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R. Santa Cruz

R. A. Cushman

C. Viñoles

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Antral follicular count is a tool that may allow the selection of more precocious Bradford heifers at weaning☆

R. Santa Cruz [a, *, 1], R.A. Cushman [b], C. Viñoles [a, 1]

[a] Programa Nacional de Investigación Producción Carne y Lana, INIA Tacuarembó, Ruta 5 km 386, 45000, Tacuarembó, Uruguay
[b] USDA, ARS, U.S. Meat Animal Research Center, Clay Center, NE, USA

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ABSTRACT

Although antral follicle count is a repeatable parameter across life that is positively associated with fertility, its use at weaning as a tool to discard less fertile heifers has not been extensively evaluated. The hypotheses of this work are: 1) maximum antral follicle count (MAFC) is repeatable between weaning and pre breeding evaluations, allowing selection of more fertile heifers at an early age, 2) heifers with high MAFC have growth and development parameters linked to an earlier puberty and pregnancy, 3) MAFC has a positive correlation with AMH concentrations, so that both could be used inter changeably. In this study, Hereford (n = 42 and n = 50) and Braford (n = 40 and n = 50) females were used in years 1 and 2; respectively, in a completely randomized experimental design. Heifers were examined for five to ten days at two different moments (post weaning and pre service), to determine MAFC. The concentrations of Anti müllerian hormone (AMH) were evaluated on the day of MAFC assessment. Growth and development parameters were evaluated post weaning and pre service. The repeatability of MAFC between post weaning and pre service evaluations was poor in three cases (Hereford Year 1 = 0.36 and 2 = 0.39 and Braford, Year 2 = 0.32) but it was high for Braford in Year 2 (0.72). The AMH repeatability between post weaning and pre service evaluations was high in one case (Braford Year 2 = 0.72) and moderate in the others (Year 1, Hereford = 0.50 and Braford = 0.52 and Year 2, Hereford = 0.50). In Year 2, Braford heifers with greater MAFC attained puberty at an earlier age ($r^2 = 0.129$; $P = 0.0196$). Also, diminished MAFC corresponded with decreased growth and development, thus less Braford heifers with low MAFC were inseminated (2/16), compared to those with medium (12/17) and high MAFC (7/17; $P < 0.01$). Moreover, Braford heifers with low AFC had less progesterone in the cycle post insemination but pregnancy rate was not affected. In Braford heifers in Year 2, there was a high correlation between MAFC and AMH concentrations (0.85 $P < 0.001$). The results of these experiments indicate that post weaning MAFC and AMH concentrations may be applied to select those Braford heifers that attain puberty at an early age, but these tools are not useful in Hereford heifers.

1. Introduction

Age at puberty and first calving affect the reproductive performance of breeding cows and sheep during the remainder of their productive life [1,2]. Heifers that conceive in the first 21 days of the breeding season, stay in the herd longer and wean heavier

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* Corresponding author.
E-mail address: rodrigo.santacruz@poloagroforestal.edu.uy (R. Santa Cruz).
[1] Present address: Polo Agroforestal, Estación Experimental Bernardo Rosengurtt, Facultad de Agronomía, Universidad de la República, Ruta 26 km 408, 37000, Cerro Largo, Uruguay.

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calves during their productive life [3]. Increased age, live weight and body condition improve the probability of attaining puberty and conceiving early [4]. Other parameters of growth and development such as hip height and body composition, have been used successfully because of their high association with live weight [5]. However, puberty is a complex process involving a series of events. Nutrition, age and genetics influence puberty, mainly as regulators of the maturation of the hypothalamic – pituitary - ovarian axis that must occur for the initiation of normal oestrous cycles [6]. The complexity of the onset of puberty explains that selecting replacement heifers based on body weight may not be enough. More recently, antral follicle count (AFC) and Anti müllerian (AMH) hormone concentration have been reported to be phenotype markers of fertility in heifers at mating [7]. Heifers that have greater AFC also have greater concentrations of AMH and progesterone and they become pregnant earlier than heifers with lower AFC [8–10]. Similarly as occurs in ewe lambs where an earlier attainment of puberty have been associated with increased circulating concentrations of AMH [11]. Greater concentrations of progesterone have a positive impact on embryo development [12], thus contributing to an earlier conception. Moreover, an earlier attainment of puberty allows the occurrence of more oestrous cycles before breeding and a greater fertility at first service [13]. Although, AFC is a repeatable parameter across the life of a cow [7], its use at weaning as a tool to discard less fertile heifers has not been extensively evaluated [14].

The hypotheses of this work are: 1) MAFC is repeatable between weaning and pre breeding evaluations, allowing selection of more fertile heifer at an early age; 2) Heifers with high MAFC have growth and development parameters related to an earlier puberty that could be associated with an earlier pregnancy; 3) AFC has a high correlation with AMH concentrations, so that both parameters would be used inter changeably.

The objectives were to evaluate MAFC repeatability between weaning and pre breeding, its correlation with AMH, parameters of growth and development, age at puberty, progesterone concentrations at the insemination cycle and pregnancy at 13–15 months in Hereford and Braford heifers.

2. Materials and methods

2.1. Location and animals

Animal experimentation was approved by the Ethics Committee for the Use of Animals (CEUA; file number 2013.13). Hereford and Braford females were used in different years (2013 2014: Year 1 and 2015 2016: Year 2), in a completely randomized experimental design. The evaluation period started with the selection of calves at weaning and ended with pregnancy diagnosis, 30 days after bull removal from the herd. The number of animals used in Year 1 was 42 Hereford and 40 Braford and in Year 2, 50 animals of each breed. The calves were daughters of at least seven different bulls per breed per year. Hereford heifers were managed in the Experimental Unit "Glencoe" and Braford heifers in the Experimental Unit "La Magnolia" both from INIA Tacuarembó.

2.2. Live weight, age at the beginning of evaluations and feeding

In Year 1, the animals were selected at weaning in April, with 189 ± 2.2 kg and 189 ± 2.5 kg of live weight and 198 ± 3 days and 182 ± 3 days of age, for Hereford and Braford calves, respectively. From weaning until middle September calves grazed on *Campos grasslands* [15] with a forage allowance of 10 kg of dry matter per kg of live weight (kg DM/kg LW) [16] and offered supplement *ad libitum* in an automatic self feeder. Thereafter, heifers grazed 4 h per day on improved pastures with a forage allowance of 7 kg DM/kg LW and the supplement was adjusted to 1% of the LW. The supplement provided 14% of crude protein (PC) and 3 Mcal of metabolizable energy (ME)/kg DM. The average weight gain between weaning and mating was 0.700 ± 0.1 kg in Hereford and 0.681 ± 0.1 kg in Braford heifers, attaining mating (at 13–15 months) with an average weight of 356 ± 3.5 kg and 322 ± 3.9 kg, respectively. During the breeding season, all heifers grazed *Campos grasslands* with a forage allowance of 7 kg DM/kg LW.

In Year 2, calves were weaned in March (Hereford) and April (Braford), with 164 ± 2.5 kg and 168 ± 3.6 kg of live weight and 165 ± 2 days and 174 ± 3 days of age; respectively. Between weaning and mating, they grazed annual winter pastures (oats and ryegrass); with an average forage allowance of 1.1 kg DM/kg LW in both systems. All females were supplemented with corn DDGS (Dried Distillers Grains with Solubles) (22.6% digestible protein and 2.8 Mcal/kg DM ME), that was adjusted according to the average LW. Supplementation was offered at 0.7–1.2% of the LW. The average daily weight gain from weaning to service was 0.855 ± 0.1 kg in Hereford and 0.669 ± 0.1 kg in Braford, reaching the beginning of mating (at 13–15 months) with an average LW of 378 ± 3.8 kg and 312 ± 4.6 kg in Hereford and Braford, respectively. During the breeding season, all heifers grazed *Campos grasslands* and sorghum in Hereford and setaria in Braford, at a minimum allowance of 7 kg MS/kg LW and 1 kg MS/kg LW in natural and annual summer pastures, respectively.

2.3. Breeding

In Year 1, all heifers were synchronized using the Ovsynch protocol [17] plus a progesterone device. They were inseminated artificially at fixed time 52–60 h after Cloprostenol, using frozen semen from two bulls per breed with more than 60% motile sperm after thawing. Ten days after artificial insemination, a single sound bull was introduced into each herd for an additional period of 50 days.

In Year 2, heifers were synchronized with synthetic prostaglandin F2 alpha (Cloprostenol D, 150 mg i.m., Dalmaprost® Laboratory Fatro, Uruguay) and the insemination was performed following heat detection for 5 days, using frozen semen from two different bulls per breed with the same conditions as in Year 1. After the end of insemination, a sound bull was introduced in each herd for an additional period of 50 days. Inseminations were performed by two trained technicians in Hereford and Braford in both years.

2.4. Animal measurements

2.4.1. Antral follicle count (AFC)

The maximum antral follicle count (MAFC) was evaluated in both ovaries, recording diameter, number, and position of all follicles ≥ 2 mm on ovarian maps. The evaluation was carried out at two different ages (post weaning = 246 days of age on average and pre service = 385 days of age on average) using a real time, B mode scanner with a transrectal probe of 7.5 MHz (Aloka Co., Ltd., Tokio, Japón). The same operator performed all measurements in each year, but the technician was different between years of the study. In Year 1, the evaluations (post weaning and pre service) were carried out 6 times over 12 days, while in Year 2, they were done during five consecutive days.

In the post weaning evaluation, a rigid probe was used and manipulated externally applying the methodology described by Viñoles et al. [18], in which the ovaries were visualized using the bladder and the uterus as references structures and rotating the probe in a clockwise direction to locate the left ovary and in a counter clockwise direction to locate the right ovary.

For the pre service evaluation, heifers were synchronized with

two doses of synthetic prostaglandin F2 alpha (Cloprostenol D, 150 mg im) administered with an 11 day interval. Evaluation started 48 h after the second PG injection. In this case, a flexible probe was used and the ovaries were palpated and placed directly under the probe.

2.4.2. Blood sampling and hormone determinations

Blood samples were collected by jugular venipuncture every day during follicular evaluation periods; then between the two evaluation periods, the frequency was reduced to twice a week. The blood collections was halted in those heifers with a corpus luteum (CL) in pre service ultrasound, but continued until the end of the mating period in those ones that did not present a CL. The serum was separated by centrifugation during 10 min at 3500 rpm (1578 g force) and samples were stored at −20 °C until progesterone and AMH determinations.

Hormone determinations were performed in the Laboratory of Endocrinology and Animal Metabolism, Facultad de Veterinaria, Montevideo, Uruguay. Progesterone concentrations were determined by a solid phase Radioimmunoassay (RIA). In Year 1, DPC kits (Diagnostic Product Co., Los Angeles, CA, EEUU) were used. The sensitivity of the assay was 0.079 ng/ml and the intra assay CV for low (1 ng/ml) and high (29 ng/ml) quality controls were 10% and 8%, respectively. In Year 2, MP (BIOMEDICALS, INC. Solon, OH 44139 USA) kits were used. The sensitivity of the assay was 0.1 ng/ml and the intra assay CV for low (0.94 ng/ml) and high (5.09 ng/ml) quality controls were 13.6% and 13.4%, respectively.

Anti Müllerian hormone concentration was determined the day of MAFC for each animal in each evaluation and in the five days of evaluation in those animals that we could follow a follicular wave (n = 37). The Bovine AMH ELISA AL 114 (Ansh Labs, Texas, EEUU) kit, which is an AMH assay designed specifically for use in the cow and previously validated in Braford [19] and Hereford [20] heifers, was used. The assay sensitivity was 0.011 ng/ml. In Year 1, the intra assay CV for low (0.177 ng/ml) and high (0.379 ng/ml) quality control were 15.7% and 6.8%, respectively; and the inter assay CV for low (0.178 ng/ml) and high (0.372 ng/ml) were 18.8% and 7.7%, respectively. While in Year 2, the intra assay CV for low (0.264 ng/ml) and high (0.945 ng/ml) were 13.6% and 8.1%, respectively. Inter assay CV for low (0.259 ng/ml) and high (0.946 ng/ml) were 20.7% and 17.0%, respectively.

2.4.3. Reproductive parameters

The onset of puberty was determined using progesterone measurements. A single sample with ≥ 1 ng/ml concentrations or two consecutives samples with ≥ 0.5 ng/ml were used as an indicator of the attainment of puberty.

Pregnancy diagnosis and the estimation of embryonic/fetal age were performed every 30 days, starting 30 days after the beginning of the breeding period until 30 days after bulls were removed from the herd. An Agroscan scanner with a dual linear transrectal probe of 5.0/7.5 MHz (Biotay SA, Montevideo, Uruguay) was used. The date of conception, the age of the heifers at conception and the moment of conception were also determined. The predicted date of conception was calculated by subtracting the age of the embryo or fetus to the date of pregnancy diagnosis. Using the date of conception and the date of birth of each heifer, its age at the moment of conception was calculated. The moment of conception was defined as the days after the beginning of service when each heifer got pregnant.

2.4.4. Parameters of growth and development

2.4.4.1. Live weight. Live weight was evaluated every two weeks, from weaning until the end of mating. The measure was always done in the morning using the same scale (True test GR 3000s, True test Corporation Limited, Montevideo, Uruguay). Using this information the daily weight gain was calculated.

2.4.4.2. Body condition. Body condition was evaluated every two weeks by the same person, using the 8 point scale described by Vizcarra et al. [21], (1 = emaciated animal and 8 = animal with excess fat).

2.4.4.3. Hip height. Hip height was measured the week after each evaluation for antral follicle count (post weaning and pre service) with a tape measure at the point directly above the hip bones while the animal was standing on a level surface.

2.4.4.4. Corporal composition. Back fat thickness (subcutaneous fat over the longissimus dorsi muscle between the 12th and 13th rib, measured in mm), rib eye area (area of the longissimus dorsi muscle measured in cm^2) and fat thickness at the rump (sub cutaneous rump fat depth at the p8 site, measured in mm), were estimated by ultrasonography, using a real time, B mode scanner with a linear array transducer of 3.5 MHz (Aloka Co., Ltd., Tokio, Japón) and stored on portable PC (Houghton and Turlington, 1992). These images were analysed using the software Biosofts (Biotronics Inc., Ames, Iowa, EEUU). These scans were performed at the same time that hip height measurements were obtained.

2.5. Statistical analysis

To identify outliers and inconsistencies in the data for all variables and to verify the normality of the residuals, analyses were performed using the UNIVARIATE procedure available in SAS (SAS 9.4, SAS Institute Inc, Cary, Carolina del Norte, EEUU, 2002) statistical package. The Shapiro Wilk test demonstrated the rejection of the normality hypothesis ($P < 0.05$) for MAFC and AMH variables, so the data was logarithmic transformed.

The maximum value of AFC (MAFC) in each observation was used to evaluate post weaning and pre service AFC repeatability by one way analysis of variance (ANOVA), using the following formula $r = S^2A/(S^2 + S^2A)$, where S^2A is the between group variance and S^2 is the within group variance. To evaluate AMH repeatability between post weaning and pre service observations, AMH samples corresponding to the greatest value of post weaning and pre service AFC were used.

The correlations between AFC and AMH were analysed separately for each breed and year, using the CORR procedure available in SAS.

Only in Year 2, in which the measurements were performed on a daily basis, the consistency of daily AFC (follicles 2–5 mm) and AMH concentrations for each evaluation (post weaning and pre service) and breed (Hereford and Braford), were analysed by ANOVA using the MIXED procedure of SAS. AMH was analysed in a subgroup of heifers (n = 37), selecting those in which the emergence of a follicular wave was determined.

To evaluate the relationship between AFC and reproductive, developmental and body composition variables, regressions were performed using the REG procedure of SAS, creating a trend line and regression equation. The regression graphs were done using JMP 10 (SAS Institute Inc., Cary, NC, USA).

The high variability of AFC among beef heifers and the large variation of the classification criteria of high, medium or low AFC causes that values considered as low AFC in one article might be considered as high AFC in other [22]. This is why, in this case MAFC classes were created for each breed using a cut point that divided the population in three thirds. The cut points were: Year 1 Hereford post weaning (low ≤ 18, medium 19 to 21 and high ≥ 22) and pre service (low ≤ 24, medium 25 to 29 and high ≥ 30); Year 1 Braford

post weaning (low ≤ 26, medium 28 to 33 and high ≥ 34 follicles) and pre service (low ≤ 33, medium 34 to 42 and high ≥ 43 follicles); Year 2 Hereford post weaning (low ≤ 23, medium 24 to 31 and high ≥ 32) and pre service (low ≤ 25, medium 26 to 34 and high ≥ 35); Year 2 Braford post weaning: follicles low ≤ 21, medium 22 to 31 and high ≥ 32 follicles) pre service (low ≤ 28, medium 29 to 36 and high ≥ 37 follicles). To evaluate their relationship with progesterone concentrations in the post insemination cycle, the MIXED procedure of SAS was used with follicle class, day and their interaction as the fixed effects in the model. The frequency of heifers artificially inseminated and conception to artificial insemination were analysed using the PROC GENMOD in SAS. The model included the fixed effect of MAFC class.

The moment and conception age were analysed by linear regression using the GLM procedure of SAS with MAFC class as the independent variable. Models were considered significant if $P < 0.05$, and trends were identified when $0.05 < P \leq 0.10$.

3. Results

3.1. MAFC repeatability post weaning and pre service

MAFC repeatability between post weaning and pre service evaluations per breed and year is shown in Table 1. The greatest repeatability was observed in Braford heifers in Year 2.

3.2. Repeatability of AMH concentrations

The repeatability of AMH concentrations comparing post weaning to pre service data is showed in Table 2. The repeatability of AMH concentrations had a similar pattern to that observed for MAFC, being greater in Braford heifers in Year 2.

3.3. Correlation between MAFC and AMH concentrations

The correlation between MAFC and AMH concentrations for each year and breed, including the post weaning and pre service values for each animal, is presented in Table 3. In all cases the relationship between the two variables was significant ($P < 0.001$), but the strength of the correlation was greater for Braford heifers in Year 2.

Table 3
Correlation between maximum antral follicle count and AMH concentrations including post weaning and pre service measurements in two different years using Hereford and Braford heifers.

Year	Breed	Correlation	P
Year 1	Hereford (n = 84)	0.38	<0.01
	Braford (n = 80)	0.44	<0.001
Year 2	Hereford (n = 100)	0.64	<0.001
	Braford (n = 100)	0.85	<0.001

3.4. Consistency of AFC and AMH measurements during daily examinations

In Hereford heifers Year 2, the consistency of the measurement of follicles from 2 to 5 mm during five consecutive days for the post weaning and pre service measurements was 0.80 (n = 50) and 0.79 (n = 50), respectively; and for Braford, the consistency of the post weaning was 0.93 (n = 50) and for the pre service measurement was 0.95 (n = 50).

The consistency of AMH measurements in each evaluation (post weaning and pre service) was also evaluated using a subgroup of heifers (n = 37). In Hereford, the consistency for post weaning and pre service evaluation was 0.89 (n = 10) and 0.95 (n = 9), respectively; and for Braford, for the post weaning evaluation was 0.92 (n = 10) and for the pre service evaluation was 0.94 (n = 8). In this subgroup, minimal daily variation in the concentration of AMH (P = 0.45; Fig. 1) and in the number of 2–5 mm follicles was observed (P = 0.4; Fig. 1).

3.5. Relation of MAFC with reproductive parameters

In Year 1 in Hereford and Braford, and Year 2 in Hereford heifers, post weaning evaluations showed no associations between MAFC and reproductive variables (age at puberty, moment of conception, age at conception and final pregnancy). In Braford heifers in Year 2, however, post weaning evaluation showed that animals with greater MAFC attained puberty at an earlier age ($r^2 = 0.129$; P = 0.0196; Fig. 2). The equation that best describe this relationship is *age at puberty* = 483.79704–27.503385 * log *MAFC*. The mean age at puberty for each group (low = 418.7 ± 9.0 days; medium = 379.2 ± 8.4 days and high = 383.4 ± 8.7 days) determined that a smaller proportion of heifers with low MAFC were inseminated (2/16), compared to those with medium (12/17) and high

Table 1
Repeatability of the maximum antral follicle count measured post weaning and pre service in two different years using Hereford and Braford heifers.

Year	Breed	Mean MAFC post weaning (n)	Mean MAFC pre service (n)	Repeatability	P
Year 1	Hereford (n = 42)	21.0 ± 0.9	26.9 ± 1.0	0.36	0.009
	Braford (n = 40)	31.0 ± 1.4	37.7 ± 1.4	0.39	0.006
Year 2	Hereford (n = 50)	29.2 ± 1.7	31.2 ± 1.5	0.32	0.01
	Braford (n = 50)	30.2 ± 2.1	38.0 ± 3.0	0.72	<0.001

Table 2
Repeatability of AMH concentrations measured post weaning and pre service in two different years using Hereford and Braford heifers.

Year	Breed	Mean AMH post weaning (ng/ml)	Mean AMH pre service (ng/ml)	Repeatability	P
Year 1	Hereford (n = 42)	0.463 ± 0.089	0.271 ± 0.050	0.50	<0.001
	Braford (n = 40)	0.462 ± 0.076	0.343 ± 0.048	0.52	<0.001
Year 2	Hereford (n = 50)	0.511 ± 0.053	0.606 ± 0.050	0.50	<0.001
	Braford (n = 50)	0.661 ± 0.060	0.887 ± 0.092	0.71	<0.001

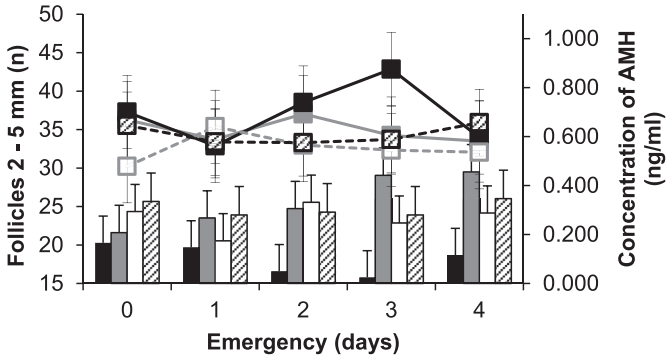

Fig. 1. Changes in the number of 2–5 mm follicles (bars) and concentration of AMH (square) during 5 consecutive days from the emergence of the follicular wave, in Hereford heifers, black bar and square (post weaning observation, n = 10) and grey bar and square (pre service observation, n = 9) and Braford heifers, white bar and square (post weaning observation, n = 10) and dashed bar and square (pre service observation, n = 8).

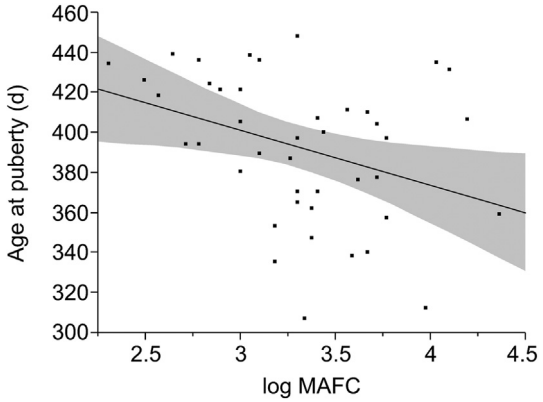

Fig. 2. Regression of age at puberty and the logarithm of maximum antral follicle count (MAFC) post weaning, in Braford heifers, during Year 2 (n = 50). The shaded area represents the 95% confidence interval.

MAFC (7/17; P < 0.01). The proportion of heifers conceiving to artificial insemination and the moment and age at conception were similar among groups (low MAFC = 0/2, 28.6 ± 4.9 days and 429.9 ± 7.1 days; medium MAFC = 6/11, 23.0 ± 4.4 days and 419.1 ± 6.5 days; high MAFC = 4/7, 24.3 ± 4.2 days and 431.2 ± 6.5 days; respectively; P > 0.05). The final pregnancy rate was similar for heifers with low (11/16), medium (13/17) and high (13/17) MAFC (P > 0.05).

In the pre service evaluation, no associations between MAFC and reproductive variables were found in any of the years or breeds.

3.6. Relationship between AMH concentrations and reproductive parameters

In Braford heifers in Year 2, we also observed a relationship between the AMH logarithm and age at puberty, such that greater concentrations of AMH corresponded with a younger age at puberty ($r^2 = 0.135$; P = 0.0168; Fig. 3). The equation that best describes this relationship is *age at puberty* = 381.56734–18.446481 * log *AMH*.

3.7. Relationship of MAFC with progesterone concentrations

In Braford Year 2, progesterone concentrations were affected by MAFC class, day and their interaction (P < 0.001), since heifers of

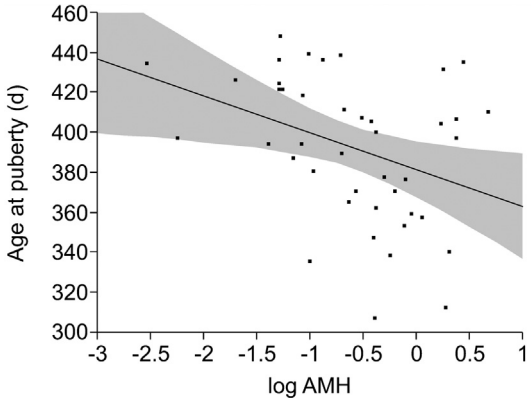

Fig. 3. Regression of age at puberty with the logarithm of AMH concentrations post weaning in Braford heifers, Year 2 (n = 50). The shaded area represents the 95% confidence interval.

the low MAFC class, had lower progesterone concentrations starting on day 10 of the cycle compared with those from the medium and high classes (Fig. 4).

3.8. Relationship of MAFC with parameters of growth and development

In Year 1, no associations were found between MAFC and parameters of growth and body composition, in none of the evaluations, for any of the breeds. However, in Year 2, an association between MAFC and growth and body composition was observed in Braford heifers at the post weaning evaluation. Diminished AFC corresponded with decreased weight ($r^2 = 0.146$; P < 0.01), hip height ($r^2 = 0.159$; P < 0.01) and rib eye area ($r^2 = 0.111$; P = 0.018; Fig. 5). The equations that best described this relationship were *weight* = 122.9377 + 23.796473 * log *MAFC*, *hip height* = 97.320422 + 3.6574946 * log *MAFC* and *rib eye area* = 26.445724 + 4.2351617 * log *MAFC*.

In the same year and breed, but in the pre service observation, heifers with low MAFC were still shorter (P = 0.0042) and had decreased rib eye area (P = 0.0307).

4. Discussion

This is one of the first studies that applied MAFC as a tool to select replacement heifers at weaning. However, the hypotheses tested in these studies were partially accepted. The MAFC between post weaning and pre breeding evaluations was repeatable in only one of four cases. In the only case that the repeatability between measurements evaluated six months apart was high, we found a relationship between MAFC and parameters that describe growth and development, so heifers with lesser MAFC grew slower and reached puberty later than those with high and intermediate values. Moreover, in the same case, a high correlation between AFC and AMH concentrations was found, suggesting that they can be use inter changeably to select Braford heifers at weaning. Our results reinforce that onset of puberty is a complex process affected by weight, growth rate and breed among other factors, and that a single measurement of MAFC or AMH concentrations at weaning may not be useful techniques to select more productive Hereford heifers.

The MAFC repeatability observed in Braford heifers in Year 2, was very good but not as high as previously reported [23–25] (0.87–0.95), indicating, in this case, that early selection of heifers at weaning may be possible. However, our results were poor in the

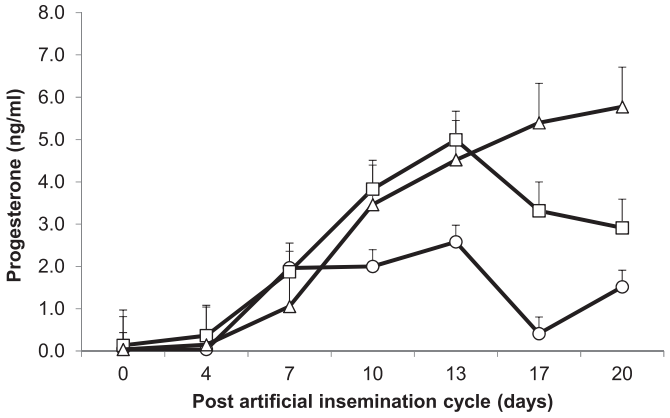

Fig. 4. Progesterone concentrations in the cycle after artificial insemination, in Braford heifers, Year 2, with low (circle, n = 2), medium (square, n = 9) and high (triangle, n = 6) antral follicles counts.

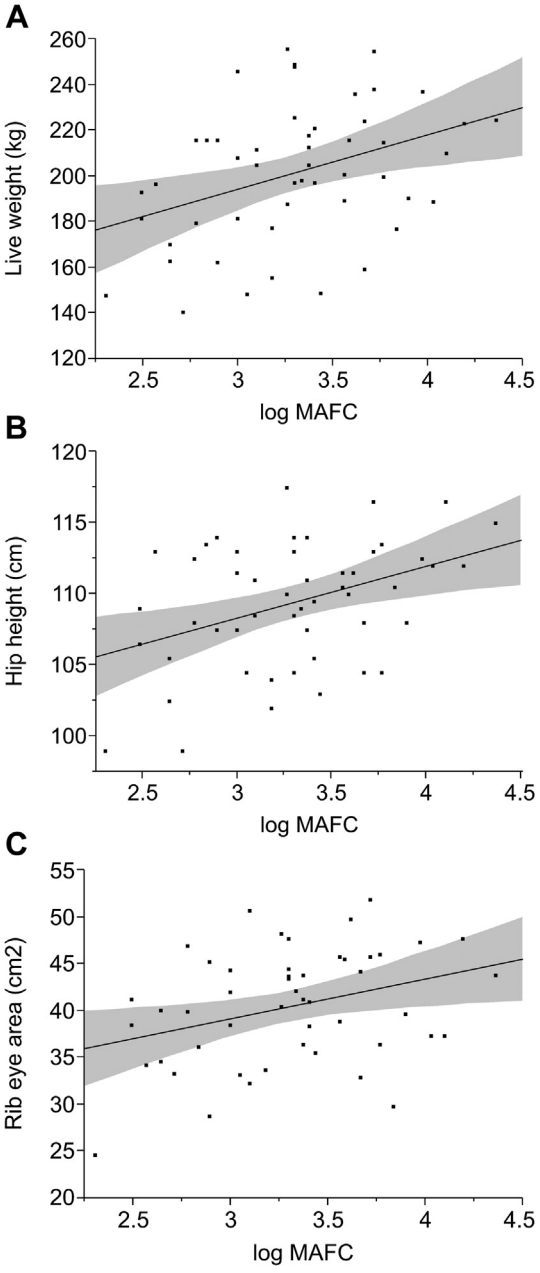

Fig. 5. Relationship of the logarithm of the maximum antral follicles count (MAFC) with live weight (A), hip height (B) and rib eye area (C) in Braford heifers, Year 2, in the post weaning evaluation (n = 50). The shaded area represents the 95% confidence interval.

remaining cases (Year 1, Hereford and Braford heifers and Year 2, Hereford heifers). Our poor results could be associated with the methodology used and with the physiological status of the heifers (pre vs peri puberal). In the post weaning evaluation a rigid rectal probe with external manipulation was used, whereas in the pre service evaluation a flexible probe was used, and that may be affecting the repeatability obtained in our study. The ovaries, in the post weaning evaluation, were visualized using the bladder and the uterus as references structures and rotating the probe in a clockwise direction to locate the left ovary and in a counter clockwise direction to locate the right ovary, as has been described for sheep [18]. In the pre service evaluation, however, the ovary was palpated, and placed directly under the probe, allowing greater definition of its anatomic structures. The decreased AFC repeatability obtained using the rigid probe in peri puberal animals has been reported before [26]. Another factor that may be affecting the results is that the repeatability was measured considering only the day of maximum AFC, in two measures separately by six months, the first in pre puberal calves and the second in peri puberal heifers. The repeatability results presented by Ireland et al. [7] were of synchronized puberal adult animals, in which the maximum count was evaluated in successive waves of the oestrous cycle, performing two ultrasounds evaluation per day. This methodological difference is important for two reasons: 1) the complex hormonal changes that occur around puberty [27] could be affecting secondary to antral follicle transition and follicle dynamics [31]; 2) the probability that two different measurements are equal is greater when they are closer in time [28]. Recent research, however, reported good results in term of AFC repeatability (0.90–0.92) at weaning and yearling age in Braford cattle [14]. This reinforces our good result obtained in Braford heifers in Year 2, and indicates that an AFC performed at weaning may be a good predictor of their reproductive performance at breeding.

Regarding the moment of the cycle in which the AFC evaluation should be performed, in our experiment, heifers were ultrasonographically examined for five to ten days, with the aim of capturing the emergence of at least one follicular wave. The first publication in this area was performed knowing the day of wave emergence [23], but the methodology has been modified in order to have greater utility in a production setting [9]. Gobikrushanth et al. [29] obtained a low repeatability (0.37) comparing the follicular count on a unique day in a known vs unknown moment of follicular growth. However, our results would strongly indicate that a single measurement at an unknown stage of the oestrous cycle can have great field application. In Year 2, the number of follicles from 2 to 5 mm evaluated during five consecutive days, was highly consistent, particularly in Braford females. The minimal daily variation in the number of follicles in this size class is in agreement with the description of follicular waves in cattle [30] and sheep [31], and reinforces the concept that a single measurement on an unknown day of the oestrous cycle is also correct from the biological point of view.

Only in Braford heifers and in the second year of evaluation, the MAFC allowed the post weaning identification of heifers that will reach puberty early. This result may be explained by two factors: 1) breed differences in age at puberty, as *Bos Indicus* and their crosses attain puberty later than *Bos Taurus* [32]; and 2) differences in the plane of nutrition between years and breeds, which caused lower daily weight gain and pre mating weights in Braford heifers. Both,

nutrition and genetics combined to stimulate an earlier attainment of puberty in Hereford heifers, which were closer to the biological limit for this breed. Since more Hereford heifers were in the peri pubertal period by the time of the pre service evaluation, the hormonal changes and its impact on follicle growth during this physiological period may have impeded to find good correlation between both measurements (post weaning and pre service). However, since in Braford heifers there is capacity for improvement in the plane of nutrition, the use of additional tools such as the MAFC may have aid the selection of more precocious females. In Year 2, Braford heifers with low MAFC had decreased growth and development and this may explain the delay in the attainment of puberty and the lower proportion of heifers artificially inseminated. Moreover, those heifers that were inseminated with diminished numbers of antral follicles had less progesterone in the cycle post insemination, compared to those with medium and high numbers of follicles. This did not result in a later average moment of conception or lower percentage pregnant at final diagnosis. It is important to reiterate the low proportion of heifers in the low AFC group ($n = 2$). Previous works in cattle did not find a beneficial relationship between AFC and puberty [33,34]. Heifers with greater AFC had increased concentrations of progesterone (current study and [8]). Heifers with increased AFC also had larger uteri and greater uterine protein content that indicates the existence of a more auspicious environment for embryo development and could explain the earlier conception [8–10,33,35]. The increased concentrations of progesterone in the heifers with high MAFC after day 10 of the oestrous cycle may contribute to increased embryo elongation and improved maternal recognition of pregnancy [36]. It is important to consider that these are the first set of data generated in our conditions, and with a very small number of heifers, particularly in the low MAFC class. In the same way, since the evaluation of reproductive variables such as the moment of conception and final pregnancy rate requires a greater number of heifers, this may be the explanation for the lack of differences among animals in the different MAFC classes in this study. Therefore, more research is required to confirm the decreased concentrations of progesterone in Braford heifers with low MAFC and its relationship with fertility.

The relationship between AFC and parameters that describe growth and development was also observed only in Braford heifers in Year 2. The results suggested that heifers with diminished AFC were lighter, had less rib eye area and were shorter, than those with medium and high AFC. These results are in agreement with previously published data [37], and suggest that pre and post natal nutrition, associated to a faster growth, could affect the ovarian reserve [34,38,39]. Parameters of growth and development are known to be associated with age at puberty [40], since heifers with increased growth potential attain the critical percentage of mature weight to start cycling at a younger age. However, it is important to consider the poor performance of large cows in pastoral systems [41], which means that selecting heavier animals does not always allow to select the most fertile ones. This is why, it is not recommended to use only body weight as a tool to select replacement heifers.

In our experiment, MAFC and AMH were positively correlated in all analyses, but with a lower coefficient compared that those previously reported [7]. The correlation observed in Year 2 in both breeds was good, but not in Year 1. Moreover, the average AMH repeatability was greater than that of MAFC, being moderate in three cases (Year 1, Hereford and Braford and Year 2, Hereford) and very good in Braford heifers in Year 2 (0.71). These results are in agreement with previous reports [24], and indicate that AMH may be used to select precocious Braford heifers at weaning. It was suggested that one of the most important advantages of AMH in contrast to AFC was its stability across the oestrous cycle [7]. However, later research indicated that AFC (counting follicles ≥ 2 mm in diameter) can be performed at any time in the oestrous cycle, since the 2 mm follicles that represents a high percentage of the follicles on the ovary [30], would not be as affected by FSH concentrations as follicles ≥ 3 mm [9]. Other relevant aspects in favour of AFC are that the information is obtained *in situ*, it also allows to evaluate the development of the reproductive tract in pre service heifers and its cost is lower than AMH analysis (3.5 vs 9.7 American dollars per animal not considering the lab fees in the case of the hormonal analysis). Moreover, some methodological aspects need to be adjusted in the AMH analysis, since an important variation in AMH concentrations have been reported for the same sample of the same animal in the same laboratory (Cushman, com. pers; Anderson, com. pers). The association between both variables suggests that they could be used inter changeably to select at weaning Braford heifers that will subsequently attain puberty earlier, although it would be useful to take into account the advantages and disadvantages of each alternative, as was previously proposed.

5. Conclusion

The results of these experiments show that MAFC and AMH concentrations are not useful to select less fertile Hereford heifers post weaning or pre service. However, post weaning MAFC and AMH concentrations may be applied to select Braford heifers that attain puberty at an early age. Additionally, examinations can be performed on any day of the oestrous cycle, making this technique more applicable to beef production systems. Braford heifers with greater MAFC had increased growth and development that may contribute to the younger age at puberty. These results are relevant for our breeding systems because decisions can be made on replacement heifers at an early age (e.g., weaning) allowing heifers with decreased fertility to be removed from the replacement herd at a young age and developed to be raised to be sold as finished heifers and improve economic returns in multiple phases of the production system (e.g., the cow herd and the feedlot). However, more studies with larger numbers of Braford heifers would be necessary to prove the validity of MAFC and AMH concentrations as early selection tools. The application of these techniques may not occur isolated, but rather, associated to a plane of nutrition that can stimulate adequate growth and development of the replacement heifers.

Conflicts of interest

The authors have no conflicts of interest to declare.

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