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Association between Pre-slaughter Udder Status and Relative Zona Pellucida Thickness in Bovine Oocytes

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ABSTRACT

Background: The physiological condition of the donor animal may influence the ovarian environment and, consequently, characteristics associated with bovine oocyte quality. The zona pellucida is an important structural component of the oocyte, yet its thickness is commonly considered as an absolute measurement and may therefore be influenced by differences in overall oocyte size. This study investigated the association between pre-slaughter udder status and the relative thickness of the zona pellucida in bovine oocytes using a normalized structural parameter.

Methods: Oocyte and donor records from 20 cows were evaluated according to pre-slaughter udder appearance. Twelve donors were classified as having an apparently normal udder and eight as having an apparently abnormal udder. Zona pellucida thickness (ZPT) was normalized to Outer oocyte diameter (OOD) to calculate the Relative zona thickness index (RZTI), expressed as ZPT divided by OOD and multiplied by 100. Between-group differences were evaluated using an exact two-sided Mann-Whitney U test. Outer oocyte diameter, ooplasm diameter, perivitelline space and cumulus-oocyte complex measurements before and after maturation were examined as additional parameters.

Result: The apparently abnormal group showed a lower median RZTI than the apparently normal group (7.70% vs. 9.97%), with a nominal exact P value of 0.0159. Absolute ZPT was also lower in the apparently abnormal group (15.01 vs. 18.16 µm; P = 0.0314), whereas outer oocyte diameter did not differ significantly (194.84 vs. 187.81 µm; P = 0.1569). Ooplasm diameter, perivitelline space and cumulus-oocyte complex measurements before and after maturation showed no significant between-group differences. Neither RZTI nor ZPT remained statistically significant after Benjamini-Hochberg adjustment (FDR-adjusted P = 0.1100 for both).

Key words: Bovine oocyte, Donor health, *In vitro* maturation, Oocyte quality, Udder status, Zona pellucida.

INTRODUCTION

The developmental competence of bovine oocytes is influenced by several factors associated with the donor animal, ovarian condition and follicular environment. Variations in these factors may affect oocyte morphology, quality and suitability for in vitro maturation and embryo production (Costa *et al.*, 2023; Pytel *et al.*, 2024). Previous studies have also demonstrated associations between ovarian characteristics, oocyte quality and maturation outcomes in cattle (Ammari *et al.*, 2022; Ammari *et al.*, 2023).

Inflammatory conditions in donor animals may influence the ovarian environment and oocyte development. In particular, mastitis has been associated with impaired oocyte developmental competence in dairy cows (Roth *et al.*, 2013; Asaf *et al.*, 2014). Experimental evidence has further indicated that inflammation during oocyte maturation may affect subsequent embryonic development (Tariq *et al.*, 2025). These findings suggest that the physiological condition of donor animals deserves consideration when evaluating bovine oocyte characteristics.

The zona pellucida (ZP) is an extracellular glycoprotein layer surrounding the mammalian oocyte. It contributes to sperm-oocyte interactions, fertilization and early embryonic development. Its structural characteristics, including thickness and organization, have been investigated as potential morphological indicators of oocyte quality and developmental potential (Santos *et al.*, 2008; Koester *et al.*, 2011; Timlin *et al.*, 2021). Absolute zona pellucida thickness (ZPT), expressed in micrometres, provides information about the physical thickness of this layer. Nevertheless, absolute thickness alone does not describe its proportion relative to the overall oocyte size.

Previous studies have also examined the influence of oocyte recovery procedures and culture conditions on bovine oocyte quality and *in vitro* maturation, highlighting the importance of recovery methods and morphological assessment in reproductive studies (Hussain *et al.*, 2015).

A relative measurement may therefore provide complementary morphological information. The relative

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zona thickness index (RZTI) expresses ZPT as a percentage of the outer oocyte diameter (OOD), calculated as follows: The relative zona thickness index (RZTI) was calculated by dividing the zona pellucida thickness (ZPT) by the outer oocyte diameter (OOD) and multiplying the result by 100 to express it as a percentage.

This index describes the proportion of the oocyte diameter occupied by the zona pellucida. It allows zona thickness to be considered in relation to oocyte size, rather than as an isolated absolute measurement. Evaluating both ZPT and RZTI may therefore provide a broader description of zona pellucida morphology.

Although previous research has examined the effects of mastitis and inflammatory conditions on bovine oocyte quality, the relationship between pre-slaughter udder appearance and the relative thickness of the zona pellucida remains insufficiently characterized. Examining this relationship may provide preliminary information about the association between an observable donor characteristic and oocyte morphology.

Therefore, our study investigated the association between pre-slaughter udder status and ZPT and RZTI in bovine oocytes. Additional morphometric characteristics, including OOD, ooplasm diameter, perivitelline space and cumulus–oocyte complex measurements, were also evaluated. Udder classification was based on the recorded external appearance before slaughter and was not considered a laboratory-confirmed diagnosis of mastitis.

MATERIALS AND METHODS

Study design and data source

This study evaluated donor-level and oocyte-related records obtained from bovine ovaries collected at a slaughterhouse in Riyadh, Saudi Arabia. The dataset comprised records from 20 donors for which udder status could be linked to the corresponding ovarian and oocyte measurements. The analysis focused on donor udder appearance and quantitative characteristics of the recovered oocytes. No new experimental intervention or treatment was applied and the analysis was based on measurements available for the identified donor records. The principal analytical endpoint was the relative zona thickness index (RZTI), a normalized parameter calculated from zona pellucida thickness and outer oocyte diameter. Additional oocyte measurements, including outer oocyte diameter, ooplasm diameter, perivitelline space and cumulus-oocyte complex measurements before and after maturation, were evaluated for comparison.

For each donor, the total number of recovered oocytes was recorded separately from the morphometric measurements. Multiple oocytes from each donor were selected for morphometric assessment and the recorded values for outer oocyte diameter (OOD), zona pellucida thickness (ZPT), ooplasm diameter and perivitelline space represented donor-level averages. Accordingly, each donor contributed one average observation to the statistical analysis and the donor was considered the experimental unit.

Classification of pre-slaughter udder status

The pre-slaughter udder status of each donor was obtained from the original health records. The recorded symbols were used to classify the external udder appearance, with the cross mark (×) indicating an apparently normal udder and the check mark (–') indicating an apparently abnormal udder. One additional record contained the explicit entry "Mastitis" in the Udder field and was assigned to the apparently abnormal group.

Based on these records, donors were categorized into two groups: apparently normal udder appearance (n = 12) and apparently abnormal udder appearance (n = 8). Classification was based on the recorded external observations before slaughter and the corresponding donor records used to link udder status with ovarian and oocyte measurements. These categories represented apparent udder status rather than confirmed mastitis diagnoses, as the available records did not include somatic cell counts, bacteriological cultures, histopathological examinations, inflammatory biomarkers, or standardized clinical diagnostic criteria.

Oocyte measurements

Oocytes were randomly selected from the apparently normal and apparently abnormal udder groups, with each group handled separately to preserve the original donor classification. Morphometric assessment was performed using an inverted microscope equipped with a digital camera. Images were captured using Leica Application Suite software (Version 4.0) and the recorded images were analyzed using ImageJ software (Version 1.50i; National Institutes of Health, USA). ImageJ measurements were calibrated using a stage micrometer before morphometric assessment to ensure accurate measurements.

The morphometric parameters assessed included outer oocyte diameter (OOD), zona pellucida thickness (ZPT), ooplasm diameter and perivitelline space (PVS). Cumulus-oocyte complex (COC) diameter was also measured before and after *in vitro* maturation. All measurements were expressed in micrometres (µm). Multiple oocytes were assessed for each donor and the resulting measurements were summarized as donor-level averages. Accordingly, each donor contributed one average observation to the statistical analysis.

Relative zona thickness index

Because absolute zona pellucida thickness may vary in relation to the overall dimensions of the oocyte, a normalized measure was calculated to express zona pellucida thickness relative to outer oocyte diameter.

For each oocyte record, the relative zona thickness index (RZTI) was calculated using the following equation:

$$\text{RZTI (\%)} = \frac{\text{ZPT}}{\text{OOD}} \times 100$$

Where,

- RZTI = relative zona thickness index;
- ZPT = zona pellucida thickness (µm);
- OOD = outer oocyte diameter (µm).

A higher RZTI value indicates that zona pellucida thickness represents a greater proportion of the measured outer oocyte diameter, whereas a lower value indicates a smaller proportional contribution of the zona pellucida. RZTI was prespecified as the principal derived endpoint because it combines two directly related structural measurements and expresses zona pellucida thickness relative to overall oocyte size. The remaining individual oocyte measurements were evaluated as secondary parameters to determine whether differences in RZTI were accompanied by changes in other oocyte dimensions.

Statistical analysis

Given the small number of donor-level observations, non-parametric methods were used for between-group comparisons. The donor, rather than the individual oocyte, was considered the independent experimental unit because multiple oocytes were assessed from each donor and their measurements were summarized as donor-level averages. Accordingly, the statistical comparisons were performed using one average observation per donor (n = 12 for the apparently normal group and n = 8 for the apparently abnormal group).

The primary comparison of the relative zona thickness index (RZTI) between the two groups was performed using an exact two-sided Mann-Whitney U test. The same test was applied to secondary comparisons of outer oocyte diameter, zona pellucida thickness, ooplasm diameter, perivitelline space and cumulus-oocyte complex (COC) measurements before and after maturation. Continuous variables were summarized using mean ± standard deviation, median and range. Exact two-sided P values were reported for all between-group comparisons. RZTI was prespecified as the principal derived endpoint, whereas the individual morphometric measurements were treated as secondary parameters.

To quantify the direction and magnitude of between-group differences, rank-biserial correlation was calculated. Median differences were expressed as the median in the apparently abnormal group minus the median in the apparently normal group. The Benjamini-Hochberg procedure was applied to adjust P values for multiple comparisons. All statistical tests were two-sided and $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Relative zona thickness index

The principal analysis showed a difference in RZTI between the two-pre-slaughter udder-status groups.

The apparently normal group had a median RZTI of 9.97%, compared with 7.70% in the apparently abnormal group. Mean RZTI values were 9.70±1.00% and 7.95±1.32%, respectively. The corresponding ranges were 7.32-10.95% for the apparently normal group and 6.03-10.33% for the apparently abnormal group. The exact two-sided Mann-Whitney test yielded P = 0.0159 (Table 1), indicating an unadjusted difference in the distribution of RZTI between the two groups. The distribution of RZTI values according to pre-slaughter udder status is presented in Fig 1.

Distribution of the relative zona thickness index (RZTI) among bovine oocytes according to pre-slaughter udder status. Each point represents one donor-level observation. The box represents the interquartile range and the horizontal line within each box indicates the median. The apparently abnormal group showed lower RZTI values than the apparently normal group (exact two-sided Mann-Whitney U test, P = 0.0159).

Individual oocyte measurements

Absolute zona pellucida thickness showed a pattern consistent with the RZTI finding. The median ZPT was 18.16 µm in the apparently normal group and 15.01 µm in the apparently abnormal group, with an exact P value of 0.0314. In contrast, outer oocyte diameter did not differ significantly between the groups. Median outer oocyte diameter was 187.81 µm in the apparently normal group and 194.84 µm in the apparently abnormal group (P = 0.1569). Ooplasm diameter and perivitelline space also showed no statistically significant between-group differences. Similarly, COC diameter before and after maturation did not differ significantly between the two groups (Table 2).

Magnitude and direction of between-group differences

The median difference in RZTI, calculated as apparently abnormal minus apparently normal, was -2.26 percentage points, with a rank-biserial correlation of -0.646 (P = 0.0159). The negative direction indicates lower RZTI values in the apparently abnormal group. Similarly, the median difference in absolute ZPT was -3.16 µm, with a rank-biserial correlation of -0.583 (P = 0.0314), indicating lower ZPT values in the apparently abnormal group. In contrast, outer oocyte diameter showed a positive median difference of +7.03 µm, indicating a higher median value in the

Table 1: Relative zona thickness index according to pre-slaughter udder status.

Pre-slaughter udder status	n	Mean ± SD (%)	Median (%)	Range (%)	Exact P value
Apparently normal	12	1.00±9.70	9.97	10.95-7.32	
Apparently abnormal	8	1.32±7.95	7.70	6.03-10.33	0.0159

RZTI was calculated as zona pellucida thickness / outer oocyte diameter × 100. The statistical comparison was performed using an exact two-sided Mann-Whitney U test.

apparently abnormal group; however, this difference was not statistically significant (P = 0.1569). Ooplasm diameter also showed a positive median difference (+3.68 µm), whereas perivitelline space and COC measurements showed smaller differences in either direction (Table 3).

Median difference was calculated as the median value in the apparently abnormal group minus the median value in the apparently normal group. Negative values indicate lower values in the apparently abnormal group, whereas positive values indicate higher values.

Multiple-testing consideration

The unadjusted exact Mann-Whitney U tests showed nominal statistical significance for RZTI (P = 0.0159) and absolute zona pellucida thickness (ZPT; P = 0.0314). However, after adjustment for multiple comparisons using

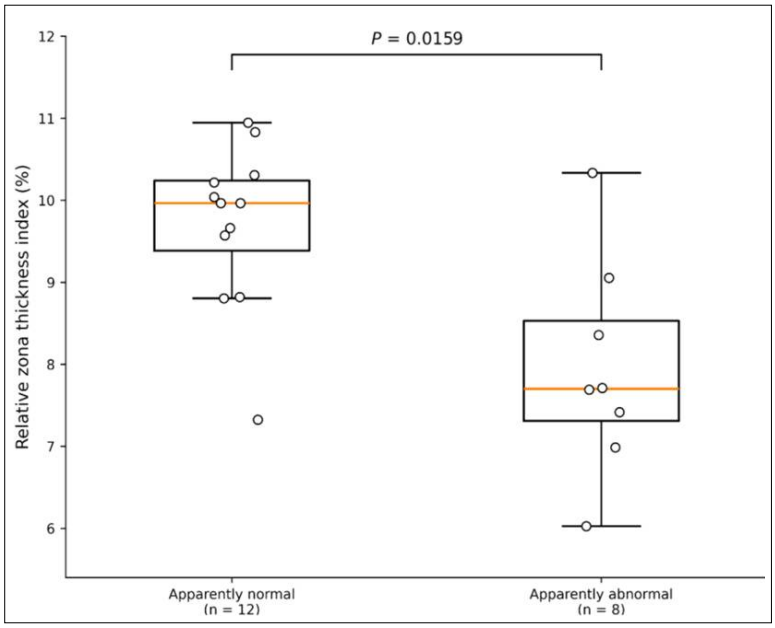


Fig 1: Distribution of the relative zona thickness index according to pre-slaughter udder status.

Table 2: Oocyte and cumulus-oocyte complex measurements according to pre-slaughter udder status.

Variable	Apparently normal, median	Apparently abnormal, median	Nominal exact *P* value	FDR-adjusted P
Outer oocyte diameter (µm)	187.81	194.84	0.1569	0.3660
RZTI (%)	9.97	7.70	0.1569	0.1100
Zona pellucida thickness (µm)	18.16	15.01	0.0314	0.1100
Ooplasm diameter (µm)	132.95	136.63	0.2380	0.4165
Perivitelline space (µm)	56.90	53.99	0.6784	0.8506
COC before maturation	41.02	46.14	0.8506	0.8506
COC after maturation	166.24	168.45	0.7345	0.8506

Values are donor-level medians. Comparisons were performed using exact two-sided Mann-Whitney U tests. FDR-adjusted P values were calculated using the Benjamini-Hochberg procedure across the seven tested endpoints.

Table 3: Between-group differences in oocyte-related measurements.

Variable	Median difference*	Rank-biserial correlation	Exact P value
RZTI (%)	-2.26	-0.646	0.0159
Zona pellucida thickness (µm)	-3.16	-0.583	0.0314
Outer oocyte diameter (µm)	+7.03	+0.396	0.1569
Ooplasm diameter (µm)	+3.68	+0.333	0.2380
Perivitelline space (µm)	-2.91	-0.125	0.6784
COC before maturation	+5.12	+0.063	0.8506
COC after maturation	+2.22	+0.104	0.7345

the Benjamini-Hochberg false discovery rate (FDR) procedure across the seven tested endpoints, the adjusted P values for both parameters were approximately 0.110. Neither association remained statistically significant after FDR adjustment. Therefore, the nominal P values are reported as unadjusted results, whereas the FDR-adjusted P values account for multiple testing. These findings should be interpreted as exploratory associations rather than statistically confirmed evidence and require further investigation in larger prospective studies.

The present study identified an association between pre-slaughter udder appearance and relative zona pellucida thickness in bovine oocytes. The median relative zona thickness index (RZTI) was lower in the apparently abnormal udder group than in the apparently normal group (7.70% vs. 9.97%; nominal P = 0.0159). This difference was accompanied by lower absolute zona pellucida thickness (ZPT) in the apparently abnormal group (15.01 vs. 18.16 µm; nominal P = 0.0314), whereas outer oocyte diameter (OOD) did not differ significantly between groups. These findings indicate that the observed variation was mainly associated with zona-related measurements rather than a detectable difference in overall oocyte diameter. The biological relevance of zona pellucida characteristics has been investigated in previous bovine oocyte studies. Santos *et al.* (2008) examined relationships between zona pellucida ultrastructure, polyspermic penetration and oocyte developmental characteristics, while Koester *et al.* (2011) investigated zona pellucida properties as potential indicators of embryonic developmental potential. Timlin *et al.* (2021) also evaluated physical characteristics of bovine oocytes and zygotes in relation to subsequent developmental outcomes. These studies provide a biological basis for examining zona pellucida morphology. In this context, RZTI offers a complementary description of zona thickness by expressing it relative to OOD, rather than considering absolute thickness alone. Nevertheless, the present study did not evaluate fertilization or embryo development and therefore cannot establish whether the observed RZTI differences have functional consequences.

The association between udder appearance and zona-related measurements may be considered in the broader context of donor physiological condition. Previous studies have reported associations between mastitis and changes in bovine oocyte characteristics. Roth *et al.* (2013) reported impaired developmental competence of oocytes from cows with naturally occurring mastitis, while Asaf *et al.* (2014) investigated the effects of experimentally induced mastitis on bovine oocytes. Previous work from our group also demonstrated associations between ovarian characteristics, oocyte quality and *in vitro* maturation outcomes (Ammari *et al.*, 2022), as well as seasonal variation in oocyte recovery, quality and maturation (Ammari *et al.*, 2023). These findings provide context for investigating donor-related characteristics in relation to oocyte morphology. However, the present study identified an association with external udder appearance and did not establish that mammary inflammation was responsible for the observed differences. The absence of significant differences in ooplasm diameter, perivitelline space and cumulus-oocyte complex measurements before and after maturation suggests that the observed variation was not accompanied by detectable differences across all measured morphometric parameters. The findings were mainly concentrated in absolute ZPT and RZTI. Although both parameters showed nominally significant between-group differences, neither remained statistically significant after Benjamini-Hochberg adjustment for multiple comparisons (FDR-adjusted P = 0.1100 for both). Therefore, these results should be interpreted as exploratory and hypothesis-generating rather than confirmatory. The consistent direction of the two zona-related measurements nevertheless supports further investigation of RZTI as a complementary morphometric descriptor.

Several limitations should be considered when interpreting these findings. First, the study included only 20 donor-level observations, comprising 12 apparently normal and 8 apparently abnormal udder records. Second, udder classification was based on recorded external appearance before slaughter rather than objective diagnostic procedures. The absence of somatic cell counts, bacteriological cultures, inflammatory biomarkers and histopathological examination means that the apparently abnormal group cannot be considered a laboratory-confirmed mastitis group. Third, the study did not include direct measurements of fertilization, cleavage, blastocyst formation, or embryo quality. Accordingly, no conclusions can be drawn regarding the effects of udder appearance or RZTI on reproductive or developmental outcomes. Future studies should include larger donor populations and standardized assessments of udder health, supported by somatic cell counts, microbiological examination and inflammatory biomarkers. Combining these assessments with detailed oocyte morphometry and subsequent in vitro maturation, fertilization and embryo-development outcomes would help determine whether variations in RZTI have functional reproductive significance. Overall, the present findings indicate an association between pre-slaughter udder appearance and relative zona pellucida thickness in bovine oocytes. The lower RZTI and absolute ZPT observed in the apparently abnormal group suggest that zona-related measurements may vary according to donor characteristics. However, the limited sample size, observational classification and absence of inflammatory and developmental measurements require cautious interpretation. Further studies are needed to establish the reproducibility and biological significance of this association.

CONCLUSION

Our study identified an association between pre-slaughter udder appearance and the relative thickness of the zona pellucida in bovine oocytes. Oocytes obtained from donors

with apparently abnormal udder appearance showed a lower median relative zona thickness index (RZTI) than those from donors with apparently normal udder appearance. This difference was accompanied by a lower absolute zona pellucida thickness, whereas outer oocyte diameter did not differ significantly between groups. The absence of significant differences in ooplasm diameter, perivitelline space and cumulus-oocyte complex measurements before and after maturation suggests that the observed variation was primarily concentrated in the zona-related parameters rather than representing a generalized alteration in oocyte dimensions. RZTI provides a normalized descriptor of zona pellucida thickness relative to overall oocyte size and may therefore offer a useful complementary parameter for quantitative assessment of bovine oocytes. However, our results should be interpreted cautiously because of the limited sample size, the observational nature of the udder classification and the absence of objective inflammatory or developmental measurements. In addition, the nominal associations did not remain significant after false-discovery-rate adjustment. Therefore, the findings are best regarded as exploratory and hypothesis-generating. Larger prospective studies incorporating standardized udder-health assessment, inflammatory biomarkers and subsequent fertilization and embryo-development outcomes are required to determine the biological and reproductive significance of variation in RZTI.

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Author contributions

Aiman Ammari: Conceptualization, Methodology, Investigation, Data curation, Writing - review and editing. Ahmad R. Alhimaidi: Supervision, Resources, Project administration, Funding acquisition, Writing - review and editing. Ramzi A. Amran: Investigation, Formal analysis, Data curation, Validation, Visualization, Writing - original draft, Experimental animal handling, Figure preparation.

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Ethics and informed consent

Ethical approval was not required for this study because it involved only bovine ovaries and associated donor records obtained from animals slaughtered at a commercial slaughterhouse. No live animals were handled, subjected to experimental procedures, or sacrificed specifically for this research. All biological materials were collected after slaughter as part of routine commercial slaughterhouse operations.

Availability of data and materials

All data analyzed during this study are included in this published article.

Use of artificial intelligence

Our team used the AI as a developmental editor. We submitted the original draft and prompted the tool (Google Gemini) to polish the language and correct nuanced syntax errors that standard spell-checkers might miss.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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