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Relationships between antral follicle counts and ovarian morphology of *Bos indicus* of different ages

[*Relações entre a contagem de folículos antrais e a morfologia ovariana de Bos indicus de diferentes idades*]

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ABSTRACT

This study aimed to evaluate the relationship between the antral follicle count (AFC) and the ovarian morphology of *Bos indicus* in different age groups: 12-23 months (G1), 24-35 months (G2), 36-47 months (G3), 48-59 months (G4), and >60 months (G5). Ovaries were collected individually and sent to the laboratory, where we measured AFC, the diameter and weight of the ovaries, dominant follicle (DF, ≥8mm), corpus luteum (CL), and small follicles (SF, <8mm). AFC were classified as high, intermediate-high, intermediate-low, and low. A group of ovaries was subjected to follicular aspiration to evaluate the morphological quality of the recovered cumulus oocyte complexes (COCs). Mean AFC did not vary between ages. The morphological characteristic that was most closely correlated with AFC was the weight of the small follicles. There was no relationship between AFC and the weight and diameter of the DF and CL. Quality of the COCs was superior in ovaries in which the CL was present, but it did not vary between the AFC classes. We conclude that AFC can be performed on a single ovary, regardless of the presence, diameter, and weight of the CL and DF in zebu between 12 and 60 months old.

Keywords: ovary, follicles, corpus luteum, heifers, cows

RESUMO

O presente estudo teve como objetivo avaliar a relação entre a contagem de folículos antrais (CFA) e a morfologia ovariana de Bos indicus *em diferentes faixas etárias: 12-23 meses (G1), 24-35 meses (G2), 36-47 meses (G3), 48-59 meses (G4) e >60 meses (G5). Os ovários foram coletados individualmente e enviados ao laboratório, onde suas características morfológicas foram medidas: CFA, diâmetro e peso dos ovários, do folículo dominante (FD, ≥8 mm), do corpo lúteo (CL) e dos folículos pequenos (SF, <8 mm). A CFA foi classificada como alta, intermediária-alta, intermediária-baixa e baixa. Um grupo de ovários foi submetido à aspiração folicular para avaliar a qualidade morfológica dos complexos cumulus oócitos (COCs) recuperados. A CFA média não variou entre as idades. A característica morfológica que se correlacionou mais intimamente com a CFA foi o peso dos folículos pequenos. Não houve relação entre a CFA e o peso e o diâmetro do FD e do CL. A qualidade dos COCs foi superior em ovários em que o CL estava presente, mas não variou entre as classes de CFA. Concluiu-se que a CFA pode ser realizada em um único ovário, independentemente da presença, do diâmetro e do peso do CL e do FD em zebuínos entre 12 e 60 meses de vida.*

Palavras-chave: vacas, ovário, folículos, corpo lúteo, novilhas

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INTRODUCTION

The bovine follicular population is established during the development of fetal gonads, and it is estimated that the population of preantral follicles at birth consists of an average of 235,000 (Betteridge *et al.*, 1989). The number and size of follicles present in the ovaries vary according to the stage of the estrous cycle and over time (Nascimento *et al*., 2003). Upon reaching puberty, the number of follicles present in the ovaries ranges from 12,000 to 86,000, and of these, 0.01% reach ovulation, with the rest suffering atresia. The number of antral follicles depends on the mobilization rate of the primordial follicular reserve, in addition to growth factors and pituitary gonadotropins (Campbell *et al*., 1995).

The population of antral follicles present in the ovaries can be measured using the antral follicle counting (AFC) technique. AFC has been identified as an indicator of fertility in cattle (Summers *et al*., 2019). However, differences in the relationship between AFC and fertility and between *Bos indicus* and *Bos taurus* have been demonstrated, and these differences are accompanied by differences in serum concentrations of anti-Müllerian hormone (AMH) and in the diameter of the ovary (Morotti *et al*., 2015).

Throughout the life of females, there are physiological variations of the estrous cycle, follicle, and oocyte quality, from the first estrous cycles after birth to senility (Cooper *et al.*, 1980; Matt *et al.*, 1987; De Paolo, 1988; Anzalone *et al.*, 2001). Despite the high variability in AFC between individuals, this characteristic has high individual repeatability (Silva-Santos *et al*., 2014). Patterns in AFC are well characterized in taurine cows throughout their useful life (Cushman *et al*., 2009). A better understanding of AFC variation and other ovarian morphological characteristics at different ages in zebus is needed.

Morotti *et al.* (2017) demonstrated that a lower AFC is related to greater fertility in zebu submitted to fixed time artificial insemination (FTAI) (Morotti *et al.*, 2017). The age at the last calving is highly related to the age at the first calving in Nelore heifers, and so the females with the highest number of births along the life are the most precocious (Caetano *et al.*, 2013). In this sense, the selection of more fertile heifers can impact the reproduction lifespan and consequently the economic return of the activity. So, it is very important that reliable and easily applicable tools, such as AFC, are available to select heifers of greater fertility.

For the AFC to be effectively adopted as a selection tool, it is important that all factors that may interfere with the characterization of the AFC are evaluated, so that AFC can be performed in a reliable way.

The ovary is an endocrine gland that produces steroid hormones through the follicles and the corpus luteum (CL), whose presence and morphological characteristics vary according to the stage of the estrous cycle (Saraiva *et al*., 2011). CL remains active between days 7 and 16 of the estrous cycle (Viana *et al.*, 1999), a period in which progesterone is secreted in high concentrations, inhibiting the production of follicle stimulating hormone (FSH) and luteinizing hormone (LH), responsible for the recruitment and growth of follicles (Giometti *et al.*, 2009). Therefore, the presence of CL in the ovary is believed to interfere with AFC; however, whether and how this occurs is not well understood.

So, the aim of the present study was to characterize the AFC of zebus of different ages and in ovaries with different morphological characteristics. In addition, we evaluated the quality of cumulus oocyte complexes (COCs) obtained from zebus with different classes of AFC and ovarian characteristics.

MATERIALS AND METHODS

The ovaries used in this study were collected at a local slaughterhouse between March and December 2016.

The age of each animal was identified by evaluating the carcass dentition according to the methodology proposed by Mcmanus *et al*. (2010). Each pair of ovaries was placed in a container labelled with the age. The ages of the zebus from which the ovaries were collected were classified as follows: G1: females aged 12-23 months (*n* = 82); G2: females aged 24-35 months (*n* = 74); G3 females aged 36-47 (*n* =

60); G4 females aged 48-59 months (*n* = 81); G5: females aged >60 months (G5; *n* = 83).

Ovaries were collected at different stages of the estrous cycle, and we evaluated the estrous cycle considering the size of the dominant follicle and CL characteristics in accord to Viana *et al.* (1999). Eighteen animals were in stage 3 of the estrous cycle (between days 18 and 21 after ovulation), 232 animals were in stage 2 of the estrous cycle (between days 5 and 17 after ovulation), and 61 animals were in stage one of estrous cycle (between days 1 and 4 after ovulation). In 68 animals it was not possible to identify with accuracy the stage of the estrous cycle, therefore, these animals were discarded from the analyses. For morphological evaluation, only females that were in stage 2 of the estrous cycle were used, as there was a greater number of animals.

The morphological variables evaluated included: AFC, ovarian diameter (OD), weight (WCL) and diameter of the CL after dissection, weight (WDF) and diameter (DDF) of the dominant follicle, the entire ovary weight (EOW), weight of the ovary after small and dominant follicle aspiration and/or CL dissection (partial ovary weight; POW), the weight of the fluid from small (< 8mm) follicles (WSF), and the weight of fluid from the dominant (≥ 8mm) follicle (WDF). The diameter of the dominant follicle located on the surface of the ovary was measured with the aid of a digital caliper. An analytical balance (Analítica® GX-200) was used for weighting, and the diameters were measured with the aid of a digital caliper (Profield® EDC-150). DDF

From 88 animals, after weighting, the follicular fluid was deposited in plates containing PBS plus 5% bovine fetal serum at 37 °C to search for and select COCs under a stereomicroscope (Nikon® SMZ 745 and model C-DSLS). The recovered COCs were classified according to the appearance and distribution of cumulus cells and cytoplasmic uniformity (Stojsin-Carter *et al.*, 2016). The rate of COCs morphological quality was calculated considering the number of COCs class 1 (>5 layers of cumulus cells and homogeneous cytoplasm) and class 2 (at least 3 layers of cumulus cells and homogeneous cytoplasm) divided to the total number of collected COCs. AFC groups were classified as follows: high ≥120 follicles; intermediate-high = 60-119; intermediate-low = 21-59; and low = ≤20 follicles. The AFC reflects the sum of all antral follicles seen in each ovary.

The residual normality of the data was tested using the Shapiro Wilk statistical procedure. To meet the criteria for homogeneity of variance, data were transformed [log10 (x+1) or log (x+1)] when considered appropriate. When a significant difference was found ($p \leq 0.05$), the Tukey test was applied at 5% probability. Analyses of the interaction between the AFC classes and the ovarian characteristics were performed using GLIMMIX in a factorial design of 5×4. Correlations between ovarian morphological characteristics were evaluated using Pearson's correlation. Logistic regression analysis was performed to evaluate the fixed effects of AFC on the characteristics with the highest correlation with AFC. To evaluate the relationship between the DF and AFC, logistic regression was performed with AFC and DF as fixed effects. All tests were performed using the statistical program SAS® On Demand for Academics.

RESULTS

We found that 82% (311/380) of the sampled females had a CL in one of the ovaries. The analyses in which zebus were classified according to their AFC included only females who had a CL in at least one of their ovaries; thus, only post-pubertal zebus were evaluated.

The mean AFC, weight of the dominant follicle, and diameter of the dominant follicle did not vary between animals in the different age groups. However, we found that zebus older than 60 months (G5) had a larger ovary diameter as well as a greater entire ovary weight and partial ovary weight. The average weight of small follicles was higher in zebus aged 36-47 months. The weight and diameter of CL did not vary between zebus in different ages (Table 1).

Table 1. Antral follicle count (± standard error) and morphological characteristics of the ovaries of zebus in different age groups

	G1 (n=53)	G2 (n=60)	G3 (n=54)	G4 (n=69)	G5 (n=76)	P-value
Antral follicle count	49.16±4.3	54.3±4.1	60.14±4.3	49.96±0.3	56.32±0.3	0.2799
Entire ovarian weight (g)	6.19±0.4b	6.24±0.4b	6.99±0.4b	7.24±0.4b	8.67±0.4a	<.0001
Ovarian diameter (mm)	23.15±0.6b	24.81±0.6b	24.78±0.6b	23.45±0.5b	27.26±0.5a	<.0001
Partial ovarian weight (g)	3.95±0.4b	3.95±0.4b	4.38±0.4b	4.85±0.3ab	5.97±0.3a	<.0001
Weight of dominant follicle follicular fluid (g)	0.57±0.1	0.68±0.01	0.76±0.1	0.75±0.1	0.88±0.1	0.4048
Diameter of the dominant follicle(mm)	10.13±0.4	10.82±0.4	9.68±0.5	9.75±0.4	10.26±0.4	0.5738
Weight of small follicles follicular fluid (g)	0.59±0.1b	0.64±0.0b	0.68±0.0b	0.75±0.0ab	0.93±0.0a	0.0036
Weight of corpus luteum (g)	2.66±0.1	2.27±0.1	2.75±0.1	2.83±0.1	3.05±0.1	0.0893
Diameter of corpus luteum (mm)	18.31±0.5	18.57±0.4	18.49±0.5	19.41±0.4	18.98±0.4	0.1858

Age groups: G1: 12-23 months; G2: 24-35 months; G3: 36-47 months; G4: 48-59 months; G5: >60 months). Different lower-case letters in the same line indicate statistically significant differences at 5% probability using the Tukey test. Data are shown as mean values.

Most females had intermediate-low AFCs (197/380, 51.84%) or intermediate-high AFCs (125/380, 32.89%), whereas 10% (38/380) had low AFCs, and only 5.26% (20/380) had high AFCs.

The diameter and weight of the whole and partial ovary, as well as the weight of dominant and small follicles are greater in females with high antral follicle count. On the other hand, the diameter of the dominant follicle and the corpus luteum diameter and weight did not show significant differences between the AFC groups (Table 2).

Table 2. Antral follicle count-AFC (± standard error) and ovarian morphological characteristics of zebu ovaries according to AFC classification (high = ≥120, intermediate-high = 60-119, intermediate low = 21–-59, and low = ≤20 follicles)

	Antral Follicle Count Classification				
	High (n=14)	Intermediate-High (n=95)	Intermediate-Low (n=168)	Low (n=35)	P-value
Antral follicle count	144.83±3.5a	80.39±1.3b	40.60±1c	14.60±2.1d	<.0001
Entire ovarian weight (g)	11.19±0.8a	8.35±0.3b	6.18±0.2c	7.14±0.5bc	<.0001
Partial ovarian weight (g)	8.06±0.7a	5.59±0.4b	3.94±0.2c	4.72±0.5bc	<.0001
Ovarian diameter (mm)	29.40±1.2a	26.24±0.4ab	23.74±0.3c	25.10±0.3bc	<.0001
Dominant follicle diameter (mm)	9.89±1.2	9.48±0.4	10.34±0.3	10.98±0.6	0.3321
Weight of dominant follicle follicular fluid (g)	2.17±0.2a	0.78±0.1ab	0.65±0.1b	0.72±0.0b	<.0001
Weight of small follicles follicular fluid (g)	1.36±0.1a	0.99±0.0b	0.52±0.0c	0.53±0.0c	<.0001
Weight of corpus luteum (g)	2.89±0.3	2.84±0.1	2.76±0.1	2.83±0.02	0.9292
Diameter of corpus luteum (mm)	18.61±1.0	18.88±0.4	18.77±0.3	19.15±0.6	0.9002

Different lower-case letters in the same line indicate statistically significant differences at 5% probability using the Tukey test. Data are shown as mean values.

We found that AFCs did not differ between ovaries with or without the CL, neither did the partial ovary weight, nor the weight of small and dominant follicles. As expected, the entire ovary weight and the ovary diameter were higher in the ovaries that had the CL. The DF was more frequently located in the ovary ipsilateral to the CL; however, the weight and diameter of DF did not vary between the ovaries with or without the CL (Table 3).

Table 3. Morphological characteristics of the ovaries and morphological quality of the cumulus oocyte complexes-COCs (± standard error) according to the location of the corpus luteum (CL, ipsilateral or contralateral)

Means	Ipsilateral CL	Contralateral CL	*P*-value
AFC	27.75±0.9	26.11±0.9	0.2211
Entire ovary weight (g)	8.04±0.2a	5.83±0.2b	<0.0001
Partial ovary weight (g)	4.58±0.2	4.77±0.2	0.4172
Ovarian diameter (mm)	25.63±0.3a	23.38±0.3b	<0.0001
Small follicles weight (g)	0.69±0.3	0.73±0.3	0.3246
Dominant follicle diameter (mm)	10.36	10.35	0.9847
Dominant follicle weight (g)	0.75±0.06	0.74±0.07	0.9432
Presence of dominant follicle (%)	28.44±0.13a(66/232)	21.55±0.14b(50/232)	-
Morphological quality COCs (%)	83.95a(272/324)	78.57b(132/168)	-

AFC: Antral follicle count. Different lower-case letters in the same line indicate statistically significant differences at a 5% probability using the Tukey test. Data are shown as mean ± standard error.

The quality of the COCs was superior in the ovaries with the CL (Table 3) but did not vary between animals with different AFCs (High: 85.3%; Intermediate-high: 74.8%; Intermediate-low: 71.9%; low: 71.7%; $P = 0.451$).

The AFC was highly positively correlated with the weight of small and dominant follicles. As well as the entire ovary weight and the weight of CL also present a very significant correlation. A weak positive correlation was observed between AFC and the weight of dominant follicle and ovary diameter. A weak negative correlation was found between AFC and the diameter of the dominant follicle. The weight and diameter of the CL were not correlated with the AFC. The only relationship of age with any morphological feature of the ovaries is a weak correlation between the weight of the ovary and the age of the animals. (Table 4).

Table 4. Correlations between the morphological characteristics of zebu ovaries

	Antral follicle count	Age	Ovarian diameter (mm)	Weight of dominant follicle	Weightof small follicles follicular fluid	Weight of corpus luteum	Entire ovarian weight
Antral follicle count	1.00000	0.00943	0.32992**	0.15527*	0.67201**	0.02250	-0.00608
Age		1.00000	-0.05281	0.18364	0.09945	0.16962*	0.12279**
Ovarian diameter			1.00000	0.20827*	-0.10818	-0.00280	0.10976
Weight of dominant follicle				1.00000	0.17701	0.02769	0.14190
Weight of small follicles follicular fluid					1.00000	0.10080	0.11848
Weight of corpus luteum						1.00000	0.40047**
Entire ovarian weight							1.00000

$^*P < 0.05$; $^{**}P < 0.01$

The logistic regression coefficient indicated that the weight of small follicles, a variable that was highly correlated with AFC, may explain 44% of the AFC variability. The ovary diameter, the characteristic with the second highest correlation with AFC, explained only 8% of the variability of AFC (Fig. 1).

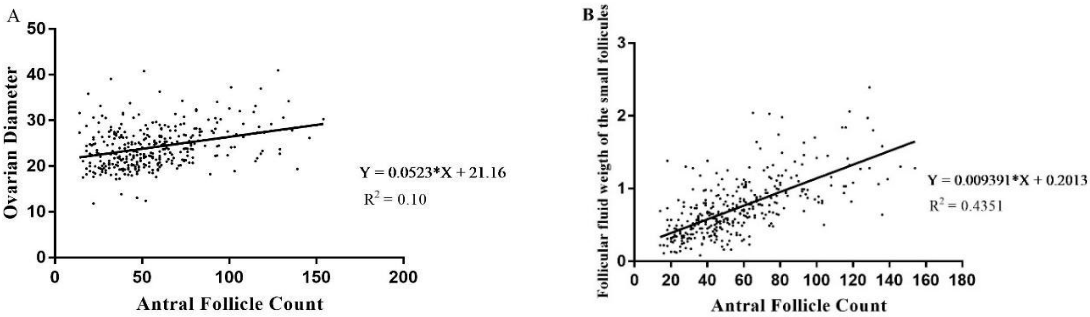

Figure 1. Prediction of variation in antral follicle count (AFC) as a function of follicular fluid weight of small follicles (a) and ovarian diameter (b).

DISCUSSION

In the present study we evaluated the influence of age and morphological characteristics of the ovary of zebu females on Antral Follicle Count, with the objective of identifying some bottlenecks in this technique that could interfere with its efficiency. For that we studied ovaries from zebus aged 12 to >60 months (based on evaluation of the tooth eruption at the time of slaughter). When we evaluated the effect of age on ovarian characteristics, all animals were included in the analysis, regardless of estrous cycle stage. Our results indicate that there was no difference in AFC between the age groups evaluated. Similar results were demonstrated in the Tabapuã cattle breed (*B. indicus*), in which the AFC reduction was observed at 16 years old (Maculan *et al*., 2017). In crossbred bovine females, AFC increased between 1 and 5 years, and then gradually decreased (Cushman *et al.*, 2009). The difference observed between breeds emphasizes the need for knowledge of reproductive characteristics in a specific way. In addition, it is important to consider that Nelore females remain in the herd on average 75.5 months (Bertazzo *et al*., 2004), therefore we believe that the old animals studied in the present study were not older than 75 months, thus comprising the average age range of zebu breeding cows. On the other hand, only 58% (29/82) females in G1 (12 to 23 months) showed a CL and were considered pubertal. In general, zebu females enter puberty at an age above 24 months, but with the association of genetic selection and adequate nutrition it is possible to significantly reduce the age of entry into reproduction (Ferraz *et al*., 2018). So, G1 consisted of pubertal and prepubertal females. Our results showed that zebu heifers seem to have stable AFCs also during peri puberty. Corroborating our data, Silva-Santos *et al.* (2014) found no variation in AFC between weanling and yearling crossbred (*B. indicus* × *B. taurus*) donors, and the authors suggested that the classification of AFC could be performed in prepubertal heifers to select donors capable of producing a larger number of embryos.

Thus, we can state that during bovine female reproductive life AFC doesn't change and can be used as a selection tool for the demand required according to age, for example, the choice of semen of higher value or the choice of replacement females.

Since no variation in AFC between ages was observed in the present study, we chose to evaluate ovarian characteristics as a function of AFC independent of age, in order to verify the best way to perform the AFC. For such analyses only females identified at stage 2 of the estrous cycle were evaluated.

Around puberty in cattle, there is a pronounced increase in the size of the reproductive tract, especially in the ovaries (Honaramooz *et al*., 2004), due to the greater release of gonadotropin-releasing hormone (GnRH) by the hypothalamus, which in turn stimulates the pituitary to produce gonadotropins (FSH and LH), which stimulate follicles to produce estradiol and increase the number and size of ovarian follicles (Fortune, 1994). In the present study, ovaries in zebus over 60 months were significantly heavier and greater in diameter. Corroborating our study, it was demonstrated that Nelore cows between 3 and 7 years old had larger ovaries than 2-years-old heifers (Monteiro *et al.*, 2008). The authors also showed that the size of the ovary varies in different reproductive stages (Nascimento *et al*., 2003), especially because of the presence of the CL (Murasawa *et al*., 2005). These studies corroborate ours that demonstrated that the ovary diameter was higher in ovaries with CL and explains why at this stage of the study we chose to evaluate animals at the same stage of the estrous cycle.

The ovary diameter (OD) was higher in females with high AFC, as previously observed for taurine females (Ireland *et al*., 2008) and zebu females (Rodrigues *et al*., 2015; Morotti *et al.*, 2018). The results of the present study demonstrate that there was a positive correlation between AFC and OD, and especially between AFC and weight of small follicles. Similar results, demonstrating the high correlation between small (<5mm) and medium (5-8 mm) follicles with the OD have been previously reported for taurine females (Murasawa *et al.*, 2005; Ireland *et al*., 2008).

We found that AFC did not vary among ovaries that had a dominant follicle >8 mm, as also seen in taurine females (Silva-Santos *et al*., 2014). It has been demonstrated that the diameter of the dominant follicle in Nelore cows, on the day of progesterone implant removal, was higher in females with low AFC (Morotti *et al*., 2018). In the present study only ovaries of females in the luteal phase of the estrous cycle were evaluated, that means the dominant follicle evaluated was not an ovulatory one. So, our results cannot be directly compared to the study by Morotti *et al*. (2018), who evaluated the preovulatory follicular diameter. However, both information are complementary: during the lutein phase, which comprises the longest period of the estrous cycle, the presence of DF does not interfere in the AFC, but females with lower AFC have greater pre-ovulatory follicles that may justify the higher fertility of these animals.

We found that AFC did not vary among ovaries with or without the CL. This contrasts with results found by Rodrigues *et al*. (2015) in Tabapuã zebus, but similar to results found by Domínguez (1995), who, as in the present study, evaluated ovaries collected from a slaughterhouse. Corroborating the results of this study, Barbosa *et al*. (2013) demonstrated that there was no variation in oocytes aspirated *in vivo* from Nelore cows between ovaries with and without the CL, regardless of whether the CL came from a cycling or pregnant cow. The authors noted, however, that the total number of COCs aspirated from cyclic cows was higher than those obtained from non-cycling donors.

Our results showed a higher incidence of DF in the ovary with the CL than in the ovary contralateral to the CL, although there was no variation in the weight and diameter of the DF between the ovaries. In Addition, the COCs collected from ovaries, which had the CL, were of higher quality than COCs collected from ovaries without the CL. These results corroborate those of Manjunatha *et al.* (2007), who demonstrated that ovaries with the CL produce better quality COCs with greater cleavage capacity and production of blastocysts. Karami Shabankareh *et al.* (2015) found that oocytes from small follicles (3-6mm) of Holstein cows showed less developmental competence up to the blastocyst stage when obtained from ovaries with the CL, whereas the competence of larger follicles (10-20mm) was higher in ovaries with the CL. A paracrine effect of the presence of the CL may be responsible for the differences described, since the biochemical composition of the follicular fluid varies between small, intermediate, and large follicles in the ovary with the CL, but not in the ovary contralateral to the CL (Karami Shabankareh *et al.*, 2013). However, further studies are needed to clarify the paracrine effect of the CL on follicular development and oocyte competence.

CONCLUSIONS

AFC does not change from heifers aged 12 – 24 months and around 60 months of age of *Bos indicus*. The ovarian characteristics found to be most closely related to AFC were the weight of small follicles and ovary diameter. The diameter of the dominant follicle during the luteal phase was found to be similar for all classes of AFC. It is possible to classify AFC in only one ovary, regardless of the presence of the CL or DF, which makes the application of AFC as an evaluation tool more flexible and minimizes the stress to the cows. The quality of the COCs was superior in the ovaries with the CL but did not vary between the AFC classes.

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