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Original Research Article

# Transcriptomic analysis of heifers according to antral follicle count

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ABSTRACT

While antral follicle count (AFC) has been associated with higher pregnancy rates, at present, our understanding of it as a reproductive parameter remains incomplete. This study aimed to characterize gene expression profile of oocytes from *crossbred* Bos taurus x *Bos indicus* heifers with high and low AFCs. Crossbred Nelore-Angus heifers (n = 50) with a mean (SD) age of 9.6 ± 0.55 months, a weight of 295.4 ± 32.6 kg, and a BCS of 3.44 ± 0.41 were studied in a feedlot system. The heifers received a hormonal protocol based on injectable progesterone and estradiol cypionate administered 12 days apart, and ovarian ultrasonography (US) was performed 12 days after to assess the AFC. Based on AFC, heifers were divided into low (≤14 follicles) and high (≥31 follicles) AFC, groups.Forty-five days after US, 14 heifers were slaughtered, and their ovaries were collected for morphological analysis and follicle aspiration. Cumulus-oocyte complexes (COCs) from the high and low AFC groups were graded according to their quality. Only best-quality COCs were stored for RNA-seq analysis. No differences were found in the presence or diameter of the dominant follicle and corpus luteum in the US, nor in the volume of the dominant follicle postmortem. The quantity of COCs recovered from high-AFC heifers was higher than that from low-AFC heifers ($P < 0.05$), and a tendency ($P = 0.07$) toward a higher amount of grade II COCs was observed. Thirty-two genes were differentially expressed between the groups, of which 30 were up-regulated and two down-regulated in the low AFC group. Among these, 22 % (7/32) were associated with fertility (CAB39, SLC2A6, CITED2, FDX1, HSD11B2, CD81, and PLA2G12B). Moreover, 9 and 2 exclusive genes were identified in the high and low AFC groups, respectively. Enrichment analyses showed that genes exclusive to oocytes from low-AFC heifers were associated with fundamental cellular processes, such as biosynthesis/biogenesis of ribosomes, peptides, amides, and nucleotides, and also with autophagy, mitophagy and mTOR signalling pathways.On the other hand, only one pathway was enriched in the high AFC group, but this cannot be related to the events studied No differences were observed in the ovarian structures after pre-synchronization of the estrus cycle of young Crossbred Nelore-Angus heifers. However, a tendency of a higher amount of grade II COCs was observed in heifers with high AFC than in those with low AFC. RNA sequencing results indicated that the main differences between high and low AFC heifers were not reflected in the genes directly related to fertility.

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## 1. Introduction

The relationship between antral follicle count (AFC) and fertility in cattle has been the focus of several studies over the years [1–9]. AFC is a highly variable trait among females but remains constant throughout the life of the animal [1,10], regardless of breed, age, or stage of the estrous cycle [1]. Thus, it is possible to determine the AFC from a single ultrasound scan and classify females according to the number of follicles found [5,10]. The ease of application of this assessment technique makes AFC a potential marker of fertility in cattle.

In *Bos taurus*, a high AFC positively influences fertility [10–13], with poorer reproductive performance observed in females with low AFC [2,4,11,12]. Among crossbred animals, high AFC was reported to be associated with a higher pregnancy rate in Girolando cows (Bos taurus x *Bos indicus* crossbreed) [14], while no relationship was observed between high AFC and reproductive performance in Braford females. However, females with low AFC were observed to have a higher follicular growth rate and larger follicular diameters than those with high AFC [15]. Although animals from the Nelore (*Bos indicus*) and Angus (*Bos taurus*) crossbreeds are present in a large part of Brazil and are of great importance owing to their adaptability to adverse conditions, such as high temperatures, parasite infection, and poor pastures [13], there are no data in the literature relating AFC to fertility in females from this crossbreed.

Identifying fertile females in herds as early as possible represents an interesting strategy if the aim is to improve fertility. However, the most widely used tool for identifying precocious females is through the exposure of young heifers to reproduction (artificial insemination, fixed-time artificial insemination (FTAI), or natural breeding) [16,17]. However, for economic and animal welfare reasons, identifying the precocity and fertility of heifers before they are bred is ideal. To date, there is no explanation for the differences in AFC among breeds or even among animals. Thus, our understanding of AFC as a reproductive tool to select fertile heifers remains incomplete [5]. Studies investigating mRNA expression by RT-PCR in oocytes from Nelore females have attempted to elucidate the mechanisms involved in the relationship between AFC and fertility but have shown that although there are differences in gene expression, these do not affect the developmental competence of embryos *in vitro* [18]. Another research have demonstrated that oocytes and cumulus cells from Nelore heifers with low AFC show distinct, but as yet unexplained, gene expression patterns [19].

Herein, we hypothesized that heifers from Angus-Nelore crosses with high AFC would exhibit phenotypes related to high fertility, which can be explained by the greater expression of fertility-related genes revealed after RNA sequencing. Therefore, we sought to assess the transcriptome of oocytes from high and low-AFC Nelore-Angus heifers, relating these to the morphological characteristics of the ovary and cumulus oocyte complexes.

## 2. Material and methods

This study was approved (number 1.077/2019) by the Ethics Committee for the Use of Animals of the State University of Mato Grosso do Sul.

### 2.1. Location and animals

The experiment was carried out at a commercial farm, located in Mato Grosso do Sul (−20°30′04.08′S 55°53′34.13′O). Heifers (½ Angus x ½ Nelore; n = 140) had an average age of 9.6 ± 0.55 months, an average weight of 295.4 ± 34.6 kg, and an average body condition score (BCS) of 3.44 ± 0.41 [20]. The heifers were born and weaned simultaneously on the farm, remaining in the extensive system for 30 days after weaning. After this period, all heifers were maintained in a confinement system and fed the same diet with a roughage-concentrate ratio of 30:70, based on corn, soybean meal, soybean hulls, kernels, and grass silage, containing 16 % crude protein, 2 % ether extract, 4 % minerals, and 48 % neutral detergent fiber.

The experimental design is shown in Fig. 1.

### 2.2. Hormonal protocol

Heifers were subjected to a hormonal protocol for pre-synchronization of the estrous cycle, characterized by the administration of intramuscular injectable progesterone (150 mg of Sincrogest®, Ourofino, São Paulo, Brazil) on a random day of the estrous cycle, followed by 1 mg of estradiol cypionate (ECP® Zoetis, São Paulo, Brazil) after 12 days, according to de Lima [21].

### 2.3. Ovarian ultrasound and antral follicle count

Ovarian assessments were performed using an ultrasound (US) device (SonoScape® A5 VET) coupled to a 5.0-MHz linear transducer. Ovarian activity was assessed on a random day of the estrous cycle, 12 days after the application of estradiol cypionate, when both ovaries of each female were evaluated to determine the presence and diameter of the corpus luteum (CL) and the dominant follicle (DF) if ≥ 8 mm. The DF and CL diameters were measured by considering the greatest distance between two points on the structure, using the ultrasound tools themselves.

On the same day, the AFC was determined [6,9], and the heifers were divided into low (≤14 follicles; n = 29) and high (≥31 follicles; n = 21) groups. Heifers with an intermediate AFC (>14 and ≤30 AFC) were not used because AFC categories at the ends of the distribution curve better allow for the identification of possible differences within the studied population.

### 2.4. Slaughtering and collecting cumulus-oocyte complexes (COCs)

The heifers reached slaughter weight approximately 45 days after AFC evaluation and were then slaughtered. Ovaries were collected from 14 heifers for analysis.

The heifers were identified by an ear tag attached immediately after bleeding, while the team waited for the carcasses on the slaughter line. Ovary pairs from each heifer were collected individually after evisceration and placed in duly labeled packets made of permeable material. The packages were placed in an isothermal container with saline solution at 35 °C until they were taken to the laboratory, which is approximately 100 km from the slaughterhouse (a ~1-h journey). In the laboratory, ovarian morphology was assessed by measuring the diameter of the ovaries, number of follicles, diameter of the dominant follicles, and diameter of the CL (Fig. 2).

All visualized follicles, except the dominant follicle, were aspirated using a 25 × 0.8 needle attached to a 3 mL syringe. The follicular fluid and COCs from each pair of ovaries were placed in Petri dishes for selection and classification based on morphology.

COCs were classified according to their quality as grades I, II, III, IV, naked, or degenerated [22]. Only COCs classified as grades I and II were used for RNA-seq analysis, in which category 1 presented homogeneous oocyte cytoplasm and a compact multilayer cumulus oophorus, while category 2 presented cytoplasm with small heterogeneous areas and more than five layers of compact cumulus [22]. After classification, they were transferred to a drop of Ca- and Mg-free DPBS (D8537, Sigma-Aldrich) and washed three times with the same solution. COCs were denuded by pipetting with a pipette set to 60 μL. Only fully denuded oocytes were used in this study. After this procedure, pools of five oocytes totaling 5 μL were stored in RNase- and DNase-free Eppendorf tubes. Immediately after processing, samples were snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

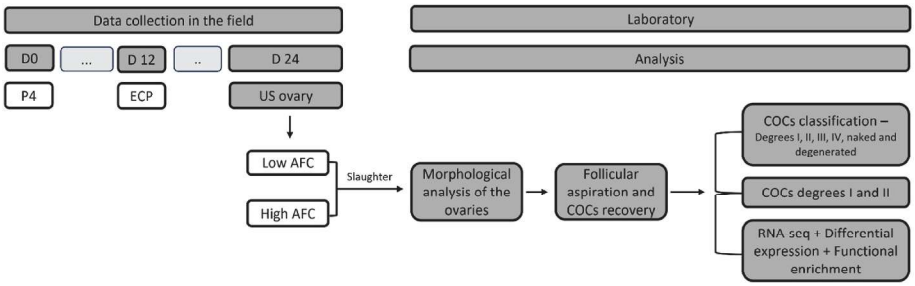


**Fig. 1.** Experimental design: Nelore-Angus heifers (n = 50) underwent pre-synchronization and were separated into high and low AFC groups according to ultrasound evaluation, from which ovarian morphology was assessed. Part of the heifers (n = 14) were slaughtered 45 days later, and morphology was assessed again, in addition to follicles being aspirated and the cumulus-oocyte complexes (COCs) evaluated and stored for later RNA-seq analysis.

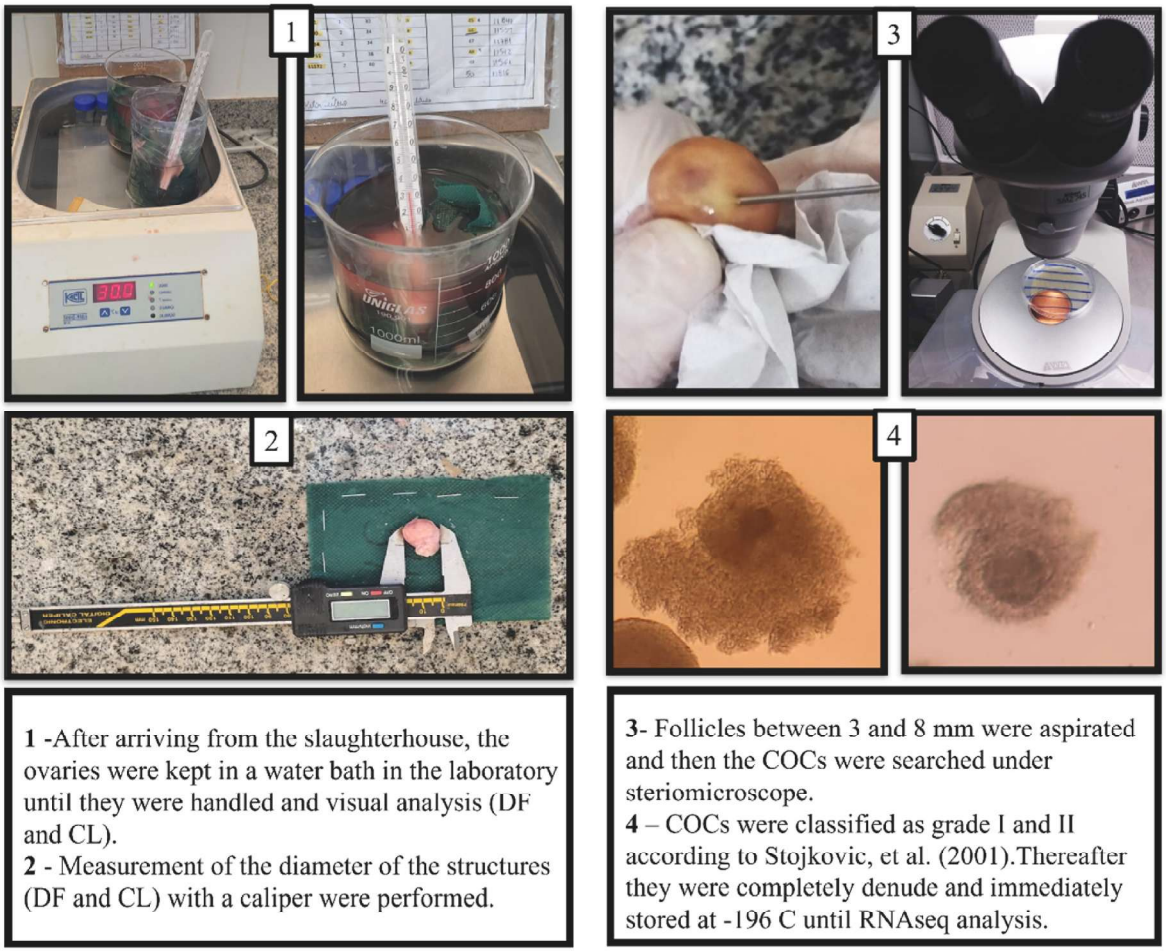


**Fig. 2.** Steps of the methodology performed after collecting the ovaries after slaughter until the classification of COCs that were later used for the RNA-seq analysis.

### 2.5. RNA sequencing

Six heifer oocyte pools, three pools from each group (low and high AFC), each containing 10 oocytes, which were the result of the union of five oocytes from two animals from each group were evaluated for gene expression differences based on RNA sequencing (RNA-seq) analysis, as previously reported [23]. Briefly, each pool of denuded oocytes was subjected to cDNA synthesis and amplification using a SMART-Seq HT kit (Takara Bio, Kusatsu, Kansai, Japan) according to the manufacturer's recommendations. Next, AMPure XP Beads (Beckman Coulter, Brea, CA, USA) were used for cDNA purification, while cDNA concentration and length were assessed using a Qubit dsDNA High Sensitivity (Thermo-Fisher Scientific, Waltman, MA, USA) and Bioanalyzer High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA), respectively. Libraries were prepared from cDNA using Nextera XT DNA Library Prep (Illumina, San Diego, CA, USA), as recommended by the SMART-Seq HT Kit. Finally, library length and concentration were assessed on a Qubit Bioanalyzer before pooling equimolar amounts to a final concentration of 4 nM. Sequencing was performed on a NextSeq 550 (Illumina) with a minimum of 15 million reads of 1 × 75 bp per sample. Raw data were deposited on the Gene Expression Omnibus (GEO) database under accession number GSE283305.

### 2.6. Pre-processing the sequencing data

The quality of the raw sequencing data was assessed using the

FastQC tool v0.12.1 (https://www.bioinformatics.babraham.ac.uk/projects/download.html#fastqc), which confirmed the presence of a small number of low-quality reads possibly containing sequencing errors.

To remove bases that were sequenced with high uncertainty at the read ends as well as reads of overall poor quality, the Cutadapt trimming tool (v4.94) was used [24]. The following parameters were employed: single-end reads; minimum read length: 20; minimum quality cutoff: 20. The quality of the preprocessed data was assessed using the FastQC tool, which confirmed that low-quality bases were removed from the reads.

### 2.7. Mapping against the bovine reference genome

To determine the genes to which the sequenced reads belonged, the reads were mapped to the bovine reference genome (version bosTau9). Mapping was performed using the STAR tool (version 2.7.10) with default settings [25]. Total reads and percentage of mapped sequence can be accessed in Supplementary File "Table S1".

### 2.8. Differential and exclusive gene expression analysis

Using the mapping results of the two groups analyzed (high and low AFC), each with three biological replicates, statistical analysis of differential gene expression was performed using the DESeq2 tool [26], implemented in the DEApp interactive interface [27]. To avoid including genes expressed at very low levels, only those with more than three reading counts from at least three libraries were considered. The Wald test was used to identify differentially expressed genes (DEGs), with selection criteria based on log2 fold-change (log2 FC) values greater in magnitude than 0.6 (log2 FC $>$ 0.6 and log2 FC $<$ −0.6) and a Benjamini-Hochberg adjusted P-value (FDR) at 5 %. Additionally, exclusive gene expression patterns were identified. Genes expressed (i.e., with TPM $>$1 counts) in all samples of one group and absent (zero counts) in all samples of the other group were considered exclusive.

### 2.9. Functional enrichment analysis

Functional enrichment analysis of differentially expressed and exclusive genes was performed to identify over-represented pathways and terms based on the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, using the clusterProfiler [28] package (v. 4.12) in RStudio (v. 4.4.1). Terms and pathways with a P-value $<$0.10 were considered enriched by Fisher's exact test. For this analysis, upregulated genes from the Low AFC *versus* High AFC comparison, together with exclusive genes from the Low AFC group, were used as input. Similarly, downregulated genes from the Low AFC *versus* High AFC comparison, along with exclusive genes from the High AFC group, comprised the second input group.

### 2.10. Statistical analyses

For all analyses (except RNA-seq), each heifer was considered an experimental unit. The average AFC, ovarian diameter, number of follicles, diameter of dominant follicles, and CL were obtained by PROC MEANS using the SAS software (University Edition). The means of the AFC groups were compared using PROC GLIMMIX SAS (University Edition). For all analyses, statistical significance was set at $P < 0.05$ and tendency at $P < 0.1$.

## 3. Results

### 3.1. AFC and ovarian morphology and COC quality

The AFC of the heifers in this study ranged from 4 to 52, corresponding to a mean of 22.2 ± 12.9 follicles, with low AFC being defined as ≤14 follicles (29/50) and high AFC as ≥31 follicles (21/50). US evaluation of ovarian structures (presence and diameter of the CL and DF) on a random day of the estrous cycle showed no association with AFC ($P > 0.05$) (Table 1), and no significant differences ($P > 0.05$) were found between AFC and structures in post-mortem ovaries (Table 2). No differences were observed in oocyte quality rate between the low and high AFC groups; however, a tendency ($P < 0.07$) was observed when we examined the COCs of grade II, which were higher in the high-AFC than in the low-AFC group.

### 3.2. Gene expression of high and low AFC heifers

Principal Component Analysis of the high and low AFC samples (Fig. 2) showed that there was a similarity between individual cases in some datasets, but there were also samples grouped into separate regions. These data did not prevent the analysis from being conducted.

Thirty-two genes were differentially expressed between low and high AFC groups (Table 3; Figs. 3 and 4). Of these, 30 were upregulated in the low AFC group (Table 3), whereas only two were downregulated in the low AFC group compared to the high AFC group (Table 3). In addition, 9 and 2 exclusive genes were identified in the high AFC and low AFC groups, respectively. A complete list is available in Supplementary File "Tables S2 and S3".

We observed that 93 % (30/32) of the DEGs were upregulated in the low AFC group, indicating a higher expression in oocytes from heifers with a lower follicle count. The low AFC group had fewer exclusive genes than the high AFC group.

### 3.3. Enrichment analysis of expressed genes

Enrichment analysis was performed using all the significant genes: upregulated genes in the low AFC group, including their exclusive genes, and upregulated genes in the high AFC group, along with their exclusive genes. The overrepresented terms and pathways are shown in Fig. 5 and Supplementary File "able S4, S5, and S6". In the low AFC group, some of the identified terms were associated with the mitochondrial matrix (GO:0005759, P = 0.02); peptide- (GO:0043043, P = 0.02), nucleotide- (GO:0009165, P = 0,002) and amide biosynthetic process (GO:0043604, P = 0,03), as well as Mitophagy (bta:04137, P = 0,01), Autophagy (bta:04140, P = 0,04) and mTOR signalling pathway (bta:04150, P = 0,03). In contrast, only one pathway was enriched in the high AFC group, organophosphate metabolic process (GO:0019637, P < 0,01), however, this could not be related to the events studied.

## 4. Discussion

This study provides new insights into the relationship between AFC and fertility in Bos taurus x *Bos indicus* crossbreed heifers through characterization of the oocyte transcriptome. Although several studies have addressed fertility in female cattle with different AFCs, few reports have studied heifers. No such reports were found for the crossbreed studied herein, which is interesting considering the controversial data between taurus and indicus breeds [3,6,29,30]. To the best of our knowledge, this is the first study to evaluate and compare the

**Table 1**
Ultrasound evaluation of crossbred Nelore-Angus heifers 12 days after pre-synchronization.

| Variable | Low AFC | High AFC | P |
|---|---|---|---|
| DF presence (%) | 50 (14/28) | 57.1 (12/21) | 0.60 |
| DF diameter (mm) | 12.2 (±1.73) | 11.56 (±2.33) | 0.16 |
| CL presence (%) | 39.3 (11/28) | 42.8 (9/21) | 0.74 |
| CL diameter (mm) | 18 (±1) | 19.5 (±2.08) | 0.95 |

DF, dominant follicle ≥8 mm; CL, corpus luteum.
* Values represent the average of the variables found per female per AFC group with a standard error.

**Table 2**
Morphology of ovary pairs obtained after slaughter of crossbred Nelore-Angus heifers. Values represent the average per pair of ovaries per AFC group.

| Variable | Low AFC | High AFC | P |
|---|---|---|---|
| Total number of COCs | 18.62 (±9.44) | 39 (±25.1) | 0.05 |
| DF volume (mL) | 0.56 (±0.55) | 0.43 (±0.2) | 0.76 |
| CL diameter (cm) | 14.4 (±9.91) | 10.3 (5.1) | 0.59 |
| OV diameter (cm) | 27.75 (±3.2) | 30 (4.35) | 0.53 |
| Number COCs DI | 5.5 (±3.89) | 11.4 (10.1) | 0.15 |
| Number COCs DII | 4.8 (±3.27) | 10.4 (6.91) | 0.076 |
| Number COCs DIII | 6.7 (±5,8) | 7.3 (±5.34) | 0.86 |
| Number COCs deg | 2.25 (±1.25) | 2.2 (±1.64) | 0.32 |
| Viability rate (%) | 37.8 | 39.8 | 0.79 |

DF, dominant follicle; CL, corpus luteum; OV, ovary; COCs, cumulus-oocyte complex; DI, grade I; DII, grade II; DIII, grade III; DIV, grade IV; nak, naked; exp, expanded; deg, degenerated (Stojovic et al., 2001).

transcriptome of high and low-AFC Nelore-Angus heifer oocytes through RNA-seq.

Similar to reports by Dominguez [31] and Silva et al. [32], we found that AFC did not vary according to the other structures observed in ovarian pairs (size-DF and CL). Data from the postmortem evaluation complemented the results of *in vivo* US ovarian evaluation of the same females, allowing us to conclude that the AFC of heifers did not vary according to the structures present in the ovaries at the time of evaluation.

As expected, the number of COCs obtained was higher in the high AFC than in the low AFC group. No differences were found in the oocyte quality rate between the low and high AFC groups. However, a tendency ($P < 0.07$) was observed when we looked at grade II COCs, which were more common in the high AFC than in the low AFC group. The oocyte quality is directly related to the quality of resulting embryos. Many reports have described a greater number of oocytes retrieved from donors with a higher AFC. Consequently, a greater number of embryos are

**Table 3**
Annotated and differentially expressed genes between the low and high AFC groups and upregulated or downregulated in the low AFC group.

| Gene ID | Description | Base Mean | Log2 (FC) | StdErr | Wald-Stats | P-value | P-adjusted | Up/Downregulated |
|---|---|---|---|---|---|---|---|---|
| AP1S2 | adaptor related protein complex 1 subunit sigma 2 | 179.3 | −1.6 | 0.3 | −5.1 | 0.00000022 | 0.0006 | Upregulated |
| CAB39 | calcium binding protein 39 | 289.6 | −1.0 | 0.2 | −3.9 | 0.00009 | 0.037 | Upregulated |
| CACHD1 | cache domain containing 1 | 326.7 | −1.3 | 0.3 | −4.3 | 0.000011 | 0.009 | Upregulated |
| UBE2H | ubiquitin conjugating enzyme E2 H | 458.4 | −0.9 | 0.2 | −4.0 | 0.000058 | 0.025 | Upregulated |
| KLRG2 | killer cell lectin like receptor G2 | 69.0 | −1.3 | 0.3 | −4.0 | 0.000048 | 0.022 | Upregulated |
| CCDC107 | coiled-coil domain containing 107 | 921.4 | −0.8 | 0.2 | −4.0 | 0.000048 | 0.022 | Upregulated |
| ISOC1 | isochorismatase domain containing 1 | 182.9 | −1.3 | 0.3 | −4.2 | 0.000025 | 0.015 | Upregulated |
| NRG2 | neuregulin 2 | 258.4 | −1.2 | 0.3 | −3.9 | 0.000073 | 0.031 | Upregulated |
| NACC2 | NACC family member 2 | 108.3 | −1.5 | 0.3 | −4.6 | 0.0000035 | 0.004 | Upregulated |
| SLC2A6 | solute carrier family 2 member 6 | 160.3 | −1.0 | 0.2 | −3.8 | 0.0001 | 0.04 | Upregulated |
| MRPS2 | mitochondrial ribosomal protein S2 | 757.2 | −1.2 | 0.2 | −4.3 | 0.000014 | 0.009 | Upregulated |
| CITED2 | Cbp/p300 interacting transactivator | 255.7 | −1.3 | 0.2 | −5.1 | 0.00000023 | 0.0006 | Upregulated |
| KCTD12 | potassium channel tetramerization domain | 39.8 | −1.6 | 0.3 | −4.6 | 0.0000041 | 0.004 | Upregulated |
| CARS2 | cysteinyl-tRNA synthetase 2, mitochondrial | 795.6 | −1.0 | 0.2 | −3.8 | 0.0001 | 0.04 | Upregulated |
| P4HA3 | prolyl 4-hydroxylase subunit alpha 3 | 1925.8 | −1.0 | 0.2 | −4.0 | 0.000046 | 0.022 | Upregulated |
| FDX1 | ferredoxin 1 | 934.1 | −1.2 | 0.2 | −4.4 | 0.0000071 | 0.006 | Upregulated |
| ESPN | espin | 63.0 | −1.7 | 0.3 | −4.9 | 0.0000007 | 0.001 | Upregulated |
| SNAP29 | synaptosome associated protein 29 | 331.9 | −1.3 | 0.2 | −4.6 | 0.0000027 | 0.004 | Upregulated |
| RANBP1 | RAN binding protein 1 | 1620.8 | −1.6 | 0.2 | −6.3 | 0.00000000017 | 0.0000023 | Upregulated |
| NADK2 | NAD kinase 2, mitochondrial | 1204.3 | −1.0 | 0.2 | −4.6 | 0.0000036 | 0.004 | Upregulated |
| MRPL36 | mitochondrial ribosomal protein L36 | 222.7 | −1.7 | 0.3 | −5.5 | 0.000000022 | 0.0001 | Upregulated |
| HSD11B2 | hydroxysteroid 11-beta dehydrogenase 2 | 197.1 | −1.1 | 0.2 | −4.0 | 0.000048 | 0.022 | Upregulated |
| MRM1 | mitochondrial rRNA methyltransferase 1 | 374.9 | −1.3 | 0.2 | −4.6 | 0.0000037 | 0.0044 | Upregulated |
| TYMS | thymidylate synthetase | 927.4 | −1.1 | 0.2 | −4.3 | 0.000012 | 0.009 | Upregulated |
| VAPA | VAMP associated protein A | 2132.3 | −1.3 | 0.2 | −4.6 | 0.0000031 | 0.004 | Upregulated |
| INPP5F | inositol polyphosphate-5-phosphatase F | 676.4 | −1.1 | 0.2 | −4.3 | 0.00001 | 0.009 | Upregulated |
| CD81 | CD81 molecule | 1485.2 | −1.1 | 0.2 | −4.1 | 0.000033 | 0.018 | Upregulated |
| HRAS | HRas proto-onco, GTPase | 584.2 | −1.3 | 0.2 | −5.2 | 0.00000016 | 0.0006 | Upregulated |
| UBFD1 | ubiquitin family domain containing 1 | 3291.2 | −1.0 | 0.2 | −4.6 | 0.0000032 | 0.004 | Upregulated |
| TSR3 | TSR3 ribosome maturation factor | 286.6 | −1.2 | 0.2 | −4.3 | 0.000013 | 0.009 | Upregulated |
| ALDOB | aldolase, fructose-bisphosphate B | 29.9 | 1.4 | 0.3 | 4.2 | 0.000026 | 0.015 | Downregulated |
| PLA2G12B | phospholipase A2 group XIIB | 943.8 | 1.2 | 0.3 | 4.0 | 0.000043 | 0.022 | Downregulated |

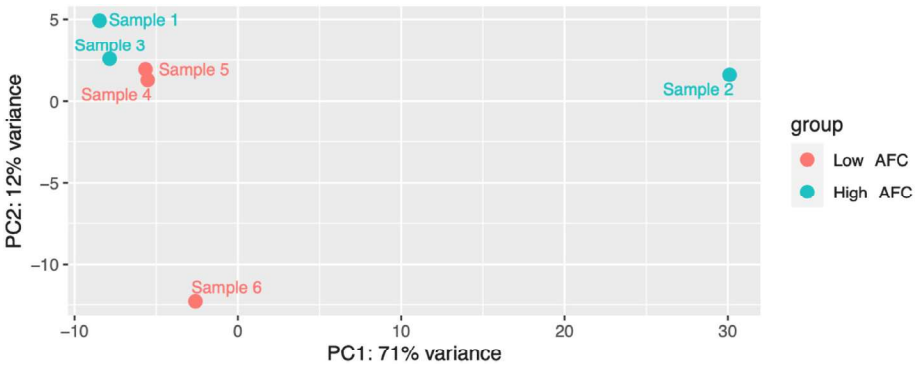


**Fig. 3.** Principal Component Analysis of high-AFC (1–3) and low-AFC (4–6) samples. Each replicate is plotted as an individual data point.

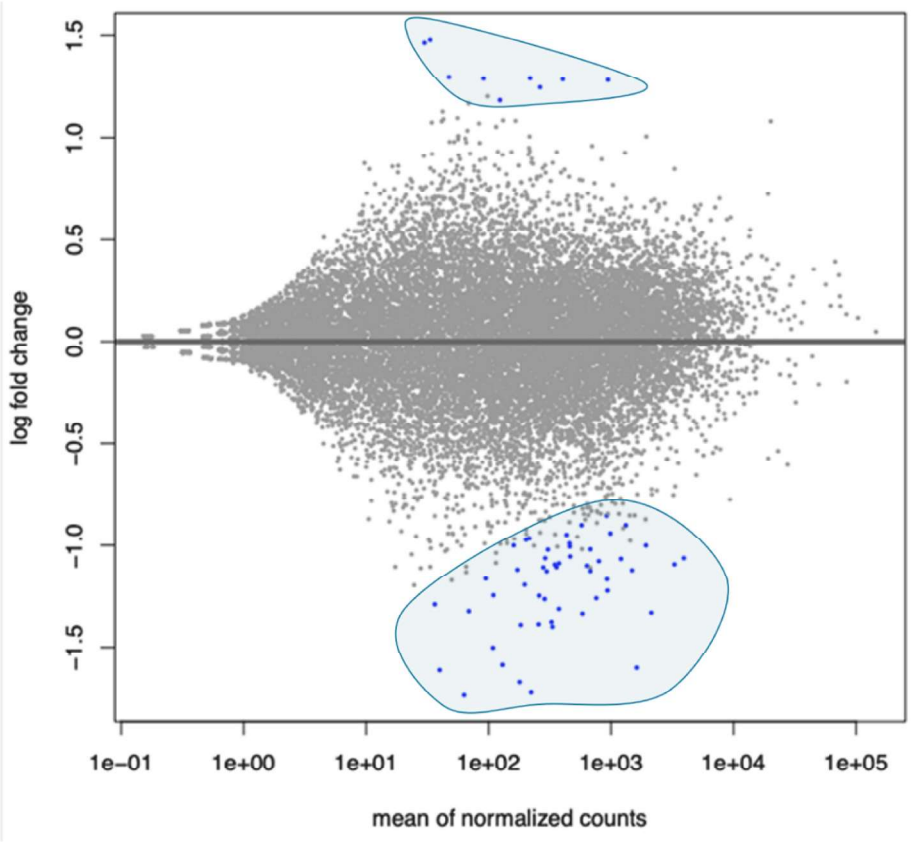


**Fig. 4.** MA-plot displaying the global view of the relationship between the expression changes (high AFC vs low AFC) on the y-axis, the average expression strength of genes on the x-axis, and the ability of the algorithm to detect differential gene expression. The genes that passed the significance threshold (adjusted p-value <0.05) are colored in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

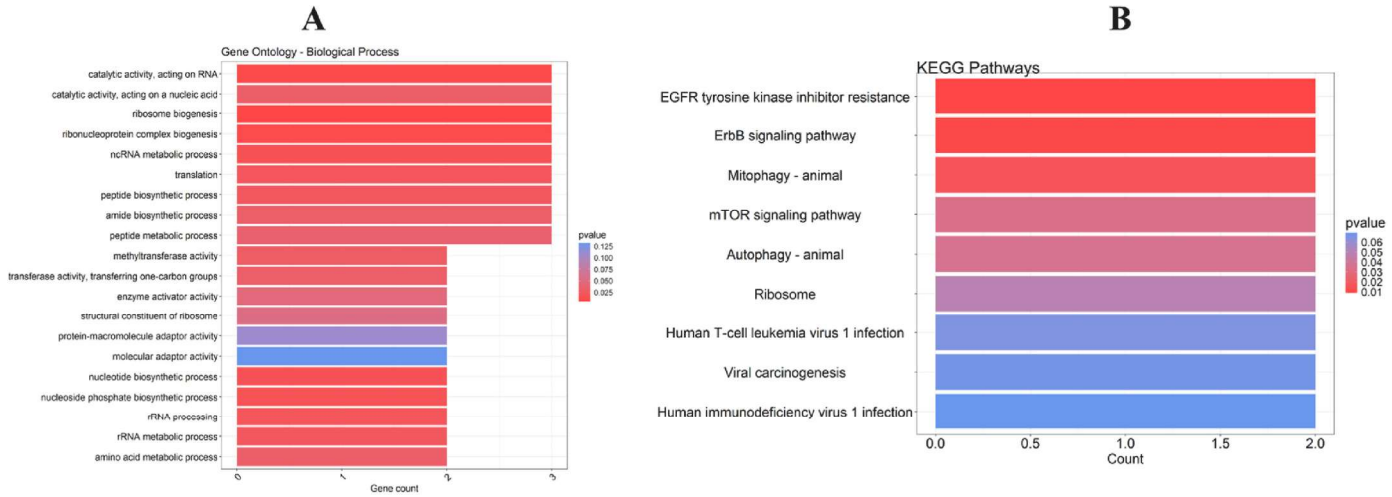


**Fig. 5.** Enrichment analysis of DEGs and exclusive genes. (A) Enrichment plot with the main pathways overrepresented in the GO database for the low AFC group, (B) Enrichment plot with the main pathways overrepresented in the KEGG database for the low AFC group.

produced *in vitro* [5,6,10]. However, there are inconsistencies in the results on oocyte recovery rates and the proportion of embryos produced *in vitro* from females with low and high AFC among different breeds [5,32,33]. For example, Garcia et al. [14] found a higher number of oocytes in Nelore females with a high AFC, whereas embryonic development rates up to the blastocyst stage did not differ. On the other hand, Silva-Santos et al. [5] reported that Braford females (Bos taurus x *Bos indicus* crossbreed) had a higher number of oocytes recovered at oocyte retrieval (OPU) and higher *in vitro* embryo production for donors with a high AFC (≥40). Therefore, the authors suggested that the use of Braford females with high AFC increases the number of embryos produced [5]. The AFC was also shown to not compromise the genetic merit trait used in genetic improvement programs [34].

The results of morphological evaluation of the ovaries and COCs support the notion that evaluation of the AFC 12 days after presynchronization, on the day corresponding to D0 in programs where heifers

are subjected to FTAI, is feasible, facilitating the use of this tool to assist in the decision-making process of the program, such as in the choice of semen.

In addition, considering the current results and previous reports by our research group and others, it is clear that the differences in *in vitro* embryo production and fertility in FTAI are related not only to follicular dynamics, but also to factors related to metabolic pathways, follicular cell development, and oocyte competence [5,7,8,32]. Therefore, we investigated gene expression patterns in oocytes of heifers with low and high AFC and performed a functional enrichment analysis of genes involved in pathways that are significant for reproductive traits.

Prior to identifying differentially expressed genes between the low-AFC and high-AFC groups, a Principal Component Analysis was carried out. As can be seen on Fig. 3, samples 2 and 6 appear relatively distant compared to the other samples. This could be the reason for the low number of DEGs identified later.

We observed that most of the DEGs (30/32) were upregulated at low AFC; that is, they were more highly expressed in oocytes from heifers with a lower compared to higher follicle count.

After analyzing follicles dissected from the ovaries of Nelore and Angus heifers with high and low AFCs, it was found that follicular development from the pre-antral phase onwards is regulated by different genes in *Bos taurus* and *Bos indicus*, this may be related to the fact that *Bos taurus* has a larger pre-antral follicle population. Based on these results, it is plausible to expect that the gene regulation of AFC in crossbred heifers is also different from that in other breeds, highlighting the complex relationship between AFC and fertility [35].

In the present study, we observed a tendency toward more grade II COCs in high-AFC heifers, a unique phenotype that we could correlate with better fertility. Further studies, including those on embryo production, are necessary to confirm these results in Nelore × Angus heifers.

Among the DEGs, 22 % (7/32) have been reported in human and bovine fertility studies, namely, CAB39 (MO25), SLC2A6 (GLUT6), CITED2, FDX1, HSD11B2, CD81, and PLA2G12B.

CAB39 regulates protein kinases responsible for intracellular communication as well as the regulation and transduction of signals through chemical and structural changes [36]. Although CAB39 is generally responsible for basic biological processes, it has been implicated in cell invasion in liver [37] and pancreatic [38] cancers and pregnancy loss in cattle [39]. SLC2A6 and GLUT6 are members of a family of genes responsible for glucose transport across the plasma membrane [40]. Other genes in this family, such as GLUT4, GLUT9, and GLUT12, regulate oocyte development and embryo implantation rates, whereas GLUT6 expression has been reported in oocytes from women with polycystic ovaries and insulin resistance [40]. Byrne et al. [41] and Maedera et al. [42] found that GLUT6 is upregulated in female endometrial cancer cells. Although this gene was identified in our study and in other studies [41,43], the mechanisms involved in fertility remain unclear.

Another gene that was differentially expressed between the AFC groups was FDX1. FDX1 is an iron-sulfur protein and monooxygenase that facilitates the enzymatic activity of cytochrome P450 [44,45]. It is highly expressed in the ovary, but its effects on granulosa cells during the developmental stages of polycystic ovary syndrome (PCOS) remain to be elucidated [46–48]. Xing et al. [47] found an association between increased FDX1 expression in granulosa cells and polycystic ovary syndrome in women, which is closely related to abnormal follicular development because it affects the regulation of cell proliferation, apoptosis, and autophagy.

CITED2 can act as a transcriptional coactivator or repressor, depending on the context [49,50]. It is thought to be involved in oocyte development, as gene expression profiling has shown that CITED2 is significantly upregulated in mouse primordial follicles at the onset of oocyte growth [51]. In bovine ovarian follicles, this gene is upregulated in large follicles compared to small follicles [52]. Fang et al. [53] evaluated CITED2 as a biomarker of oocyte quality and showed that oocytes with lower levels of CITED2 expression had higher rates of fertilization, transferable embryos, and implantation. Meanwhile, higher expression of this gene was associated with lower oocyte quality and pregnancy rates. Similarly, positive regulation of CDB1 is strongly associated with embryonic loss in humans, as high levels can inhibit cytotrophoblast invasion and impair placentation [54,55].

Corroborating our findings, genes such as CAB39, CITED2, NACC2, and SNAP29, were elevated in cows with low AFC, indicating higher cellular activity linked to energy metabolism and stress response [36,49,50,56,57], both pathways identified in our enrichment analysis. To compensate for the low ovarian reserve, these genes may be involved in adaptive responses to demanding cellular environments characterized by heightened metabolism and cellular signaling. To compensate for insufficient energy in the low AFC context, mitochondrial genes, such as MRPS2, CARS2, and FDX1, play critical roles in energy generation, which is crucial for oocyte quality [4,45,58,59].

The enrichment of genes corroborated the above discussion and revealed a significant relationship with mitophagy, autophagy, carcinogenesis pathways, as well as fundamental cellular processes such as biosynthesis/biogenesis of ribosomes, peptides, amides, and nucleotides. Autophagy is a recycling pathway that provides energy sources and building blocks for macromolecule synthesis. The mTOR pathway, a key regulator of autophagy, negatively regulates it by phosphorylating and inactivating the ULK1 complex, with anabolic inputs like energy, nutrients, amino acids, growth factors, and hypoxia influencing mTOR activity. Autophagy plays a role in follicular pool establishment and follicular cell survival, suggesting its potential as a target for ovarian failure treatment in women. However, increased autophagy may also contribute to follicular atresia and granulosa cell death, and while its impairment is linked to ovarian failure. So, it was suggested that autophagy is activated in low-quality eggs as an energy compensatory mechanism [60].

Genes such as PLA2G12B and ALDOB were downregulated in the low AFC group, indicating that these heifers have lower energy metabolism and a lower level of inflammatory signaling [61,62]. Among the two genes, PLA2G12B should be further discussed. This gene belongs to the phospholipase A2 family and has a secretory function that helps regulate the amount of lipoproteins in blood [63]. Furthermore, A2 phospholipases, including PLA2G12B, act together with cyclooxygenase (COX) to form two prostaglandins, which, in turn, have important regulatory functions in reproductive processes. Series 2 prostaglandins are of great importance in childbirth. They are involved in the regulation of various aspects of cellular function, including pregnancy and birth in mammals, and are recognized as factors that promote myometrial contractions, cervical dilation, and membrane rupture [61].

Corroborating the results obtained in the present study, in women with a diminished ovarian reserve, mitochondrial dysfunction and translational impairment were shown to be the primary defects present in oocytes; consequently, decreased PLAG1-IGF2 axis activity was observed [64].

HSD11B2, INPP5F, VAPA, CD81, and FDX1, as genes implicated in lipid metabolism, were upregulated in the low AFC group, indicating a compensatory adaptation of lipid metabolism to promote ovarian function and oocyte quality. These genes are crucial for oocyte viability and energy because they regulate oxidative stress, cellular signaling, and mitochondrial function [44,45,65,66].

CARS2, FDX1, and NDK2, which were upregulated, are directly linked to mitochondrial metabolism and ATP production, which are essential for sustaining oocyte viability under increased energy demands [44,45,58]. Upregulation of MRPS2 and MRPL36 suggests an increased need for mitochondrial protein synthesis, whereas TYMS and MRM1 may support mitochondrial DNA synthesis and repair. Taken together, these changes in gene expression suggest that in animals with low follicular counts mitochondrial resources are mobilized to sustain oocyte function and support biosynthesis/biogenesis of various

substrates necessary for the maintenance of oocyte health.

We acknowledge that the limited number of oocytes available for analysis prevented us from validating the RNA-seq results using quantitative PCR (qPCR). However, several studies have argued that RNA-seq methods and data analysis approaches are sufficiently robust and do not always require validation via qPCR and/or other approaches [67,68]. Another limitation of our study is that field studies with larger numbers of animals are needed to confirm the current data, especially for production systems that have been adopted in beef cattle farming, where there is a high selection pressure on fertility with the culling of young heifers that do not become pregnant after the first artificial insemination. In these cases, herd fertility tends to increase rapidly, as infertile animals are discarded before leaving any offspring.

We hypothesized that heifers from Angus-Nelore crosses with high antral follicle counts (AFC) would display phenotypes associated with high fertility, characterized by a greater expression of fertility-related genes. However, our findings revealed only a tendency towards better cumulus-oocyte complex (COC) quality in high AFC heifers. RNA sequencing results indicated that the main differences between high and low AFC heifers were not reflected in the genes directly related to fertility. Although oocytes from low AFC heifers showed the expression of genes associated with poor oocyte quality, further studies are necessary to confirm these findings. Additional clarifications should be explored at the proteomic or metabolomic levels.

## 5. Conclusion

No differences were observed in the ovarian structures after presynchronization of the estrus cycle of young Nelore Angus heifers. However, a tendency of a higher amount of grade II COCs was observed in heifers with high AFC than in those with low AFC. RNA sequencing results indicated that the main differences between high and low AFC heifers were not reflected in the genes directly related to fertility.

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## CRediT authorship contribution statement

**Janaina Menegazzo Gheller:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation. **Wilian Aparecido Leite da Silva:** Formal analysis, Data curation. **Mirela Brochado Souza-Cáceres:** Writing – review & editing, Methodology. **Aldair Félix da Silva:** Methodology, Data curation. **Mariane Gabriela Cesar Ribeiro Ferreira:** Methodology. **Taynara dos Santos Santana:** Methodology. **Angélica Camargo dos Santos:** Methodology. **Sérgio Antonio Pereira-Junior:** Data curation. **Ériklis Nogueira:** Formal analysis, Data curation. **Sérgio Amorim de Alencar:** Writing – original draft, Methodology, Formal analysis, Data curation. **Gustavo Guerino Macedo:** Resources, Methodology, Funding acquisition. **Marcelo Marcondes Seneda:** Writing – review & editing, Conceptualization. **Marcos Roberto Chiaratti:** Validation, Supervision, Resources, Methodology. **Fabiana de Andrade Melo-Sterza:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2025.02.024.

Declaration of interests.

☒ 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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