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Effect of Genetic and Environmental Factors on Twin Pregnancy in Primiparous Dairy Cows

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Simple Summary: The biological and economic impacts of pregnancy loss in dairy herds are well recognized. Twin pregnancies compromise the health and wellbeing of a cow. The use of genomic testing for production traits and fitness traits to select replacement heifers is increasing in commercial dairy farms**.** Recently, a genomic prediction for twin pregnancies has been developed. The twin pregnancies trait (TWIN) was defined as a pregnancy resulting in birth or abortion of twin calves. However, the incidence of twins at abortion or at parturition are not a true reflection of twin pregnancy. A high incidence of pregnancy loss during the first trimester of gestation has been described in cows carrying twins following pregnancy diagnosis. The objective of this retrospective cohort study was to assess cow, environmental and management risk factors affecting the incidence of twin pregnancies in high-producing dairy cows in their first lactation, with special emphasis placed on the genomic prediction values for twin pregnancy. Our study population of primiparous cows proved valuable in identifying factors other than genomic predictive values influencing the twin pregnancy rate. The factors photoperiod, milk production and estrus synchronization protocol for affixed-time artificial insemination were found to significantly influence the rate of twin pregnancy.

Abstract: Twin pregnancies are highly undesirable in dairy cattle; they compromise the health and wellbeing of a cow, and its incidence dramatically impairs the farm economy. Recently, a genomic prediction for twin pregnancies has been developed. The objective of this study was to assess cow, environmental and management risk factors affecting the incidence of twin pregnancies in high-producing dairy cows in their first lactation, with special emphasis placed on the genomic prediction values for twin pregnancy. Our study population of primiparous cows proved valuable in identifying factors other than genomic predictive values influencing the twin pregnancy rate. The odds ratio for twin pregnancy was 0.85 (p <0.0001) for each unit of a prediction value increase, 3.5 (p = 0.023) for cows becoming pregnant during the negative photoperiod, and 0.33 (p = 0.016) for cows producing ≥42 kg of milk at AI, compared to the remaining cows producing <42kg of milk. As a general conclusion, the practical implication of our findings is that genomic prediction values can identify the risk of twin pregnancy at herd level. Given the cumulative effect of genomic selection, selecting for animals with reduced genetic risk of twin pregnancies can contribute to reduce its incidence in dairy herds.

Keywords: breeding strategy; dark-light cycles; dead co-twin; early fetal loss; selection index; spontaneous twin reduction; standardized transmitting ability

1. Introduction

Twin pregnancies are highly undesirable in dairy cattle, they compromise the health and wellbeing of a cow. Compared with cows carrying singletons, cows carrying twins have a risk of pregnancy loss that is seven times higher during the first 90 days of gestation [1,2], a higher than 20% incidence of abortions during the second and third trimester of gestation [3], and a higher risk of perinatal mortality and postpartum reproductive disorders after twin delivery [4–7]. As a consequence, twin pregnancy reduces the lifespan of a cow (by 200 days) [4,8,9], and its incidence dramatically impairs the farm economy [10–13]. For example, the economic burden was estimated at $ 97–$ 225 per pregnancy depending on twin pregnancy laterality (unilateral vs. bilateral), parity, and the days in milk when the twin pregnancy occurs [12]. Twin pregnancies, more frequent in older cows, are classified into bilateral (one fetus in each uterine horn: 44%) and unilateral (both fetuses in the same uterine horn, right or left: 56%), and may account for almost 30% (1827/6463) of all pregnancies on Day 60 of gestation in cows in their third lactation or more [14,15]. Strategies such as induced twin reduction, transfer of a single embryo, or puncture and drainage of subordinate follicles at the time of insemination are available to prevent twin pregnancies [16,17].

The use of genomic testing for production traits and fitness traits to select replacement heifers is increasing in commercial dairy farms. In fact, current selection indices include both production and lifetime profitability traits [18–21]. A multi-trait selection index, including among others cow and calf wellness, milk production, fertility, abortion and longevity has been validated in commercial dairy herds to select healthier cows with a more lifetime merit [22–24]. Recently, a genomic prediction for twin pregnancies has been developed. The twin pregnancies trait (TWIN) was defined as a pregnancy resulting in birth or abortion of twin calves [25]. However, the incidence of twins at abortion or at parturition are not a true reflection of twin pregnancy. A high incidence of pregnancy loss during the first trimester of gestation has been described in cows carrying twins following pregnancy diagnosis [1,2]. Twin pregnancy loss can exceed 50% during the warm period of the year [15,26]. No less important is the fact that natural twin reduction usually occurs following a positive pregnancy diagnosis, between days 28 and 60 of pregnancy, with an incidence of cows maintaining gestation after experiencing twin reduction up to 28% [1]. Both early fetal loss and spontaneous twin reduction often escape clinical diagnosis. Thus, there may be a wide difference between the rate of twin pregnancies and the subsequent rates of abortion of twin calves or twinning. Diagnosis of twins towards the end of the embryonic period, i.e. days 30 to 40 of gestation, is the most appropriate target when investigating factors affecting the twin pregnancy rate [1,14,15].

Lactation number, photoperiod, season, and management related to estrus synchronization protocols for fixed-time artificial insemination (FTAI) influenced the twin pregnancy rate in an extensive previous study [27]. As it was demonstrated that genetic correlations between TWIN and other traits were low [25], it should be expected that genomic prediction values for twin pregnancy could be used as a variable in studying factors influencing the twin pregnancy rate. The objective of this retrospective cohort study was to assess cow, environmental, and management risk factors affecting the incidence of twin pregnancies in high-producing dairy cows, with special emphasis placed on the genomic prediction values for twin pregnancy. Our hypotheses were that twin pregnancy rate would be influenced by genetic value, photoperiod and milk production.

2. Materials and Methods

2.1. Cows and Herd Management

The study population was derived from a Holstein dairy herd in north-eastern Spain (latitude 41.13 N, longitude -2.4 E). During the study period (March 2022 to February 2023), the mean number of lactating cows in the herd was 3,580 and mean annual milk production was 15,695 kg per lactating cow. Cows were milked three times daily and the mean annual culling rate for the study period was 30%. Cows were grouped according to age (primiparous, secundiparous or cows in their third lactation or more) and fed complete rations. Walking activity values were recorded at the milking parlor (three times daily) and analyzed automatically using a herd management computer program (AfiFarm System; Afikim. Israel). Herd management included the use of fans and water sprinklers. Fans were placed throughout cubicle and feeding areas, whereas water sprinklers were set up in the feeding area with water spray directed towards the cows. Fans and water sprinklers were activated automatically when the temperature reached approximately 23ºC and 25ºC, respectively.

Only primiparous cows delivering singletons experiencing their first postpartum pregnancy and with complete information from insemination to pregnancy diagnosis were included in the study. Cows were only included if they were healthy, as indicated by a body condition score of 2–3.5 on a scale of 1 to 5 [28], produced more than 30 kg of milk per day at the time of AI, and were free of clinical signs of disease during the study period (Days -20 to 34 of insemination). Exclusion criteria were the following disorders: mastitis, lameness, digestive disorders and pathological abnormalities of the reproductive tract detectable by ultrasonography. All animals were reared within the herd.

2.2. Artificial Insemination and Ultrasound Exams

All cows were artificially inseminated and the herd was maintained on a weekly reproductive health program as described elsewhere [14,15]. The waiting period for primiparous cows was 85 days. Cows showing spontaneous estrus following this time were inseminated. Cows reaching 80 days in milk with no estrous signs for at least 21 days were synchronized for FTAI using a prostaglandin (PG)$F_{2\alpha}$-based protocol for cows with a mature corpus luteum (CL), or a progesterone(P4)-based protocol for cows with no luteal structures or with an immature CL. The presence of one or more CL of at least 15 mm in diameter (mature CL) was assessed by ultrasonography. Corpus luteum size was taken as the mean of two measurements approximating the greatest length and width. A lack of high pixel intensity associated with a young CL [29,30] was used as reference to confirm the state of CL maturity. As the presence of a central cavity is not functionally important [31–33], cavity CL were measured just like solid CL.

Cows with a mature CL received cloprosternol (500 µg im; Cyclix bovino, Virbac España, Esplugues de Llobregat, Barcelona, Spain); 2 d, GnRH (100 µg im; Cystoreline, CEVA Salud Animal, Barcelona, Spain); 7 d, cloprostenol; 56 h, GnRH; 16–20 h, FTAI. Cows in absence of a mature CL were treated with a controlled intravaginal progesterone-releasing device (CIDR) (containing 1.38 g of progesterone; Zoetis Spain SL, Alcobendas, Madrid, Spain) plus GnRH upon CIDR insertion. The CIDR was left in place for 5 d, and these animals were also given cloprostenol on CIDR removal. Twenty-four and 36 h later, the cows received a second cloprostenol dose and a second GnRH dose, respectively, and were FTAI 68–72 h after CIDR removal.

Pregnancy was diagnosed by transrectal ultrasonography 31 ± 3 days post-AI using a portable B-mode ultrasound scanner equipped with a 5-10 MHz transducer (E.I. Medical IBEX LITE, E.I. Medical Imaging, Loveland CO, USA). Each ovary was scanned in several planes by moving the transducer along its surface to identify luteal structures, and the number and location of CL were recorded. Scanning was then performed along the dorso/lateral surface of each uterine horn. The presence of twins was established through the observation of two embryos in different positions within one uterine horn on two screen scans or two embryos simultaneously present on the screen (unilateral twin pregnancy), or one embryo in each uterine horn (bilateral twin pregnancy). The final study population comprised 775 cows: 29 carrying twins (11 unilateral plus 18 bilateral twins) and 746 carrying singletons. Because twin reduction by amnion rupture is routinely performed in this herd at pregnancy diagnosis [16,17], the number of fetuses was only confirmed in cows carrying unilateral twins or singletons by ultrasound 49 to 55 days post-AI. All gynecological exams and pregnancy diagnoses were performed by the same operator.

2.3. Evaluation of Genetic Merit

Ear tissue samples were collected from all animals between six and ten months of age and submitted for genomic testing using the Clarifide Plus evaluation (Zoetis Genetics, Kalamazoo, MI, USA). The Clarifide Plus evaluation includes the CDCB predictions plus exclusive health and fertility traits [21,22,24,25], and profitability indexes [23]. Within the fertility traits Clarifide Plus provides a genomic prediction for risk of twin pregnancies which has been previously described by McGovern et al. [25]. Genomic predictions for twin pregnancies are expressed as standardized transmitting abilities (STA) with mean 100 and standard deviation (S.D.) of 5. Greater values represent a lesser risk of having a twin pregnancy.

2.4. Data Collection and Statistical Analyses

The twin pregnancy rate was defined as the percentage of pregnant cows carrying twins at pregnancy diagnosis. The following data were recorded in each animal: parturition and AI dates; estrus at AI (spontaneous estrus, $PGF_{2\alpha}$-based synchronization or P4-based synchronization); days in milk at AI; milk production at AI (mean production in the seven days before AI) (low producers <42 kg vs. high producers ≥42 kg); genomic prediction values for twin pregnancy; pregnancy 28–34 days post-AI; and presence of twins in pregnant cows. The threshold for milk production was set as the mean value of production recorded. AI dates were used to assess the effects of photoperiod (positive or negative) and season on the incidence of twin pregnancies. The positive photoperiod extends from December 22 to June 21. In our geographical region, there are only two clearly differentiated weather periods: warm (May to September) and cool (October to April) [26,27]. Temperatures for the study period were: 29 days of minimum temperatures <0ºC and 5 days of maximum temperatures >25ºC for March to April 2022 plus October 2022 to February 2023; and 0 days of minimum temperatures <0ºC and 101 days of maximum temperatures >25ºC for May to September 2022.

The software package PASW Statistics for Windows Version 18.0 (SPSS Inc., Chicago, IL, USA) was used for data processing. Significance was set at $p < 0.05$. Variables are ex-pressed as the mean ± S.D. A binary logistic regression

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analysis was performed using twin pregnancy as the dependent variable. The factors entered in the model were genomic prediction values and days in milk as continuous variables; season of AI (warm), photoperiod (positive) and milk production (high production) as dichotomous variables (where "1" denotes presence and "0" absence); and estrus at AI (spontaneous estrus, $PGF_{2\alpha}$-based synchronization or P4-based synchronization) as a class variable. Possible interactions between milk production and season or photoperiod were also investigated. Regression analyses were conducted according to the method of Hosmer and Lemeshow [34]. Basically, this method consists of five steps as follows: preliminary screening of all variables for univariate associations; construction of a full model using all the significant variables arising from the univariate analysis; stepwise removal of non-significant variables from the full model and comparison of the reduced model with the previous model for model fit and confounding; evaluation of plausible interactions among variables; and assessment of model fit using Hosmer-Lemeshow statistics. Variables with univariate associations showing P values < 0.25 were included in the initial model. Model reduction continued until only significant terms according to the Wald statistic remained in the model at P < 0.05.

3. Results

Of the 775 cows, 29 (3.7%) carried twins, 330 (42.6%) became pregnant during the warm period and 268 (34.6%) during the positive photoperiod. At least one CL ipsilateral to the embryo was observed in all twin pregnancies. Triplets or quadruplets were not recorded. Milk production and days in milk at the time of AI, as well as genomic prediction values were 42 ± 5.8 (32–59) kg, 134 ± 42 (85–330) days, and 100 ± 4.5 (82—110) units, respectively (mean ± SD; ranges between parentheses).

Table 1 summarizes the twin pregnancy rate, odds ratio and 95% confidence interval for all cows. The final model included the effect of genomic prediction values, photoperiod, milk production and reproductive treatment. The warm period of the year and days in milk were not significant and were not included in the model. Reproductive treatment-season, reproductive treatment-photoperiod or reproductive treatment-milk production interactions were not found. The odds ratio for twin pregnancy was 0.85 (p <0.0001) for each unit increase in genomic STA, 3.5 (p = 0.023) for cows becoming pregnant during the negative photoperiod, and 0.33 (p = 0.016) for cows producing ≥42 kg of milk at AI, compared to cows producing <42kg of milk. Using spontaneous estrus as reference, the odds ratio for twin pregnancy was 3.1 (p = 0.019) for cows receiving the $PGF_{2\alpha}$-based protocol. Influence of the P4-based protocol was not significant (Table 1).

Table 1. Odds ratios of the twin pregnancy rate variables included in the final logistic regression model (n = 775 primiparous pregnant cows).

Factor	Class	n	Twin pregnancy	Odds Ratio	95% Confidence Interval	p
ZTWINS [(a)]	Continuous	29/775	3.7%	0.85	0.78–0.95	<0.0001
Photoperiod	Positive	4/268	1.5%	Reference		
	Negative	25/507	4.9%	3.5	1.2–10.5	0.023
Milk production (kg)	<42	22/458	4.8%	Reference		
	≥42	7/317	2.2	0.33	0.13–0.81	0.016
Estrus [(b)]	Spontaneous	16/578	2.8%	Reference		
	$PGF_{2\alpha}$ protocol	7/102	6.9%	3.1	1.2–1.81	0.019
	P4 protocol	6/95	6.3%	1.8	0.6–5.3	0.22

Hosmer and Lemeshow Goodness-of-fit test = 23.6; 3 df, p = 0.92.
R^2 Nagelkerke = 0.14.
[(a)] Genomic prediction values for twin pregnancy.
[(b)] Type of estrus at pregnant insemination: Spontaneous; following a prostaglandin (PG)$F_{2\alpha}$-based protocol; following a progesterone(P4)-based protocol.

4. Discussion

The incidence of twin pregnancies continues on the rise in parallel with increased milk production [1,4,25,35,36]. In fact, already in 1924 Hunt wrote: "There is a definite relationship between high fertility and high milk production… This relationship seems to hold true when high fertility is evidenced in the production of twins" [37]. However, younger females have remained over time the lowest risk group for producing twins. Primiparous cows rarely reach a twinning rate of 2% [8,38]. This is expected, given that maturity of the reproductive tract and the highest milk production is achieved in multiparous cows, which experience a higher incidence of multiple pregnancies [4,8,35,36,38]. With a 3.7% (29/775) of twin pregnancies, our study population of primiparous cows proved valuable in identifying factors other than genomic predictive values influencing the twin pregnancy rate. The factors photoperiod, milk production and estrus synchronization protocol for FTAI were found to significantly influence the rate of twin pregnancy.

Our results are in agreement with McGovern et al. [25], who observed a reduction of twin pregnancies in those cows with greater genomic STA values. Interestingly, in contrast to what we observed in the present study, the authors observed an interaction be-tween genomic predictions and herd reproductive management, with increased risk of twin pregnancy in those herds that relied more on heat detection. A possible explanation for these differences might be that those herds with the lowest incidence of twin pregnancies used Double Ovsynch whereas the protocol used in the current study for cows with CL decreased P4 concentrations during follicular development.

Regardless of genomic prediction values, the length of the photoperiod influenced the incidence of twin pregnancies by a factor of 3.5. Although cows are not strict season breeders, a negative photoperiod leaded to higher twin pregnancy rates (4.9%) compared to positive photoperiod (1.5%) reinforcing previous results [27]. We tested a possible interaction between genomic prediction values and photoperiod on the twin pregnancy rate. A further binary logistic regression analysis was performed including genomic prediction values as a dichotomous variable (low prediction values <100 units vs. high prediction values ≥100 units). The final model (not presented) was practically the same from that presented in Table 1. However, the model was worse fitted ($p = 0.60$) than that including genomic prediction values as a continuous variable ($p = 0.92$), and a genomic prediction value-photoperiod interaction was not found. Therefore, under our work conditions, we considered that the independent variable genomic prediction values considered as a continuous variable was a better predictor of twin pregnancy than as a dichotomous variable.

Turning the point to the influence of a decreasing photoperiod length on the increased incidence of twin pregnancies, such effect may well spring from remains of an ancient strategy in mammals increasing the chances of parturition and numbers of offspring when feed availability is higher [39–41]. The fact that the warm period of the year had no effects on the twin pregnancy rate in the present study reinforces this idea.

Heat stress is a great problem in animals and humans [42], especially in high producing dairy cows [43–45]. Although the warm period of the year greatly impairs maintenance of twin pregnancies [15,26], season had no effects on the incidence of twins in the present study. In a previous study performed between 2010 and 2011 in herds in our geographical area with a mean annual milk production around 11,500 kg per cow, both warm period and positive photoperiod were associated with a significant decrease in the incidence of twin pregnancies [27]. As the warm period of the year (April to September) includes part of negative and positive photoperiod, although interactions were not found, it is possible that the effects of one of the two factors masked the effects of the other in the referred report [27]. In a subsequent experiment conducted on cows in their third lactation or more in the same farm as the present study, with a mean annual milk production of 12,230 kg per cow from 2014 to 2018, the twin pregnancy rate was similar for both warm (26.5%) and cool (26.3%) periods [15]. Currently, the herd is producing 15,695 kg of milk per cow and year (present study). It seems clear that management practices associated with high milk production and heat abatement mitigate the negative effects of heat stress on twin pregnancy rate. Therefore, the independence of season and photoperiod shown here indicates a strong effect of photoperiod on the incidence of twins.

The likelihood of twin pregnancy decreased in high-producing cows by a factor of 0.33 in this study of first lactation cows. Although high milk production has been considered the most important single contributor to the increase of multiple ovulations and so the twinning rate during the last decades [1,4,35,36], the percentage of twin pregnancies was 2.2% in high producers (≥42 kg per day) in contrast to the 4.8% recorded in cows with low production (<42 kg). This is not entirely new. In the previous study cited above [27], cows carrying twins produced less milk than cows carrying singletons: 40.5 ± 9.3 kg vs. 41.7 ± 9.1 kg, respectively, $p = 0.032$ using univariate analyses. However, after adjusting for other factors in the logistic regression analysis, milk production was not a factor associated with the likelihood of twin pregnancy [27]. These findings question the hypothesis that high-producing dairy cows may be associated with double ovulation, and so twin pregnancies [46–49]. It is clear that multiple ovulations can lead to twin pregnancies. More than 90% of twins derive from two or more ovulations (that is, dizygotic twins) [50,51]. However, the association between milk production and multiple ovulations has also been questioned in a recent review [52]. Improvements in management and nutrition practices, along with genetic progress, that have resulted in a steady increase in milk production [36,53,54] have probably reduced the incidence of pregnancy loss of twin pregnancies favoring so an increased twinning rate through the years. On the other hand, the fact that high-producing cows may show a lower twin pregnancy rate than their low producer partners may have clinical implications. The ratio of percentage of twins between low and high producers was 2.2 (4.8/2.2) in the present study. The question that arises is why the ratio of twins did favor the rate of low producers? Management practices on this farm probably controlled the heat stress of the warm period but not the metabolic stress of high milk production. If so, when assessing the reproductive performance of a herd, a ratio of twins between low and high producers close to one could be a good indicator of cow wellbeing. This should be assessed in more extensive studies. Of course, another problem would be, as in the case of low producers, how to prevent multiple pregnancies.

3. Conclusions

As a general conclusion, the practical implication of our findings is that genomic prediction values can identify the risk of twin pregnancy at herd level. Given the cumulative effect of genomic selection, selecting for animals with reduced genetic risk of twin pregnancies can contribute to reduce the incidence in dairy herds. Furthermore, being able to identify those animals at greater risk may allow to perform precision management to prevent twin pregnancies, such as transfer of a single embryo, puncture and drainage of subordinate follicles at the time of AI or use of more sophisticated synchronization protocols.

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References

1. López-Gatius, F.; Andreu-Vázquez, C.; Mur-Novales, R.; Cabrera, V.E.; Hunter, R.H.F. The dilemma of twin pregnancies in dairy cattle. A review of practical prospects. *Liv. Sci.* **2017**, *197*, 121–126. http://dx.doi.org/10.1016/j.livsci.2017.01.001
2. Szenci, O. Recent Possibilities for the diagnosis of early pregnancy and embryonic mortality in dairy cows. *Animals* **2021**, *11*, 1666. https://doi.org/10.3390/ani11061666
3. López-Gatius, F.; Garcia-Ispierto, I.; Hanzen, C. Unilateral twin pregnancy: A non-infectious factor required for the etiological diagnosis of abortion in dairy herds. *J. Reprod. Dev.* **2021**, 67, 337–339. https://doi.org/10.1262/jrd.2021-090
4. Nielen, M.; Schukken, Y.H.; Scholl, D.T.; Wilbrink, H.J.; Brand, A. Twinning in dairy cattle: A study of risk factors and effects. *Theriogenology* **1989**, *32*, 845–862. https://doi.org/10.1016/0093-691x(89)90473-1
5. Echternkamp, S.E.; Gregory, K.E. Effects of twinning on gestation length, retained placenta, and dystocia. *J. Anim. Sci.* **1999**, *77*, 39–47. https://doi.org/10.2527/1999.77139x
6. Sawa, A.; Jankowska, M.; Neja, W.; Krężel-Czopek, S. Effect of single and multiple pregnancies and calf sex on parturition process and perinatal mortality. *Ann. Anim. Sci*. **2014**, *14*, 851-858. https://doi.org/10.2478/aoas-2014-0031
7. Cuttance, E.; Laven, R. Perinatal mortality risk factors in dairy calves. *Vet. J.* **2019**, *253*, 105394. https://doi.org/10.1016/j.tvjl.2019.105394
8. Andreu-Vázquez, C.; Garcia-Ispierto, I.; Ganau, S.; Fricke, P.M.; López-Gatius, F. Effects of twinning on the subsequent reproductive performance and productive lifespan of high-producing dairy cows. Theriogenology 2012, 78, 2061–2070. https://doi.org/10.1016/j.theriogenology.2012.07.027
9. Bicalho, R.C.; Cheong, S.H.; Galvao, K.N.; Warnick, L.D.; Guard, C.L. Effect of twin birth calvings on milk production, reproductive performance, and survival of lactating cows. *J.A.V.M.A.* **2007**, *231*, 1390-1397. https://doi.org/10.2460/javma.231.9.1390
10. Eddy, R.G.; Davies, O.; David, C. An economic assessment of twin births in British dairy herds. *Vet. Rec.* **1991**, *129*, 526–529.
11. Beerepoot, G.M.; Dykhuizen, A.A.; Nielen, M.; Schukken, Y.H. The economics of naturally occurring twinning in dairy cattle. *J. Dairy Sci.* **1992**, *75*, 1044–1051. https://doi.org/10.3168/jds.s0022-0302(92)77848-5
12. Mur-Novales, R.; Lopez-Gatius, F.; Fricke, P.M.; Cabrera, V.E. An economic evaluation of management strategies to mitigate the negative effect of twinning in dairy herds. *J. Dairy Sci.* **2018**, *101*, 8335–8349. https://doi.org/10.3168/jds.2018-14400
13. Cabrera, V.E.; Fricke, P.M. Economics of twin pregnancies in dairy cattle. *Animals* **2021**, *11*, 552. https://doi.org/10.3390/ani11020552
14. Garcia-Ispierto, I.; López-Gatius, F. The effects of a single or double GnRH dose on pregnancy survival in high producing dairy cows carrying singletons or twins. *J. Reprod. Dev.* **2018**, *64*, 523–527. https://doi.org/10.1262/jrd.2018-057
15. López-Gatius, F.; Garcia-Ispierto, I.; Hunter, R.H.F. Twin pregnancies in dairy cattle: Observations in a large herd of Holstein-Friesian dairy cows. *Animals* **2020**, *10*, 2165. https://doi.org/10.3390/ani10112165

16. López-Gatius, F. Twins in dairy herds. Is it better to maintain or reduce a pregnancy? *Animals* **2020**, *10*, 2006. https://doi.org/10.3390/ani10112006
17. Garcia-Ispierto, I.; López-Gatius, F. Benefits and risks of preventing twin pregnancies. *Animals* **2021**, *11*, 148. https://doi.org/10.3390/ani11010148
18. Wiggans, G.R.; VanRaden, P.M.; Cooper, T.A. The genomic evaluation system in the United States: Past, present, future. *J. Dairy Sci.* **2011**, *94*, 3202–3211. https://doi.org/10.3168/jds.2010-3866
19. Weigel, K.A.; Hoffman, P.C.; Herring, W.; Lawlor, T.J.Jr. Potential gains in lifetime net merit from genomic testing of cows, heifers, and calves on commercial dairy farms. *J. Dairy Sci.* **2012**, *95*, 2215–2225. https://doi.org/10.3168/jds.2011-4877
20. García-Ruiz, A.; Cole, J.B.; VanRaden, P.M.; Wiggans, G.R.; Ruiz-López, F.J.; Van Tassell, C.P. Changes in genetic selection differentials and generation intervals in US Holstein dairy cattle as a result of genomic selection. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E3995–E4004. https://doi.org/10.1073/pnas .1519061113
21. Vukasinovic, N.; Bacciu, N.; Przybyla, C.; Boddhireddy, P.; DeNise, S. Development of genetic and genomic evaluation for wellness traits in US Holstein cows. *J. Dairy Sci.* **2017**, *100*, 428–438. https://doi.org/10.3168/jds.2016-11520
22. McNeel, A.K.; Reiter, B.C.; Weigel, D.; Osterstock, J.; Di Croce, F.A. Validation of genomic predictions for wellness traits in US Holstein cows. *J. Dairy Sci.* **2017**, *100*, 9115–9124. https://doi.org/10.3168/jds.2016-12323
23. Fessenden, B.; Weigel, D.J.; Osterstock, J.; Galligan, D.T.; Di Croce, F. Validation of genomic predictions for a lifetime merit selection index for the US dairy industry. *J. Dairy Sci.* **2020**, *103*, 10414–10428. https://doi.org/10.3168/jds.2020-18502
24. Wijma, R.; Weigel, D.J.; Vukasinovic, N.; Gonzalez-Peña, D.; McGovern, S.P.; Fessenden, B.C.; McNeel, A.K.; Di Croce, F.A. Genomic prediction for abortion in lactating Holstein dairy cows. *Animals* **2022**, *12*, 2079. https://doi.org/10.3390/ani12162079
25. McGovern, S.P.; Weigel, D.J.; Fessenden, B.C.; Gonzalez-Peña, D.; Vukasinovic, N.; McNeel, A.K.; Di Croce, F.A. Genomic prediction for twin pregnancies. *Animals* **2021**, *11*, 843. https://doi.org/10.3390/ani11030843
26. López-Gatius, F.; Santolaria, P.; Yániz, J.L.; Garbayo, J.M.; Hunter, R.H.F. Timing of early foetal loss for single and twin pregnancies in dairy cattle. *Reprod. Domest. Anim.* **2004**, *39*, 429–433. https://doi.org/10.1111/j.1439-0531.2004.00533.x
27. Andreu-Vázquez, C.,;Garcia-Ispierto, I.; López-Gatius, F. Photoperiod length and the estrus synchronization protocol used before AI affect the twin pregnancy rate in dairy cattle. *Theriogenology* **2012**, *78*, 1209–1216. https://doi.org/10.1016/j.theriogenology.2012.05.014
28. Edmondson, A.J.; Lean, I.J.; Weaver, C.O.; Farver, T.; Webster, G. A body condition scoring chart for Holstein dairy cows. *J. Dairy Sci.* **1989**, *72*, 68–78. https://doi.org/10.3168/jds.S0022-0302(89)79081-0
29. Townson, D.H.; Ginther, O.J. Ultrasonic echogenicity of developing corpora lutea in pony mares. *Anim. Reprod. Sci.* **1989**, *20*, 143–153. https://doi.org/10.1016/0378-4320(89)90071-7
30. Singh, J.; Pierson, R.A.; Adams, G.P. Ultrasound image attributes of the bovine corpus luteum: Structural and functional correlates. *J. Reprod. Fert.* **1997**, *109*, 35–44. https://doi.org/10.1530/jrf.0.1090035
31. Kito, S.; Okuda, K.; Miyazawa, K.; Sato, K. Study on the appearance of the cavity in the corpus luteum of cows by using ultrasonic scanning. *Theriogenology* **1986**, *25*, 325–333. https://doi.org/10.1016/0093-691x(86)90068-3
32. Kastelic, J.P.; Pierson, R.A.; Ginther, O.J. Ultrasonic morphology of corpora lutea and central luteal cavities during the estrous cycle and early pregnancy in heifers. *Theriogenology* **1990**, *34*, 487–498. https://doi.org/10.1016/0093-691x(90)90006-f
33. Perez-Marin, C. Formation of corpora lutea and central luteal cavities and their relationship with plasma progesterone levels and other metabolic parameters in dairy cattle. *Reprod. Domest. Anim.* **2009**, *44*, 384–389. https://doi.org/10.1111/j.1439-0531.2007.01021.x
34. Hosmer, D.W.; Lemeshow, S. *Applied Logistic Regression*; Wiley: New York, NY, USA, 1989.
35. Kinsel, M.L.; Marsh, W.E.; Ruegg, P.L.; Etherington, W.G. Risk factors for twinning in dairy cows. *J. Dairy Sci.* **1998**, *81*, 989–993. https://doi.org/10.3168/jds.S0022-0302(98)75659-0
36. Johanson, J.M.; Bergert, P.J.; Kirkpatrick, B.W.; Dentine, M.R. Twinning rates for North American Holstein sires. *J. Dairy Sci.* **2001**, *84*, 2081–2088. https://doi.org/10.3168/jds.s0022-0302(01)74653-x
37. Hunt, G.A. Relation of milk production to the twinning tendency. *J. Dairy Sci.* **1924**, *7*, 262–266. https://doi.org/10.3168/jds.S0022-0302(24)94021-5
38. Jones, S.V.H.; Rouse, J.E. The relation of age of dam to observed fecundity in domesticated animals: I. Multiple births in cattle and sheep. *J. Dairy Sci.* **1920**, *3*, 260–290. https://doi.org/10.3168/jds.S0022-0302(20)94273-X
39. Mohawk, J.A.; Green, C.B.; Takahashi, J.S. Central and peripheral circadian clocks in mammals. *Annu. Rev. Neurosci.* **2012**, *35*, 445–462. https://doi.org/10.1146/annurev-neuro-060909-153128
40. Dardente, H.; Lomet, D.; Robert, V.; Decourt, C.; Beltramo, M.; Pellicer-Rubio, M.T. Seasonal breeding in mammals: From basic science to applications and back. *Theriogenology* **2016**, *86*, 324–332. https://doi.org/10.1016/j.theriogenology.2016.04.045
41. Korf, H.W. Signaling pathways to and from the hypophysial pars tuberalis, an important center for the control of seasonal rhythms. *Gen. Comp. Endocrinol.* **2018**, *258*, 236–243. https://doi.org/10.1016/j.ygcen.2017.05.011
42. Boni, R. Heat stress, a serious threat to reproductive function in animals and humans. *Mol. Reprod. Dev*. **2019**, *86*, 1307–1323. https://doi.org/10.1002/mrd.23123

43. Dikmen, S.; Hansen, P.J. Is the temperature-humidity index the best indicator of heat stress in lactating dairy cows in a subtropical environment? *J. Dairy Sci.* **2009**, *92*, 109–116. https://doi.org/10.3168/jds.2008-1370
44. Collier, R.J.; Renquist, B.J.; Xiao, Y. A 100-Year Review: Stress physiology including heat stress. *J. Dairy Sci.* **2017**, *100*, 10367–10380. https://doi.org/10.3168/jds.2017-13676
45. Wolfenson, D.; Roth, Z. Impact of heat stress on cow reproduction and fertility. *Anim. Front.* **2019**, *9*, 32–38. https://doi.org/10.1093/af/vfy027
46. Fricke, P.M.; Wiltbank, M.C. Effect of milk production on the incidence of double ovulation in dairy cows. *Theriogenology* **1999**, *52*, 1133–1143. https://doi.org/10.1016/S0093-691X(99)00205-8
47. Wiltbank, M.C.; Fricke, P.M.; Sangsritavong, S.; Sartori, R.; Ginther, O.J. Mechanisms that prevent and produce double ovulations in dairy cattle. *J. Dairy Sci.* **2000**, *83*, 2998–3007. https://doi.org/10.3168/jds.S0022-0302(00)75201-5
48. Sangsritavong, S.; Combs, D.K.; Sartori, R.; Armentano, L.E.; Wiltbank, M.C. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17 β in dairy cattle. *J. Dairy Sci.* **2002**, *85*, 2831–2842. https://doi.org/10.3168/jds.S0022-0302(02)74370-1
49. Lopez, H.; Caraviello, D.Z.; Satter, L.D.; Fricke, P.M.; Wiltbank, M.C. Relationship between level of milk production and multiple ovulations in lactating dairy cows. *J. Dairy Sci.* **2005**, *88*, 2783–2793. https://doi.org/10.3168/jds.S0022-0302(05)72958-1
50. Silva-del-Río, N.; Kirkpatrick, B.W.; Fricke, P.M. Observed frequency of monozygotic twinning in Holstein dairy cattle. *Theriogenology* **2006**, *66*, 1292–1299. https://doi.org/10.1016/j.theriogenology.2006.04.013
51. Young, A.S.; Kirkpatrick, B.W. Frequency of leukochimerism in Holstein and Jersey twinsets. *J Anim Sci* **2016**, *94*, 4507–4515. https://doi.org/10.2527/jas.2016-0623
52. Macmillan, K.; Kastelic, J.P.; Colazo, M.G. Update on multiple ovulations in dairy cattle. *Animals* **2018**, *8*, 62. https://doi.org/10.3390/ani8050062
53. Masuda, Y.; Baba, T.; Suzuki, M. Genetic analysis of twinning rate and milk yield using a threshold-linear model in Japanese Holsteins. *Anim. Sci. J.* **2015**, *86*, 31–36. https://doi.org/10.1111/asj.12236
54. De Vries, A. Symposium review: Why revisit dairy cattle productive lifespan? *J. Dairy Sci.* **2020**, *103*, 3838–3845. https://doi.org/10.3168/jds.2019-17361