledge no studies have empirically assessed its frequency. In the present study, MZ twins were definitively identified in the sampled population using molecular biology techniques. Although, errors in identification of MZ and DZ twin pairs using the methodology described in this study are possible, they are expected to be of low frequency. The most likely cause of an erroneous classification would result from an error in genotype scoring. Because a single genotyping error could lead to the conclusion that genotypes were not identical, the most likely consequence would be the erroneous classification of MZ twins as DZ. The examination of genotypes by more than one individual and the reanalysis of genotyping for samples with ambiguous initial results were intended to reduce the likelihood of such an error. The consequence of such errors, if any, would be an underestimation of MZ twin frequency. Another less likely possibility for erroneous classification of MZ twin pairs as DZ would occur in the instance of a mutational event. Such an event would have had to occur early after blastocyst cleavage for the mutant allele to be detectable. Although dinucleotide microsatellite DNA mutation rates are high relative to other classes of mutations ($\sim 1 \times 10^{-3}$ per site per generation) [18], the likelihood of such a source of error for this study is remote. Erroneous classification of DZ twins as MZ is possible, but would require a rare set of circumstances. Either all microsatellite DNA markers used would have to be uninformative (i.e., both parents homozygous for each marker), or the DZ twins would have had to fortuitously inherit the same alleles for all informative microsatellite DNA markers. The high level of polymorphism for the markers chosen to determine zygosity in the present study makes this possibility highly unlikely (Table 1).

In the present study, despite the lack of concordance in monozygosity between both methodologies (i.e., DNA fingerprinting versus Bonnier's equation), Bonnier's estimates are in agreement with the reported 23.8% incidence of MZ twins among all same-sex twins estimated by Ryan and Boland [9]. Interestingly, in both studies the population of twins sampled deviated significantly from the expected sex distribution for DZ twins. Conversely, two studies published in the 1970s estimated a lower frequency of MZ twinning of 9.0 and 13.5% for same-sex twins [7,8]. In these latter studies, no significant deviation from the 25:50:25 sex ratio among twins was detected. However, the use of Bonnier's equation with field data may be open to biased estimation with selective under reporting of mixed-sex twin data that would lead to overestimation of the frequency of monozygotic births, both as a percentage of like-sex and total twin births. On the contrary, in the present study, the empirical estimation of the percentage of same-sex twins that are monozygous is unbiased by sampling or selective reporting of twin births.

The observed MZ frequency by calving event is similar to the 0.4% frequency of MZ twins per birth reported for humans [19]. Interestingly, the observed correlation between twinning rate and the frequency of MZ twinning when estimated using Bonnier's equation may indicate an association between risk factors for DZ and MZ twins. However, since Bonnier's estimate of MZ twinning is based on an excess of same-sex twins within a population, an increase in same-sex twins, regardless of whether they are MZ or DZ, would result in an overestimation of MZ twinning. Although, our results indicate that MZ twinning occurs infrequently in Holstein cows, an extrapolation to the prenatal stage could underestimate conception of MZ twins considering that MZ twins in humans are 18-fold more likely to undergo spontaneous abortion early during gestation than DZ twins [20]. Monozygotic twins would result in unilateral twin pregnancies in cattle, and pregnancy loss and spontaneous reduction of one of two twin fetuses during the first trimester of gestation was 8- and 2.3-fold greater, respectively, for Holstein cows with unilateral versus bilateral twin pregnancies [21].

Twinning is a complex trait with multiple causative factors. Risk factors for twinning include multiple ovulation rate, milk production, parity and genetics [1]. Multiple ovulation rate, a trait strongly associated with twinning [9,22] increases with parity [23–25] and has increased over time [2,3,26,27]. Several studies have shown a positive association between milk production and conception of twins in dairy cattle [2,28]. In addition, milk production near the time of artificial insemination was associated with a greater incidence of double ovulation in Holstein cows [12,29]; however, rbST use, which has been related to both increased milk production and twinning rate, may also play a role in increasing the incidence of double ovulation. At present, data supporting a relationship between twinning, double ovulation, and milk production is associative rather than causative. Because of the high occurrence of multiple ovulations in multiparous cows, an increase in DZ twinning over time is likely responsible for the increase in twinning over time observed in dairy cattle [2,3,30] and not because of an increase in the frequency of MZ twin births.

Seasonal effects on twinning have been reported for dairy cattle peaking from April to September in the Netherlands [28], from May to July in the U.S. [7], and from May to June in Saudi Arabia [9]. In those studies, the increase in twinning was speculated to occur due to high environmental temperatures near conception, a decrease in day length and/or nutritional flushing. In the present study, the lower conception of twins from April to June corresponds with the seasonal increase in environmental temperatures in Wisconsin. By contrast, the greater twinning incidence when conception occurred from July to December could be explained by an increased frequency of multiple ovulations due to summer heat stress as reported by [23] or due to a nutritional flushing in autumn when higher quality forages are fed.

In conclusion, based on DNA fingerprinting, MZ twinning occurred infrequently in the population of twin Holstein calves evaluated in the present study, and less frequently than that predicted using Bonnier's equation if reporting of data is biased by twin birth type. We inferred that Bonnier's equation may overestimate the frequency of MZ twinning in larger epidemiologic studies of twinning in dairy cattle. We concluded that MZ twinning occurred infrequently in Holstein cattle and perhaps less frequently than that reported in studies using Bonnier's equation to estimate MZ twinning.

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