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Relationship between follicle population, AMH concentration and fertility in cattle

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Abstract

The size of ovarian follicular population evaluated by direct antral follicular count or endocrine markers can help determine the success of reproductive biotechnologies in cattle. However, although highly repeatable within animal, the antral follicular population (AFP) appears to be greatly variable across individuals. Therefore, laboratory methods that reliably predict AFP could have a significant value to select donor-cows for use in reproductive biotechnology and for genomic selection of animals with greater reproductive potential. Accordingly, the circulating levels of anti-Müllerian hormone (AMH) have been found to be associated with AFP and, thereby, identified as an important endocrine marker of superovulation response and *in vitro* embryo production in cattle. Moreover, a number of recent publications and ongoing studies are trying to determine whether circulating levels of AMH are correlated with fertility. This review summarizes recent information concerning AFP and its association with AMH, and the possibility of utilizing AMH as a marker for reproductive technologies and ultimately to enhance cattle fertility.

Keywords: anti-Müllerian, antral follicle, artificial insemination, embryo transfer, ovarian response.

Introduction

Genetic selection and reproductive efficiency are key factors for the success of the dairy and beef industries. Reproductive technologies, such as ovum-pick-up (OPU) and *in vitro* embryo production (IVEP) can rapidly enhance genetics of dairy and beef cattle through both the female and male linage. However, in females, the success of these techniques is highly dependent upon individual physiological characteristics of the animal such as ovarian antral follicle population (AFP). Therefore, the efficiency of IVEP can be compromised by the large variability of AFP among donors, despite the high repeatability within animal (Burns, 2005; Ireland *et al*., 2007).

Ovarian antral follicle numbers are positively associated with a variety of indirect measures of fertility in cattle such as ovarian function (Ireland *et al*., 2008, 2009; Jimenez-Krassel *et al*., 2009), superovulation responses (Kawamata, 1994; Cushman *et al*., 1999; Singh *et al*., 2004), *in vitro* blastocyst production (Taneja *et al*., 2000; Pontes *et al*., 2009), fertility (Erickson *et al*., 1976; Maurer and Echternkamp, 1985; Oliveira *et al*., 2002; Mossa *et al*., 2012) and herd longevity (Jimenez-Krassel *et al*., 2015). Interestingly, AFP has also been associated to several blood compounds, including circulating concentrations of insulin, insulin-like growth factor I (IGF-1), and anti-Müllerian hormone (AMH; Alvarez *et al*., 2000; Fortune *et al*., 2010; Satrapa *et al*., 2013; Batista *et al*., 2014; Sales *et al*., 2015)

In cattle, circulating AMH concentration can help field veterinarians to predict AFP in ovaries (Ireland *et al*., 2008; Rico *et al*., 2009; Batista *et al*., 2014), response to superovulation treatments (Rico *et al*., 2009; Monniaux *et al*., 2010; Souza *et al*., 2015), and more recently as a marker to predict IVEP performance of *Bos taurus* (Gamarra *et al*., 2014; Guerreiro *et al*., 2014; Vernunft *et al*., 2015) and *Bos indicus* breeds (Guerreiro *et al*., 2014). In addition, studies performed in the last decade have also indicated that cows with lower number of antral follicle counts have lower fertility (Mossa *et al*., 2012). Therefore, because circulating AMH is an indirect measure of ovarian reserve, represented by the size of the ovarian follicle pool, later studies have explored the use of AMH to predict field fertility in cattle (Ribeiro *et al*., 2014; Jimenez-Krassel *et al*., 2015). However, the value of AMH on predicting field fertility may vary according to the type of reproductive management employed in the farm, since it appears that AMH was not associated to field fertility in cows bred following the use of timed AI protocols (Ribeiro *et al*., 2014). Hence, the present review aims to discuss some key points related to AMH and antral follicle population, superovulation responses, OPU-IVEP and field fertility.

Anti-Müllerian hormone (AMH) and antral follicular population (AFP)

AMH is a member of the TGFβ superfamily of growth factors (Cate *et al*., 1986) first described to have an important role in sex differentiation in early fetal life (Lee *et al*., 1996; Rajpert-De Meyts *et al*., 1999). In females, AMH is produced by granulosa cells mainly from pre-antral and early antral follicles (Durlinger *et al*., 2002). Therefore, despite the fact that the number of ovarian follicles is variable across females and yet highly repeatable within individuals, AMH is a reliable endocrine marker of ovarian reserve (entire population of follicles in ovaries; (Ireland *et al*., 2007, 2008;

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Monniaux *et al*., 2012). In this context, AMH have been correlated with AFP in cattle of different genetic groups (Fig. 1 and 2; Baldrighi *et al*., 2013; Batista *et al*., 2014) and categories (Fig. 3; Batista *et al*., 2015; FMVZ/USP, São Paulo, SP, Brazil; unpublished data). Moreover, AMH concentration appears to vary a lot across cattle breeds following the pattern in terms of AFP (Batista *et al*., 2014; Ribeiro *et al*., 2014) and there seem to be a different AMH-threshold for the differing genetic groups when trying to classify animals into differing classes of AFP (Batista *et al*., 2014; Guerreiro *et al*., 2014). For example, in a recent study *Bos indicus* heifers showed greater AFP and AMH concentration compared to *Bos taurus* heifers (Batista *et al*., 2014).

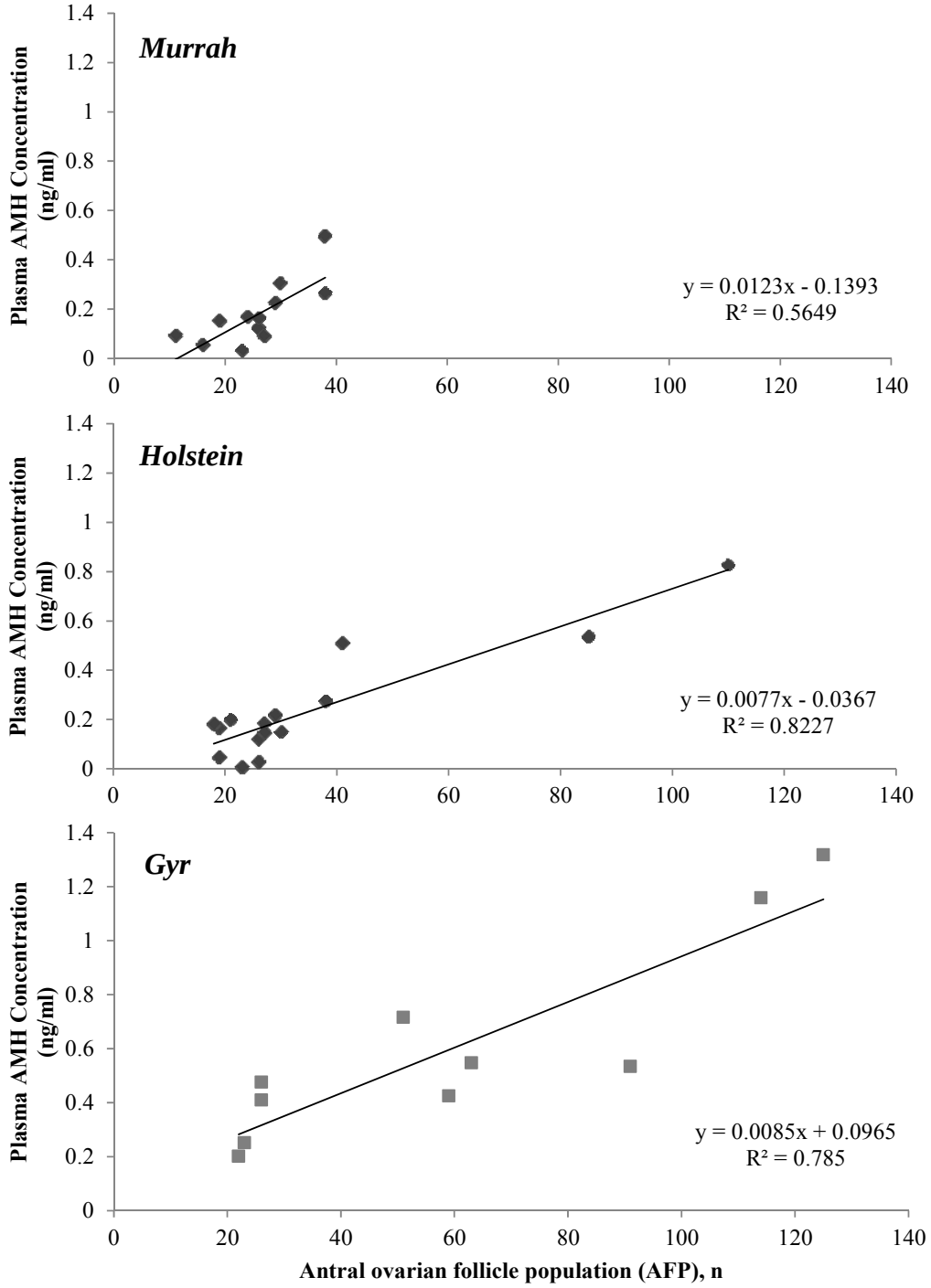

Figure 1. Relationship between the ovarian antral follicle population (AFP) counted at day of ovulation and the plasma anti-Mullerian hormone (AMH) concentration in Murrah (*Bubalus bubalis*, n = 13), Holstein (*Bos taurus*, n = 15) and Gyr (*Bos indicus*, n = 10 heifers). Adapted from Baldrighi *et al*. (2013).

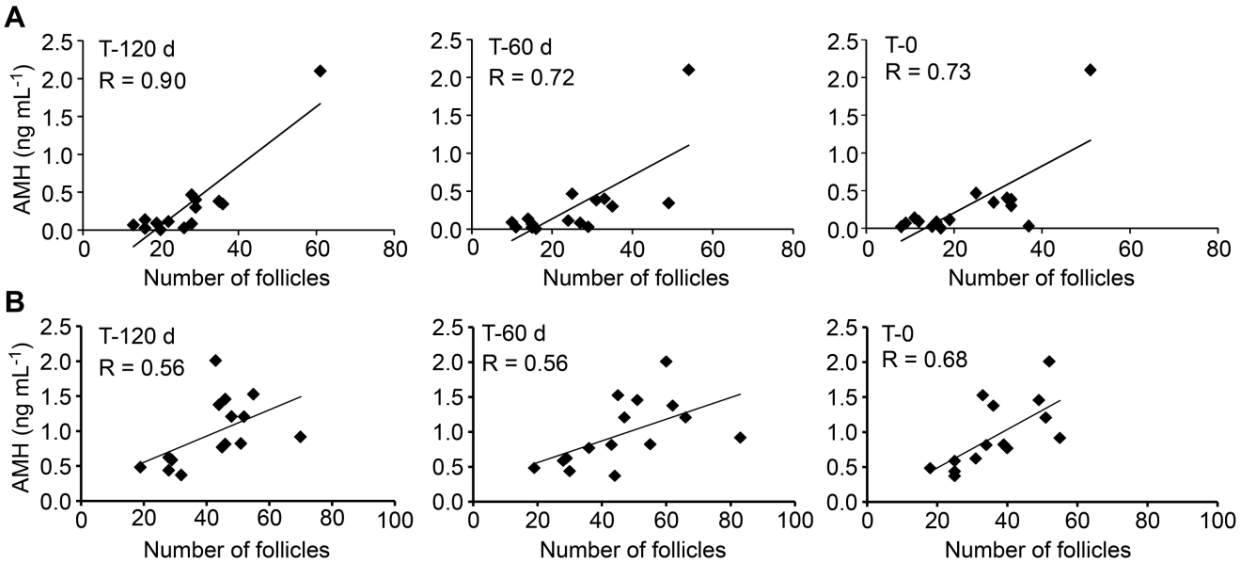

Figure 2. Relationship between the numbers of follicles counted at T-120, T-60, and T0 and plasma AMH concentration (ng/ml; T0) in Holstein heifers (n = 16; A) and Nelore heifers (n = 16; B). Ovarian antral follicular population (AFP) was evaluated three times at 60-day (d) intervals (T-120, 120 days before plasma AMH determination; T-60, 60 days before; and T0, at the time of plasma AMH determination). Blood samples were collected by jugular venipuncture on day T0 of the experimental design. Adapted from Batista *et al*. (2014).

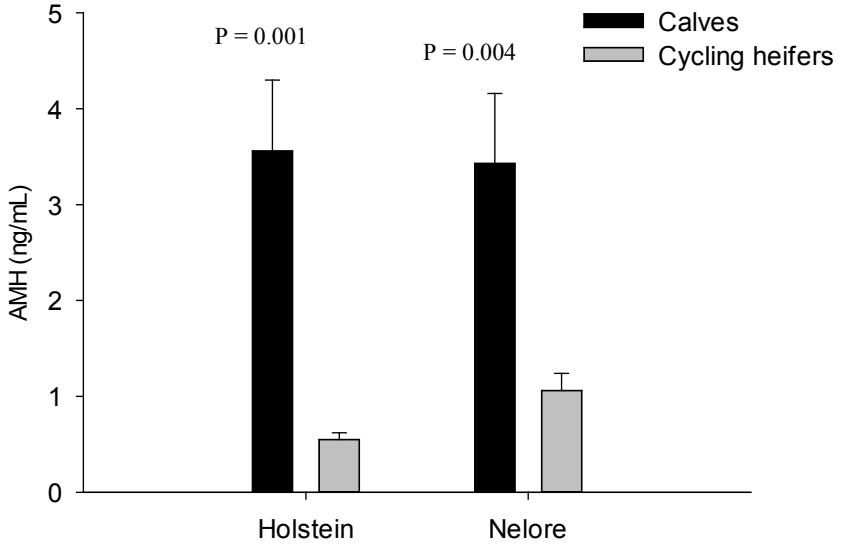

Figure 3. AMH plasma concentration (ng/ml) in calves (aging 2 to 4 months, Holstein: n = 24 and Nelore: n = 30) and cycling heifers (Holstein: n = 10 and Nelore: n = 12). Adapted from Batista *et al*. (2015); FMVZ/USP, São Paulo, SP, Brazil; unpublished data.

A practical important advantage in utilizing AMH instead of direct follicle count with an ultrasound to predict AFP is that AMH levels will vary minimally during the estrous cycle (Rico *et al*., 2009; Ireland *et al*., 2010; Souza *et al*., 2015), therefore blood samples can be taken at any time to evaluate circulating AMH. One exception would be the period just after superstimulatory treatments with FSH, in which the plasma AMH concentration appears to be greater than normal physiological levels (Rico *et al*., 2009, 2012). It is believed that this increase in AMH concentration following FSH treatment may be due to growth of small follicles that were not detected by ultrasonography (Rico *et al*., 2009, 2012). Alternatively, we can't rule out the possibility that FSH treatment may have increased AMH secretion by granulosa cells. However, this hypothesis needs further investigation (Rico *et al*., 2009, 2012).

Additionally, although AMH is produced mostly by early antral follicles, another intriguing biological aspect of AMH in the reproductive cycle in females is its involvement in mechanisms that inhibit activation of primordial follicles from entering the wave emergence pool (Durlinger *et al*., 2002; Fortune *et al*., 2010). Despite the larger AFP and AMH concentration observed in *Bos indicus* (Nelore) than in *Bos taurus* (Holstein) cattle (Batista *et al*., 2014), a previous report demonstrated that the primordial follicular populations were lower in *Bos indicus* than *Bos taurus* heifers (Silva-Santos *et al*., 2011). Additionally, ovaries of AMH null mice contained significantly more early atretic follicles (Visser *et al*., 2007). Therefore, it may be that the high AMH plasma concentration in *Bos indicus* heifers contributes to lower rates of follicular atresia.

Based on the finding that AMH can inhibit activation of primordial follicles from entering the wave emergence pool, it seems to avoid the premature depletion of the follicular population in the ovary. AMH concentrations decrease in parallel to the number of ovarian follicles as rodents (Kevenaar *et al*., 2006) and women (Piltonen *et al*., 2005) age. In agreement with that, in a recent study from our research group we have found greater plasma AMH concentrations in calves compared to cycling heifers in *Bos indicus* and *Bos taurus* cattle (Batista *et al*., 2015, FMVZ/USP, São Paulo, SP, Brazil, unpublished data).

Nutritional factors such as vitamin-D status in humans (Dennis *et al*., 2012) as well as negative energy balance in cattle (Souza *et al*., 2014) seem to have some influence in circulating AMH. Recently propylene glycol drenches administrated in Holstein heifers increased the number of follicles and blastocyst quality in heifers that had greater AMH concentration, but it had no impact in heifers with lower AMH concentration (Gamarra *et al*., 2014). Obviously, more research is urgently needed to try to manipulate circulating AMH through different feeding strategies.

AMH and superovulation/IVF response

Previous studies observed a strong positive relationship between circulating AMH and *in vivo* embryo production following superovulation in dairy cattle (Monniaux *et al*., 2010; Rico *et al*., 2012; Souza *et al*., 2015). AMH has been correlated with number of large follicles after superstimulation, number of CL after superovulation and number of embryos produced (Monniaux *et al*., 2010; Souza *et al*., 2015) in primiparous and multiparous cows (Souza *et al*., 2015) - shown in Fig. 4. Additionally, the type of blood anti-coagulant factor may influence AMH measurements and it appears to be an important detail when trying to interpret AMH results. For instance, a threshold of 87 pg/ml (samples collected with heparin) and 123 pg/ml (samples with EDTA) have been proposed to identify dairy cows producing less than 15 ovulatory follicles after FSH treatment and near the time of estrus. Thus, measuring AMH before enrolling cows in FSH programs will likely allow practitioners to improve numbers of embryos produced and, thereby, reduce costs per embryo produced.

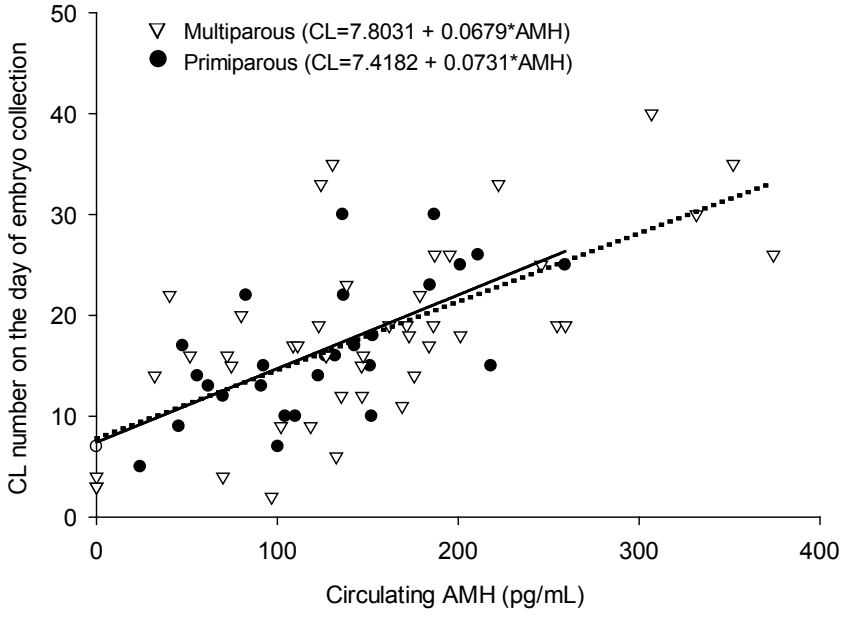

Figure 4. Average circulating AMH (pg/ml) and number of CL structures on the day of embryo collection for primiparous and multiparous dairy cows. Adapted from Souza *et al*. (2015).

In vitro embryo production has astonishingly increased in the last decade. Currently, 40.6% of the total embryo production in the world (1,275,874 embryos) are represented by *in vitro* embryo production (International Embryo Transfer Society - IETS, 2014). Therefore, ovum pick-up (OPU) and *in vitro* embryo production (IVEP) are important tools that can be used to drive genetic progress. However, success of IVF technology can be limited in animals that have lower numbers of follicles in the ovary. In that regard, recent studies have identified that AMH can be an interesting endocrine marker to select donors with the greatest potential to serve as donors for *in vitro* embryo production (Gamarra *et al*., 2014; Guerreiro *et al*., 2014; Vernunft *et al*., 2015). In agreement with these findings, plasma AMH in *Bos indicus* and *Bos taurus* heifers have been found to have a positive correlation with total follicles aspirated, total cumulus oocytes complexes (COCs) retrieved, number of COCs cultured, and number of embryos produced per OPU session (Fig. 5 and 6, and Table 1). However, some variables related to *in vitro* embryo development (cleavage and blastocyst rates) do not seem to have any correlation with circulating AMH (Fig. 5, and 6 and Table 1; Guerreiro *et al*., 2014).

Because genomic information allows producers to know genetic merit of their animals at early ages, we have recently explored the possibility of producing embryos retrieved from young female calves that were only 2-4 months old. Our preliminary results indicate that AMH concentration is once again a very useful marker to predict IVP performance of *Bos taurus* and *Bos indicus* calves (Batista *et al*., 2015; FMVZ/USP, São Paulo, SP, Brazil; unpublished data). In most situations, because examining ovaries from very young calves with an ultrasound can be difficult and unpractical, the determination of circulating AMH concentration in this animal category can be an important tool to select best oocyte-donors for *in vitro* embryo production, overcoming some of the technical limitations involved in utilizing an ultrasound in young calves. Therefore, we forecast that with the availability of genomic technology for the identification of animals with superior genetics at early ages and AMH measurement to facilitate identification of best oocyte-donors, that the use of calves as oocyte donors has its place in IVP programs and will allow faster genetic gains by dramatically decreasing generation intervals (Armstrong *et al*., 1992; Lohuis, 1995; Camargo *et al*., 2005).

Altogether, an increasing body of evidence to date have shown that circulating AMH is highly correlated with AFP, and it appears to be an interesting endocrine marker to identify best donors for IVEP, regardless of the genotype background and animal age.

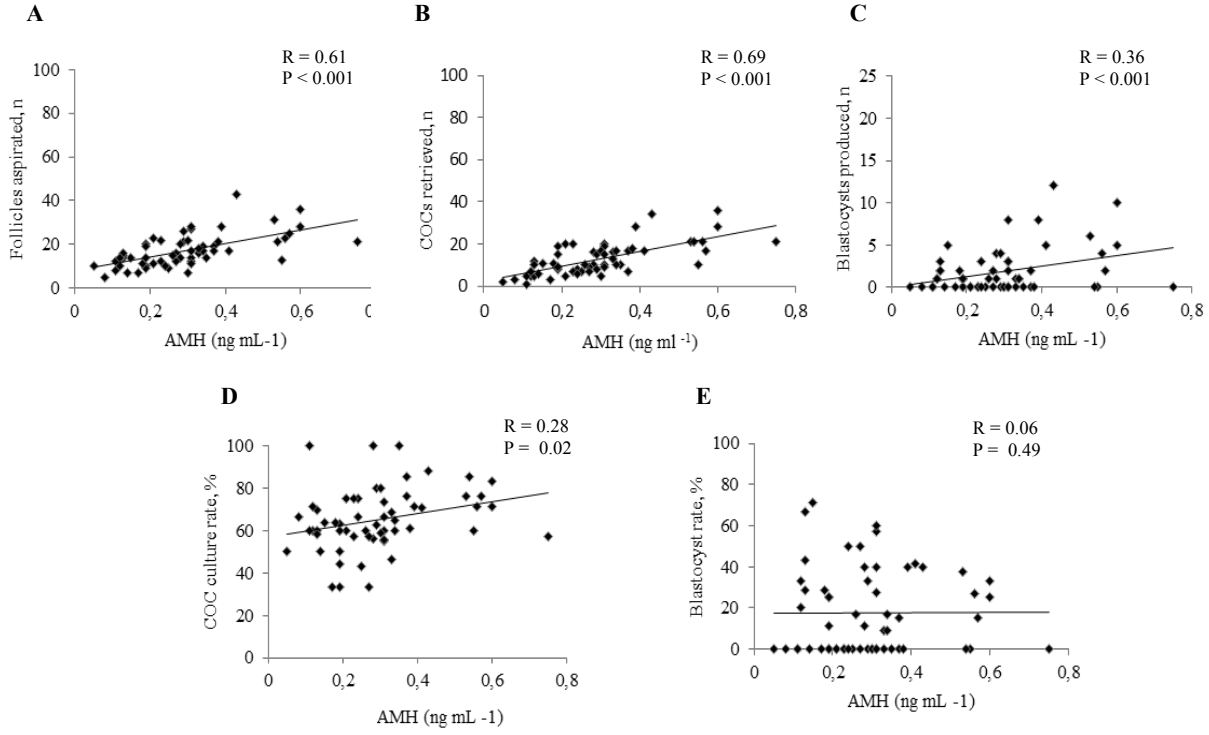

Figure 5. Correlation between plasma AMH concentrations and variables related to ovum pick-up (OPU) and *in vitro* embryo production (IVP) in *Bos taurus* donors. Relationship between the number of follicles aspirated (A), the total COCs retrieved (B), the number of blastocysts produced (C), the COC culture rate (%, D) and the blastocyst rate (%, E) and the plasma anti-Mullerian hormone (AMH) concentration in Holstein (*Bos taurus*) donors. Blood samples for plasma AMH determination were collected by coccygeal venipuncture immediately before the OPU session. Adapted from Guerreiro *et al*. (2014).

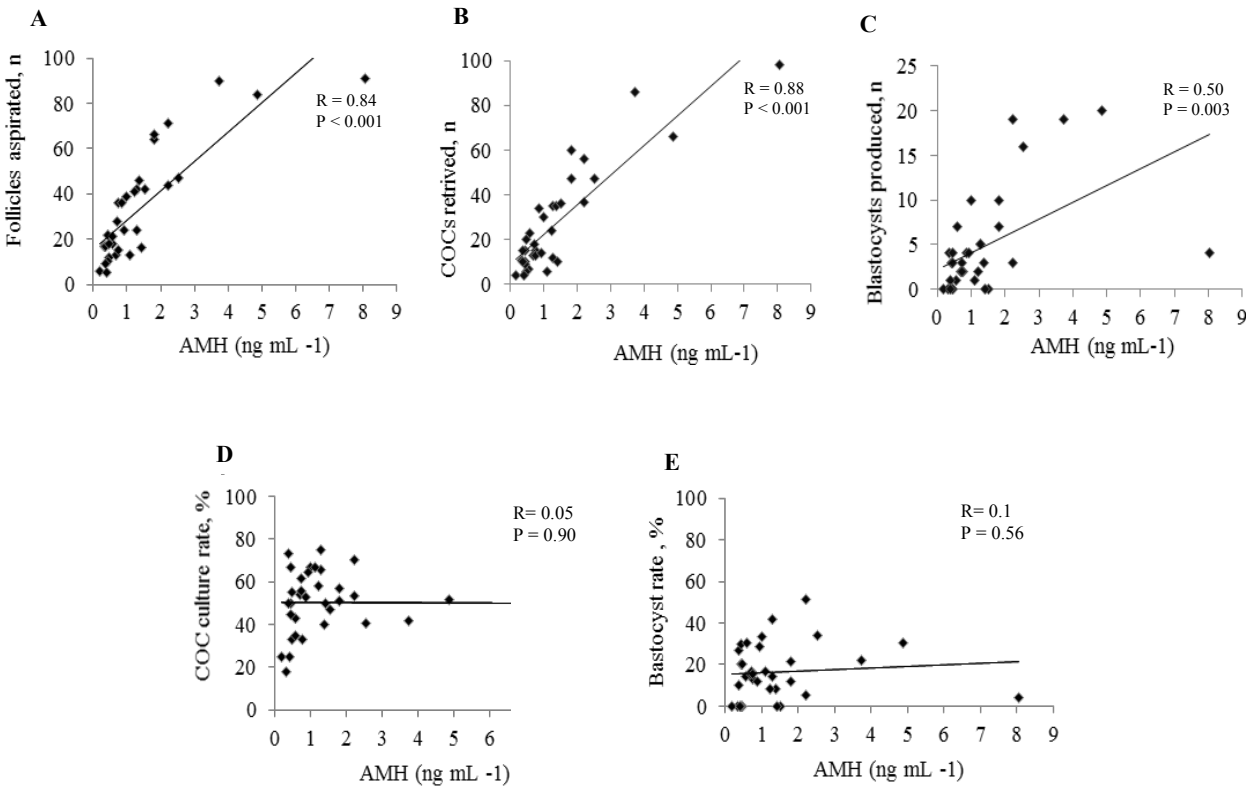

Figure 6. Correlation between plasma AMH concentrations and variables related to ovum pick-up (OPU) and *in vitro* embryo production (IVP) in *Bos indicus* donors. Relationship between the number of follicles aspirated (A), the total COCs retrieved (B), the number of blastocysts produced (C), the COC culture rate (%, D) and the blastocyst rate (%, E) and the plasma anti-Mullerian hormone (AMH) concentration in Nelore (*Bos indicus*) donors. Blood samples for plasma AMH determination were collected by coccygeal venipuncture immediately before the OPU session. Adapted from Guerreiro *et al*. (2014).

Table 1. Plasma AMH concentrations and embryo results (mean ± SE) after OPU-IVP in *Bos taurus* (Holstein) and *Bos indicus* (Nelore) donors classified into two AMH categories.

	Plasma AMH concentration		P value
	Low	High	
Bos taurus			
Number of animals	32	27	-
Plasma AMH (ng/ml)	0.2 ± 0.01	0.4 ± 0.02	<0.0001
Cleavage rate (%)	54.5 ± 5.6	58.6 ± 5.3	0.98
Blastocysts produced per OPU	1.2 ± 0.3	3.0 ± 0.7	0.04
Blastocyst rate (%)	19.8 ± 4.2	20.6 ± 4.0	0.60
Bos indicus			
Number of animals	18	16	-
Plasma AMH (ng/ml)	0.5 ± 0.05	2.0 ± 0.3	<0.0001
Cleavage rate (%)	76.0 ± 8.2	89.8 ± 4.0	0.14
Blastocysts produced per OPU	2.2 ± 0.5	7.0 ± 1.7	0.0067
Blastocyst rate (%)	27.4 ± 5.5	33.7 ± 6.5	0.41

Adapted from Guerreiro *et al*. (2014).

AMH and fertility

Recent reports observed a positive association between AMH and fertility in dairy cows (Ribeiro *et al*., 2014; Jimenez-Krassel *et al*., 2015). For example, a large study done in Florida, USA reported that cows with low AMH concentrations had lower pregnancy results following first service and greater incidence of pregnancy loss between day 30 and 65 of gestation (Ribeiro *et al*., 2014). Moreover, dairy cows with relatively low circulating AMH concentrations as heifers also had the lowest survival rate after freshening

for the first time compared with age-matched herdmates having greater AMH concentrations (Jimenez-Krassel *et al*., 2015). Interestingly though it appears that the type of AI may determine the value of using AMH in a breeding program. For example, an elegant study done by Ribeiro *et al*. (2014) showed positive association between circulating AMH and P/AI in cows inseminated after estrus detection. However, no association between circulating AMH and P/AI was observed when cows had their ovulation synchronized for timed AI (TAI; Ribeiro *et al*., 2014). Thus, it appears that the use of TAI protocols may override possible associations of AMH with field fertility and that may help explain some of contrasting results we have recently observed when working with Nelore cows. For instance, accordingly with other study (Santos *et al*., 2014), we have found no correlation of a greater antral follicular population (animals likely having greater AMH) and conception results following timed AI protocols in mature Nelore cows (n = 758) or heifers (n = 1,113, Table 2; Baruselli *et al*., 2015; USP/FMVZ, São Paulo, SP, Brazil; unpublished data). Additionally, although antral follicle population has been associated with embryo production, no effect of donor antral follicle population was observed on pregnancy establishment after transferring the produced embryos (Fig. 7; Bragança *et al*., 2014; Fig. 8; Guerreiro *et al*., 2015; USP/FMVZ, São Paulo, SP, Brazil; unpublished data).

Table 2. Number of animals enrolled in the trial, body condition score (BCS), age (months), ovulation and/or pregnancy rate after TAI in Nelore heifers or cows according to the antral follicle population (AFP; measured at day 4 of TAI protocol; the expected moment of wave emergence) category in which animals were assigned. Data presented as percentage or average ± standard error of the mean (SEM).

	Antral follicle population categories			Total	P value
	Low	Medium	High		
Cows					
Number of animals	255	250	253	758	-
BCS (1 - 5)	3.00 ± 0.02	3.02 ± 0.02	3.02 ± 0.02	3.01 ± 0.01	0.29
Antral follicle population (n)	24.5 ± 0.5^{C}	39.2 ± 0.9^{B}	56.3 ± 1.4^{A}	40.0 ± 0.7	<0.0001
Pregnancy rate (%)	47.1	53.6	45.5	48.7	0.89
Heifers					
Number of animals	371	371	371	1,113	-
Age, months	15.0 ± 0.1	14.9 ± 0.1	14.8 ± 0.1	14.9 ± 0.1	0.22
BCS (1 - 5)	3.27 ± 0.02	3.27 ± 0.02	3.31 ± 0.02	3.28 ± 0.01	0.74
Antral follicle population, n	7.1 ± 0.1^{C}	11.3 ± 0.1^{B}	17.2 ± 0.2^{A}	11.8 ± 0.2	<0.0001
Ovulation rate (%)	82.5	78.3	79.8	80.2	0.56
Pregnancy rate (%)	39.6	36.4	36.7	37.6	0.57

$^{A \neq B \neq C}$Data with different superscripts in the same line differ with P ≤ 0.05.

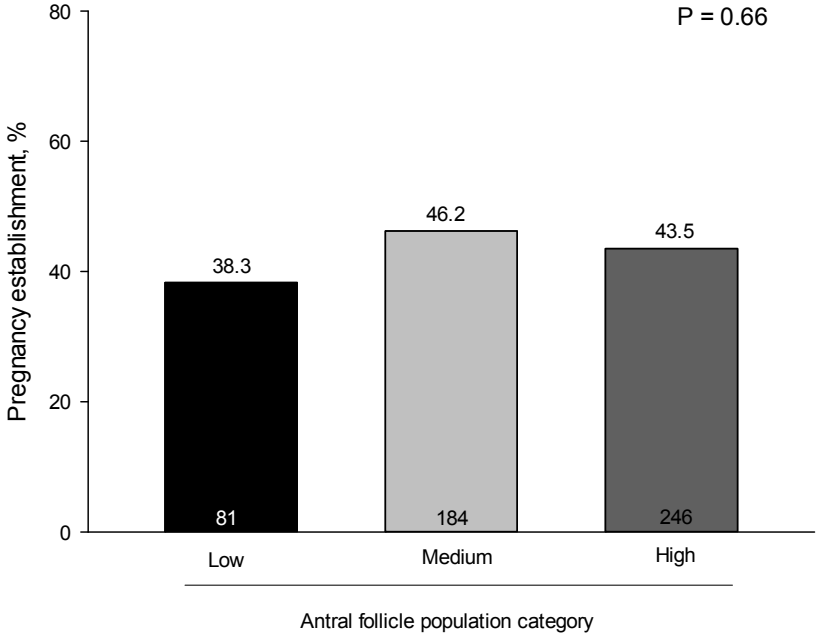

Figure 7. Pregnancy establishment in crossbred (*Bos indicus x Bos taurus)* recipients after embryo transfer of *in vitro* produced embryos according to the non-lactating Holstein donor antral follicle population category (low, medium or high). Adapted from Bragança *et al*. (2014).

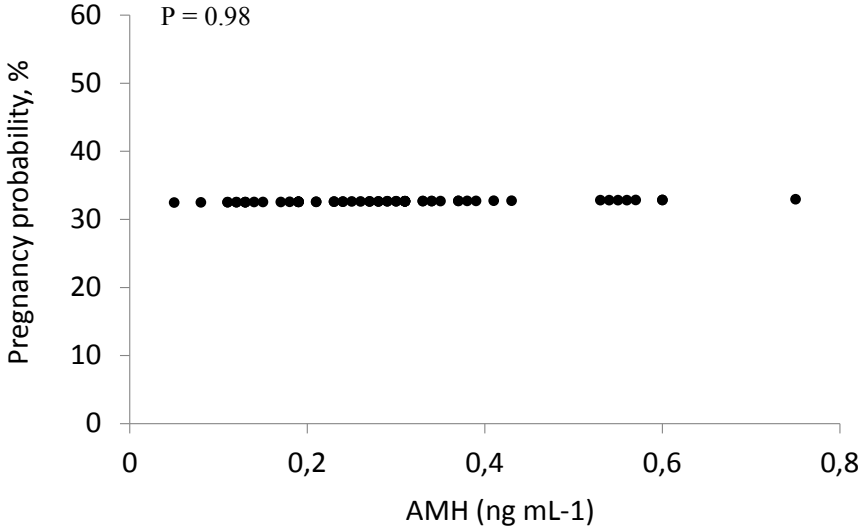

Figure 8. Relationship between circulating levels of AMH (ng/ml) and pregnancy probability in crossbred receptors after transferring *in vitro* produced embryos from Holstein donors (n = 107). Adapted from Guerreiro *et al*. (2015); USP/FMVZ, São Paulo, SP, Brazil; unpublished data.

Similarly, we failed to observe any association between circulating AMH and age at conception in Holstein heifers or interval from calving to conception in lactating Holstein cows (Carvalho *et al*., 2015; University of Wisconsin-Madison, Wisconsin, Madison, USA; unpublished data; Fig. 9). One important aspect to be discussed in our study though is that we have not taken into account heifers and/or cows that had been culled before pregnancy confirmation. This was a retrospective analysis rather than a manipulative or observational study.

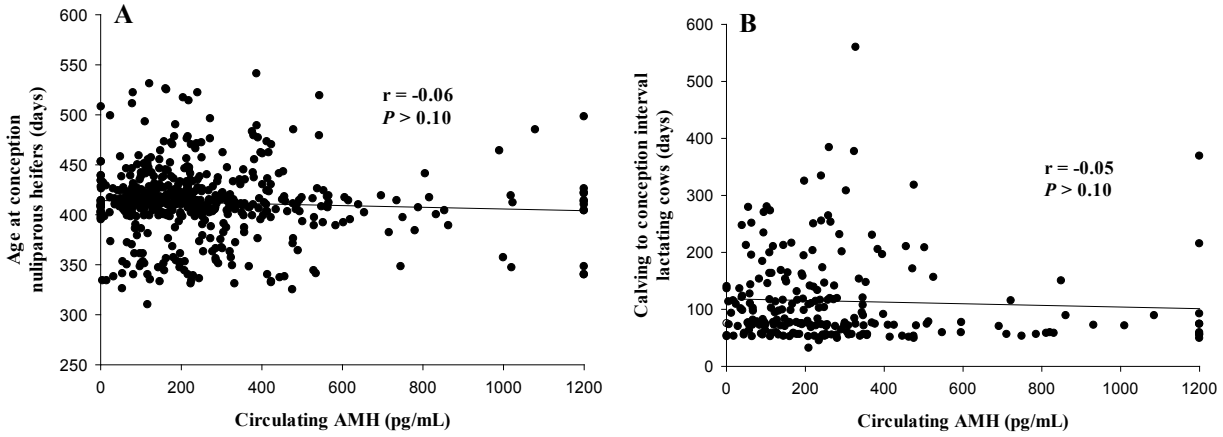

Figure 9. Relationship between circulating levels of AMH (pg/ml) and age (days) at conception in heifers (n = 528; panel A); and interval from calving-to-conception (days open) in lactating cows (n = 223; panel B).

Furthermore, because number of ovarian antral follicles appears to be correlated in cattle dam-daughter pairs (Walsh *et al*., 2014), and that may allow for selection of animals with greater AFP, we have recently looked into possible associations between circulating AMH in dam-daughter pairs in Holstein and Jersey breeds (Fig. 10; Batista *et al*., 2015; USP/FMVZ, São Paulo, SP, Brazil; unpublished data). The correlation in circulating AMH in dam-daughters although significant, was somewhat low (r = 0.18). Although, AFP in cattle is moderately heritable (0.31; (Walsh *et al*., 2014), epigenetic factors such as levels of negative energy balance during early fetal life (Evans *et al*., 2012) as well as dam-age and lactation status (Walsh *et al*., 2014) might likely influence antral follicle count in offspring. These epigenetic factors might then explain the poor correlation found in circulating AMH between dam-daughter pairs. Overall, the value of using AMH measurement to predict field fertility is still controversial and further studies using large numbers of animals are needed to draw final conclusions.

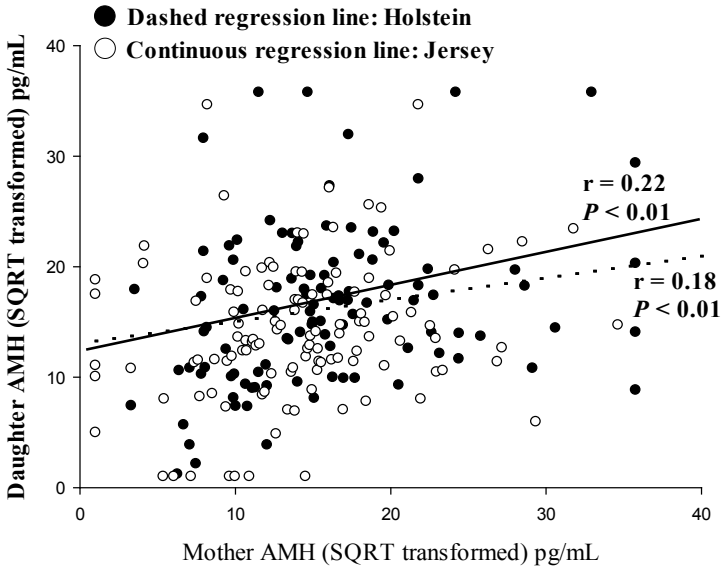

Figure 10. Correlation between circulating levels of AMH (pg/ml) in Holstein (n = 116) and Jersey (n = 106) dam-daughter pairs. AMH values are squared-root transformed. Data from Batista *et al*. (2015); USP/FMVZ, São Paulo, SP, Brazil; unpublished data.

Conclusions and future directions

Measuring circulating AMH in cattle can be useful to identify animals most likely to have improved superovulatory responses to gonadotropin treatment as well as best oocyte-donors for *in vitro* embryo production. In addition, although we cannot rule out the possibility of utilizing AMH to select cows with improved fertility; further basic and applied research are needed to elucidate whether AMH can be used by beef and dairy operations to identify animals with greater fertility and productive life.

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