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Brangus cows have ovarian reserve parameters more like Brahman than Angus cows*

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ABSTRACT

Bos indicus females have more surface antral follicles than *Bos taurus* females; however, histological studies demonstrated no difference in total number of primordial follicles between these two biological types of cattle. Primordial follicle density in the ovary was less in Nelore ovaries compared to Angus ovaries, but no studies have examined the primordial follicle density in *Bos indicus* cross-bred females. It, therefore, was hypothesized that primordial follicle density in the ovary would decrease as percentage *Bos indicus* increased. Ovaries were collected from cross-bred Angus (n = 32, no *Bos indicus* influence), Brangus (n = 15), or Brahman (n = 9) cows and prepared for histological evaluation. There was no difference in total number of primordial follicles per ovary between breeds ($P > 0.10$). When numbers of primordial follicles were expressed on a per gram of ovarian tissue basis, there were fewer primordial follicles per gram of ovarian tissue in Brangus and Brahman cows than in Angus cows ($P < 0.05$). Brangus cows did not differ from Brahman cows in primordial follicle density ($P > 0.10$). Differences in primordial follicle density could indicate differences in capacity of ovarian stroma to produce factors necessary for oogonial proliferation and primordial follicle formation among breeds. Identifying these factors could improve the aprroach for culturing pre-antral follicles of cattle. Furthermore, these results explain why ultrasonographic antral follicle counts may need to be adjusted to a greater threshold

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to predict size of the ovarian reserve and determine ovarian reserve related reproductive traits in *Bos indicus* females.

1. Introduction

During the last several years, there has been a focus on the ovarian reserve parameters of *Bos indicus* females. While *Bos indicus* females have greater numbers of antral follicles than *Bos taurus* contemporaries (Segerson et al., 1984; Alvarez et al., 2000; Batista et al., 2014), no differences in total numbers of microscopic pre-antral follicles (e.g., primordial, primary, and secondary follicles) per ovary were reported in a series of histological studies (Silva-Santos et al., 2011, 2014). This raised questions as to how ovarian ultrasonography can be applied to determine ovarian reserve parameters in *Bos indicus* females. Results of recent studies indicate there are beneficial relationships between ultrasonographic antral follicle count and post-partum reproductive performance in *Bos indicus* influenced females, such that as antral follicle number increased, both post-partum interval (Quail et al., 2018) and re-breeding interval (Maculan et al., 2018) decreased. To detect response differences in *Bos indicus* females, investigators had to use regression analysis or set thresholds at a greater level than that typically set for *Bos taurus* females. This implies a larger number of antral follicles are required to predict beneficial ovarian reserve parameters in *Bos indicus* females.

The total number of microscopic follicles in the ovaries is not the only method to measure ovarian reserve parameters histologically. Differences in the number of pre-antral follicles per gram of ovarian tissue have been reported in cattle differing in antral follicle number (Ireland et al., 2008; Modina et al., 2014), suggesting an actual difference in tissue density of follicles. Results of a recent study indicated there was a difference in primordial follicle density per unit of area when comparing ovaries of Nelore heifers to ovaries of Angus heifers. Nelore heifers had fewer primordial follicles per equivalent area of ovarian tissue (Favoreto et al., 2019). This could explain why a greater threshold in ultrasonographic antral follicle count is required in *Bos indicus* females for there to be beneficial effects on reproductive traits.

In the United States, a popular strategy for *Bos indicus* germplasm has been to develop composites that contain some percentage of *Bos indicus* influence, such as the Brangus (3/8 Brahman), Beefmaster (4/8 Brahman), and Santa Gertrudis (3/8 Brahman) breeds of cattle. Crossing Brahman with *Bos taurus* cattle results in animals that have a greater adaptation capacity for the climate of the southern regions of the United States while retaining carcass quality and reproduction advantages of *Bos taurus* cattle. To our knowledge, however, there are no studies examining ovarian reserve parameters in *Bos indicus* crosses. We hypothesized, therefore, that as the percentage of *Bos indicus* breed type decreased in these Brahman crossbred cattle that primordial follicle density in the ovary would increase. To test this hypothesis, we compared ovarian reserve parameters between Brahman, Brangus, and Angus crossbred cattle. Brangus cattle were included in the present study because these were animals with a *Bos indicus* genotype with a lesser percentage of Brahman breeding and a large breed composition similarity with Angus cattle.

2. Materials and methods

2.1. Collection and processing of ovaries

All procedures were approved by the New Mexico State University and South Dakota State University Animal Care and Use Committees in accordance with the FASS guidelines for the care and use of agricultural animals in research. Ovaries were collected from Angus cross-bred (n = 32, no *Bos indicus* influence) in South Dakota, and from a stabilized line of Brangus (n = 15) or Brahman (n = 9) cows in New Mexico at slaughter or by flank laparotomy (Summers et al., 2014). All were mature cows (> 2 y); however, most of the Angus cross-bred cows were purchased and the exact age was, therefore, unknown. The same was true of the pedigree of these purchased animals but most would have been either Angus or an Angus Hereford F1 crossbred cows with no *Bos indicus* breeding. Immediately upon collection, the ovary contralateral to the CL was weighed and measured, and all surface antral follicles (≥1 mm) were counted. A representative cross section from the middle of the ovary perpendicular to the length of the ovary (˜5 mm) was fixed in 4% paraformaldehyde overnight as described previously (Amundson et al., 2015; Tenley et al., 2019). The next day the fixed tissues were dehydrated through graded ethanol concentrations and embedded in paraffin for histological evaluation. Paraffin blocks were sectioned at 6 μm thickness and five sections were collected on slides to determine microscopic follicle numbers. Between each section that was collected, at least ten sections were discarded to ensure that the same follicles were not counted twice in subsequent sections. Furthermore, follicles were only counted if the nucleus of the oocyte was present. The combination of a 60-micrometer distance between each section counted and only counting follicles when the nucleus of the oocyte is present eliminates the possibility of counting the same secondary follicle twice. Sections were then stained with hematoxylin and eosin.

2.2. Histological evaluation

Pre-antral follicles were classified as described previously (van Wezel and Rodgers, 1996; Fortune et al., 2000; Cushman et al., 2001). Primordial follicles were defined as an oocyte surrounded by a single layer of squamous pre-granulosa cells. These were prolate with an estimated maximum to minimum diameter ratio of approximately 1.3 as described previously (van Wezel and Rodgers, 1996). A follicle with one or more cuboidal granulosa cells up to a single layer of cuboidal granulosa cells surrounding the oocyte was classified as a primary follicle. Secondary follicles were an oocyte surrounded with two or more layers of cuboidal

granulosa cells without an antrum. The technician performing the histological evaluations did not know the breed associated with each set of slides.

2.3. Statistical analyses

The average number of primordial, primary, and secondary follicles per section was determined by taking the average of the five sections within a cow. The length of the ovary in micrometers was divided by six (the thickness of the sections) to determine the correction factor, and the average number of follicles per section within each class within each cow was multiplied by that cow's unique correction factor to estimate the number of primordial, primary, and secondary follicles per ovary. To determine the number of follicles per gram of tissue, the number of follicles per ovary within a follicle class was divided by the total weight of the ovary in grams (Ireland et al., 2008; Modina et al., 2014). To determine number of follicles per mm^2 of tissue, the area of the section was determined and the number of follicles per section was divided by the area of the section (Tenley et al., 2019). Data were analyzed using the GLM procedure of SAS with breed (Angus, Brangus, or Brahman) as a class effect. Differences were considered significant when $P \leq 0.05$ and a tendency when $P > 0.05$ and ≤ 0.1. Data are presented as the mean ± S.E.M.

3. Results

As has been reported previously, the total number of surface antral follicles counted when the ovary was collected differed by breed with ovaries from Angus cows having fewer antral follicles than ovaries from Brangus or Brahman cows ($P < 0.0001$; Fig. 1). There was no difference in the number of surface antral follicles per ovary between Brangus or Brahman cows ($P > 0.10$). In contrast to antral follicle numbers, there was no effect of breed on total number of primordial follicles per ovary between ovaries from Angus, Brangus, or Brahman cows ($P > 0.10$; Fig. 2). Similarly, there was no difference in total number of primary follicles per ovary ($P > 0.10$; 11,646.0 ± 2,772.2, 21,187.0 ± 4211.2, and 11,487.0 ± 5,368.3 for Angus, Brangus, and Brahman, respectively) or total number of secondary follicles per ovary ($P > 0.10$; 4,403.8 ± 921.8, 5,821.6 ± 1400.2, and 4,248.8 ± 1,785.0 for Angus, Brangus, and Brahman, respectively) among the three breeds.

Ovaries from Brangus and Brahman cows weighed more and were longer than ovaries from Angus cows ($P < 0.01$; Table 1). There was no difference in the ovarian height when comparing Angus cows to Brangus and Brahman cows ($P = 0.14$).

When results were expressed on a per gram of ovarian tissue basis, there were marked differences in primordial follicle number. The number of primordial follicles per gram of ovarian tissue was less in Brangus and Brahman ovaries compared to Angus ovaries ($P < 0.05$; Fig. 3). There was no difference in number of primary follicles per gram of ovarian tissue ($P > 0.10$; 1,957.0 ± 407.7, 2,231.0 ± 619.3, and 1,673.0 ± 789.4 for Angus, Brangus, and Brahman, respectively) or number of secondary follicles per gram of ovarian tissue ($P > 0.10$; 675.6 ± 134.1, 656.7 ± 203.7, and 550.0 ± 259.7 for Angus, Brangus, and Brahman, respectively) due to breed.

There were similar results when data were expressed on a per mm^2 of ovarian tissue basis. Number of primordial follicles per mm^2 of ovarian tissue was less in the Brangus and Brahman ovaries compared to Angus ovaries ($P < 0.05$; Fig. 4). There was no difference in number of primary follicles per mm^2 of ovarian tissue ($P > 0.10$; 0.01 ± 0.002, 0.01 ± 0.003, and 0.01 ± 0.004 for Angus, Brangus, and Brahman, respectively) or number of secondary follicles per mm^2 of ovarian tissue ($P > 0.10$; 0.003 ± 0.001, 0.003 ± 0.001, and 0.002 ± 0.001 for Angus, Brangus, and Brahman, respectively) due to breed.

4. Discussion

Inconsistent with our hypothesis, Brangus cows did not have an ovarian morphology that was intermediate to Angus and Brahman cows. Brangus cows were, in fact, very similar in ovarian morphology to Brahman cows, although Brangus are only 3/8 Brahman. The finding that there was a lesser primordial follicle density in ovaries of Brahman and Brangus cows is consistent with results reported for Nelore cows (Favoreto et al., 2019) and provides biological support for the concept that greater numbers of antral follicles detectable by ultrasonography are required to predict increased ovarian reserves in cattle with *Bos indicus* breeding (Quail et al.,

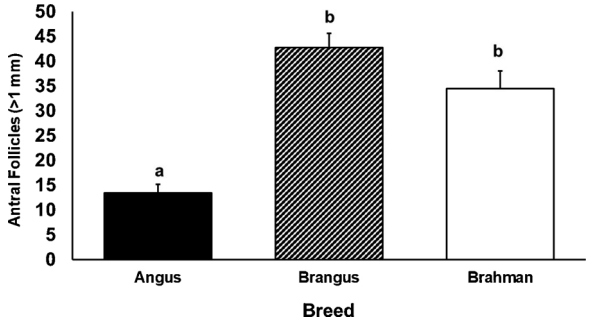

Fig. 1. Number of surface antral follicles (≥1 mm) in the ovaries of Angus, Brangus, or Brahman cows; Angus cows had fewer antral follicles per ovary than Brangus or Brahman cows ($P < 0.0001$); Data are presented as the mean ± S. E. M.

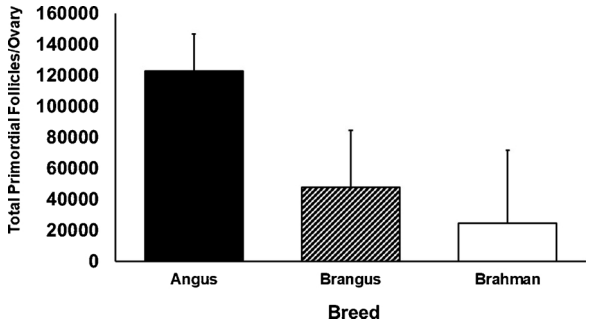

Fig. 2. Total number of primordial follicles in the ovaries of Angus, Brangus, or Brahman cows; Breed did not affect the total number of primordial follicles ($P > 0.10$); Data are presented as the mean ± S. E. M.

Table 1
Effect of biological type on ovarian phenotype in Angus, Brangus, and Brahman cows (mean ± S.E.M.).

Ovarian Phenotype	Biological Type			*P*-value
	Angus	Brangus	Brahman	
N	32	15	9	–
Ovarian Weight, g	7.0 ± 0.6[a]	10.1 ± 0.9[b]	11.5 ± 1.1[b]	0.0006
Ovarian Length, mm	29.4 ± 0.9[a]	32.7 ± 1.3[b]	35.4 ± 1.8[b]	0.005
Ovarian Height, mm	19.7 ± 0.6	20.7 ± 1.0	20.3 ± 1.3	0.65

[ab]Within a row, means with different superscript are different.

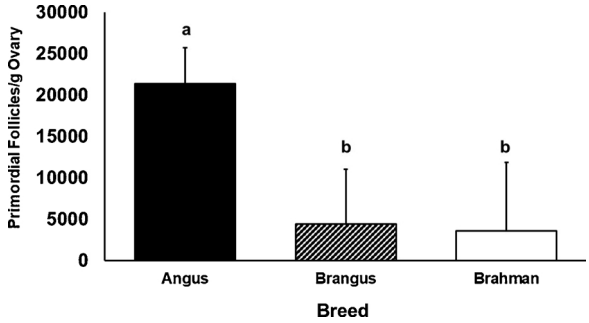

Fig. 3. Number of primordial follicles per gram of ovarian tissue in Angus, Brangus, or Brahman cows; Angus cows had more primordial follicles per gram of ovarian tissue than Brangus or Brahman cows ($P < 0.05$); Data are presented as the mean ± S. E. M.

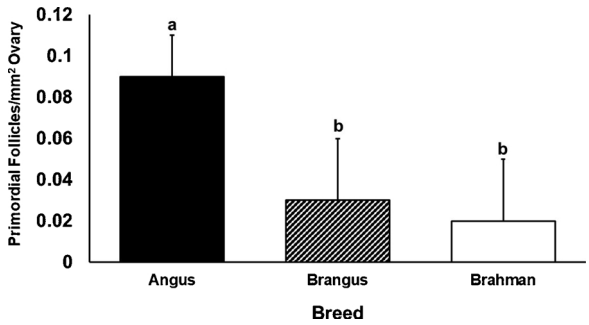

Fig. 4. Number of primordial follicles per mm^2 of ovarian tissue in Angus, Brangus, or Brahman cows; Angus cows had more primordial follicles per mm^2 of ovarian tissue than Brangus or Brahman cows ($P < 0.05$); Data are presented as the mean ± S. E. M.

2018).

While the environment differed for the two biological types in the current study and while the exact age of all the cows was not known, it should be kept in mind that the results between the Angus and the Brahman cows were similar to those described by Favoreto et al. (2019), comparing Angus and Nelore heifers managed in the same environment. Angus and Brahman cows can,

therefore, be considered the controls to which the Brangus cows were compared in the current study. The findings in the present study indicate the difference between *Bos taurus* and *Bos indicus* ovarian phenotype is regulated to a greater extent by genetic mechanisms than by environmental factors. An interesting question is whether development of the Brangus ovaries would be similar to that of Angus ovaries if the animals were located and developed in the South Dakota environment. While we cannot currently answer this question directly, ultrasonographic phenotypes of Brangus-sired heifers are different from Angus-sired heifers and similar to Brahman-sired heifers in the Germplasm Evaluation population at the U.S. Meat Animal Research Center (Cushman unpublished data). Thus, it is concluded that the mechanism that leads to these differences between *Bos taurus* and *Bos indicus* ovaries are still very active in Brangus females and are not greatly affected by environment or age.

The lesser primordial follicle density in *Bos indicus* females leads to asking the question of why ovarian stroma in *Bos indicus* females cannot support greater numbers of germ cells to a density per gram of ovarian tissue comparable to *Bos taurus* females. In mice, primordial follicles have been isolated in culture and grown to a stage where oocytes could be harvested and used to produce live progeny (Eppig and O'Brien, 1996). In cattle, it has not been possible to isolate primordial or primary follicles and successfully grow these follicles in culture systems (Wandji et al., 1996a). These follicles in the earliest stages of development will only grow in culture when contained within ovarian cortex (Wandji et al., 1996b; Fortune et al., 2000). This implies that the ovarian cortex is producing factors that are necessary to support oogonial proliferation and formation of primordial follicles. The lack of capacity of *Bos indicus* ovaries to support primordial follicles at a similar tissue density as *Bos taurus* ovaries implies that there may be differences in the function of the ovarian tissue between the two biological types of cattle.

In future studies, it will be important to examine biological differences between the ovarian stroma of *Bos taurus* and *Bos indicus* cattle. Transcriptomic or proteomic analyses contrasting *Bos indicus* and *Bos taurus* ovarian stroma during development might provide insight that would help elucidate biological pathways involved in development of the ovary, establishment of the ovarian reserve, and pre-antral follicle development in cattle. Identifying these pathways could improve on the capacity to grow pre-antral follicles of cattle in culture, which would allow for greater propagation of the genetics of cows with superior genetic merit of all biological types. Understanding these differences may also aid in improving reproductive management of *Bos indicus* cattle, because these cows respond differently to estrous synchronization and ovulation induction protocols (Scarpa et al., 2019).

Overall, there is still inconsistency of thought about the use of antral follicle counts of cattle to predict fertility. Starbuck-Clemmer et al. (2007) reported there was no relationship between antral follicle number and fertility in Angus cows in the first study to examine the phenotype in cattle. Since then, results of studies have been reported indicating both positive and negative effects of increased numbers of antral follicles detectable by ultrasonography on fertility (Mossa et al., 2012; McNeel and Cushman, 2015; Morotti et al., 2018) and reproductive longevity (Jimenez-Krassel et al., 2017) in cattle. Interestingly, with two studies there were reports of antagonistic genomic relationships between antral follicle number and heifer pregnancy rate in *Bos taurus* (Snelling et al., 2012) and *Bos indicus* (Oliveira et al., 2017) females. One conclusion that could be drawn from these results is that there still is not enough known about the basic mechanisms involved to consistently and reliably use the number of antral follicles detected by ultrasonography as a diagnostic technique for fertility in cattle.

There is some evidence that antral follicle number may be positively associated with fertility traits in *Bos indicus* females. Cavalieri et al. (2018) reported that conception to artificial insemination was less in *Bos indicus* females with very low (≤ 4) numbers of antral follicles. This is an extremely low threshold and would be within the range of what has been reported for *Bos taurus* females with poor fertility, so it does not support the need for a greater threshold in *Bos indicus* females. Results of two studies indicate there is an improved post-partum reproductive performance in *Bos indicus* females with larger numbers of antral follicles (Maculan et al., 2018; Quail et al., 2018). The mechanisms contributing to differences in fertility remain unclear but may be due to increased circulating concentrations of progesterone in cattle with larger numbers of follicles (Jimenez-Krassel et al., 2009; Santa Cruz et al., 2018). These increased concentrations of progesterone could be related to shorter post-partum interval to estrus (Quail et al., 2018) and could result in an improved uterine environment (McNeel et al., 2017; Fontes et al., 2018) that is more suitable for supporting early embryonic development.

While there are no reports comparing uterine luminal proteins in *Bos indicus* females differing in follicle numbers, results of a recent study indicated there was a differential transcript abundance and greater protein concentrations in the oviduct ipsilateral to the CL in *Bos indicus* females with increased numbers of antral follicles compared to *Bos indicus* females with lesser numbers of antral follicles (Fontes et al., 2018). These results may suggest that an increased number of antral follicles could be indicative of a uterine environment that is more supportive of early embryonic development in *Bos indicus* females, as reported in *Bos taurus* females (McNeel et al., 2017). Future research will need to be conducted in which there is examination of uterine luminal protein concentrations in *Bos indicus* females with differing follicle numbers and its relationship with primordial follicle density in the ovary will be necessary to elucidate the possible effects of uterine proteins on follicle numbers.

In conclusion, the results of the current study indicate that, similar to Nelore females, Brahman cows have a lesser primordial follicle density in their ovaries compared to Angus cows and, perhaps more interestingly, that Brangus cows were similar to Brahman cows in primordial follicle density. Inconsistent with our hypothesis, decreasing of the percentage of *Bos indicus* breeding to 37.5% in the Brangus did not lead to an increase the primordial follicle density in ovarian tissue. This finding suggests that the mechanisms affecting oogonial proliferation or primordial follicle formation are present in cattle with a relatively small percentage of *Bos indicus* breeding. Further research is necessary to enhance the understanding of how this relates to ovarian function, uterine protein concentrations, fertility, and reproductive longevity in females with *Bos indicus* breeding.

Declaration of Competing Interest

The authors have no conflicts of interest to report.

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