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Research Article

Trio, a novel bovine high fecundity allele: III. Acquisition of dominance and ovulatory capacity at a smaller follicle size†

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Abstract

The acquisition of dominance and ovulatory capacity was evaluated in follicles from cows that were carriers or half-sibling noncarriers of the Trio allele. Follicle size at acquisition of follicular dominance was determined by evaluating whether follicles ovulate after GnRH challenge (ovulatory capacity—experiment 1) and by determination of intrafollicular concentrations of estradiol and free insulin like growth factor 1 (IGF1) and relative mRNA expression of cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*), luteinizing hormone/choriogonadotropin receptor (*LHCGR*), and pappalysin 1 (*PAPPA*, previously known as pregnancy-associated plasma protein A, pappalysin 1) in granulosa cells from follicles of different sizes (experiment 2). Ovulatory capacity developed in follicles at 8.3 mm (50% ovulatory capacity) in noncarriers but at smaller sizes (5.5 mm) in Trio carriers. Similarly, in experiment 2, follicles of Trio carriers acquired a dominant phenotype, as determined by intrafollicular estradiol and *CYP19A1*, *LHCGR,* and *PAPPA* mRNA expression in granulosa cells, at significantly smaller sizes but at a similar time after wave emergence. Overall, dominance/ovulatory capacity was acquired when follicles of Trio carriers were ~30% the size (volume basis) of follicles in noncarriers. In addition, follicles in Trio carriers appear to acquire dominance in a hierarchal manner, as demonstrated by the progressively greater number of follicles with a dominant phenotype between days 2 and 4 after wave emergence. Thus, results from this study provide further support for a physiological model in which selection of multiple follicles in Trio allele carriers is characterized by acquisition of dominance at a smaller follicle size but at a similar time in the follicular wave with multiple follicles acquiring dominance in a hierarchal sequence.

Summary Sentence

Results support a model for selection of multiple follicles in Trio allele carriers due to acquisition of dominance at a smaller follicle size but similar time in the follicular wave with multiple follicles acquiring dominance in a hierarchal sequence.

Key words: ruminants, follicle, follicular development, LH receptor, ovulation.

Introduction

Determination of which follicle or follicles are selected for ovulation from a larger cohort of available follicles is a remarkable biological process with substantial evolutionary implications, as genetic information only from the oocytes of those chosen follicles will be passed on to the next generation. The pivotal events leading to selection of a particular follicle from a growing cohort has been the subject of substantial whole animal, cellular, and molecular research particularly in monovular species such as, cattle, horses, and humans. From a morphological point of view, follicle selection is identified as continuous growth of the future dominant follicle while the subordinate follicles slow or cease to grow, termed follicle diameter deviation [1–4]. The deviation mechanism is activated rapidly (<8 h) when the future dominant follicle is ~8.5 mm (*Bos taurus*), ensuring continuous growth of the selected follicle while preventing growth of subordinate follicles because the "door" that would allow acquisition of follicular dominance has been closed [1–3, 5].

Follicle deviation and the acquisition of dominance have also been associated with marked changes in the follicular environment, including follicular fluid composition and gene expression in granulosa cells (GC). First, intrafollicular estradiol (E2) has been shown to significantly increase in the future dominant follicle near the time of follicular deviation [6–8]. Increased E2 in follicular fluid is associated with increased mRNA expression for cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*) in GC [8]. Second, the insulin like growth factor 1 (IGF1) system has also been implicated in the deviation process, with a decrease in free IGF1 in follicular fluid of subordinate follicles after deviation while free IGF1 remained at somewhat constant levels in the dominant follicle through the deviation time period [7, 9–11]. The decrease in free IGF1 in subordinate follicles is related to an increase in specific insulin like growth factor binding proteins (IGFBPs) [12]. In contrast, the relatively constant levels of free IGF1 in the dominant follicle are due to the activity of IGFBP proteases such as pregnancy-associated plasma protein A (PAPPA) [13]. Expression of *PAPPA* mRNA and the presence of PAPPA proteolytic activity are one of the early biochemical signatures of a dominant follicle, increasing the availability of free IGF1 that may synergize with follicle-stimulating hormone (FSH) to enhance follicular E2 synthesis [8, 12, 14, 15]. Finally, induction of luteinizing hormone (LH) receptors in GC has also been reported to be one of the initial signatures of the dominant follicle phenotype [7, 8]. Consistent with a role for LH pulses in dominant follicle growth after deviation, heifers treated with acyline, a GnRH antagonist [8] to eliminate LH pulses, had normal follicle growth until near the time of follicle deviation but had no growth of follicles past deviation [16]. Interestingly, treatment with acyline also suppressed the expression of luteinizing hormone/chorionic gonadotropin receptor (*LHCGR*) mRNA in GC, indicating an involvement of LH pulses in induction of LH receptors during follicle deviation [8]. Acquisition of LH receptors can also be evaluated in vivo by challenging the animal with an LH surge, and follicles will ovulate in response to an LH surge (ovulatory capacity) if they have acquired LH receptors and the dominant follicle phenotype [17]. Previous studies have determined that ovulatory capacity is attained by 10 mm in *Bos taurus* and by 7 mm in *Bos indicus* cattle [17–19]. Thus, multiple markers can be used to determine whether a given follicle has attained the dominant follicle phenotype, ranging from determination of ovulatory capacity in vivo to evaluation of follicular fluid E2 and free IGF1, and determination of expression of *CYP19A1*, *PAPPA*, and *LHCGR* in GC.

The discovery of high fecundity genotypes in sheep provided a multiple ovulation model to study follicle selection and led to the identification of members of the transforming growth factor beta (TGF-ß) family as key regulators of ovulation rate [20–25]. Mutations identified in high fecundity ovine genotypes have as common phenotypic features the ovulation of multiple smaller-sized follicles with fewer GC per follicle [26–28]. In addition, follicles of ewes carrying the Booroola/FecB mutation reached peak aromatase activity and follicular fluid E2 concentrations at progressively smaller diameters related to whether they were heterozygous or homozygous for the mutated allele [27]. In addition, follicles from ewes carrying one or multiple different mutations had GC responsive to LH stimulation at a smaller follicle size and in a greater number of follicles compared to wild-type controls [28–30]. More recently, a novel high fecundity, high ovulation rate bovine allele, named Trio was described [31]. Although it is unclear whether the Trio genotype will be practical due to problems with twins and triplets in current production systems, the Trio allele provides a new model for the study of follicle selection and in particular provides the possibility for precisely monitoring the dynamics of follicular and hormonal changes with the changes in intrafollicular composition and GC gene expression. Diameter deviation in carriers of the Trio allele was found to occur at a similar time after follicle wave emergence but at a significantly smaller individual follicle size as compared to single-ovulating, noncarrier controls [32]. The finding of ovulatory follicles that are less than 8.5 mm in carriers of the Trio allele supports the idea that acquisition of LH receptors and ovulatory capacity may occur at a smaller follicle size [32].

Thus, the objective of this study was to evaluate the follicle size at which a dominant phenotype is acquired in cattle that are carriers of a high fecundity bovine allele. In order to achieve this objective, we evaluated acquisition of dominance through two experimental approaches: (1) follicle size at which ovulatory capacity is achieved after an exogenous ovulatory stimulus and (2) comparing follicle size to follicular fluid composition and GC gene expression of previously identified dominance markers (i.e. E2, *CYP19A1*, *LHCGR*, *PAPPA*). We hypothesized that Trio carriers would acquire a dominant follicle phenotype and ovulatory capacity at a smaller size as compared to noncarrier controls but that the dominant follicle phenotype (i.e. estradiol, *LHCGR*, etc.) would be similar between Trio carriers and noncarrier controls.

Materials and methods

All animal procedures were approved by the Animal Care and Use Committee of the College of Agriculture and Life Sciences at the

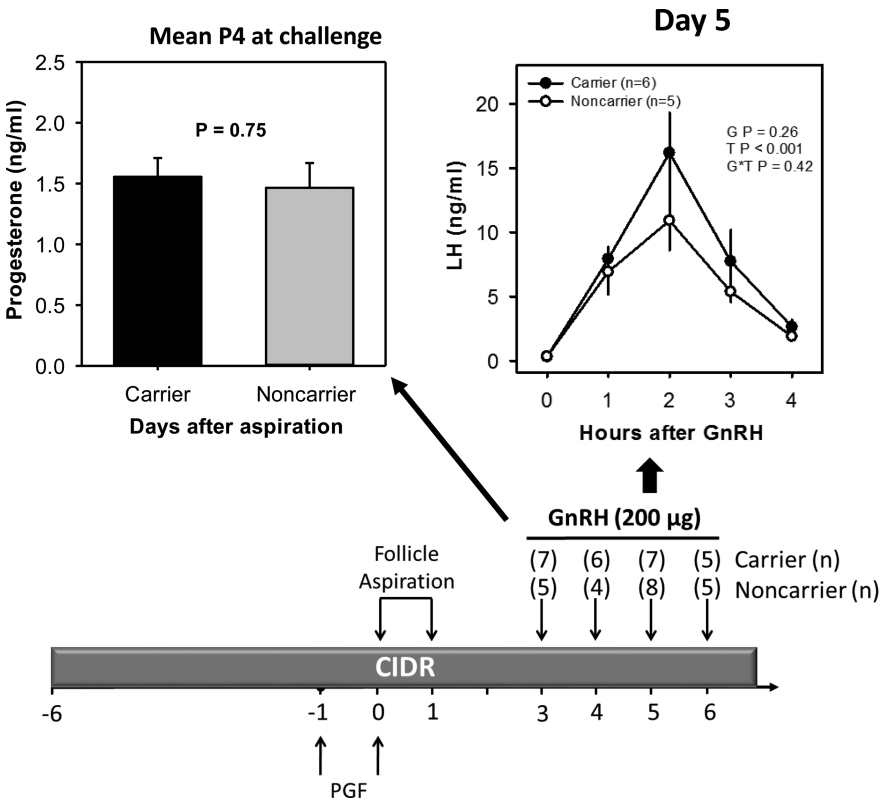

Figure 1. Treatment schedule utilized to evaluate the acquisition of ovulatory capacity in Trio carrier and noncarrier control heifers in experiment 1. The upper left panel shows the mean circulating progesterone for both Trio carrier and noncarrier control heifers at the time of GnRH challenge. The upper right panel indicates the circulating LH profile after challenge with 200 μg of GnRH (gonadorelin acetate) on D5 for both genotypes. G, genotype; T, time; G*T, genotype by time interaction.

University of Wisconsin–Madison. All animals used in this study were kept in outdoor paddocks with free access to a run-in shelter and water. Cattle were fed mixed hay ad libitum and had access to a standard mineral mix.

Females used in the following studies were produced by mating (through artificial insemination) bulls which were carriers of the Trio allele with cows that in most cases were of predominantly Angus breeding (noncarriers). Presence of the Trio allele was inferred by analysis of haplotypes based on three linked markers as described previously [31]. All females were half-sibs and either homozygous wild-type or heterozygous carriers of the Trio allele.

Experiment 1: acquisition of dominance based on ovulatory capacity

Experiment 1 evaluated the acquisition of ovulatory capacity of cattle carrying the Trio high fecundity allele under a controlled progesterone (P4) environment. Nulliparous crossbred beef heifers 21 to 34 months of age, heterozygous for the high fecundity allele (n = 13), or age-matched, half-sib controls (n = 11) weighing 472 ± 10 and 481 ± 17 kg, respectively were utilized. Heifers were synchronized as follows: all animals received a P4 intravaginal controlled internal drug release (CIDR, Eazi-Breed, Zoetis, Florham Park, NJ) device on D−6 (D0 = day of first follicular aspiration) and the CIDR was left in place throughout the experiment (until 48 h after GnRH challenge). Prostaglandin F2α (PGF, 500 μg of cloprostenol, Estroplan, Parnell, Overland Park, KS) was administered twice 24 h apart on D−1 and D0 (Figure 1). One carrier heifer in one replicate lost the CIDR during the protocol, and therefore was removed from the study.

To synchronize the emergence of a new follicular wave, all heifers had ablation of all follicles ≥4 mm on D0, as previously described [33]. Briefly, transvaginal ultrasound-guided follicle ablation was performed using an 8 MHz microconvex array transducer and a B-mode ultrasound scanner (Mylab30 vet, Esaote, Indianapolis, IN) with 2″ 18G hypodermic needle. On D1, follicle ablation was repeated in those animals in which aspirated follicles had refilled. Heifers from each genotype were challenged on days 3, 4, 5, or 6 (Figure 1) with 200 μg gonadorelin acetate i.m. (GnRH, Gonabreed, Parnell, Overland Park, KS). Each animal underwent the outlined protocol twice and each time was assigned to a different day for treatment; interval between the start of each replicate was 16 days. Ultrasound examinations of the ovaries were performed by a single operator at the time of GnRH administration and 48 h after treatment using an ultrasound scanner equipped with a 7.5 MHz transducer. Ovulation was determined as the disappearance of a previously identified follicle within 24–72 h after GnRH and was confirmed 5–7 days later by the presence of a corresponding corpus luteum. Blood collection was performed via coccygeal venipuncture into evacuated tubes at the time of GnRH challenge for determination of circulating P4 and FSH concentrations. In addition, blood samples for LH determinations were collected from 0 to 4 h after GnRH treatment at hourly intervals, from a subset of animals (n = 11) treated on D5.

Experiment 2: dominance based on intrafollicular factors and granulosa cell mRNA

Experiment 2 evaluated the acquisition of a dominant phenotype by means of intrafollicular hormones and GC gene expression analysis in follicles of Trio carriers and noncarrier controls. Nulliparous crossbred beef heifers, heterozygous for the Trio high fecundity allele (n = 14), or age-matched, half-sib controls (n = 11) were utilized. Heifers were 22 to 36 months of age with a mean weight of 500 ± 10 and 504 ± 17 kg for Trio carriers and noncarrier controls, respectively. Heifers were synchronized as described in experiment 1. Data from seven collections (Trio carrier = 4 and noncarrier = 3) were not analyzed due to failure of the synchronization procedure, as evidenced by regrowth of previously aspirated follicles and/or the presence of a follicle with a diameter exceeding 3 standard deviations (SDs) of the mean follicle size for a given day and genotype.

Follicular fluid and GC were collected from the three (carriers) or four (noncarriers) largest follicles, on D2, D3, D4, or D5 after follicle ablation (D0). Heifers were randomly assigned to be collected on a day (2, 3, 4, or 5). Samples were collected from each follicle individually, as described [34], using a transvaginal ultrasound-guided follicle aspiration system as in experiment 1 but with a 2" 21G hypodermic needle fitted to the transvaginal probe guide. Each individual follicle was sampled separately by collecting follicular fluid and GC into a 0.25-ml straw connected to the hub of a disposable needle. To minimize loss of sample and avoid cross-contamination, a new straw and needle were utilized for each individual follicle. Samples could not be obtained from 11 follicles (9 and 2 for Trio carrier and noncarrier controls, respectively) due to failure of the collection system.

Immediately after collection, the contents of the straw were placed in a microcentrifuge tube and centrifuged at 700 g for 10 min. The supernatant, i.e. follicular fluid, was removed and stored at –20°C until assayed. The GC pellet was resuspended in sterile saline and centrifuged at 700 g for 10 min. The supernatant was discarded and the pellet was resuspended in 20 to 50 μl of RNAlater (#AM7020, Ambion, Thermo Fisher Scientific, Wilmington, DE) and kept overnight at 4°C. Samples were then stored at –80°C until RNA extraction was performed. The collection procedure was repeated at 16-day intervals to obtain a total of 37 and 32 collections for Trio carriers and noncarrier control heifers, respectively. Each collection corresponded to an independently synchronized follicular wave with three or four follicles sampled at each collection. Blood samples were obtained, for P4 and FSH, via coccygeal venipuncture at time of follicle collection in each animal.

Hormone assays

Blood samples were centrifuged at 1300 g at 4°C for 20 min, and serum was transferred into vials, frozen, and stored at –20°C until assayed. Assay of P4 was performed using a solid-phase radioimmunoassay (RIA) kit (ImmuChem coated tube Progesterone; MP Biomedicals, Costa Mesa, CA). Mean assay sensitivity, calculated as 2 SD less than the mean counts per minute of maximum binding, was 0.05 ng/ml. Intra- and interassay coefficients of variation were 3.8% and 6.3%, respectively. The FSH assay incorporated the primary antibody NIDDK-anti-oFSH-I-2 and the radiolabeled and standard antigen USDA-bFSH-I-2. Mean assay sensitivity and intra- and interassay coefficients of variation were 0.04 ng/ml, 4.6%, and 2.8%, respectively. Luteinizing hormone concentrations were determined in duplicate samples by RIA as described in [35]. The standard curve ranged from 0.15 to 20 ng/ml of LH. Samples with percentage binding less than 10% due to high LH concentrations were diluted 1:4 and reanalyzed. The average sensitivity of the assays was 0.07 ng/ml. Two quality control samples, low (0.41 ng/ml) and high (5.6 ng/ml), were evaluated in each assay. For the low sample, the average intra-assay coefficient of variation was 6.4%, whereas the interassay coefficient of variation was 8.8%. For high-LH sample, average intra-assay coefficient of variation was 4.0% and the interassay coefficient of variation was 5.2%.

Intrafollicular E2 concentrations were determined using a commercially available competitive ELISA (E2 Serum EIA Kit, #KB30-H1, Arbor Assays, Ann Arbor, MI). Samples were diluted in assay buffer provided by the manufacturer at 1:5,000 or 1:20,000. Intra- and interassay coefficients of variation were 4.1% and 9.7%, respectively. Progesterone concentrations in follicular fluid samples were determined by RIA as described above for serum samples, and follicular fluid was diluted 1:100 in assay buffer. All samples were evaluated in a single assay, and the intra-assay coefficient of variation was 3.0%. Follicular fluid concentrations of dimeric inhibin A were determined by a commercially available sandwich ELISA (Inhibin A ELISA, #Al-123, Ansh Labs, Webster, TX). Samples were diluted 1:1000 using the 0 calibrator provided with the kit; intra-assay and interassay CVs were 2.4% and 13.7%, respectively. Intrafollicular free IGF1 was determined by a commercially available sandwich ELISA (Human Free IGF1 Immunoassay, #DFG100, R&D Systems, Minneapolis, MN). Samples were diluted 1:8 in the calibrator diluent provided with the kit; intra-assay and interassay CVs were 2.5% and 6.2%, respectively. Intrafollicular concentrations of inhibin A and free IGF1 were assayed in a subset of samples that included only the F1 and F2 in noncarrier controls, while for Trio carriers all four follicles, F1 to F4, were included whenever available.

Granulosa cell RNA extraction and quantitative real-time RT-qPCR

Granulosa cell gene expression was evaluated in a subset of samples that included only the F1 and F2 in noncarrier controls, while for Trio carriers all four follicles, F1 to F4, were included whenever available. RNA was extracted from GC stored in RNAlater solution using the RNeasy Plus Micro Kit (#74034, Qiagen, Germantown, MD) as per the manufacturer's instructions. RNA was quantified using the NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A maximum of 1 μg RNA was used in reverse transcription to cDNA by means of a commercially available kit (#4368814, Applied Biosystems, Wilmington, DE) with RNase inhibitor (#M0307L, New England Biolabs Inc. Ipswich, MA) following the manufacturer's instructions. Resulting cDNA was diluted 1:4 with nuclease-free water prior to use in quantitative RT-qPCR. Steady-state concentrations of mRNA for ring finger protein 20 (*RNF20*), *LHCGR*, splicing factor 3a subunit 1 (*SF3A1*), follicle stimulating hormone receptor (*FSHR*), *CYP19A1*, MAD family member 6 (*SMAD6*, previously known as MAD, mothers against decapentaplegic homolog 6), and *PAPPA* were measured with the CFX Connect Real-Time System (Biorad, Hercules, CA, USA) using 6.25 μL of SsoFast EvaGreen Supermix (#172–5203, BioRad), 500 nM each of forward and reverse primers (Supplemental Table S1), 2 μL of cDNA product, and water to bring the reaction mixture up to 10 μL. The thermal cycling conditions were as follows: 95°C for 3 min, then 40 cycles of 95°C for 10 s and 58°C for 30 s. Amplification efficiencies of primers were evaluated and ranged from 95% to 105% efficiency, and primer specificity was assessed by the presence of a single temperature dissociation peak. The geometric average of

the mean cycle threshold values of RNF-20 and SF3A1 was used as housekeeping genes, and the $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression [36].

Terminology, data arrangement, and statistical analyses

Follicles were designated as F1, F2, and so on in decreasing order based on their diameter at the time of GnRH challenge (experiment 1) or collection (experiment 2). Follicle volume was calculated assuming the shape of a sphere with the following formula: $V = \frac{4}{3}\pi r^3$. For both experiments, each challenge or collection, for experiment 1 and 2 respectively, was assumed to be independent due to being performed on an independently synchronized follicular wave.

In experiment 1, analysis of circulating LH measured after GnRH challenge was performed by analysis of variance for repeated measures using the MIXED procedure (Statistical Analysis System, SAS Institute, Version 9.4 Cary, NC, USA) with an autoregressive covariance structure. Genotype, time, and genotype by time interaction were included in the model as fixed effects. Preplanned comparisons were performed between genotypes for specific time points.

In experiment 2, the analysis of continuous variables in follicles was performed by mixed linear models using the MIXED procedure. The repeated statement with a compound symmetry covariance structure was used to account for clustering of multiple follicles within a heifer being collected at any given day. For analysis of measurements over follicle size classes, the analysis was conducted separately by genotype due to absence of certain size categories in a genotype. Comparisons between genotypes for follicle size were conducted separately, and the model included genotype, size class, and their interaction as fixed effects. Comparisons of follicle parameters between genotypes for follicles that were predominant or dominant were conducted using genotype, dominance status, and their interaction as fixed effects.

In experiment 2, the acquisition of dominance for an individual follicle was determined based on intrafollicular E2 concentrations. The cut-off point was determined for both genotypes as the intrafollicular E2 concentrations that exceeded 3 SD above the mean for the smallest follicle size class available for each genotype combined, 3 and 5 mm, for Trio carriers and noncarrier controls respectively. Determination of a cut-off value to establish dominance based on other GC markers (i.e. *CYP19A1*, *LHCGR*, and *PAPPA*) was performed by means of a receiver operating characteristic (ROC) curve by using the LOGISTIC procedure in SAS. An ROC curve analysis was performed for each marker, and the cut-off value that maximized both sensitivity and specificity was selected.

Analysis of the effect of follicle size on the probability of ovulation or dominance, for experiment 1 and experiment 2 respectively, was evaluated by logistic regression using the LOGISTIC procedure. Follicle size (diameter) and genotype were included as fixed effects, and the interaction was evaluated and retained if significant. The resulting regression equation was utilized to obtain predicted probabilities based on follicle size.

Analysis of circulating P4 and FSH, for both experiments, was performed by analysis of variance for repeated measures using the MIXED procedure with a compound symmetry covariance structure. Genotype, time, and genotype by time interaction were included in the model as fixed effects. Preplanned comparisons between genotypes for specific time points were done by least square difference.

Binomial outcomes such as proportion of heifers ovulating (at least one follicle) on each day to the GnRH (experiment 1) or the proportion of heifers with at least one dominant follicle (experiment 2) were compared between genotypes by the Fisher exact test. Number of ovulations and dominant follicles were compared using the Wilcoxon rank sum test.

Assumptions of normality and homogeneity of variance were evaluated for all continuous outcomes, and transformations were performed if assumptions were not met. Outliers were identified and removed from the analysis of a given outcome when they exceeded the threshold defined as: the third quartile limit plus three times the interquartile range. Data are presented as mean (±SEM). Values were considered statistically different when $P \leq 0.05$.

Table 1. Percentage of Trio carrier and noncarrier heifers that ovulated to a GnRH challenge on each day after follicle aspiration and mean (±SEM) number of ovulations in heifers that ovulated.

End point		Carrier	Noncarrier	*P*-value
Ovulation (%)				
	Day 3	0% (0/7)	40% (2/5)	0.15
	Day 4	66.7% (4/6)	75% (3/4)	0.99
	Day 5	100% (7/7)	100% (8/8)	NA
	Day 6	100% (5/5)	100% (5/5)	NA
Number of ovulations[a]				
	Day 3	NA	1.0 ± 0.0	NA
	Day 4	3.0 ± 0.4	1.0 ± 0.0	0.05
	Day 5	3.0 ± 0.3	1.0 ± 0.0	0.0002
	Day 6	2.8 ± 0.4	1.2 ± 0.2	0.02

[a]Number of ovulations are calculated from those heifers in each genotype that ovulated in response to GnRH for a given day. NA = not applicable.

Results

Experiment 1: acquisition of dominance based on ovulatory capacity

Figure 1 shows the experimental design used in experiment 1 and the comparison of hormonal concentrations between groups. Serum P4 concentration was not different between genotypes ($P = 0.75$) and day ($P = 0.42$), nor was there a genotype by day interaction ($P = 0.36$). Circulating LH in response to the GnRH challenge was determined in a subset of heifers treated on D5 from both genotypes and is shown in Figure 1. There was a main effect of time ($P < 0.001$), but no genotype ($P = 0.26$) or genotype by time interaction ($P = 0.42$). Circulating LH increased after GnRH injection and peaked at 2 h with a subsequent decline to basal concentrations.

The percentage of heifers ovulating to the GnRH challenge on each day (Table 1) was not different between genotypes ($P > 0.10$). All heifers ovulated in response to GnRH on D5 and D6, regardless of genotype. Trio carrier heifers had significantly more ovulations than noncarrier heifers on each day in which ovulation occurred for both groups (Table 1). The largest follicle (F1) diameter for both Trio carrier and noncarrier heifers on each day after follicle aspiration is shown in Figure 2A. Visual inspection of the distribution indicates that the proportion of follicles ovulating in response to GnRH increases with day in both genotypes, despite follicles in Trio carriers being of smaller diameter.

Analysis of the four largest follicles (F1, F2, F3, and F4) in each heifer and whether each follicle ovulated in response to the GnRH challenge, regardless of day, allowed evaluation of ovulatory capacity as a function of follicle size in each genotype (Figure 2B). The probability of ovulation of follicles increased with size ($P < 0.001$), differed between genotypes ($P < 0.001$), but no genotype by follicle

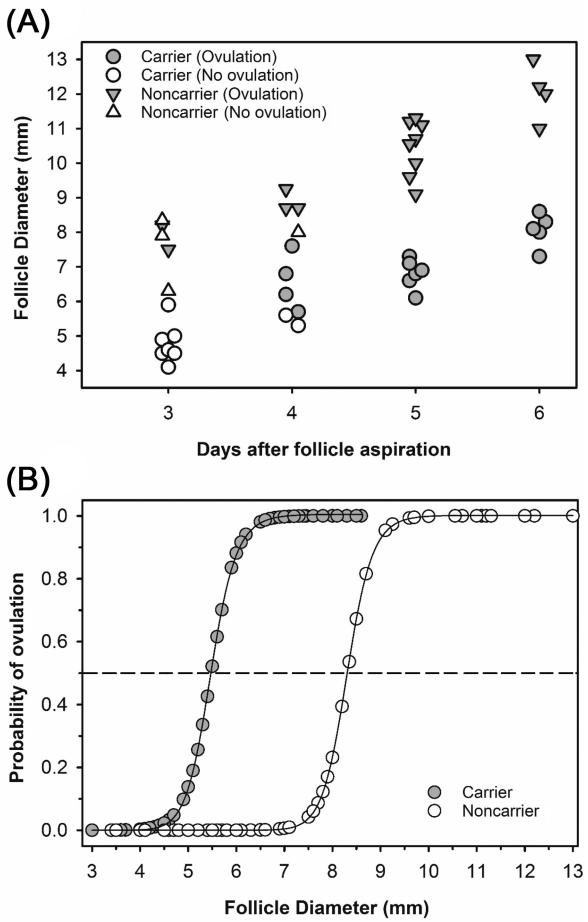

Figure 2. (**A**) Follicle size distribution of the largest follicle (F1) on each day in which the GnRH challenge was performed in Trio carriers (n = 25) and noncarrier control (n = 22) heifers. Open and closed symbols indicate whether that follicle ovulated or not in response to GnRH. In some cases, symbols are slightly offset to allow readers to visualize the data for all animals. (B) Predicted probability of ovulation as a function of observed follicle size (diameter) for Trio carriers (n = 100 follicles) and noncarrier control (n = 87 follicles) heifers. Each point represents the computed predicted probability of ovulation for an identified follicle based on its observed diameter. The dashed line indicates 50% probability of ovulation. There was a significant effect of follicle size ($P < 0.001$) and genotype ($P < 0.001$).

size interaction was detected ($P = 0.55$). As seen in Figure 2B, follicles from Trio carriers acquire ovulatory capacity at a smaller size, as illustrated by the calculated 50% probability of ovulation having a mean follicle diameter of 8.3 mm in noncarrier heifers and 5.5 mm in Trio carriers. On a volume basis, this represents 301 and 86 mm^3 for noncarriers and Trio carriers respectively, which translates into a 3.5-fold greater volume in noncarriers.

Experiment 2: dominance based on intrafollicular factors and granulosa cell mRNA

Overall 33 and 29 collections were available for Trio carriers and noncarrier controls, which generated 123 and 96 individual follicles, respectively. Serum P4 concentration was not different between genotypes ($P = 0.99$) and day ($P = 0.31$) with no genotype by day interaction ($P = 0.39$). Average P4 concentrations were 2.1 ± 0.2 ng/ml and 2.1 ± 0.2 ng/ml for Trio carriers and noncarrier control heifers, respectively. Analysis of circulating FSH concentrations showed no effect of genotype ($P = 0.30$), day ($P = 0.11$), or genotype by day interaction ($P = 0.18$).

The comparison of follicles based on hierarchy (F1, F2, F3, etc.) and day of collection after follicle aspiration is shown in Figure 3. In the upper graphs, the follicle diameter is shown with a single dominant follicle (F1) being selected D4 in noncarriers, whereas three dominant follicles are present by D4 and D5 in Trio carriers, based on follicle diameter. In the lower graphs, intrafollicular E2 concentrations are evaluated in the F1, F2, F3, and F4 (only for carriers) on the different days after aspiration. In noncarriers, there is clear selection of a single dominant follicle with follicular fluid E2 increasing in the F1 on D3 and increasing to dominant follicle concentrations by D4 and D5, whereas E2 in F2 and F3 remains at lower concentrations. In contrast, follicular fluid E2 in Trio carriers (Figure 3, lower right) increased in the F1 by D3 but a more gradual increase was observed in F2 and F3, as a subset of these follicles had elevated E2 on D2. On D4 and D5, all F1, F2, and F3 follicles had similar elevated follicular fluid E2 concentrations and they were greater than the E2 in the F4 follicles.

Intrafollicular hormone concentrations by follicle size class

In order to evaluate the effect of follicle size on hormonal concentrations within the follicular fluid, follicles were grouped into size categories from 2.5 to 9.49 mm by 1-mm increments, thus yielding classes with a mean diameter of approximately 3, 4, 5, 6, 7, 8, and 9 mm. Follicles equal or greater than 9.5 mm, only present in the noncarrier control group, were combined into the size class of 10 mm. The resulting follicle size classes with mean follicle diameter, number of samples per class, and range are shown in Table 2. As expected, mean diameter of follicles in each size category did not differ between genotypes ($P > 0.10$).

Estradiol concentration in the follicular fluid increased with follicle size in both Trio carriers ($P < 0.001$) and noncarrier controls ($P < 0.001$; Figure 4). However, the first significant E2 increase in Trio carriers was observed between 5 and 6 mm follicles ($P < 0.01$), while in noncarrier controls a similar E2 increase was observed between 7 and 8 mm ($P < 0.01$). These increments represented ~1.4-fold and ~2.1-fold increases in intrafollicular E2. After the initial increase in E2, there were further increases in E2 in the 7 and 8 mm follicles for Trio carriers and in the 9 and 10 mm follicles for noncarriers. Between genotypes, E2 concentrations were different ($P < 0.001$) for follicles in the 6, 7, and 8 mm size categories; however, 5 mm follicles were not different ($P = 0.26$).

Intrafollicular concentrations of inhibin A and free IGF1 were available for a subset of samples, as indicated in Figure 5. Inhibin A concentrations were not different among follicle size categories for Trio carriers ($P = 0.56$) and noncarrier controls ($P = 0.16$; Figure 5A). Comparisons between genotypes indicated that noncarrier follicles of 7 and 8 mm had greater ($P < 0.05$) concentrations of inhibin A than Trio carrier follicles of similar diameter, while follicles of 6 mm were not different between genotypes ($P = 0.51$).

Free IGF1 concentrations were different among follicle size categories in noncarrier controls ($P = 0.008$) and tended to be different in Trio carriers ($P = 0.09$; Figure 5B). In noncarrier controls, follicles in the larger size categories, 9 and 10 mm, had greater ($P < 0.05$) free IGF1 concentrations versus smaller size categories with the exception of 5 mm follicles. Within Trio carriers, follicles of 8 mm had greater ($P < 0.03$) free IGF1 concentrations than 4 and 5 mm follicles and tended to be greater ($P < 0.06$) than 6 mm follicles. Between genotypes, follicles in the 6 to 8 mm size categories were not different ($P > 0.10$) while 5 mm follicle of noncarriers had greater

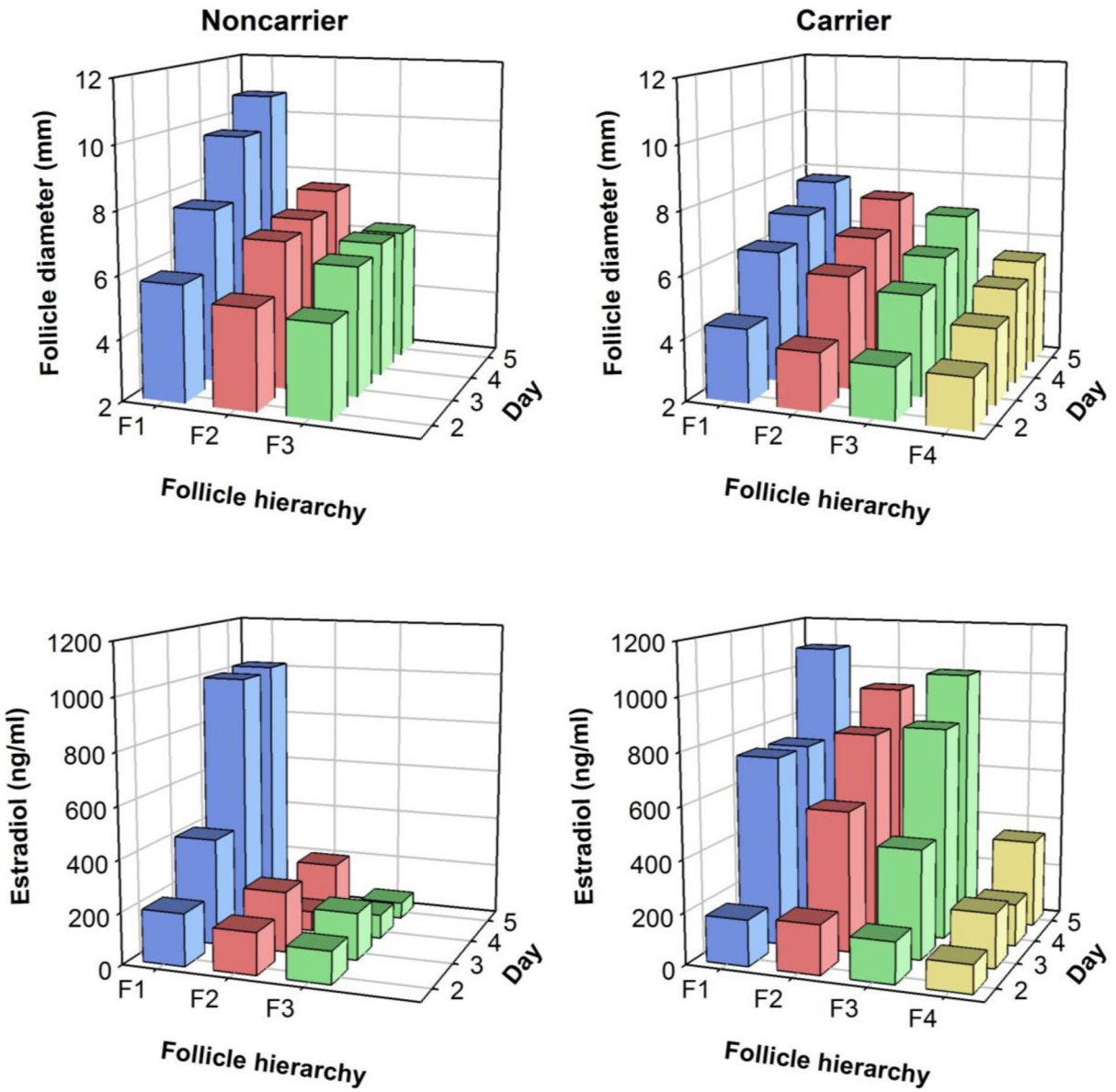

Figure 3. Follicle diameter and follicular fluid concentration of estradiol in individual follicles ordered by their hierarchy (F1, F2, etc.) on different days after follicle wave emergence synchronization in Trio carrier and noncarrier control heifers. Data are presented as means.

($P = 0.03$) free IGF1 than Trio carriers. Progesterone concentrations in the follicular fluid (data not shown) were not different among follicle size categories in Trio carriers ($P = 0.86$) or noncarrier controls ($P = 0.42$). In addition, in follicle categories 5 to 8 mm, no differences in follicular fluid P4 concentrations between genotypes were detected ($P = 0.30$).

Gene expression in granulosa cell by follicle size class

Granulosa cell mRNA expression was evaluated in a subset of samples that included the F1 to F4 in Trio carriers and F1 and F2 in noncarrier controls (Figure 5). Relative mRNA expression was calculated using the 5 mm size class of noncarrier controls as the reference group. Granulosa cell mRNA expression of *CYP19A1* increased with follicle diameter in both Trio carriers ($P < 0.001$) and noncarrier controls ($P < 0.001$; Figure 5C). The first significant increase (~3.3-fold) in Trio carriers was observed between 3 and 6 mm follicles ($P < 0.01$), while in noncarrier controls the increase (~4-fold) was observed between 8 and 9 mm ($P < 0.001$). However, the increase in *CYP19A1* mRNA appeared to be more abrupt in noncarrier heifers as no differences were seen among follicles of 5 to 8 mm ($P > 0.10$) and a sharp increase was observed between 8 and 9 mm, as indicated previously. In contrast, in Trio carriers there appeared to be a more gradual increase, as indicated by the lack of significant differences between adjacent follicle size categories, although GC mRNA concentrations increased steadily. Granulosa cell mRNA for *CYP19A1* was greater in Trio carrier follicles of 6 to 8 mm in diameter ($P < 0.05$) than in similar size follicles of noncarrier controls, while no differences ($P = 0.52$) between genotypes were observed for 5 mm follicles.

Granulosa cell mRNA concentrations of *LHCGR* increased with follicle diameter in both Trio carriers ($P < 0.001$) and noncarrier controls ($P < 0.001$; Figure 5D). The first significant increase in

Table 2. Follicle diameter (mean ± SEM) and range for each follicle size class in Trio carrier and noncarrier control heifers (experiment 2).

	Follicle size class (mm)							
	3	4	5	6	7	8	9	10
Carrier								
N	5	23	21	33	27	14	–	–
Mean diameter	3.3 ± 0.2	4.1 ± 0.1	4.9 ± 0.1	6.0 ± 0.1	6.9 ± 0.1	7.9 ± 0.1	–	–
Range	2.7–3.5	3.6 -4.4	4.5–5.4	5.5–6.4	6.5–7.4	7.5–8.9	–	–
Noncarrier								
N	–	–	13	27	18	11	7	9
Mean diameter	–	–	5.0 ± 0.1	5.9 ± 0.1	6.9 ± 0.1	7.8 ± 0.1	9.0 ± 0.1	10.8 ± 0.4
Range	–	–	4.6–5.4	5.5–6.4	6.5–7.4	7.5–8.2	8.6–9.2	9.7–13.3
P-value	NA	NA	0.20	0.12	0.34	0.30	NA	NA

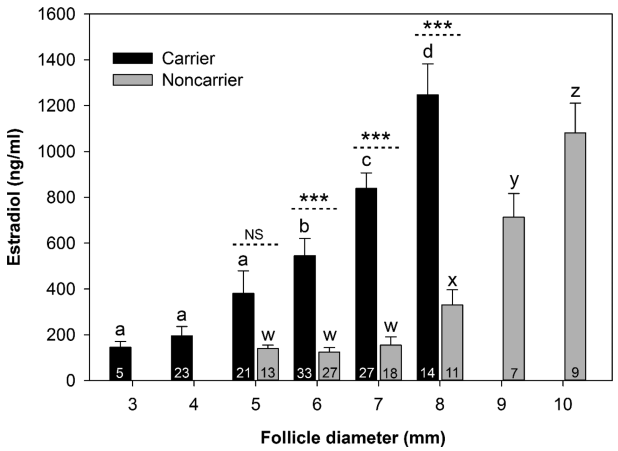

Figure 4. Intrafollicular estradiol concentrations (mean ± SEM) in follicles of different size categories of Trio carrier and noncarrier control heifers. Numbers at the base of each bar indicate the number of follicles in each size category. [a,b/y,z] Means with different superscripts within a genotype differ ($P < 0.05$). *Means between genotypes at a specified follicle size class differ (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS $P > 0.05$).

LHCGR (~2.7-fold relative to 5 mm; ~220-fold greater mRNA relative to reference group) in Trio carriers was observed between 5 and 6 mm follicles ($P < 0.01$) with further dramatic increases in *LHCGR* mRNA at 7 (523-fold relative to reference group) and 8 mm (~2000-fold relative to reference group). In noncarrier controls, the increase in *LHCGR* mRNA (~9-fold) was observed between 5 and 7 mm ($P < 0.001$) with a further dramatic increase in LHCGR mRNA in 9 and 10 mm follicles (>500-fold relative to reference group). Granulosa cell mRNA for *LHCGR* was greater in Trio carrier follicles of 5, 6, 7, or 8 mm in diameter ($P < 0.001$) than in similar size follicles of noncarrier controls.

The GC mRNA expression of *PAPPA* was influenced by follicle size in both Trio carriers ($P < 0.001$) and noncarrier controls ($P < 0.001$; Figure 5E). The first significant increase (~1.4-fold) in Trio carriers was observed between 5 and 6 mm follicles ($P < 0.01$), while in noncarrier controls the increase (~3.6-fold) was observed between 8 and 9 mm ($P < 0.001$). No further increases in *PAPPA* mRNA were observed with larger follicle size categories in Trio carriers and noncarrier controls ($P > 0.10$). Granulosa cell mRNA expression of *PAPPA* was greater in follicles from Trio carriers than noncarrier controls at 6, 7, and 8 mm ($P < 0.01$), while no difference was observed in 5 mm follicles ($P = 0.71$).

The mRNA expression of *FSHR* in GC was not influenced by follicle size in Trio carriers ($P = 0.72$), while a follicle size effect was detected in noncarrier controls ($P = 0.003$; Figure 5F). The size effect observed in noncarriers is due to an increase in *FSHR* mRNA between follicles of 8 and 9 mm in size ($P < 0.05$). There were no differences in *FSHR* mRNA between genotypes for follicles of 5 to 8 mm ($P > 0.12$).

Acquisition of dominance in individual follicles

In order to determine if an individual follicle had acquired dominance and thereby investigate the follicle size at which dominance was acquired in each genotype, we determined an intrafollicular E2 concentration cut-off point. The resulting cut-off value was 309 ng/ml of E2 and was determined as 3 SD above the mean for small follicles (3 mm for Trio carriers and 5 mm for noncarriers) assumed to be predominant. Thus, follicles with concentrations above this threshold were considered dominant. This approach allowed for the analysis of follicle size and the probability of acquisition of a dominant phenotype based on intrafollicular E2 concentrations for each genotype (Figure 6A). The probability of dominance, based on intrafollicular E2, increased with follicle size ($P < 0.001$) and was different between genotypes ($P < 0.001$), but no genotype by follicle size interaction was detected ($P = 0.69$). As shown in Figure 6A, follicles from Trio carriers acquire dominance at a smaller size with the calculated 50% probability of dominance, based on intrafollicular E2, being at a follicle diameter of 7.9 mm in noncarrier heifers and 5.5 mm in Trio carriers. On a volume basis, this represents 257 mm^3 and 85 mm^3 for noncarriers and Trio carriers respectively, which translates into a 3.0-fold greater volume in noncarriers.

A similar approach was taken to determine the acquisition of a dominant phenotype based on GC cell mRNA of selected markers. The selected cut-off value for the relative mRNA expression of *LHCGR*, based on ROC analysis, was 48.6 and exhibited 88.9% sensitivity and 82.1% specificity. Based on this cut-off value, the probability of dominance (Figure 6B) was greater with increasing follicle size ($P < 0.001$) and was different between genotypes ($P < 0.001$); however, no follicle size by genotype interaction was detected ($P = 0.23$). Trio carriers acquired dominance, based on expression of *LHCGR* mRNA, at a significantly smaller size than noncarrier control heifers. The follicle size (diameter) at which a 50% probability of achieving dominance was calculated to be 8.1 mm in noncarriers (279 mm^3) and 5.3 mm in carriers (79 mm^3) represents a 3.5-fold greater volume for noncarriers than Trio carriers.

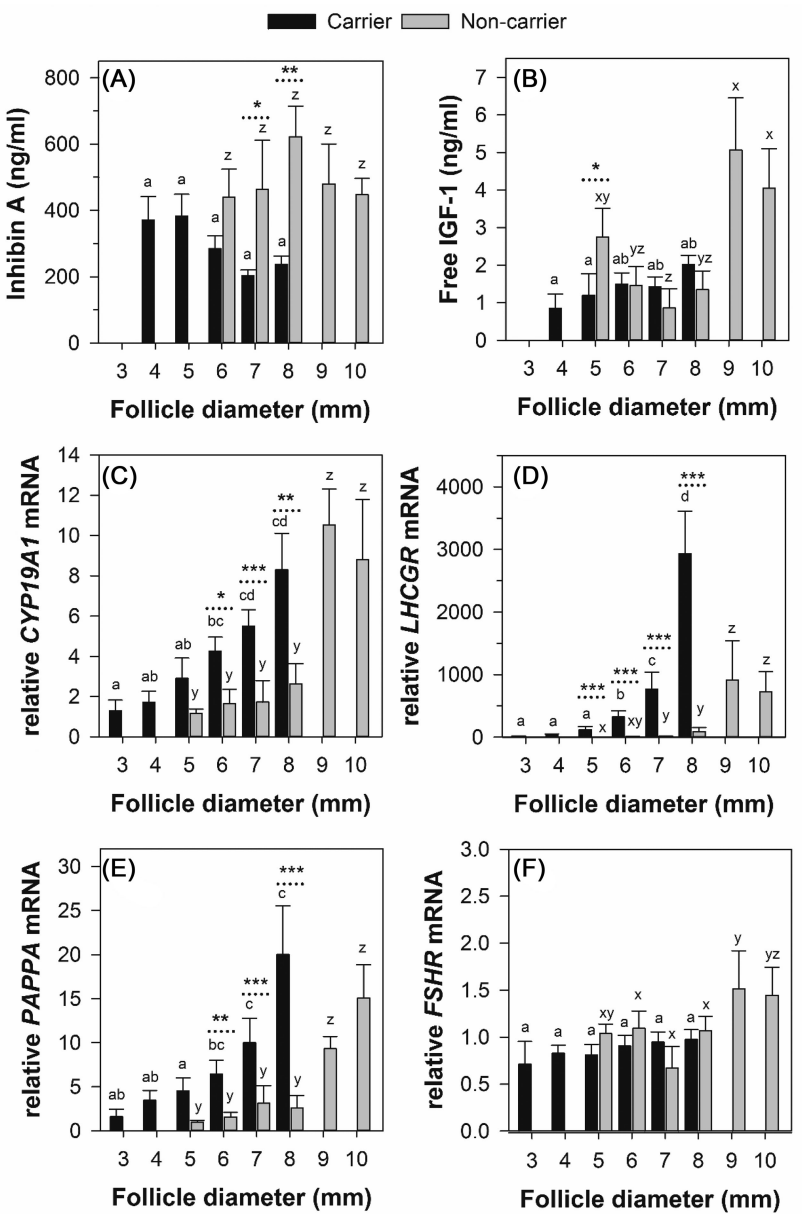

Figure 5. Intrafollicular inhibin A (A) and free IGF1 (B) concentrations (mean ± SEM) in follicles of different size categories of Trio carrier and noncarrier control heifers. Granulosa cell mRNA expression of cytochrome P450, family 19, subfamily A, polypeptide 1 (*CYP19A1;* C), luteinizing hormone/choriogonadotropin receptor (*LHCGR;* D), pappalysin 1 (*PAPPA*, previously known as pregnancy-associated plasma protein A, pappalysin 1; E), and follicle stimulating hormone receptor (*FSHR;* F) in follicles of different size categories of Trio carrier and noncarrier control heifers. Data on granulosa cell gene expression are presented as mean (±SEM) fold change in relation to the reference group, 5 mm size class of noncarriers.[a,b/y,z] Means with different superscripts within a genotype differ ($P < 0.05$). *Means between genotypes at a specified follicle size class differ (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

The selected cut-off value for the relative mRNA expression of *CYP19A1*, based on ROC analysis, was 2.3 and exhibited 81.9% sensitivity and 78.8% specificity. Based on this cut-off, the probability of dominance was greater with increasing follicle size ($P < 0.001$) and was different between genotypes ($P < 0.001$); however, no follicle size by genotype interaction was detected ($P = 0.73$). Trio carriers acquired dominance, based on expression of *CYP19A1* mRNA, at a significantly smaller size than noncarrier control heifers (Figure 6C). The follicle size (diameter) at which a 50% probability of achieving dominance was 7.6 mm in noncarriers (231 mm^3) and 5.5 mm in carriers (88 mm^3) represents a 2.6-fold greater volume for noncarriers than Trio carriers.

Finally, the selected cut-off value for the relative mRNA expression of *PAPPA*, based on ROC analysis, was 2.3 and exhibited 83.3% sensitivity and 81.0% specificity. The probability of dominance, based on *PAPPA* mRNA, of follicles increased with size ($P < 0.001$) and was different between genotypes ($P < 0.001$), but no genotype by follicle size interaction was detected ($P = 0.30$). As shown in Figure 6D, follicles from Trio carriers acquired dominance at a smaller size, with the calculated 50% probability of dominance being at a mean follicle diameter was 8.0 mm in noncarrier heifers and 5.4 mm in Trio carriers. On a volume basis, this represents 268 mm^3 and 84 mm^3 for noncarriers and Trio carriers respectively, which translates into a 3.2-fold greater volume in noncarriers.

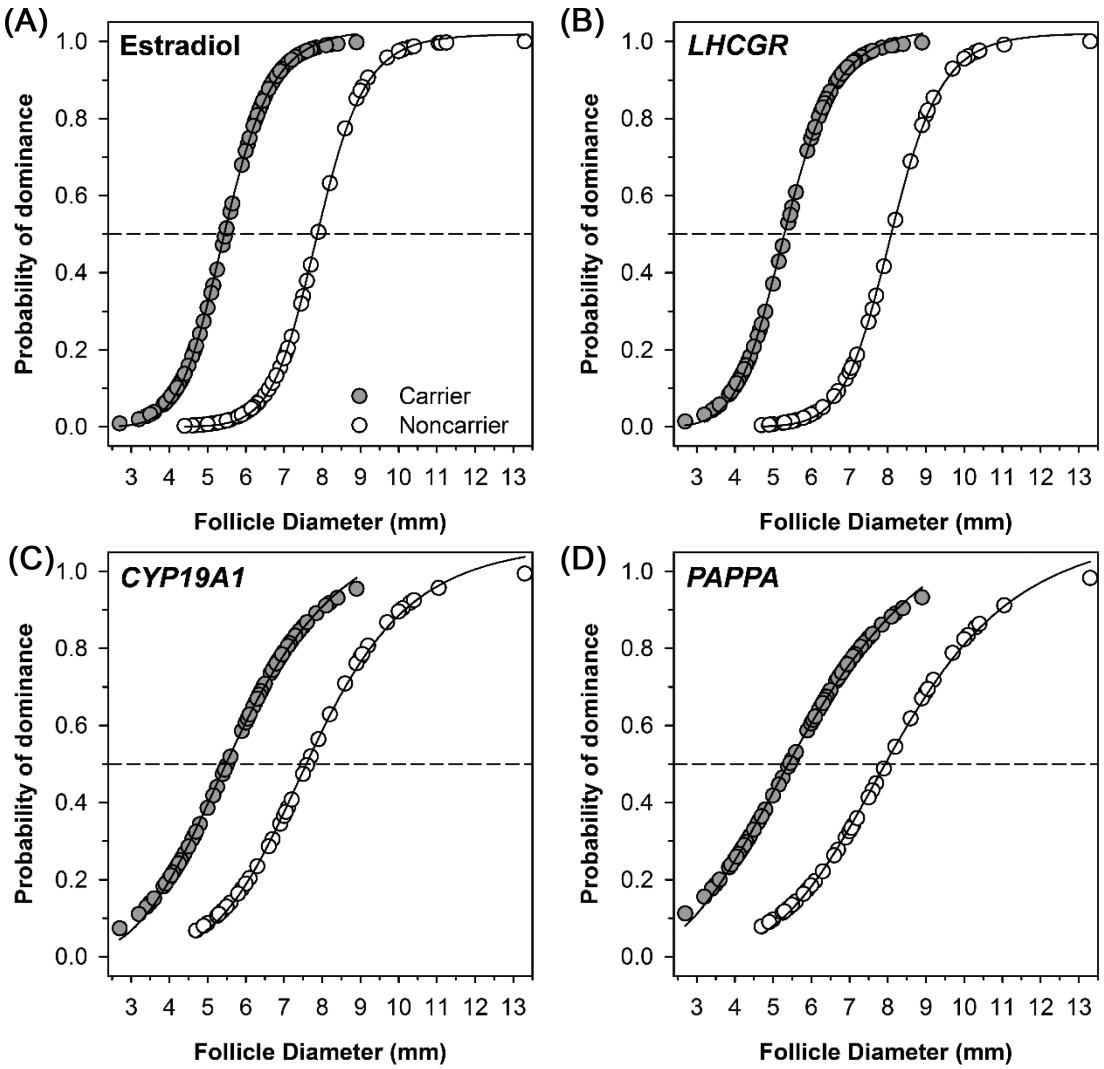

Figure 6. Predicted probability of dominance, based on intrafollicular estradiol (A), relative LHCGR mRNA (B), CYP19A1 mRNA (C), and PAPPA mRNA (D), as a function of observed follicle size (diameter) for Trio carriers and noncarrier control heifers. The dashed line indicates 50% probability of dominance. Each point represents the computed predicted probability of dominance for a collected follicle based on its diameter. There was significant effect of follicle size ($P < 0.001$) and genotype ($P < 0.001$) on the probability of dominance for all factors evaluated. The number of follicles in panel A was 122 and 85 follicles and for panels B to D 103 and 54 follicles for Trio carrier and noncarrier controls, respectively.

The percentage of heifers with at least one dominant follicle, based on intrafollicular E2, on days 2, 3, 4, and 5 for Trio carrier vs noncarrier heifers (all $P > 0.10$) was as follows: D2, 28.6% (2/7) vs 14.3% (1/7); D3, 75% (6/8) vs 77.8% (7/9); D4, 100% (9/9) vs 100% (6/6); and D5, 100% (9/9) vs 100% (7/7). The number of dominant follicles between genotypes was not different on D2 (1.0 ± 0.0 vs 1.0 ± 0.0; $P > 0.10$), but were greater in Trio carriers than in noncarrier controls on D3 (2.3 ± 0.4 vs 1.3 ± 0.2; $P = 0.05$), D4 (2.9 ± 0.2 vs 1.0 ± 0.0; $P < 0.001$), and D5 (3.1 ± 0.2 vs 1.1 ± 0.2; $P < 0.001$). The percentage of heifers of both genotypes that had 0, 1, 2, or ≥3 dominant follicles, based on intrafollicular E2 concentrations, on each day is shown in Figure 7. On D2, the majority of heifers do not have any dominant follicles (85.7% and 71.4%, for noncarrier and Trio carriers, respectively). Interestingly, the remaining heifers had only one dominant follicle, regardless of genotype. On subsequent days, the percentage of heifers with at least one dominant follicle increased. In noncarriers, the increase is mainly due to a decrease in percentage of heifers with no dominant follicles and an increase in percentage of heifers with one dominant follicle, although some heifers (22% on D3; 12% on D5) had two dominant follicles, based on intrafollicular E2. In contrast, Trio carriers displayed a progressive shift in the number of dominant follicles. On D2, most Trio carriers had no dominant follicles, although 28.6% of heifers had one dominant follicle. On D3, there was a distribution of Trio carriers across categories with some having zero dominant follicles (25%), some with one (12.5%), some with two (37.5%), and some with ≥3 (25%). By D4, most Trio carriers had ≥3 dominant follicles (77.8%) with the rest having two (22.2%). Similarly on D5, most carriers had ≥3 dominant follicles (88.9%).

Intrafollicular factors and granulosa cell mRNA in predominant and dominant follicles

In order to evaluate changes associated with follicles in the transition from predominance to dominance, follicles were classified within genotype into predominant and dominant based on the intrafollic-

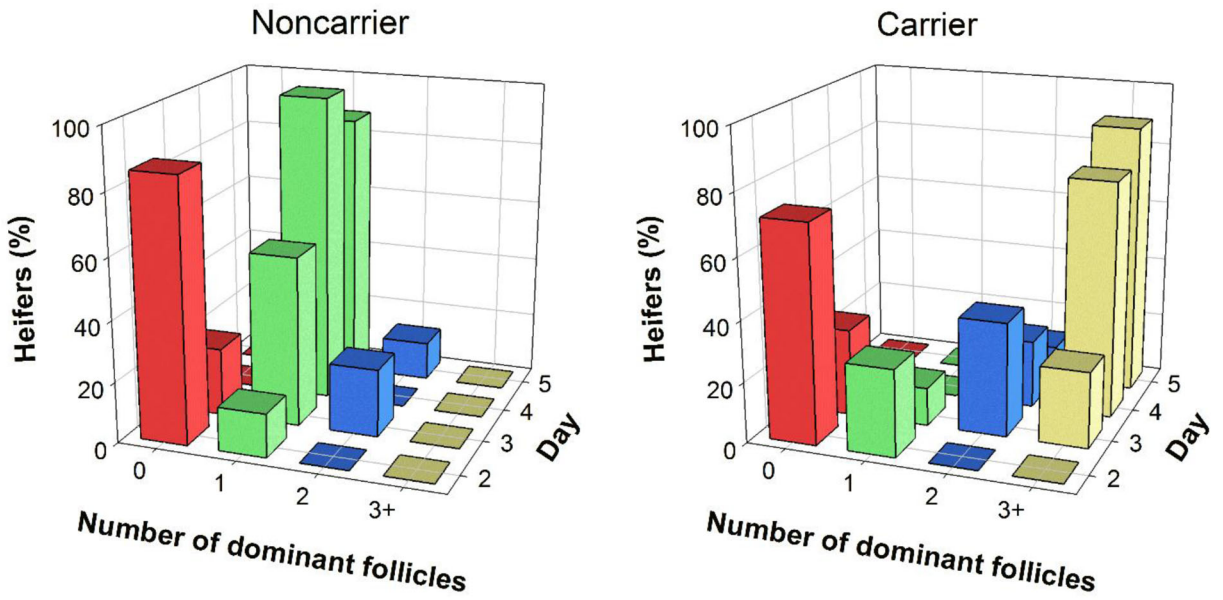

Figure 7. Percentage of Trio carrier and noncarrier control heifers on each day after wave emergence synchronization with 0, 1, 2, or ≥3 dominant follicles based on intrafollicular estradiol concentrations (>309 ng/ml = dominant). The number of heifers on each day was as follows: D2 (7 vs 7), D3 (8 vs 9), D4 (9 vs 6), and D5 (9 vs 8) for Trio carrier and noncarrier controls, respectively.

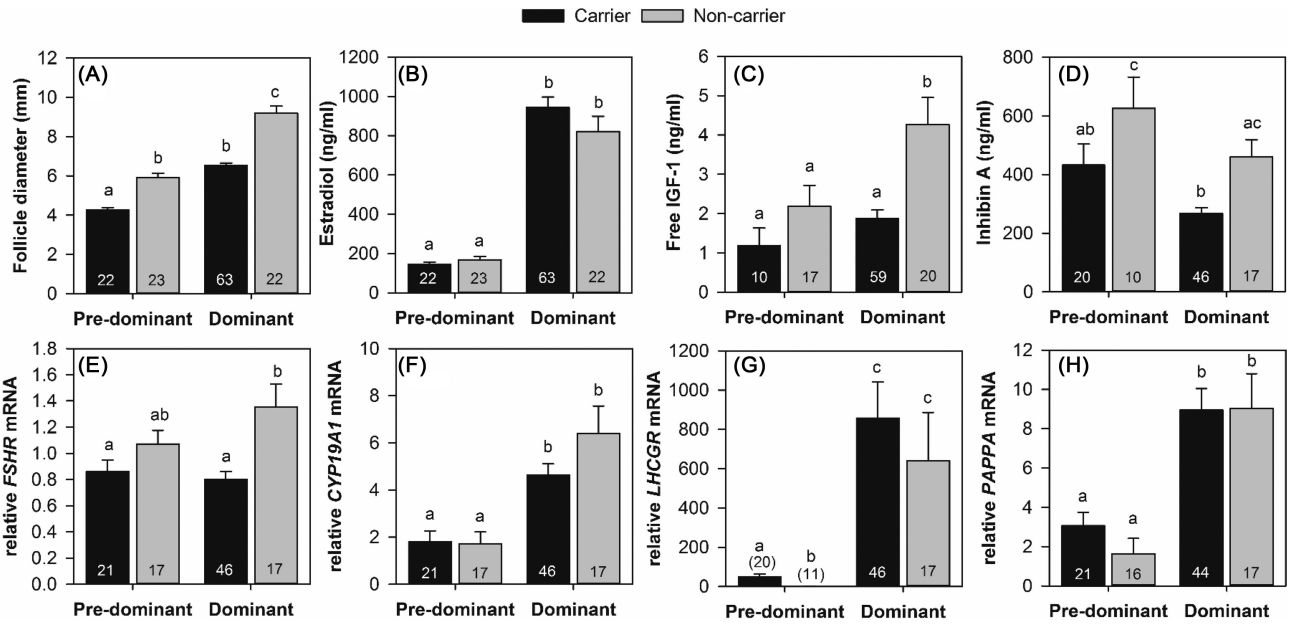

Figure 8. Follicle diameter (A), intrafollicular estradiol (B), free IGF1 (C), and inhibin A (D) concentrations and granulosa cell mRNA expression of follicle stimulating hormone receptor (*FSHR; E),* cytochrome P450, family 19, subfamily A, polypeptide 1 (*CYP19A1; F*), luteinizing hormone/choriogonadotropin receptor (*LHCGR; G),* and pappalysin 1 (*PAPPA*, previously known as pregnancy-associated plasma protein A, pappalysin 1; *H*) in predominant or dominant follicles of Trio carrier and noncarrier control heifers. Predominant follicles include follicles of heifers in which intrafollicular estradiol of any given follicle did not exceed 309 ng/ml, while dominant follicles represent follicles that had exceeded that threshold. Data are presented as mean (±SEM), and numbers at the base of each bar indicate number of follicles in any given category. [a,b]Means with different superscripts differ ($P < 0.05$).

ular E2 concentrations, as described above. However, in order to avoid the inclusion of subordinate follicles, after dominance had been acquired, follicles were classified as follows. The follicles, irrespectively of their hierarchy, within a collection for any given heifer were included in the predominant group if each follicle had not exceeded the E2 threshold. In contrast, only follicles that had exceeded the E2 threshold within a collection in any given heifer were included in the dominant group. As a result, follicles with E2 lower than the threshold that originated from a collection in which at least one of the follicles had acquired dominance were not included.

Analysis of follicle diameter showed a main effect of genotype ($P < 0.001$) and dominance status ($P < 0.001$), but no genotype by dominance status interaction ($P = 0.76$; Figure 8A). Follicle diameter increased ($P < 0.001$) between predominant and

dominant follicles for both Trio carriers and noncarrier controls; however, at both stages noncarrier follicles had a greater diameter ($P < 0.001$). Intrafollicular E2 had a main effect of dominance status ($P < 0.001$) but no effect of genotype ($P = 0.93$) or genotype by dominance status interaction ($P = 0.39$; Figure 8B). The acquisition of dominance was associated with a 6.5-fold and 4.9-fold increase in E2 concentrations for Trio carriers and noncarrier controls, respectively.

Analysis of free IGF1 concentrations in follicular fluid showed a main effect of genotype ($P = 0.01$) and dominance status ($P = 0.04$) but no genotype by dominance status interaction ($P = 0.29$; Figure 8C). Free IGF1 concentrations were not different between genotypes in predominant follicles ($P = 0.37$) but were greater ($P = 0.004$) in noncarriers than in Trio carriers in dominant follicles. Within genotype, there was an increase in free IGF1 in dominant follicles as compared to predominant follicles in noncarrier controls ($P = 0.01$), while no change was observed in Trio carriers ($P = 0.48$).

Intrafollicular inhibin A concentrations exhibited a main effect of genotype ($P = 0.006$), but no effect of dominance status ($P = 0.17$) and no interaction ($P = 0.63$; Figure 8D). Inhibin A concentrations were greater in noncarrier controls than in Trio carriers regardless of whether follicles were predominant or dominant ($P < 0.04$).

Analysis of *FSHR* mRNA expression in GC of predominant and dominant follicles of each genotype indicated a main effect of genotype ($P = 0.009$) but no effect of dominance status ($P = 0.15$) or genotype by dominance status interaction ($P = 0.75$; Figure 8E). Dominant follicles of noncarrier controls had greater mRNA for *FSHR* than the predominant and dominant follicles of Trio carriers ($P < 0.02$). The *CYP19A1* mRNA analysis showed a main effect of dominance status ($P = 0.0002$) but no genotype effect ($P = 0.26$) and no genotype by dominance status interaction ($P = 0.23$; Figure 8F). Dominant follicles had greater ($P < 0.04$) expression of *CYP19A1* than predominant follicles regardless of genotype.

Granulosa cell mRNA expression for *LHCGR* showed a main effect of genotype ($P = 0.02$) and dominance status ($P < 0.001$), as well as a trend ($P = 0.06$) for a genotype by dominance status interaction (Figure 8G). Trio carrier predominant follicles had greater ($P = 0.01$) mRNA expression for *LHCGR* than noncarrier predominant follicles. In addition, dominant follicles had greater ($P < 0.001$) mRNA expression of *LHCGR* than predominant follicles regardless of genotype.

Analysis of mRNA concentrations of *PAPPA* indicated a main effect of dominance status ($P < 0.001$) but no effect of genotype ($P = 0.73$) or genotype by dominance status interaction ($P = 0.36$; Figure 8H). Dominant follicles had greater ($P < 0.02$) mRNA for *PAPPA* as compared to predominant follicles in both Trio carriers and noncarrier controls.

Granulosa cell SMAD6 mRNA expression in relation to follicle size and dominance status

Analysis of mRNA concentrations for *SMAD6* by follicle size within each genotype indicated an effect of follicle size in Trio carriers ($P = 0.02$), but not in noncarrier controls ($P = 0.61$; Figure 9A). Expression of *SMAD6* mRNA in follicle size categories in which data were available for both genotypes (5–8 mm) indicated that Trio carriers had much greater expression of *SMAD6* mRNA than noncarrier controls ($P < 0.001$). Analysis of mRNA expression of *SMAD6* in relation to follicle dominance status indicated a main effect of genotype ($P < 0.0001$) but no dominance status ($P = 0.12$) or genotype by dominance status interaction ($P = 0.75$; Figure 9B). Trio carriers had greater expression of *SMAD6* mRNA than noncarrier controls ($P < 0.0001$) in follicles that were predominant (9.7-fold) or dominant (7.3-fold).

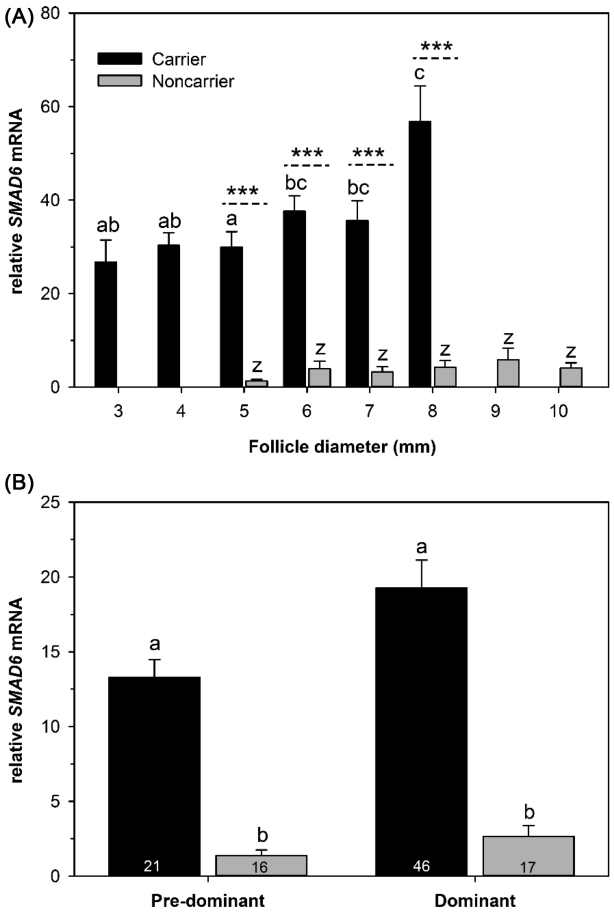

Figure 9. Granulosa cell mRNA expression of MAD family member 6 (*SMAD6*, previously known as MAD, mothers against decapentaplegic homolog 6) in different follicle size classes (A) and predominant or dominant follicles (B) of Trio carrier and noncarrier control heifers. The number of follicles included in each size class was as follows: 2.5–3.49 mm (4 vs 0), 3.5–4.49 mm (22 vs 0), 4.5–5.49 mm (18 vs 8), 5.5–6.49 mm (26 vs 11), 6.5–7.49 mm (20 vs 8), 7.5–8.49 mm (9 vs 10), 8.5–9.49 mm (0 vs 6), ≥9.5 mm (0 vs 6). Predominant follicles include follicles of heifers in which intrafollicular estradiol of any given follicle did not exceed 309 ng/ml, while dominant follicles represent follicles that had exceeded that threshold. Data are presented as mean (±SEM), and numbers at the base of each bar indicate number of follicles in any given category. $^{a,b/y,z}$ Means with different superscripts differ ($P < 0.05$). *Means between genotypes at a specified follicle size class differ (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS $P > 0.05$).

Discussion

This study provides further insight into the mechanisms underlying selection of multiple follicles in the recently discovered bovine high fecundity allele, Trio [31]. Granulosa cells from dominant follicles of Trio carriers have been shown to have greater expression of *SMAD6* mRNA, an inhibitory member of the bone morphogenetic protein 15 (BMP15) signaling pathway [37]. In this study, we confirmed the overexpression of SMAD6 in GC from Trio carrier follicles by analysis of more than 200 individual follicles. It is clear that there is a large difference (>7-fold) with no overlap in concentrations between

SMAD6 expression in Trio carriers compared to noncarriers, regardless of the size of the follicle or their dominance status. Thus, overexpression of SMAD6 continues to be the most reasonable explanation for the differences in physiology and ovulation rate that define the Trio genotype. In addition, we recently reported that follicles of Trio carriers had a reduced size throughout the follicular wave compared to noncarrier controls; however, the total dominant follicle volume (all dominant follicles) was similar to noncarrier controls [32]. Furthermore, Trio carriers undergo diameter deviation at a similar time after wave emergence albeit at a much smaller follicle size. Based on these results, we propose a physiological model that was tested, in part, in this study. The key component of this model being tested in this study was the acquisition of dominance at a smaller follicle size and in a hierarchal manner in carriers of the Trio high fecundity allele. Follicles of Trio carriers acquired a dominant phenotype, as determined by intrafollicular E2 and *CYP19A1* concentrations in GC, at significantly smaller size than noncarriers. Noncarriers acquired the dominant phenotype near 8.5 mm, which has been shown to be the time of acquisition of follicle dominance in *Bos taurus* cattle in previous studies [1, 2]. Moreover, ovulatory capacity in response to GnRH and increased LHCGR in GC occurred at a reduced follicle size in Trio carriers than in noncarriers. Overall, the different measures of follicle dominance all occurred at a similar time in both Trio carriers and noncarrier controls but follicles of Trio carriers were approximately 1/3 the size, on a volume basis, compared to noncarriers. The smaller size of the dominant follicles in carriers of the Trio allele may be related to overexpression of SMAD6 in GCs, producing tonic inhibition of pathways activated by oocyte-derived TGFβ family members, such as BMP15, and reduced proliferation of GCs. The timing of acquisition of the dominant phenotype does not appear to be altered by activation of this inhibitory pathway and, interestingly, follicles in Trio carriers appear to acquire dominance in a hierarchal manner, as shown by the progressively greater number of follicles with a dominant phenotype between days 2 and 4 after wave emergence.

The increase in expression of *CYP19A1* in GC and the associated increase in intrafollicular E2 are characteristic features of the acquisition of dominance [6–8]. Intrafollicular E2 and relative mRNA expression of *CYP19A1* in GC were greater in Trio carriers than in noncarrier controls in follicles from 6 to 8 mm, and the first increase in E2 within each genotype was first detected in follicles of 6 mm for Trio carriers while a similar increase was not observed until 8 mm in noncarrier controls. In agreement with our results, greater aromatase activity and E2 concentrations in the follicular fluid of smaller size follicles (2–3 mm) have been reported in ewes carrying the high fecundity Booroola/FecB mutation, and maximal concentration was achieved at 3–4.5 mm [26, 27, 38]. However, similar E2 concentrations were only achieved in control ewes when they reached >5 mm, indicating that follicles of Booroola/FecB carriers develop a dominant phenotype at a smaller size. More interestingly, when dominant follicles of both genotypes were compared, regardless of size, E2 concentrations in the follicular fluid were similar. Thus, follicle dominance, as determined by intrafollicular E2 and GC *CYP19A1* expression, was clearly attained in follicles of smaller sizes in carriers of Trio, consistent with the Booroola/FecB fecundity genotype.

The evaluation of LH receptors in GC, through the relative mRNA expression of *LHCGR*, is a definitive marker of dominance since only follicles with LH receptors in their GC are able to continue to grow past deviation and to ultimately ovulate [16, 17]. Follicles of 5 to 8 mm in Trio carriers had significantly greater expression of *LHCGR* mRNA in GC than similar sized follicles in noncarrier controls. However, much like what was observed for E2 and *CYP19A1* expression, dominant follicles of both genotypes had similar elevated expression of *LHCGR* mRNA although this phenotype was attained at 6 mm in Trio carriers and at 9 mm in noncarriers. Similarly, follicles of high fecundity ovine genotypes are able to produce cAMP, in response to LH, at a smaller size but in a similar manner as observed in large presumptive preovulatory follicles of wild-type controls [30, 38, 39]. Furthermore, small and medium sized follicles of ewes heterozygous for both Booroola/FecB and Inverdale/FecX^{I} were shown to have greater *LHCGR* mRNA than wild-type controls [40]. Ovulation of multiple, smaller-sized follicles is the hallmark feature of high fecundity genotypes in sheep [28, 41]. In this regard, we performed an evaluation that had not previously been done in high ovulation rate genotypes of any species by evaluating the presence of LH receptors in GC by determining ovulatory capacity in follicles of different sizes, by a challenge with GnRH. The use of acquisition of ovulatory capacity has been found to be a valuable tool to determine timing of acquisition of follicle dominance in vivo and has been utilized both in *Bos taurus* and *Bos indicus* cattle [17, 19]. Ovulatory capacity was acquired at a significantly smaller follicle size in Trio carriers (5.5 mm) than in noncarrier controls (8.3 mm); this size difference translates into ~3.5-fold greater volume in noncarriers that is in agreement with our previous finding of the follicle size at diameter deviation [32].

Our results with the IGF1 system are not definitive, with *PAPPA* mRNA showing a clear increase in expression in dominant follicles of both genotypes and at a smaller size in Trio carriers, whereas free IGF1 in follicular fluid showed a corresponding increase in noncarriers after acquisition of follicle dominance but no corresponding increase in free IGF1 was observed in Trio carriers. The role of the IGF1 system in selection of multiple follicles has been proposed based on results obtained with the USDA-MARC twinner cows. Twinner cows have been found to have greater plasma and intrafollicular IGF1 than unselected control cattle [42, 43]. In addition, follicles of MARC twinner cows have been shown to have greater binding activity of insulin like growth factor binding protein 3 (IGFBP3) and one form of insulin like growth factor binding protein 5 (IGFBP5), but lower binding activity of one form of insulin like growth factor binding protein 4 (IGFBP4) [43]. Another suggestive piece of information regarding the role of the IGF system in the selection of multiple follicles in the MARC twinner cattle was that small antral follicles (1–5 mm) of twinner cows had less insulin like growth factor 2 receptor (IGF2R) mRNA in GC [44]. The IGF2R has been shown to sequester insulin like growth factor 2 (IGF2), since this receptor lacks tyrosine kinase activity, and thus lower levels of IGF2R could indicate a greater availability of IGF-2 [45]. The results in Trio carriers obtained in this study do not support a causative role for the IGF system in the occurrence of multiple ovulations as seen by the similar mRNA expression of *PAPPA* mRNA in Trio carriers and noncarrier controls both in predominant and dominant follicles. Furthermore, the intrafollicular concentrations of free IGF1 were not different between genotypes in pre dominant follicles but more surprisingly Trio carriers had lower free IGF1 in dominant follicles. All of these results suggest that the mechanisms underlying selection of multiple follicles in USDA-MARC twinner cattle and Trio carriers are different.

A key insight provided by the present results relates to the acquisition of dominance and ovulatory capacity in relation to follicle size. The analysis of these data by logistic regression is a novel approach that has not been employed before in the evaluation of acquisition of dominance and ovulatory capacity. This strategy has allowed us

to obtain probability curves for either dominance (by each evaluated factor) or ovulatory capacity. The 50% probability point is perhaps the most meaningful point in the curve and thus was used to draw comparisons. The methodology utilized to determine cut-off values for gene expression in GC was based on E2 due to the reliability of the E2 assay for measurements of E2 in follicular fluid and to the consensus within the literature as to the increase in follicular fluid E2 during acquisition of dominance [7, 11, 15]. The follicle diameter at which there is a 50% probability of dominance, calculated based on analysis of each of the different dominant phenotypes, is strikingly similar within a genotype with a similar dramatic difference between genotypes based on determination of dominance by any of the criteria. Particularly striking is the almost identical size for acquisition of follicular dominance obtained in the completely independent evaluation of dominance based on timing of acquisition of ovulatory capacity in vivo. Comparison between all of the different methods produced a calculated size at 50% acquisition of follicle dominance with less than 0.2 mm difference between measures for Trio carriers and less than 0.7 mm difference for noncarrier controls. Overall, follicles of noncarrier controls acquire dominance at a follicle size that is 3-fold to 3.5-fold greater (on a volume basis) than that of Trio carriers. Thus, by using multiple measures of follicular dominance we have definitively supported our primary hypothesis of this study; follicles of Trio carriers acquire dominance at a much smaller size than age-matched, half-sib noncarriers.

Follicles in Trio carriers appeared to acquire dominance in a hierarchal manner, such that the "window" for acquiring dominance remains open for a longer period in Trio carriers due to the inability of smaller-sized dominant follicles in Trio carriers to provide final suppression of FSH. This was supported, in part, by the observation that acquisition of dominance, based on intrafollicular E2 in individual follicles of Trio carriers, occurred in only single follicles on D2, proceeding to two or three follicles on D3, and culminating in three or more follicles in nearly all (88.9%) Trio carriers by D5 (Figure 9). Nevertheless, no support for the hierarchal pattern was provided by the results of experiment 1 evaluating ovulatory capacity, since Trio carriers either had three follicles or no follicle ovulating in response to GnRH treatment on any given day. The discrepancy may be due to differences in state of the follicles in experiment 1 at GnRH treatment vs. experiment 2 when contents of dominant follicles were collected, since the two experiments were completely independent. Alternatively, the differences in method for measurement of dominance may account for the discrepancy, in particular GnRH administration will induce both an FSH surge and an LH surge, and these could provide extra stimulation for follicles that are relatively close to acquisition of follicular dominance. Nevertheless, previous studies indicate an interval of <8 h for execution of follicular deviation during selection of a single dominant follicle [3] and, although the time is likely to be longer for selection of multiple follicles, it seems probable that this process will occur in <24 h preventing subsequent follicles from attaining dominance. Obviously, future research that focuses more definitively on this time period and research question is needed in order to resolve these concepts.

Greater FSH sensitivity of GC from high fecundity genotypes has been proposed as a potential mechanism underlying the selection of multiple follicles [46, 47]. This model has been recently updated particularly in relation to the Booroola/FecB mutation and links the decreased BMP signaling with lower AMH production which in turn modulates FSH sensitivity [46]. In this study, we evaluated the abundance of *FSHR* mRNA and found no difference in similar sized follicles or predominant follicles between Trio carriers and noncarrier controls. Unexpectedly, in dominant follicles *FSHR* mRNA was greater in noncarriers than Trio carriers, indicating that, at least from a receptor standpoint, Trio carriers do not have an increased advantage in FSH sensitivity. In contrast, in our previous study there was a slight increase in circulating FSH encompassing the time of diameter deviation in Trio carriers and we proposed that this could be the result of smaller follicles acquiring dominance in a hierarchal manner which in turn prevents the final suppression of FSH until a sufficient total dominant follicle volume is reached by the acquisition of dominance in multiple follicles. In this regard, it may be noteworthy that intrafollicular inhibin A was lower in Trio carriers at both the predominant and dominant follicle stages, as compared to controls. Decreased inhibin A output by follicles is likely to result in lower circulating inhibin A which may decrease inhibition of FSH secretion. Unfortunately, we were unable to determine circulating inhibin A concentrations because the assay used in this study was not sensitive enough to detect inhibin A in bovine serum (data not shown). Previous research in Booroola/FecB ewes failed to detect differences in circulating immunoreactive inhibin or dimeric inhibin A [48, 49]. In agreement with the Booroola/FecB data, RNA-seq analysis of GC from Trio carriers and noncarrier controls failed to detect differences in the mRNA expression for the inhibin A subunits, inhibin alpha subunit (INHA) and inhibin beta A subunit (INHBA) [37].

The precise mechanism by which multiple ovulations occur in high fecundity genotypes, both in cattle and sheep, has not been completely resolved. This study provides key information that should allow development of a physiological model for increased ovulation in high fecundity genotypes such as (1) follicles of Trio carriers acquire a dominant phenotype (i.e. E2, *CYP19A*, *LHCGR*, and *PAPPA*) and ovulatory capacity at smaller follicle size but at a similar time after emergence of the follicular wave compared to age-matched, half-sib noncarrier controls; (2) *SMAD6* is overexpressed in GC of Trio carriers at all follicles sizes and regardless of dominance status; (3) follicles in Trio carriers may acquire dominance in a hierarchal manner; and (4) characteristics of dominant follicles in Trio carriers are similar in almost every property to the dominant follicle phenotype observed in noncarriers with the exception of free IGF1 which was lower in Trio carriers.

Supplementary data

Supplementary data are available at *BIOLRE* online.

Supplemental Table S1. Primer sequences utilized for the evaluation of granulosa cell gene expression in Experiment 2.

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