J. Dairy Sci. TBC
https://doi.org/10.3168/jds.2025-26726


# Association of fertility traits with markers of oocyte competence in dairy cattle

**B. Chasi,[1] J. P. Andrade,[2] P. L. J. Monteiro,[3] B. Castro,[1] E. Moreno,[1] E. Anta,[1] N. N. Ali,[4] J. B. Cole,[5,6,7] M. C. Wiltbank,[1] F. Peñagaricano,[1] A. Z. Balboula,[4] and M. S. Ortega[1]***

[1]Department of Animal and Dairy Sciences, University of Wisconsin, Madison, WI, 53706
[2]Department of Agricultural Sciences, West Texas A&M University, Canyon, TX, 79016
[3]Department of Large Animal Clinical Sciences, University of Florida, Gainesville, FL, 32608
[4]Division of Animal Sciences, University of Missouri, Columbia, MO, 65211
[5]Council on Dairy Cattle Breeding, Bowie, Maryland 20716
[6]Department of Animal Sciences, Donald Henry Barron Reproductive and Perinatal Biology Research Program, and the Genetics Institute, University of Florida, Gainesville, FL 32611-0910
[7]Department of Animal Science, North Carolina State University, Raleigh, NC 27695-7621

## ABSTRACT

Fertility traits such as daughter pregnancy rate (DPR), cow conception rate, and heifer conception rate are key predictors of reproductive performance in dairy herds. However, their low heritability, likely due to their multifactorial nature and difficulty in measuring phenotypes, poses challenges for genetic improvement. Oocyte competence, encompassing nuclear and cytoplasmic maturation, is a critical factor influencing fertility. Errors in chromosome segregation during nuclear maturation can result in aneuploidy, which leads to embryonic mortality. During cytoplasmic maturation, mitochondrial replication is vital for energy production to support subsequent embryo development. The objective of this work was to investigate the association between fertility traits and markers of oocyte competence after in vitro maturation in dairy cattle. To do this, 105 Holstein cows with 32 to 52 d of pregnancy and ranging from 1 to 6 lactations were enrolled in the study. Cumulus oocyte complexes (COC) were collected by ovum pickup (OPU). Each cow was subjected to OPU 3 times, and collected COC were classified by grade following International Embryo Transfer Society guidelines. For maturation, COC were split into groups by grade as either grade 1 and 2 or grade 3 and 4. After maturation, COC were used for 2 analyses: aneuploidy screening and mitochondrial copy number. Low-fertility cows, based on DPR, might harbor genetic variants associated with elevated oocyte aneuploidy rates, potentially contributing to their reduced fertility. No association was found between mitochondria copy number and fertility traits. Further research needs to be performed on mitochondria functionality and its relationship with fertility. In conclusion, high-fertility cows based on DPR had fewer oocytes with chromosomal abnormalities, which could explain improved fertility.

**Key words:** aneuploidy, fertility traits, mitochondria, oocyte competence

## INTRODUCTION

Dairy cattle breeding programs are designed to improve the farm's profitability and sustainability. In the case of fertility, the most widely used traits are daughter pregnancy rate (**DPR**), cow conception rate (**CCR**), and heifer conception rate (**HCR**). Daughter pregnancy rate is a reflection of days open, whereas HCR and CCR measure the ability of heifers and cows, respectively, to conceive or become pregnant at each insemination (VanRaden et al., 2004; Kuhn et al., 2006; Gobikrushanth et al., 2020). Fertility traits have low heritability (4% for DPR, 2% for CCR, and 1% for HCR), meaning that genetic selection for fertility is difficult and their progress tends to be slow (VanRaden et al., 2021). Despite this, they can be valuable for selecting animals of better reproductive performance, where cows with high genetic merit for fertility have fewer services per conception and higher pregnancy rates per insemination compared with those with lower fertility values (Ortega et al., 2017a; Lima et al., 2020), regardless of their reproductive management (Sitko et al., 2023).

Selection for fertility is challenging because the phenotypes involved in reproductive processes are difficult to measure. For example, studies are often limited to resulting pregnancies or services per conception, which do not provide much information on earlier reproductive events, such as fertilization and embryonic development. Indeed, it has been estimated that during the first week

Received April 7, 2025.
Accepted August 11, 2025.
*Corresponding author: sofia.ortega@wisc.edu

The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-25. Nonstandard abbreviations are available in the Notes.

after insemination, the period of preimplantation embryonic development, 10% to 50% of pregnancies fail (Wiltbank et al., 2016). One of the key factors affecting early embryonic development is oocyte competence, which is defined as the ability of an oocyte to mature, be fertilized, and develop into a viable embryo capable of establishing pregnancy (Conti and Franciosi, 2018; Aguila et al., 2020). The acquisition of this competence is regulated by various processes during nuclear and cytoplasmic maturation.

During nuclear maturation, the chromosomes condense and align at the metaphase plate, and then half of the chromosomes are extruded in the polar body. The oocyte then enters and arrests at the metaphase-II (**MII**) stage until fertilization. Oocytes must reach the MII stage of meiosis to be fertilized and subsequently develop into blastocysts. (Sirard et al., 1989; Combelles and Albertini, 2001; Landim-Alvarenga and Maziero, 2014). Chromosome segregation errors during oocyte meiosis, can lead to aneuploidy—where the oocyte contains an abnormal number of chromosomes—which subsequently associates with fertilization defects or early embryonic arrest. In humans, aneuploidy is a major cause of infertility, with its incidence increasing with maternal age (Tyc et al., 2020). In cattle, most cases originate from errors during oocyte meiosis (Silvestri et al., 2021). Aneuploidy can originate from various causes, including incorrect kinetochore–microtubule attachments, a defective spindle assembly checkpoint, premature loss of cohesion, or elevated levels of DNA damage (Balboula et al., 2020; Ma et al., 2020; Kincade et al., 2023; Sun et al., 2024).

Cytoplasmic maturation is another key hallmark of oocyte competence. During this process, organelles reorganize, and maternal mRNA needed to support protein synthesis during early embryonic development accumulates (Mao et al., 2014; Conti and Franciosi, 2018). Here, the mitochondria play a central role by generating ATP through oxidative phosphorylation to meet the energy demands of oocyte maturation and early embryo development (Dumollard et al., 2007; Sutton-McDowall et al., 2010; Wai et al., 2010; Ge et al., 2012). During cytoplasmic maturation, mitochondrial replication occurs exponentially, ranging between 100,000 and 1,000,000 mitochondria in a mature oocyte (Iwata et al., 2011). Oocytes with higher mitochondrial content exhibit greater competence and improved embryonic development following fertilization (Wai et al., 2010; Ge et al., 2012), and it has been estimated that at least 150,000 mitochondrial DNA copies are needed for an oocyte to support embryonic development (St John et al., 2023).

Genes associated with mitochondria function have been associated with fertility in dairy cattle. For example, an SNP in *COQ9*, which is necessary for the electron transport system, has been positively associated with DPR in Holstein cows (Ortega et al., 2017b). However, it is still not clear if fertility traits are associated with specific oocyte phenotypes. As such, the objective of this study was to evaluate whether DPR and CCR are associated with key oocyte competence parameters, including aneuploidy incidence and mitochondrial content. It was hypothesized that oocytes from females with higher genetic merit for fertility traits have increased mitochondrial content and also a lower incidence of aneuploidy.

## MATERIALS AND METHODS

All animal procedures were conducted in accordance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin.

### *Follicular Aspiration and Oocyte Collection*

A total of 105 cows were enrolled in the study, ranging from 32 to 52 d pregnant, with less than 160 DIM, and ranging from 1 to 6 lactations. Cows were split into 3 groups: first, second, and third or later lactations. The experiment was performed at the Emmons Blaine Arlington Dairy Research Center (Arlington, WI). All cows had genetic evaluations (March 2023, CLARIFIDE Plus) and recorded PTA for DPR and CCR.

The day after the routine pregnancy diagnosis (d 32 after AI), any follicle larger than 5 mm in diameter was ablated to synchronize a new follicular wave (Hayden et al., 2025). After 3 d, and every 3 d thereafter, follicular aspiration or ovum pick up (**OPU**) was performed to retrieve cumulus oocyte complexes (**COC**) from follicles between 2 to 8 mm, recording the total number of follicles aspirated. A total of 3 OPU sessions were performed in each cow. Before starting the OPU session, the sacrococcygeal area was shaved and cleaned with 70% ethanol, and an epidural injection of 5 mL of 2% lidocaine solution (Aspen Veterinary Resources, Loveland, CO) was administered. The vulvar area was thoroughly washed and disinfected with 70% ethanol (vol/vol), and transrectal palpation was used to reach the ovaries and bring them near the vaginal wall. An IBEX EVO II ultrasound (E.I. Medical Imaging, Loveland, CO) coupled with a microconvex probe inside an OPU handle was used to image the ovaries. Within the handle, an OPU system (Watanabe Applied Technology, São Paulo, Brazil) in a rod with a 50-mm 21-gauge needle at the tip was used to perform the aspirations transvaginally.

The OPU system was connected to a 50-mL tube containing 15 mL of oocyte collection medium (**OCM**) and a follicular aspiration pump (Watanabe Applied Technology, São Paulo, Brazil) set at a pressure of 90 mm

Hg. The pump was turned on to create negative pressure when the needle punctured a follicle, so the follicular fluid would be collected into the 50-mL tube. Once the OPU procedure was completed for a cow, the medium with follicular fluid was filtered through a 100-µm cell strainer (Fisher Scientific, Waltham, MA) and rinsed with fresh OCM. The strainer was then backwashed into grid plates (Fisher Scientific) to search for COC under a stereoscope (Leica Microsystems Inc., Deerfield, IL). The OCM and oocyte maturation medium (**OMM**) were prepared as routinely done by our group (Ortega et al., 2017b, 2020). One liter of OCM without supplements consisted of 9.5 g of Medium 199 Modified (Sigma, St. Louis, MO), 350 mg of sodium bicarbonate (Sigma), 2,380 mg of HEPES (Sigma), and 5 mL of Glutamax (Fisher Scientific). When used, OCM was supplemented with 20 mL of adult bovine serum (Sigma), 20 mL of penicilin streptomicin (Sigma), and 600 µL of heparin (Sigma) at a concentration of 4 mg/mL. The OMM is composed of TCM-199 Earle's salts (Fisher Scientific) supplemented with 10% fetal bovine serum (Sigma), 2 µg/mL of estradiol-17B, 25 µg/mLof porcine FSH, 22 µg/mL of sodium pyruvate (Sigma), 50 µg/mL of gentamicin and 1 m*M* of L-glutamine (Fisher Scientific).

Retrieved COC were classified by the International Embryo Transfer Society guidelines into grades 1 to 4 (Demetrio and Barfield, 2022). For maturation, COC were split into 2 groups as grades 1 and 2 (higher quality) and grades 3 and 4 (lower quality). It was decided to separate them that way because grade 1 and 2 oocytes had higher embryo development rates compared with oocytes classified as grade 3 and 4, with no differences on embryo development rate between grade 1 and 2 oocytes and between grade 3 and 4 oocytes (Demetrio et al., 2022).

The COC were matured for 24 h in 5-well plates (Watanabe Applied Technology, São Paulo, Brazil), with each well containing 500 µL of OMM overlaid with 300 µL of light mineral oil (Irvine Scientific, Santa Ana, CA), in an incubator set at 38.5°C with 5% (vol/vol) $CO_2$. At the end of maturation, cumulus cells were removed from the oocytes by vortexing the COC for 5 min in 100 µL of a hyaluronidase (from bovine testis-Sigma) resuspended at 10,000 units/mL in saline. Denuded oocytes were split to evaluate mitochondria copy number or chromosome number.

### Mitochondrial Copy Number

Individual oocytes were collected into 6 µL of embryo lysis buffer (Ortega et al., 2020) and lysed in a T100 96-well gradient thermal cycler (Bio-Rad, Hercules, CA) at 60°C for 30 min, followed by 85°C for 10 min. The lysis buffer consisted of 40 m*M* Tris (pH 8.9), 0.9% (vol/vol) Triton X-100, 0.9% (vol/vol) Nonidet P-40, and 32 U/mL proteinase K. The lysed product was used as template DNA. Mitochondria number in individual oocytes was determined by qPCR of the mitochondrial gene cytochrome c oxidase subunit I (*COX1*) following procedures previously described (May–Panloup et al., 2005; Ortega et al., 2017b). The primers sequences were as follows: forward 5′-AAATAATATAAGCTTCTGACTCC-3′, and reverse 5′TCCTAAAATT-GAGCAAACTCC-3′, resulting in a 190-bp amplicon. The slope of the regression was −3.6, and the primer efficiency was 99.6%.

To determine the mitochondrial (**mt**) copy number, a standard curve with 6 points through a 10-fold serial dilution was developed using synthetic gene fragments "gBlock" (IDT Technologies, Coralville, IA) containing the partial sequence of *COX1* amplified by the primers. The standard curve ranged from 11.2 to $1.12 \times 10^6$ copies of *COX1* (Supplemental Figure S1A, see Notes). In each PCR run, a standard curve was performed. Each PCR reaction consisted of 5 µL of Universal SYBR Green Supermix (Bio-Rad, Hercules, CA), 0.5 µL of each primer, 1 µL of nuclease-free water, and 3 µL of template DNA (either oocyte DNA or gblock). Amplification conditions were 95°C for 7 min, 40 cycles at 95°C for 1 s, 56°C for 5 s, and 72°C for 13 s. Copy numbers were determined by comparing the cycle threshold values of each sample to those of the standard curve, with all samples and standards analyzed in duplicate.

### Aneuploidy Screening and Oocyte Maturation

To alter the spindle structure from bipolar to monopolar and disperse the chromosomes to allow counting, denuded oocytes were incubated for 3 h in OMM with 100 m*M* monastrol (Sigma, St. Louis, MO). Oocytes were then fixed with 2% paraformaldehyde, placed in a blocking buffer (Balboula et al., 2020; Sun et al., 2024), and shipped to the University of Missouri (Columbia, MO) for staining and imaging. Immunostaining was performed by incubating the samples in human anticentromere primary antibody (Antibody Inc., Davis, CA, #15-234) at a dilution of 1:30 for 1 h at room temperature, followed by incubation in the secondary antibody Alexa fluor 568 goat anti-human (Invitrogen, Waltham, MA, #A21090) at a dilution of 1:200 for 1 h at room temperature. Oocytes were then mounted onto slides with Vectashield mounting medium containing DAPI (Vector Laboratories Inc., Newark, CA).

Oocytes were imaged under 63× oil objective using a Leica Stellaris 5 confocal microscope at 0.5-µm intervals to capture all kinetochores. Kinetochores were counted using Image J 1.54g (ImageJ, National Institutes of

Health, Bethesda, MD) and were categorized as euploid (containing 60 kinetochores) or aneuploid when the number of kinetochores was different from 60 (Supplemental Figure S1C). If chromosomes were not clearly dispersed for proper counting, the oocyte was not considered for analysis. Oocytes were considered to have reached MII stage or matured if there was a visible polar body (Supplemental Figure S1B).

### Statistical Analysis

All statistical analyses were performed using R v.4.4.0. The following model was used to evaluate the effect of fertility traits:

$$y = \beta_0 + \beta_1 \times PTA + e,$$

where y is the response variable, $\beta_0$ is the intercept, $\beta_1$ is the coefficient for the effect of the PTA, with PTA represented as a continuous predictor for DPR or CCR, and e is the residual deviance. The response variables were follicular count, the proportion of grade 1 and 2 or 3 and 4 oocytes collected, mitochondrial content, maturation rate, and aneuploidy incidence. Average follicle number and mitochondria copy number per cow were evaluated with the "lm" function to fit a linear model. To achieve normality, the number of follicles was transformed using natural logarithm (log base *e*), and mt copy number was square root transformed. To evaluate oocyte grade, aneuploidy incidence, and maturation rate, a generalized linear model with a binomial distribution and logit link function was fitted using the "glm" function, with successful versus nonsuccessful events modeled as the response variable. To model oocyte grade, aneuploidy incidence, and maturation rate, oocytes that were labeled as grade 1 and 2, aneuploid, and MII stage were considered as successful events. Analyses were weighted by the number of oocytes collected per cow.

A similar model was used to evaluate the effect of lactation:

$$y = \beta_0 + LACT + e,$$

where y is the response variable, $\beta_0$ is the intercept, LACT is the fixed effect representing the lactation of the cow, and e is the residual deviance.

To further determine the effect of oocyte grade, the following model was applied:

$$y = \beta_0 + GRADE + ID + e,$$

where y is the response variable, $\beta_0$ is the intercept, and GRADE is the fixed effect representing oocyte grade classification. The ID of the cow (ID) is treated as a random intercept, and e is the residual deviance. Response variables were mitochondrial content, aneuploidy incidence, and maturation rate, and mixed-effects models were fitted using the "lmer" and "glmer" functions from the "lme4" package.

## RESULTS

### Number of Follicles

Fertility traits were not associated ($P = 0.69$ for DPR; $P = 0.16$ for CCR) with the average number of follicles present in the ovaries (Figure 1A, B). It was estimated that for each one-unit increase in DPR, there was a reduction of 0.12 follicles, and for each one-unit increase in CCR, there was a reduction of 0.36 follicles, but these values were not significant. First-lactation cows exhibited a lower ($P = 0.006$) follicular count compared with cows that had 3 or more lactations (Supplemental Figure S2A, see Notes). The first aspiration had more follicles ($P < 0.001$) than the second and third aspirations (Supplemental Figure S2B).

### Oocyte Grade

The proportion of grade 1 and 2 oocytes decreased linearly ($P < 0.05$) with increasing DPR and CCR values (Figure 2A, B). Specifically, each one-unit increase in DPR was associated with an estimated 2.67% reduction in grade 1 and 2 oocytes, and each one-unit increase in CCR corresponded to a 3.16% reduction. First-lactation cows also had a lower ($P = 0.001$) percentage of oocytes classified as grade 1 and 2 and a higher proportion of oocytes classified as grade 3 and 4 compared with second-lactation cows (Supplemental Figure S2C). However, oocyte grades remained consistent across aspirations ($P = 0.63$; Supplemental Figure S2D).

### Mitochondria Copy Number

We found no association between mitochondria copy number and the fertility traits ($P = 0.78$ for DPR; $P = 0.20$ for CCR). Additionally, no association was found between cow lactation ($P = 0.47$) or oocyte quality groups ($P = 0.11$) and mitochondrial DNA content (Supplemental Figure S3A–B, see Notes). The CV of the transformed data was 25.81%, ranging from 4.19% to 63.70% (Supplemental Table S1, see Notes). (Figure 3).

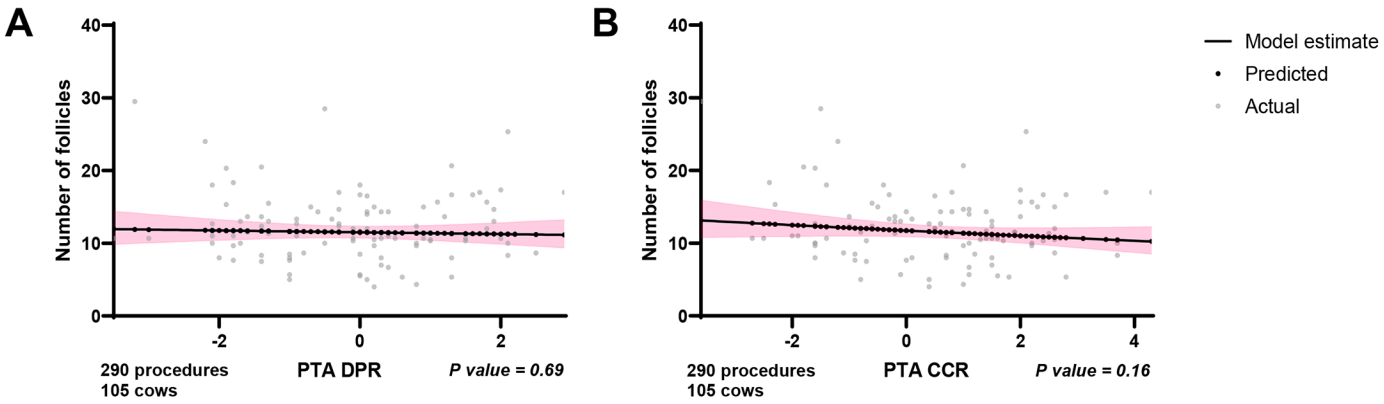


**Figure 1.** Association between fertility traits and follicular count at the time of aspiration. (A) Association between follicle number and DPR. (B) Association between follicle number and CCR. Black points represent predicted values, and gray points are the average follicular count per cow. The CI is presented in pink.

### *Maturation Rate*

The ability of the oocytes to achieve nuclear maturation was not associated with DPR or CCR ($P = 0.89$ for DPR; $P = 0.49$ for CCR). Interestingly, visual oocyte grading was not associated with the oocyte's ability to reach the MII stage ($P = 0.51$; Supplemental Figure S4B, see Notes). However, lactation had an effect ($P = 0.001$) on oocyte nuclear maturation, with fewer oocytes from second-lactation cows reaching the MII stage compared with those from first and third or later lactations (Supplemental Figure S4A).

### *Aneuploidy Incidence*

Aneuploidy incidence in oocytes decreased linearly with increasing DPR values (P = 0.03; Figure 4A). For each one-unit increase in DPR, the proportion of aneuploid oocytes was estimated to decline by 5.36%. No significant effect was observed when evaluating fertility by CCR ($P = 0.25$; Figure 4B). Additionally, we found no influence of cow lactation ($P = 0.56$) and oocyte grade ($P = 0.55$) in the incidence of aneuploidy (Supplemental Figure S4C–D).

## DISCUSSION

In this study, fertility traits were not associated with the oocyte's ability to reach the MII stage, a widely used marker of nuclear maturation and developmental competence. However, a significant negative association was observed between aneuploidy incidence and DPR, suggesting that higher genetic merit for fertility may

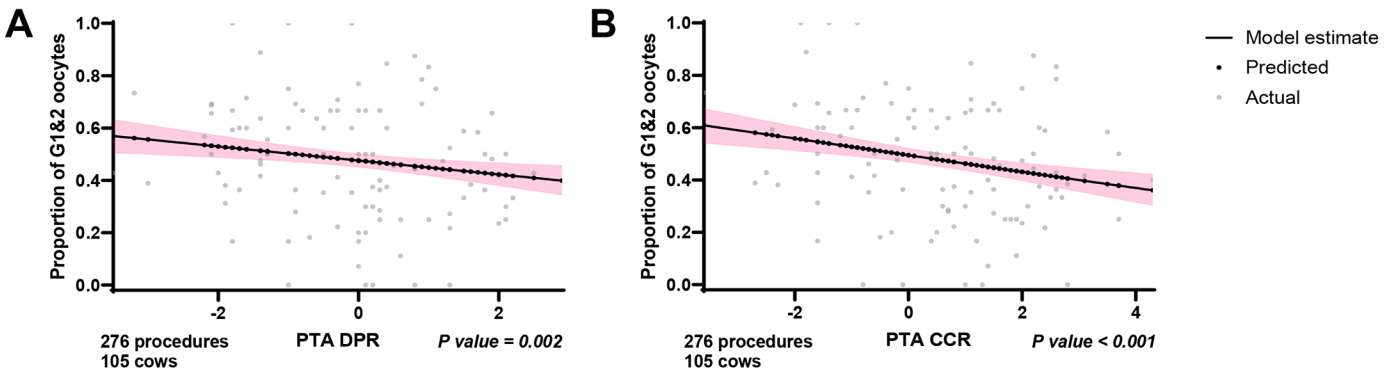


**Figure 2.** Association of visual oocyte quality grade and fertility traits. (A) Association between the proportion of grade 1 or 2 oocytes (G1&2) and DPR. For each increase in one DPR value, there was an estimated decrease of 2.67% in grade 1 or 2 oocytes observed ($P = 0.002$). (B) Association between the proportion of grade 1 or 2 oocytes and CCR. For each increase in one CCR value, there was an estimated decrease of 3.16% in grade 1 and 2 oocytes observed ($P < 0.001$). Black points represent predicted values, and gray points are the total proportion of grade 1 and 2 oocytes collected per cow across the 3 procedures. The CI is presented in pink.

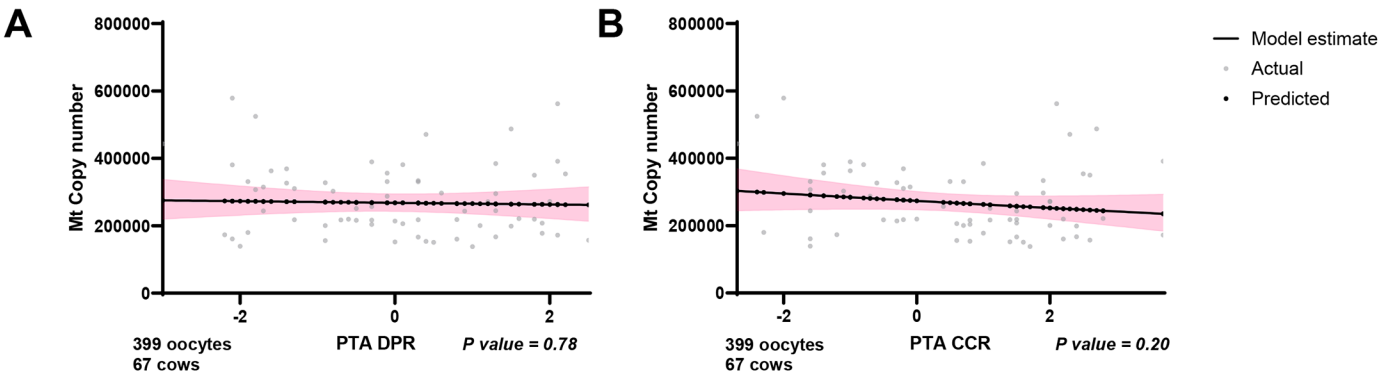


**Figure 3.** Association between oocyte mitochondrial content and fertility traits. (A) Association between mitochondria copy number per oocyte and DPR. (B) Association between mt copy number per oocyte and CCR. Dots presented in gray are the average mt copy number of the oocytes per cow. Black points represent predicted values, and gray points are the average mitochondria content from oocytes collected per cow. The CI is presented in pink.

be linked to enhanced chromosomal stability during meiosis. Aneuploidy can result from various meiotic disruptions, including improper kinetochore–microtubule attachments, a malfunctioning spindle assembly checkpoint, premature loss of chromosomal cohesion, or increased DNA damage (Balboula et al., 2020; Ma et al., 2020; Kincade et al., 2023; Sun et al., 2024). These findings raise the possibility that fertility-associated genetic variation influences the fidelity of these processes.

Supporting this hypothesis, a recent study in humans identified genetic variants in the kinesin protein family, key mediators of microtubule–kinetochore interactions, as being associated with increased aneuploidy incidence, independent of maternal age (Biswas et al., 2024). This evidence highlights a potential heritable component to aneuploidy risk. It is therefore plausible that variants in kinesin-related or other meiotic regulatory genes could also contribute to fertility traits in cattle. Although our current dataset does not allow for a direct genetic association analysis, these results lay the groundwork for future studies. The use of candidate gene approaches has previously been effective in improving the reliability of fertility traits evaluations (Ortega et al., 2016) and may help identify genetic markers linked to oocyte chromosomal integrity in future bovine studies. It should be noted this study was performed using in vitro maturation, and further evaluation using in vivo maturation should also be explored.

Mitochondrial content and function are critical determinants of oocyte competence and early embryo development. Insufficient mtDNA content has been linked to fertilization failure and early developmental arrest, whereas restoration of mtDNA levels has been shown to improve developmental outcomes and normalize gene expression

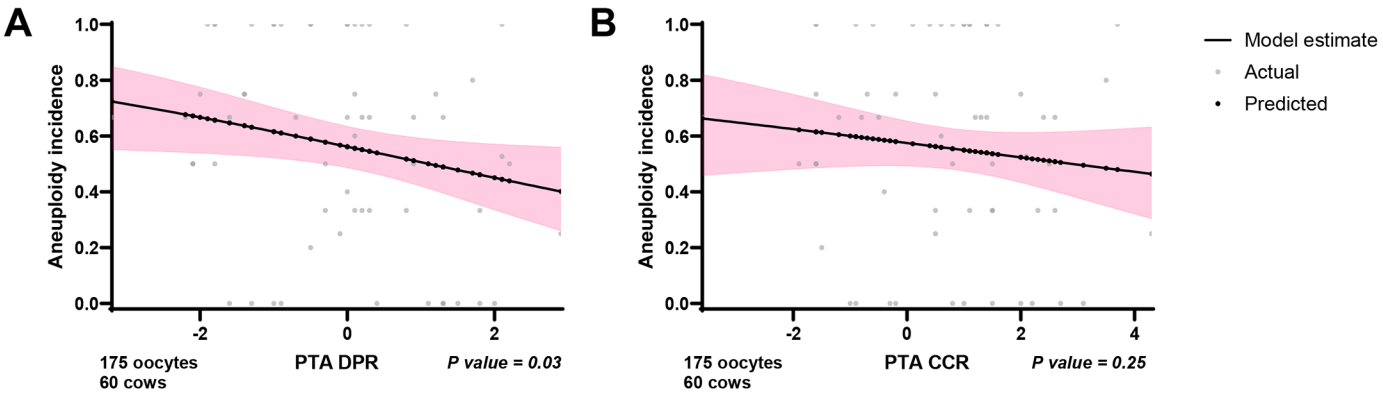


**Figure 4.** Association between aneuploidy incidence in oocytes matured in vitro and fertility traits. (A) Association between aneuploidy incidence and DPR. For each increase in one DPR value, there was an estimated decrease of 5.36% of aneuploid oocytes observed ($P = 0.03$). (B) Association between oocyte aneuploidy incidence and CCR. For each increase in one CCR value, there was an estimated decrease of 2.53% of aneuploid oocytes observed ($P = 0.25$). Black points represent predicted values, and gray points are the total proportion of oocytes with aneuploidy collected per cow across the 3 procedures. The CI is represented in pink.

profiles (St. John et al., 2023). In cattle, higher mtDNA content has been associated with increased expression of genes linked to high-grade blastocysts (Traut et al., 2022), and our previous work reported a relationship between mitochondrial content and fertility (Ortega et al., 2017b). However, in the present study, no association was found between mtDNA content and fertility traits; the variability in mtDNA content was higher than initially estimated, limiting our ability to detect differences with the current sample size.

Across species, competent oocytes are characterized not only by higher mtDNA content but also by specific patterns of mitochondrial redistribution during meiotic progression (Kirillova et al., 2021). Reaching a critical mtDNA threshold is especially important in cattle (~150,000 mt per oocyte), where deficiencies compromise early embryonic development (St. John et al., 2023). In this study, all the oocytes evaluated were above this threshold (Supplemental Table S1).

Furthermore, mitochondrial functionality, including ATP production, reactive oxygen species regulation, regulation of nuclear-mitochondrial genomic interactions, and calcium homeostasis are essential for meiotic integrity, cytoplasmic maturation, and embryo development. Disruptions in these processes can impair gene expression, DNA methylation, spindle formation, and chromosomal alignment in bovine, porcine, and murine models, ultimately reducing developmental competence (Kirillova et al., 2021; St. John et al., 2023).

Here, only mitochondrial copy number was measured, but future studies could explore mitochondrial function to assess differences between cows with higher and lower fertility phenotypes. Although mtDNA content alone may not currently serve as a practical or predictive marker for donor selection, our data revealed a within-cow coefficient of variation of ~25%, with values ranging from 4% to 63% across cows. This variation may reflect inherent differences in oocyte quality at the individual level, and warrant further investigation, in a way that such phenotypes could be integrated into multitrait models could improve the predictive accuracy of fertility-related breeding values and help refine donor selection criteria as our understanding of oocyte biology evolves.

No association was found between lactation number and mtDNA content or aneuploidy incidence in this study. Previous work has shown that maternal age is associated with reduced mitochondrial content in bovine oocytes when comparing females from 20 to 200 mo old (Iwata et al., 2011), but our use of young animals likely limited the detection of such effects. Higher values of DPR and CCR were associated with a reduced proportion of grade 1 or 2 oocytes and, consequently, an increased proportion of grade 3 or 4 oocytes. However, no associations were found between oocyte grade and mitochondrial DNA copy number or aneuploidy incidence. This finding was somewhat unexpected, as oocyte and embryo quality are generally presumed to correlate positively with fertility traits. Morphological grading, which is largely based on cumulus cell investment and cytoplasmic appearance, has traditionally been used as a proxy for developmental competence; however, its predictive value is limited.

Previous studies have shown that morphological assessment alone may not accurately reflect the full biological potential of the oocyte, particularly in in vitro systems (De Santis et al., 2005). Although grade 1 oocytes are typically associated with higher blastocyst rates, and grade 1 embryos with greater pregnancy success compared with lower grades (Hasler et al., 1995; Chebel et al., 2008; Demetrio et al., 2020, 2022), visual classification does not account for underlying molecular or chromosomal defects that may impair developmental potential. Moreover, there is limited evidence supporting a direct link between oocyte morphology and the likelihood of producing high-grade embryos. Therefore, the negative association observed between the proportion of grade 1 and 2 oocytes and fertility traits such as DPR may reflect the limitations of visual grading in capturing intrinsic oocyte quality. To balance biological relevance with experimental feasibility, oocytes were classified into 2 broader categories: grade 1 and 2, and grade 3 and 4, based on established morphological criteria. This grouping strategy is supported by Demetrio et al. (2022), who found that grade 1 and 2 oocytes have higher embryo development rates than grade 3 and 4 oocytes, with no significant differences observed between the subgrades within each group. Pooling oocytes in this way helped reduce subjectivity in grading while still preserving developmental contrasts relevant to oocyte quality.

In this study, first-lactation cows had a lower follicular count compared with multiparous cows. This is in agreement with other studies where primiparous cows have fewer follicles compared with multiparous cows per OPU session (Snijders et al., 2000; Grimard et al., 2013). The variability in follicular counts between aspirations may have been influenced by small follicles present at the time of ablation. Because only follicles ≥5 mm were aspirated, smaller follicles remaining from the ongoing wave could have been included in the subsequent session, artificially increasing the follicle count in the first aspiration procedure. Although follicular size was not measured before each aspiration, we attempted to standardize follicular development by conducting aspirations on d 3 of the follicular wave, when most follicles are expected to be in the 6 to 8 mm range (Gomez-Leon et al., 2022), thereby minimizing size-related variation (Pytel et al., 2024).

In this work, first-lactation cows yielded fewer high-quality (grade 1 and 2) oocytes, which was unexpected

given that primiparous cows are often reported to have higher fertility than multiparous cows under synchronization protocols such as Double-Ovsynch (Souza et al., 2008; Herlihy et al., 2012). However, it is important to acknowledge that fertility is a complex, multifactorial trait, and oocyte morphology alone does not fully explain reproductive outcomes. Factors such as the uterine and oviductal environment (Wiltbank et al., 2016; Tríbulo et al., 2019), as well as sire effects on embryo quality and pregnancy success (Ledoux et al., 2015; Ortega et al., 2018), also play critical roles in determining fertility.

Although visual oocyte grading is the most widely used and practical method to assess oocyte quality in both research and clinical settings, its subjective nature and limited resolution may not capture more subtle indicators of developmental competence. In this study, oocyte grade was not associated with fertility traits or molecular indicators such as mitochondrial DNA content or aneuploidy, suggesting that morphology alone may not reliably predict oocyte potential. Nonetheless, in the absence of validated noninvasive alternatives, visual grading remains a valuable tool for routine oocyte assessment.

In summary, cows with higher genetic merit for fertility, as measured by DPR, exhibited a lower incidence of oocyte aneuploidy, suggesting that genetic factors influencing fertility may also contribute to chromosomal stability during oocyte maturation. Although mitochondrial DNA copy number was not associated with fertility traits in this study, the observed variability across individuals and the limited sample size warrant further investigation with larger cohorts. Additionally, visual grading of oocyte quality based on cumulus cell investment did not reliably reflect developmental competence, highlighting the limitations of current morphological assessment methods and the need for more objective indicators of oocyte quality.

## NOTES

This work was supported by the Agriculture and Food Research Initiative, project award no. 2022-67015-38938, from the US Department of Agriculture's National Institute of Food and Agriculture. Thanks are extended to Brianna Meyer who helped count the kinetochores in the oocytes for aneuploidy screening, and to Jackson Gille and Livia Martino for their help at the farm. Supplemental material for this article is available at [URL]. All animal procedures were conducted in accordance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of the University of Wisconsin. The authors have not stated any conflicts of interest.

**Nonstandard abbreviations used:** DPR = daughter pregnancy rate; CCR = cow conception rate; HCR = heifer conception rate; OPU = ovum pickup; COC = cumulus oocyte complex; MII = metaphase-II; OCM = oocyte collection medium; OMM = oocyte maturation medium; mt = mitochondrial.

## REFERENCES

Aguila, L., F. Treulen, J. Therrien, R. Felmer, M. Valdivia, and L. C. Smith. 2020. Oocyte selection for in vitro embryo production in bovine species: Noninvasive approaches for new challenges of oocyte competence. Animals (Basel) 10:2196. https://doi.org/10.3390/ani10122196.

Balboula, A. Z., K. Schindler, T. Kotani, M. Kawahara, and M. Takahashi. 2020. Vitrification-induced activation of lysosomal cathepsin B perturbs spindle assembly checkpoint function in mouse oocytes. Mol. Hum. Reprod. 26:689–701. https://doi.org/10.1093/molehr/gaaa051.

Biswas, L., K. M. Tyc, M. Aboelenain, S. Sun, I. Dundović, K. Vukušić, J. Liu, V. Guo, M. Xu, R. T. Scott Jr., X. Tao, I. M. Tolić, J. Xing, and K. Schindler. 2024. Maternal genetic variants in kinesin motor domains prematurely increase egg aneuploidy. Proc. Natl. Acad. Sci. USA 121:e2414963121. https://doi.org/10.1073/pnas.2414963121.

Chebel, R. C., D. G. B. Demétrio, and J. Metzger. 2008. Factors affecting success of embryo collection and transfer in large dairy herds. Theriogenology 69:98–106. https://doi.org/10.1016/j.theriogenology.2007.09.008.

Combelles, C. M. H., and D. F. Albertini. 2001. Microtubule patterning during meiotic maturation in mouse oocytes is determined by cell cycle-specific sorting and redistribution of gamma-tubulin. Dev. Biol. 239:281–294. https://doi.org/10.1006/dbio.2001.0444.

Conti, M., and F. Franciosi. 2018. Acquisition of oocyte competence to develop as an embryo: integrated nuclear and cytoplasmic events. Hum. Reprod. Update 24:245–266. https://doi.org/10.1093/humupd/dmx040.

De Santis, L., I. Cino, E. Rabellotti, F. Calzi, P. Persico, A. Borini, and G. Coticchio. 2005. Polar body morphology and spindle imaging as predictors of oocyte quality. Reprod. Biomed. Online 11:36–42. https://doi.org/10.1016/S1472-6483(10)61296-5.

Demetrio, D., and J. Barfield. 2021. Appendix 2: Photographic illustrations of bovine cumulus–oocyte complexes. International Embryo Technology Society Manual. 5th ed. IETS, Champaign, IL.

Demetrio, D., C. Demetrio, M. Oliveira, R. Reis, and R. Santos. 2022. From oocyte to calf: Practical aspects of bovine in vitro embryo production. Clin. Theriogenology 14:193–201. https://doi.org/10.58292/ct.v14.9671.

Demetrio, D. G. B., E. Benedetti, C. G. B. Demetrio, J. Fonseca, M. Oliveira, A. Magalhaes, and R. M. D. Santos. 2020. How can we improve embryo production and pregnancy outcomes of Holstein embryos produced in vitro? (12 years of practical results at a California dairy farm). Anim. Reprod. 17:e20200053. https://doi.org/10.1590/1984-3143-AR2020-0053.

Dumollard, R., M. Duchen, and J. Carroll. 2007. The role of mitochondrial function in the oocyte and embryo. Curr. Top. Dev. Biol. 77:21–49. https://doi.org/10.1016/S0070-2153(06)77002-8.

Ge, H., T. L. Tollner, Z. Hu, M. Dai, X. Li, H. Guan, D. Shan, X. Zhang, J. Lv, C. Huang, and Q. Dong. 2012. The importance of mitochondrial metabolic activity and mitochondrial DNA replication during oocyte maturation in vitro on oocyte quality and subsequent embryo developmental competence. Mol. Reprod. Dev. 79:392–401. https://doi.org/10.1002/mrd.22042.

Gobikrushanth, M., K. Macmillan, D. Hipkin, and M. G. Colazo. 2020. The relationships among sire's predicted transmitting ability for daughter pregnancy rate and cow conception rate and daughter's reproductive performance in Canadian Holstein cows. Theriogenology 149:117–122. https://doi.org/10.1016/j.theriogenology.2020.03.026.

Gomez-Leon, V. E., A. D. Beard, O. J. Ginther, and M. C. Wiltbank. 2022. Effect of elevating luteinizing hormone action using low

doses of human chorionic gonadotropin on double ovulation, follicle dynamics, and circulating follicle-stimulating hormone in lactating dairy cows. J. Dairy Sci. 105:7023–7035. https://doi.org/10.3168/jds.2021-21767.

Grimard, B., B. Marquant-Leguienne, D. Remy, C. Richard, F. Nuttinck, P. Humblot, and A. Ponter. 2013. Postpartum variations of plasma IGF and IGFBPs, oocyte production and quality in dairy cows: relationships with parity and subsequent fertility. Reprod. Domest. Anim. 48:183–194. https://doi.org/10.1111/j.1439-0531.2012.02127.x.

Hasler, J. F., W. B. Henderson, P. J. Hurtgen, Z. Q. Jin, A. D. McCauley, S. A. Mower, B. Neely, L. S. Shuey, J. E. Stokes, and S. A. Trimmer. 1995. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology 43:141–152. https://doi.org/10.1016/0093-691X(94)00020-U.

Hayden, C. B., J. C. L. Motta, R. V. Sala, N. M. Bello, M. A. Coutinho Da Silva, and A. García-Guerra. 2025. Efficacy of methods to synchronize follicular wave emergence in pregnant heifers. JDS Commun. 6:154–159. https://doi.org/10.3168/jdsc.2024-0629.

Herlihy, M. M., J. O. Giordano, A. H. Souza, H. Ayres, R. M. Ferreira, A. Keskin, A. B. Nascimento, J. N. Guenther, J. M. Gaska, S. J. Kacuba, M. A. Crowe, S. T. Butler, and M. C. Wiltbank. 2012. Presynchronization with Double-Ovsynch improves fertility at first postpartum artificial insemination in lactating dairy cows. J. Dairy Sci. 95:7003–7014. https://doi.org/10.3168/jds.2011-5260.

Iwata, H., H. Goto, H. Tanaka, Y. Sakaguchi, K. Kimura, T. Kuwayama, and Y. Monji. 2011. Effect of maternal age on mitochondrial DNA copy number, ATP content and IVF outcome of bovine oocytes. Reprod. Fertil. Dev. 23:424–432. https://doi.org/10.1071/RD10133.

Kincade, J. N., A. Hlavacek, T. Akera, and A. Z. Balboula. 2023. Initial spindle positioning at the oocyte center protects against incorrect kinetochore-microtubule attachment and aneuploidy in mice. Sci. Adv. 9:eadd7397. https://doi.org/10.1126/sciadv.add7397.

Kirillova, A., J. E. J. Smitz, G. T. Sukhikh, and I. Mazunin. 2021. The role of mitochondria in oocyte maturation. Cells 10:2484. https://doi.org/10.3390/cells10092484.

Kuhn, M. T., J. L. Hutchison, and G. R. Wiggans. 2006. Characterization of Holstein heifer fertility in the United States. J. Dairy Sci. 89:4907–4920. https://doi.org/10.3168/jds.S0022-0302(06)72541-3.

Landim-Alvarenga, F. C., and R. R. D. Maziero. 2014. Control of oocyte maturation. Anim. Reprod. 11:150–158.

Ledoux, D., C. Ponsart, B. Grimard, J. Gatien, M. C. Deloche, S. Fritz, R. Lefebvre, and P. Humblot. 2015. Sire effect on early and late embryonic death in French Holstein cattle. Animal 9:766–774. https://doi.org/10.1017/S1751731114003140.

Lima, F. S., F. T. Silvestre, F. Peñagaricano, and W. W. Thatcher. 2020. Early genomic prediction of daughter pregnancy rate is associated with improved reproductive performance in Holstein dairy cows. J. Dairy Sci. 103:3312–3324. https://doi.org/10.3168/jds.2019-17488.

Ma, J. Y., S. Li, L. N. Chen, H. Schatten, X. H. Ou, and Q. Y. Sun. 2020. Why is oocyte aneuploidy increased with maternal aging? J. Genet. Genomics 47:659–671. https://doi.org/10.1016/j.jgg.2020.04.003.

Mao, L., H. Lou, Y. Lou, N. Wang, and F. Jin. 2014. Behaviour of cytoplasmic organelles and cytoskeleton during oocyte maturation. Reprod. Biomed. Online 28:284–299. https://doi.org/10.1016/j.rbmo.2013.10.016.

May-Panloup, P., X. Vignon, M. F. Chrétien, Y. Heyman, M. Tamassia, Y. Malthièry, and P. Reynier. 2005. Increase of mitochondrial DNA content and transcripts in early bovine embryogenesis associated with upregulation of mtTFA and NRF1 transcription factors. Reprod. Biol. Endocrinol. 3:65. https://doi.org/10.1186/1477-7827-3-65.

Ortega, M. S., A. C. Denicol, J. B. Cole, D. J. Null, and P. J. Hansen. 2016. Use of single nucleotide polymorphisms in candidate genes associated with daughter pregnancy rate for prediction of genetic merit for reproduction in Holstein cows. Anim. Genet. 47:288–297. https://doi.org/10.1111/age.12420.

Ortega, M. S., A. C. Denicol, J. B. Cole, D. J. Null, J. F. Taylor, R. D. Schnabel, and P. J. Hansen. 2017a. Association of single nucleotide polymorphisms in candidate genes previously related to genetic variation in fertility with phenotypic measurements of reproductive function in Holstein cows. J. Dairy Sci. 100:3725–3734. https://doi.org/10.3168/jds.2016-12260.

Ortega, M. S., A. M. Kelleher, E. O'Neil, J. Benne, R. Cecil, and T. E. Spencer. 2020. NANOG is required to form the epiblast and maintain pluripotency in the bovine embryo. Mol. Reprod. Dev. 87:152–160. https://doi.org/10.1002/mrd.23304.

Ortega, M. S., J. G. N. Moraes, D. J. Patterson, M. F. Smith, S. K. Behura, S. Poock, and T. E. Spencer. 2018. Influences of sire conception rate on pregnancy establishment in dairy cattle. Biol. Reprod. 99:1244–1254. https://doi.org/10.1093/biolre/ioy141.

Ortega, M. S., S. Wohlgemuth, P. Tribulo, L. G. B. Siqueira, D. J. Null, J. B. Cole, M. V. Da Silva, and P. J. Hansen. 2017b. A single nucleotide polymorphism in COQ9 affects mitochondrial and ovarian function and fertility in Holstein cows. Biol. Reprod. 96:652–663. https://doi.org/10.1093/biolre/iox004.

Pytel, A. T., K. Żyżyńska-Galeńska, Z. Gajewski, and K. Papis. 2024. Factors defining developmental competence of bovine oocytes collected for in vitro embryo production. Biol. Reprod. 111:1–10. https://doi.org/10.1093/biolre/ioae065.

Silvestri, G., C. Canedo-Ribeiro, M. Serrano-Albal, R. Labrecque, P. Blondin, S. G. Larmer, G. Marras, D. A. R. Tutt, A. H. Handyside, M. Farré, K. D. Sinclair, and D. K. Griffin. 2021. Preimplantation genetic testing for aneuploidy improves live birth rates with in vitro produced bovine embryos: A blind retrospective study. Cells 10:2284. https://doi.org/10.3390/cells10092284.

Sirard, M. A., H. M. Florman, M. L. Leibfried-Rutledge, F. L. Barnes, M. L. Sims, and N. L. First. 1989. Timing of nuclear progression and protein synthesis necessary for meiotic maturation of bovine oocytes. Biol. Reprod. 40:1257–1263. https://doi.org/10.1095/biolreprod40.6.1257.

Sitko, E. M., M. M. Perez, G. E. Granados, M. Masello, F. Sosa Hernandez, E. M. Cabrera, E. M. Schilkowsky, F. A. Di Croce, A. K. McNeel, D. J. Weigel, and J. O. Giordano. 2023. Effect of reproductive management programs that prioritized artificial insemination at detected estrus or timed artificial insemination on the reproductive performance of primiparous Holstein cows of different genetic merit for fertility. J. Dairy Sci. 106:6476–6494. https://doi.org/10.3168/jds.2022-22673.

Snijders, S. E. M., P. Dillon, D. O'Callaghan, and M. P. Boland. 2000. Effect of genetic merit, milk yield, body condition and lactation number on in vitro oocyte development in dairy cows. Theriogenology 53:981–989. https://doi.org/10.1016/S0093-691X(00)00244-2.

Souza, A. H., H. Ayres, R. M. Ferreira, and M. C. Wiltbank. 2008. A new presynchronization system (Double-Ovsynch) increases fertility at first postpartum timed AI in lactating dairy cows. Theriogenology 70:208–215. https://doi.org/10.1016/j.theriogenology.2008.03.014.

St John, J. C., T. Okada, E. Andreas, and A. Penn. 2023. The role of mtDNA in oocyte quality and embryo development. Mol. Reprod. Dev. 90:621–633. https://doi.org/10.1002/mrd.23640.

Sun, F., N. N. Ali, D. Londoño-Vásquez, C. A. Simintiras, H. Qiao, M. S. Ortega, Y. Agca, M. Takahashi, R. M. Rivera, A. M. Kelleher, P. Sutovsky, A. L. Patterson, and A. Z. Balboula. 2024. Increased DNA damage in full-grown oocytes is correlated with diminished autophagy activation. Nat. Commun. 15:9463. https://doi.org/10.1038/s41467-024-53559-w.

Sutton-McDowall, M. L., R. B. Gilchrist, and J. G. Thompson. 2010. The pivotal role of glucose metabolism in determining oocyte developmental competence. Reproduction 139:685–695. https://doi.org/10.1530/REP-09-0345.

Traut, M., I. Kowalczyk-Zieba, D. Boruszewska, J. Jaworska, K. Lukaszuk, and I. Woclawek-Potocka. 2022. Mitochondrial DNA content and developmental competence of blastocysts derived from pre-pubertal heifer oocytes. Theriogenology 191:207–220. https://doi.org/10.1016/j.theriogenology.2022.07.017.

Tríbulo, P., L. Balzano-Nogueira, A. Conesa, L. G. Siqueira, and P. J. Hansen. 2019. Changes in the uterine metabolome of the cow during the first 7 days after estrus. Mol. Reprod. Dev. 86:75–87. https://doi.org/10.1002/mrd.23082.

Tyc, K. M., R. C. McCoy, K. Schindler, and J. Xing. 2020. Mathematical modeling of human oocyte aneuploidy. Proc. Natl. Acad. Sci. USA 117:10455–10464. https://doi.org/10.1073/pnas.1912853117.

VanRaden, P. M., J. B. Cole, M. Neupane, S. Toghiani, K. L. Gaddis, and R. J. Tempelman. 2021. Net merit as a measure of lifetime profit: 2021 revision. AIPL-USDA Research Reports. NM$8(05–21). USDA.

VanRaden, P. M., A. H. Sanders, M. E. Tooker, R. H. Miller, H. D. Norman, M. T. Kuhn, and G. R. Wiggans. 2004. Development of a national genetic evaluation for cow fertility. J. Dairy Sci. 87:2285–2292. https://doi.org/10.3168/jds.S0022-0302(04)70049-1.

Wai, T., A. Ao, X. Zhang, D. Cyr, D. Dufort, and E. A. Shoubridge. 2010. The role of mitochondrial DNA copy number in mammalian fertility. Biol. Reprod. 83:52–62. https://doi.org/10.1095/biolreprod.109.080887.

Wiltbank, M. C., G. M. Baez, A. Garcia-Guerra, M. Z. Toledo, P. L. J. Monteiro, L. F. Melo, J. C. Ochoa, J. E. P. Santos, and R. Sartori. 2016. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. Theriogenology 86:239–253. https://doi.org/10.1016/j.theriogenology.2016.04.037.