Preantral Follicle Numbers and Size in Heifers Carrying the Bovine High Fecundity Allele Trio

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ABSTRACT

The bovine high fecundity allele, Trio, results in overexpression of SMAD6 and a 3-fold increase in ovulation rate compared to wild-type non-carrier cattle. In addition, Trio carriers have similar number of antral follicles as non-carrier cattle; however, antral follicles develop at a slower growth rate and acquire ovulatory capacity at a smaller size. The present study was designed to test the following hypotheses: 1) Trio carrier cattle have similar preantral follicle numbers as non-carriers; and 2) preantral follicles of Trio carriers are smaller in size than non-carriers. Ovarian tissue from Trio carrier (n = 12) and non-carrier (n = 12) heifers were obtained by laparotomy and a 1 x 1 cm section was fixed and paraffin embedded. Sixty consecutive sections (6 μm) were obtained and every tenth (6 total) mounted and stained with hematoxylin-eosin. Follicle numbers were determined for each stage of development (primordial, primary, and secondary) using a 5 x 5 grid overlay. Follicle dimensions were determined from 10 random follicles of each follicle stage and heifer using ImageJ. Differences between genotypes were assessed by t-test or Wilcoxon's rank test. Number of primordial and secondary follicles were not different between genotypes ($P > 0.1$). Trio carriers had greater ($P < 0.01$) number of primary follicles per square mm (0.27 ± 0.04) than non-carriers (0.11 ± 0.01). Primordial follicle and oocyte volume were not different between genotypes ($P > 0.2$). Primary follicle and oocyte volume were 1.2-fold and 1.1-fold larger in Trio carrier than non-carriers ($P < 0.05$). Secondary follicle volume was not different ($P > 0.5$), however, oocyte volume was 1.8-fold larger in Trio carriers than non-carriers ($P < 0.03$). Granulosa cell number per cross section was not different between genotypes at any stage ($P > 0.4$). In conclusion, follicle numbers were similar for most preantral follicle stages, however, Trio carriers had greater number of primary follicles. In addition, primary follicle and oocyte and secondary oocyte size were greater in Trio carriers. These results suggest that, once activated, Trio carrier follicles, have reduced progression through the primary stage, hence the larger oocyte and greater number.

INTRODUCTION

A novel high fecundity allele in cattle, known as Trio, has been recently identified and linked to an increase in ovulation rate. The Trio allele was discovered after a cow named Treble gave birth to three sets of triplets, an extremely rare occurrence in cattle. Only about 1% of cattle births are twins, with significant variation between breed and across geographical regions, with triplets occurring at even lower rates [1]. One of Treble's sons, Trio, sired daughters with twin and triple calvings, indicating the transmission of a genetic factor with a significant contribution to prolificacy. Further studies using 131 half sibs sired by Trio at the University of Wisconsin-Madison, provided strong evidence for segregation of a major gene affecting ovulation rate in bovine chromosome 10 [2]. While multiple ovulations and multiple births are often considered undesirable in cattle due to numerous risks such as an increased risk of abortion, retained placenta, and less milk production [3], the knowledge gained from studying its causes can provide valuable information on follicle growth and selection of a single follicle in monovular species.

Follicular selection is a biological process that occurs in the female ovary where a single follicle is selected from a group of growing follicles and allowed the capacity to ovulate [12]. It has been observed that cattle that are heterozygous for the Trio allele have greater ovulation rate and smaller sized ovulatory follicles than wild-type cattle. Among cattle that are non-carriers of the Trio allele, there is on average one ovulation per ovulatory period, while cattle that are Trio carriers have an average of three to four ovulations per ovulatory period [4]. Furthermore, preovulatory follicles of non-carriers are approximately 4 times larger, on a volume basis, than preovulatory follicles in their Trio counterparts [12]. In addition, Trio carriers have an increase in circulating follicle-stimulating hormone encompassing the time of follicular deviation [4] but have similar antral follicle counts and circulating anti-Müllerian hormone levels [8]. The Trio allele also has been linked to increased expression of the protein SMAD6, which inhibits the bone morphogenetic protein 15 (BMP15) and growth and differentiation factor 9 (GDF9) pathways. Both, BMP15 and GDF9, members of the TGF-β superfamily, have been shown to play an important role in granulosa cell proliferation [12]. In that regards, there are multiple mutations in the BMP15 and GDF9 pathways identified in sheep that lead to the occurrence of multiple ovulations [12]. Currently there are two proposed models to explain the occurrence of multiple ovulations in cattle. The one model proposes that the underlying mechanism responsible for these

phenomena is an inherently greater complement of follicles within the ovaries [5]. On the other hand, the second model proposes that follicles in cattle with multiple ovulations have reduced growth rate and thus are smaller in size. Because of their smaller size, hormonal output, important for follicle selection, is lessened and thus multiple follicles are required to achieve similar output to that observed in single ovulating cattle. Thus, leading to the presence of multiple smaller sized preovulatory follicles in cattle carrying the Trio allele that, on a volume basis, are equivalent to one larger sized follicle in single ovulating non-carrier cattle.

The earliest stage in follicular development is the primordial stage, which is characterized by a single layer of squamous granulosa cells surrounding the oocyte [6]. Primordial follicles, once activated, will develop through a series of stages until becoming the ovulatory follicle or alternatively undergoes atresia. The first stage of development, after primordial follicle activation is the primary stage, which is characterized by presenting a single layer of cuboidal granulosa cells surrounding the oocyte [6]. Growth of the primary follicle leads to transition into the next stage, the secondary follicle, in which the follicle contains 2 or more layers of cuboidal granulosa cells surrounding the oocyte and the presence of the zona pellucida [6]. Further development involves additional multiplication of granulosa cells, the formation of a fluid filled cavity in between the granulosa cell layers, and the oocyte assuming an eccentric position leading to the formation of a tertiary follicle or antral follicle [6]. Primordial, primary, and secondary follicles are further classified as pre-antral follicular stages because an antrum is not yet present. The development of the follicle through the aforementioned stages involves a progressive increase in follicle size, such that primordial follicles are smaller than primary follicles which are smaller than secondary follicles. Granulosa cells play a key role in the growth and maturation of the follicle and oocyte. Granulosa cells produce estradiol which is essential for the development of the follicle and for follicular selection and apoptosis [6,7].

Previously identified mutations in sheep, that increase ovulation rate, have been shown to also alter the characteristics of preantral follicle development. For example, one study compared follicle populations in Boorola sheep, a high fecundity ovine breed to control merino sheep. Results indicated that in Booroola sheep there were 2 to 4 times more primordial follicles (< 60 μm) and 1.5 to 2 times more preantral follicles (> 60 μm without an antrum) compared to control Merino sheep, however, the number of antral follicles was not different. In addition, Booroola

ewes had less granulosa cells per follicle compared to control Merino ewes [15]. Results from a second study conducted on Booroola ewes indicated that oocytes of primordial follicles were similar in size to those in wild-type control ewes [16]. However, when follicles in subsequent stages were categorized by size, it was found that oocytes in Boorola ewes were larger than in control ewes. These results taken together indicate that mutations involving the TGF-β superfamily affect folliculogenesis by altering the relationship between the oocyte and granulosa cells beginning at preantral stages.

Thus, the objectives of the present thesis were to evaluate potential differences in pre-antral (primordial, primary and secondary) follicle numbers between cattle that are carriers and non-carriers of the Trio allele and to determine granulosa cell proliferation at different stages of folliculogenesis by determining the size of the follicle, number of granulosa cells, and size of the oocyte. It was hypothesized that there will be no difference in the number of pre-antral follicle between carriers and non-carriers of the Trio allele. This hypothesis is supported by the previously observed lack of differences in antral follicle count (AFC) between carriers and non-carriers of the Trio allele [8], as well as previous studies conducted on ewes carrying high fecundity alleles [9]. Based on the observation that antral follicles of Trio carrier cattle have reduced growth rate and ovulatory size [4,8], it was hypothesized that pre-antral follicle diameter at each stage of development will be reduced due to fewer granulosa cells, while the size of the oocyte will be similar.

MATERIALS AND METHODS

Animals and tissue Collection

Ovarian tissue from Trio allele carrier (n = 12) and non-carrier control (n = 12) heifers collected during a previous study [10], were used in this study. Briefly, heifers were pre-synchronized by receiving a progesterone intravaginal controlled internal drug releasing device (CIDR) on D-8 and removed 5 days later, on D-3. On D-3 and D-2, each heifer received Prostaglandin F2α. In order to synchronize the emergence of a new follicular wave, animals had ultrasound-guided follicular ablation of all follicles ≥ 4 mm on D-2, -1, and 0. A new CIDR was placed on D0 and was removed on D5. The ovary containing the largest follicle was collected 12

hours after the removal of the CIDR via flank laparotomy [10]. Immediately after retrieving the ovary a section of the ovary, measuring 1 cm x 1 cm x 350 μm, was excised and placed into a fixative solution (10% buffered formaldehyde). Subsequently tissue samples were embedded in paraffin following standard procedures.

Tissue processing for histology

The ovarian tissue samples were sectioned using a microtome and sectioning was performed as follows: for each ovary tissue sample 50 sections were sectioned at 6 μm beginning at the outer surface of the ovary, and each section was numbered. Every 10th section was mounted and stained with a standard hematoxylin and eosin stain, as described below, to be utilized for follicle counting and size determination. Every 10th section was selected so that 60 μm would be between each slide to ensure that follicles were not counted twice. In addition, follicles were only counted when the nucleus of the oocyte was visualized. As a result, a total of 6 slides were mounted, stained and evaluated for each animal.

The hematoxylin eosin staining procedure was performed in the following steps. First, deparaffinization was used to remove the paraffin wax. The slides were placed in xylene 3 times for 4 minutes each. The slides were then placed in 100% ethanol twice for 3 minutes each, 95% ethanol for 3 minutes, 70% for 3 minutes, and then deionized water for 3 minutes. Subsequently, slides were blotted to remove excess water and placed in hematoxylin for 8 minutes. They were then placed under running tap water for 8 minutes to develop the stain, dipped in acid ethanol 3 times to destain the slides, and placed in deionized water for 2 minutes to rinse. In the next step slides were placed in a bluing regent for 1-minute, deionized water for 2 minutes to rinse and then 95% ethanol for 2 minutes. Next the slides were placed in eosin for 2 minutes and rinsed with alcohol by placing the slides in 95% ethanol for 2 minutes and into 100% ethanol for 3 minutes twice. The slides were cleared, to remove the alcohol, by placement into xylene for 3 minutes three times. Finally, a coverslip was mounted onto the slide and the slide was dried overnight.

Histological Evaluation

Evaluation of tissue sections was performed using a compound inverted microscope (Invitrogen™ EVOS™ XL Digital Inverted Brightfield and Phase Contrast Microscope), 10

randomly selected follicles of each preantral follicle stage (primordial, primary, and secondary) were chosen for each heifer in the control (n = 12) and carrier group (n = 12), and images were taken of the follicles. The open source software, Image J, was used to analyze captured images.

Follicular diameter was determined by taking two perpendicular measurements from the outer edges of the granulosa cell layer of each follicle. Similarly, oocyte diameter was determined by taking two perpendicular measurements from the inner edge of the granulosa cell layer. Both the follicle and oocyte were assumed to be spherical and thus the volume of each structures was calculated using the formula $V = (4/3)\ \pi\ r^3$, where V is the volume in μm^3, and r is the radius. The oocyte to follicle ratio was calculated by dividing the oocyte volume by the follicle volume of each follicle, as a means to determine what percentage of the follicle volume was occupied by the oocyte. Number of granulosa cells for each follicle were marked and quantified using the built-in count function in Image J software.

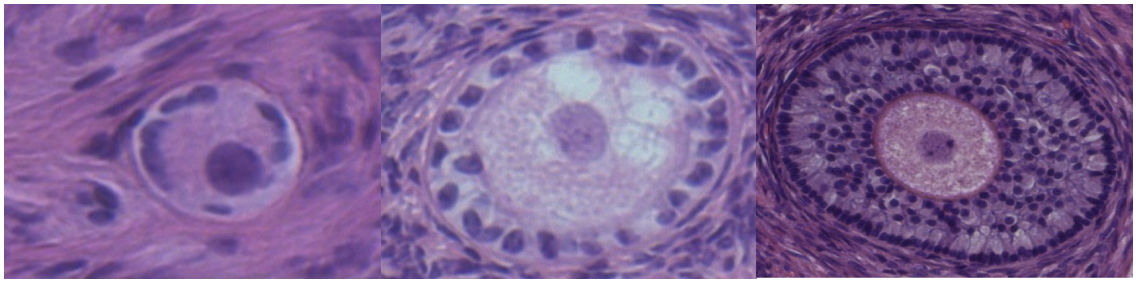

Figure 1: *From left to right, primordial follicle (single layer of squamous granulosa cells surrounding the oocyte), primary follicle (single layer of cuboidal granulosa cells surrounding the oocyte), secondary follicle (two or more layers of cuboidal granulosa cells surrounding the oocyte)*

Determination for the number of follicles for each stage of development was performed using a 5 x 5 grid overlay and a 100x objective in a bright field compound microscope (Nikon Eclipse E400 with Insight Firewire Spot model 11.2 color mosaic camera). Follicle numbers were determined for each preantral follicle stage in both carries and non-carriers, characteristics used to identify the stage of development of each follicle are shown in Figure 1. Follicles were only counted if they were located inside the overlay grid and that portion of the grid was filled with tissue. The grid was overlay multiple times over any given section ensuring that each portion of

the tissue section was evaluated once, to avoid duplication. The number of grid placements for each animal was recorded so that the total area of tissue evaluated for each animal could be determined, and results are reported as follicle numbers of each developmental stage per mm^2.

Statistical Analysis

All statistical analyses were performed using SAS software, version 9.4 (SAS Institute Inc., Cary, NC). Values from each variable obtained for the multiple follicles of the same stage within an individual animal where averaged. Differences between genotypes were assessed using a two-sample t-test or Wilcoxon's rank sum test when the assumptions of normality were not met. Associations between follicle numbers of different follicle stages in each genotype were evaluated by linear mixed models using PROC MIXED. A significant difference between treatment groups was considered when $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered a tendency. Data are presented as means ± standard error of the mean (SEM), obtained using PROC MEANS of SAS.

RESULTS

Follicle numbers for each preantral stage of development are shown in Table 1. There was no significant difference in the number of primordial follicles/mm^2 in Trio carrier compared to non-carrier heifers. Similarly, there was also not a significant difference in the number of secondary follicles/mm^2 between Trio carriers and non-carriers. There was, however, a significant difference in the number of primary follicles/mm^2 among genotypes. Trio carrier heifers had a greater number of primary follicles compared to their non-carrier counterparts.

Table 1. *Number of primordial, primary and secondary follicles in heifers with or without the high fecundity allele Trio. Data are presented as mean (± SEM).*

	Trio carrier	Non-carrier	P-value
N	12	12	
Primordial Follicles/mm^2	2.63 ± 0.3	2.10 ± 0.5	0.18
Primary Follicles/mm^2	0.27 ± 0.04	0.11 ± 0.01	0.0008
Secondary Follicles/mm^2	0.036 ± 0.005	0.027 ± 0.007	0.18

Associations between follicle numbers of different stages in both Trio carrier and non-carrier control heifers are shown in Figure 2. The number of primordial follicle was positively associated with the number of primary follicles in non-carrier heifers (r = 0.73; P = 0.007). Conversely, there was no association between primordial and primary follicle numbers in Trio carrier heifers (r = 0.01; P = 0.96). Similarly, primordial follicle numbers were positively associated with the number of secondary follicles in non-carrier heifers (r = 0.92; P < 0.0001), while no association was found in Trio carrier heifers (r = -0.31; P = 0.33). The number of secondary follicles was positively associated with the number of primary follicles in non-carrier heifers (r = 0.87; P = 0.0002), while it tended to be positively associated in Trio carrier heifers (r = 0.50; P = 0.09).

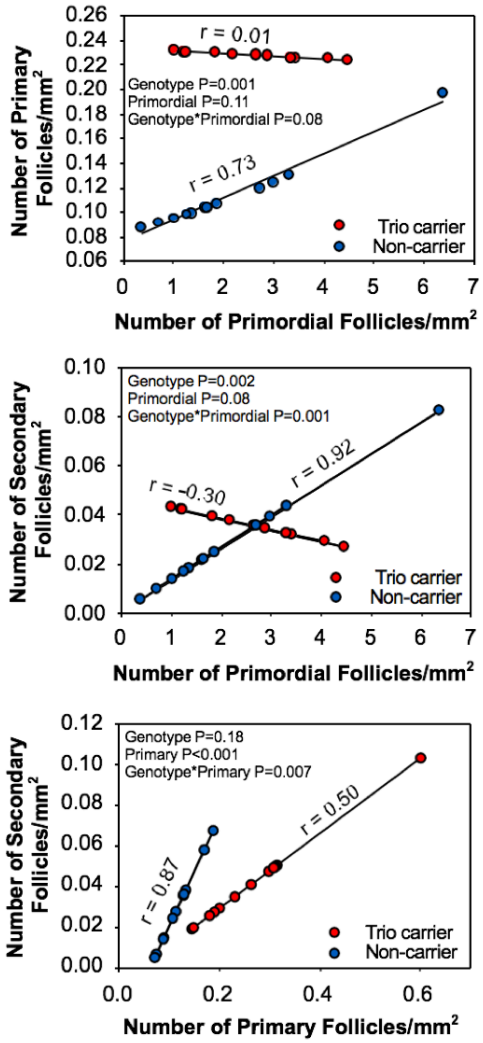

Figure 2: *Associations between Primordial, Primary and Secondary Follicles in heifers with or without the high fecundity allele Trio. The main effect of genotype, follicle type and their interaction are shown. r = correlation coefficient*

Follicle and oocyte size, on a volume basis, for each preantral follicle stage in both Trio carrier and non-carrier control heifers is shown in Figure 3. There was no difference ($P > 0.2$) between genotypes in follicle and oocyte volume among primordial follicles. In addition, there was no significant difference between genotypes in the oocyte to follicle ratio of primordial follicles. Conversely, primary follicle and oocyte volume were 1.2-fold and a 1.1-fold larger respectively in Trio carriers than non-carrier control heifers ($P < 0.05$), however, oocyte to follicle ratio was not different between genotypes for primary follicles ($P > 0.10$). Secondary follicle volume was not different ($P > 0.5$) between genotypes, however, oocyte volume in secondary follicles was 1.8-fold larger in Trio carrier than non-carrier control heifers ($P < 0.03$). As a result, oocyte to follicle ratio tended ($P < 0.10$) to be greater in Trio carriers than non-carrier controls.

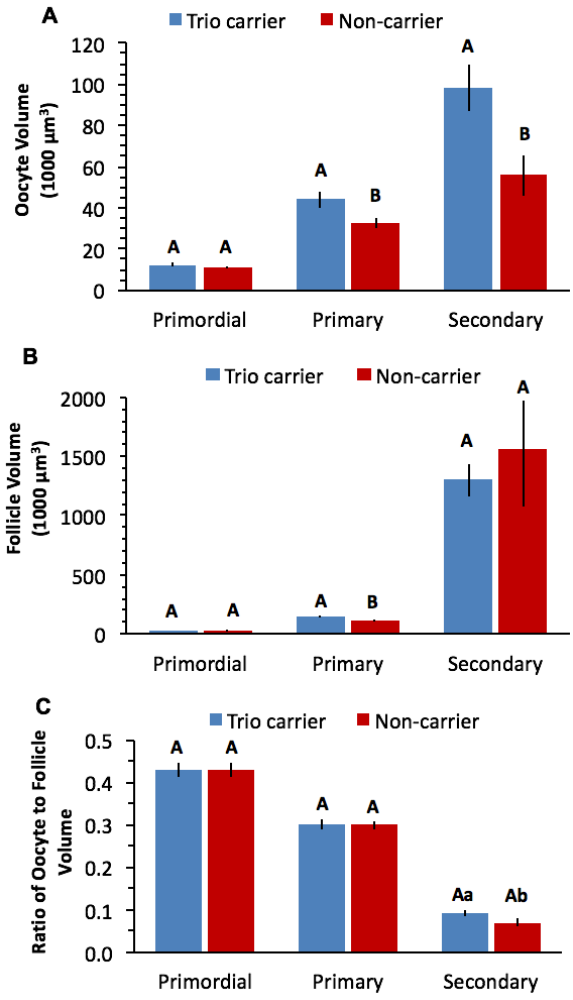

***Figure 3**: Oocyte Volume (A), Follicle Volume (B) and Oocyte to Follicle Ratio (C) in heifers with or without the high fecundity allele Trio .[A,B] Means within a follicle stage with different superscripts differ (P<0.05) [a,b] or tended to differ (P<0.10).*

Granulosa cell number per cross section was evaluated for each preantral follicle stage and are shown in Figure 4. There were no differences in the number of granulosa cells between Trio carrier and non-carrier control heifers neither in primordial, primary nor secondary follicles ($P > 0.10$).

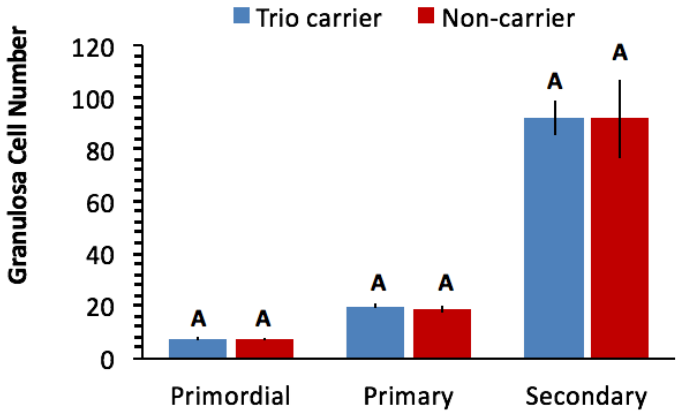

Figure 4*: Number of Granulosa Cells per Follicle in heifers with or without the high fecundity allele Trio.* ^{A,B} *Means within a follicle stage with different superscripts differ (P<0.05).*

DISCUSSION

The present study constitutes the first charcterization of pre-antral follicle development in heifers that are carriers of the high fecunidty bovine allele, Trio. The current mechanism underlying the effect of the increased expression of SMAD6, in Trio allele cattle, remains poorly understood. Results from previous studies in Trio allele carrier cattle have provided insight on the physiological characteristics of antral follicle development and the corresponding endocrine regulation [4], however, information on the early stages of folliculogenesis is lacking and could provide insight into the precise role of SMAD6 in folliculogenesis. As stated previously, two models have been proposed in an attempt to explain the occurrence of multiple ovulations in otherwise monovular species. The first model postulates that the size of the follicle population plays an important role, and thus in the present study we carefully characterized pre-antral follicle numbers of each developmental stage by evaluation of ovarian histological sections. The second model, on the other hand, postulates that reduced follicle growth rate, due to reduced granulosa cell proliferation, leads to development of smaller sized follicles and thus multiple follicles are needed in order to achieve a similar hormonal input. As a result, in the present study, we evaluated

both follicle size and number of granulosa cell at each pre-antral follicle stage to further evaluate the validity of this model. Furthermore, we utilized volume as a measure of size as results from previous research indicate that volume provides a more accurate representation of follicle functionality [12].

It has been proposed that the increase in ovulation rate observed in ruminants carrying high fecundity alleles could be due to increasing the number of follicles that become gonadotrophin responsive, which in turn could be accomplished by increasing the overall follicle population [5]. Results from previous research in Trio carrier cattle indicated that antral follicle number and circulating AMH, a marker for antral follicle population, was similar to that in single ovulating controls without the Trio allele [8]. Thus, our first hypothesis was that there will be no difference in the number of pre-antral follicle numbers between carriers and non-carriers of the Trio allele. This hypothesis was partially supported as there was no significant difference in the number of follicles between carriers and non-carriers in the primordial or secondary stage in the present study. Conversely, we identified a greater number of primary follicles in cattle carrying the Trio allele. These data differ from a previous study conducted on cattle selected for twinning over 20 years, at the USDA-MARC, which had similar numbers of primordial and primary follicles compared to single ovulating control cattle, however, the number of secondary follicles and surface antral follicles was greater in twinner cattle [11]. The observed differences between results from the present study and those reported for the USDA MARC twinner cattle may be based on the differences in the underlying mutation. On the other hand, results obtained herein with Trio carriers also contrast previous reports in ewes carrying a TGF-β mutation, such as Boorola ewes. Boorola ewes were found to have 2 to 4 times more primordial follicles and 1.5 to 2 times more preantral follicles compared to wild-type control ewes, while the number of antral follicles were not different [15]. It is unclear if the differences in the results obtained herein and those observed in Boorola ewes are due to physiological differences between the Boorola and the Trio alleles, or alternatively if these are due to differences in the definitions used to categorize follicles. For example, follicles in the Boorola study were defined primarily based on size rather than morphological characteristics, and primary and secondary stages were not quantified separately.

It is expected that the number of follicles during each stage of preantral development to be correlated with the number of follicles in subsequent stages of development. Results from previous

research in wild-type cattle indicated that the number of primordial follicles was positively correlated with the number of both primary and secondary follicles, and the number of primary follicles was positively correlated with the number of secondary follicles [14]. In the present study, number of primordial, primary and secondary follicles were highly correlated between each other in non-carrier control heifers, in agreement with previous research [11]. Conversely, in Trio carrier heifers, there was no correlation between the number of primordial and primary follicles and primordial and secondary follicles, while the number of primary and secondary follicles tended to be positively correlated. These findings differ from previous reports in twinner cattle at USDA MARC, where preantral follicle number at each stage were positively correlated among them [11]. One reason for this may be because the mutation present in Trio cows is located on a single gene while twinner cows were selected over generations for increased ovulation and twinning, thus involving multiple genes and a different mechanism. Unfortunately, correlations between follicle number at different pre-antral stages have not been reported for ewes carrying high fecundity alleles. The data suggests that primordial activation rate and the transition into primary follicle is not related to primordial follicle numbers in Trio carriers but is related in non-carriers. The greater number of primary follicles and the absence of a relationship between primordial follicles and primary and secondary follicles in Trio carriers suggests that the growth rate and rate of atresia of follicles beginning in the primary stage is different than in non-carrier wild-type cattle. Furthermore, these data indicate that SMAD6 and the TGF-β superfamily play an important role in primordial follicle activation and the initial stages of folliculogenesis.

A second model has been proposed to explain the occurrence of multiple ovulations in animals with mutations involving the TGF-β superfamily, such as cattle carrying the Trio allele. This alternative model suggests that the increase in ovulation rate is due to a decrease in the growth rate and size of the antral follicles, as supported by previous observations [4]. Thus, our second hypothesis was that there would be a decrease in the size of preantral follicles among Trio carriers, which could explain the decrease in the size of antral follicles. Results from the present study, do not support our hypothesis, as indicated by the following observations: 1) there was no difference in primordial follicle size between Trio carrier and non-carrier heifers; 2) primary follicles were larger in Trio carriers compared to non-carriers; 3) secondary follicle size was not different between genotypes. The follicle sizes observed in both genotypes in the present study are,

however, comparable to follicle sizes in wild-type cattle previously reported [6]. Interestingly, oocyte volume was found to be similar between genotypes in primordial follicles, however it was larger in Trio carriers than non-carrier heifers at both the primary and secondary stages. Taking the follicle and oocyte size data together suggests that the larger primary follicle size, observed in Trio carriers, is due primarily because of a larger oocyte, considering that at this stage the oocyte contributes 30% of the total volumes of the follicle. Conversely, during the secondary stage follicle size was not different despite oocytes in Trio carrier being larger than in controls, because the oocyte only contributes < 10% of the follicle size. Comparisons of pre-antral follicle characteristics between our results and those reported in ewes carrying high fecundity alleles, such as the Boorola, are hampered by the different definitions used to classify follicles. Nevertheless, it is noticeable, that oocyte size in Boorola ewes was similar to that in control ewes in primordial follicles, while larger oocytes were found in Boorola ewes at stages equivalent to the primary and secondary stages in agreement with our results [16]. These results taken together suggest that high fecundity alleles, do not affect primordial follicle development nor oocyte growth through any of the stages. On the other hand, once a primordial follicle is activated, transition through the primary stage may be delayed, possibly due to reduced granulosa cell proliferation, while the oocyte continues to grow thus explaining the larger oocyte observed in Trio carriers. Furthermore, the delayed progression through the primary stage may also explain the greater number of primary follicles observed in Trio carriers.

The decreased growth rate of antral follicles observed in cattle carrying the Trio allele as well as in sheep carrying the Booroola mutation appears to be due to reduced granulosa cell proliferation [12]. This led to the hypothesis that granulosa cell numbers of preantral follicles in Trio carriers would be reduced compared to non-carriers. This hypothesis was not supported as no difference in the number of granulosa cells among any of the follicular stages (primordial, primary, and secondary) was identified between Trio carriers and non-carries. The number of granulosa cells observed in the present study are consistent with the typical number of granulosa cells for each follicle stage in cattle, < 10 for primordial follicles, 10 to 40 cells in primary follicles and 41 to 250 cells in secondary follicles [6]. Results from previous research in high fecundity Booroola ewes indicated that antral follicles had fewer granulosa cells per follicle when compared to control ewes, however, granulosa cell numbers for pre-antral follicles were not reported [15]. A limitation

of the present study is that follicle classification into primordial, primary, and secondary follicles relies in the shape and number of granulosa cells layers [5]. Thus, if the Trio allele affects granulosa cell proliferation during pre-antral development the classification used may result in the comparison of follicles, between genotypes, that ultimately are in two different physiological states. In addition, this may potentially explain the observed differences in follicle size and oocyte size. For example, if granulosa cells proliferate at a slower rate in Trio carriers then it is likely that a primary follicle would require a greater amount of time to increase the number of granulosa cells and acquire multiple layers to become a secondary follicle. In addition, because oocyte growth appears to be unaffected by the Trio allele, oocytes are larger in Trio carriers. These conclusions are consistent with previous observations in sheep carrying high fecundity alleles. Thus, future research may consider utilizing a different pre-antral follicle classification that allow comparisons between genotypes of follicles that are in similar physiological states.

CONCLUSION

The present study provides novel information on the physiology of early folliculogenesis in cattle carrying the bovine high fecundity allele Trio. Results presented herein suggest a slowed progression of follicular growth beginning in the primary stage leading to a greater quantity of larger primary follicles, due to a larger oocyte, in Trio carriers and the presence of a larger oocyte in secondary follicles. Furthermore, these results further support that increased expression of SMAD6, the underlying molecular component in trio allele carriers, reduces follicle growth from early folliculogenesis, as well as later stages as previously described [12]. Future research should focus on validating our proposed model by investigating the time required for primary follicles to transition into secondary follicles through in vitro follicle culture. In addition, research into the size and area of individual granulosa cells of Trio carriers may provide additional evidence of the underlying alterations in granulosa cell growth in Trio carriers.

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