Reproduction in Domestic Animals

Reprod Dom Anim doi: 10.1111/rda.12430
ISSN 0936–6768

# Anti-Mullerian Hormone Concentration and Antral Ovarian Follicle Population in Murrah Heifers Compared to Holstein and Gyr Kept Under the Same Management

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**Contents**

This study was performed to evaluate plasma concentrations of anti-Mullerian hormone (AMH) and the ovarian antral follicle population (AFP) in different genetic groups. Cyclic heifers (13 *Bubalus bubalis* [Murrah]; 15 *Bos taurus* [Holstein] and 10 *Bos indicus* [Gyr]) were maintained under the same management and were synchronized with two doses of 150 μg IM d-cloprostenol administered 14 days apart. After the second d-cloprostenol treatment, heifers had their ovaries scanned daily by ultrasound to define the day of ovulation. On the same day, the AFP was determined and a plasma sample was collected to measure AMH. Murrah heifers had less AFP (25.6 ± 2.1 follicles; $p = 0.01$) and plasma AMH concentration (0.18 ± 0.03 ng/ml; $p < 0.001$) than Gyr (60.0 ± 12.2 follicles and 0.60 ± 0.12 ng/ml of AMH); however, data were similar when compared to Holstein (35.9 ± 6.8 follicles and 0.24 ± 0.06 ng/ml of AMH) heifers. Regardless of genetic background, there was a positive relationship between the AFP and plasmatic AMH concentration (Murrah [$r = 0.62$; $p < 0.01$], Holstein [$r = 0.66$; $p < 0.001$] and Gyr [$r = 0.88$; $p < 0.001$]). Also, when heifers were classified according to high- or low-AMH concentration based on the average within each genetic group, high-AMH heifers had greater ($p < 0.0001$) AFP than low-AMH heifers. In conclusion, both Murrah and Holstein heifers presented lower plasma AMH concentration and AFP when compared to Gyr.

## Introduction

In females, anti-Mullerian hormone (AMH) is produced in the granulosa cells of ovarian follicles (Vigier et al. 1984), and its mRNA expression was detected in granulosa cells of primary follicles immediately after their formation in neonatal rats and mice (Baarends et al. 1995). The expression of AMH mRNA was subsequently detected in granulosa cells of all secondary pre-antral stage follicles; small antral follicles during the first pre-pubertal wave of development; and during oestrous cycle thereafter (Durlinger et al. 1999; Rico et al. 2011). Circulating AMH concentrations are positively associated with the ovarian antral follicle population (AFP) in mice (Durlinger et al. 2002) and women (Fanchin et al. 2003). AMH is also a reliable endocrine marker the small antral gonadotropin-responsive follicle population in *Bos taurus* (Rico et al. 2009; Monniaux et al. 2010, 2012), *Bos indicus* (Batista et al. 2014) and goats (Monniaux et al. 2011).

*Bubalus bubalis* have subtle differences in their reproductive biology compared with *Bos taurus* and *Bos indicus* breeds (Figueiredo et al. 1997; Sartori et al. 2001; Bó et al. 2003; Neglia et al. 2003; Escalona et al. 2008; Gimenes et al. 2008; Sartori and Barros 2011; Baldrighi et al. 2013). Some important differences among these genetic backgrounds in AFP have been demonstrated. Buffalo females present lower number of primordial (Van Ty et al. 1989) and antral follicles (Baruselli et al. 1997; Gimenes 2010; Baldrighi et al. 2013) when compared to cattle. Furthermore, *Bos indicus* cattle have greater numbers of follicles recruited per follicular wave than *Bos taurus* (Baruselli et al. 1997; Gimenes 2010; Baldrighi et al. 2013). Therefore, the knowledge of the AFP between the different genetic backgrounds may be useful to explain different responses to hormonal treatments associated with *in vivo* and *in vitro* embryo production programmes.

Several impacts of nutrition and climate on reproductive parameters were reported (Santos et al. 2008; Campanile et al. 2010a,b). However, most of these studies have been performed in countries with a temperate climate (Rensis and Scaramuzzi 2003; Hansen 2004). Therefore, to develop specific reproductive strategies in different breeds managed under tropical climate, it is important to analyse reproductive parameters among different genetic backgrounds maintained in the same nutritional and climate conditions.

As mentioned previously, AMH is also a reliable endocrine marker of the AFP in *Bos taurus* (Ireland et al. 2008; Rico et al. 2009; Monniaux et al. 2012) and *Bos indicus* (Batista et al. 2014) cattle. A recent study (Batista et al. 2014) evaluated the correlation between the AMH and AFP in Nelore (*Bos indicus*) and Holstein (*Bos taurus*) heifers, demonstrating high-AMH plasma concentration and AFP in Nelore heifers. However, there is limited information about correlation between AMH concentration and AFP in other *Bos indicus* breeds, such as the Gyr breed. Furthermore, there is a lack of scientific reports in regard to AMH and AFP correlation in *Bubalus bubalis* females, especially when compared to other genetic backgrounds exposed to the same nutrition and environmental management.

Thus, this study was designed to simultaneously evaluate the AFP (number of ovarian antral follilcles equal or larger than 3 mm) and plasma AMH concentration in Murrah (*Bubalus bubalis*), Gyr (*Bos indicus*) and Holstein (*Bos taurus*) heifers kept under the same environmental and nutritional conditions. The hypotheses evaluated were (i) that AFP is positively associated with plasma AMH concentrations in *Bubalus bubalis* (Murrah) similar that *Bos indicus* (Gyr) and *Bos taurus* (Holstein) heifers and (ii) that both AFP and plasma AMH concentrations are lower in Murrah (*Bubalus bubalis*) and Holstein (*Bos taurus*) than Gyr (*Bos indicus*) heifers.

## Materials and methods

### Location and heifers

The experiment was conducted at the University of Sao Paulo (USP), Pirassununga *campus* (−21°59′46″; −47°25′33″), SP, Brazil, between June and August 2010. Three genetic groups were used: Holstein heifers (*Bos taurus*), aged 24–30 months and weighing 430.0 ± 21.1 kg (n = 15); Gyr heifers (*Bos indicus*), aged 18–24 months and weighing 358.1 ± 46.5 kg (n = 10); and Murrah heifers (*Bubalus bubalis*), aged 24–30 months and weighing 546.1 ± 35.12 kg (n = 13). All the heifers were determined to be cyclic (defined by the presence of *corpus luteum* during at least one of two consecutive ultrasound examinations performed 14 days apart). The heifers were kept in a single *Brachiaria decumbens* pasture and were provided shredded sugar cane (20 kg/heifer/day), commercial feed (2 kg/heifer/day) and *ad libitum* access to water and trace-mineralized salt. Heifers were handled in accordance with the Ethic Committee in the use of animals of the School of Veterinary Medicine and Animal Science of University of Sao Paulo, Brazil (protocol number 1758/2009).

### Oestrous synchronization and ovarian antral follicle population count

All heifers were previous synchronized with 150 μg IM d-cloprostenol (Ciosin®, MSD Animal Health, Cravinhos-SP, Brazil) administered 14 days apart. After the second d-cloprostenol treatment, heifers had their ovaries daily examined by an Aloka® SSD-500 linear array trans-rectal probe (7.5-MHz transducer, Hitachi Aloka Medical, Ltd.) to determine the day of ovulation (Day 0). On the same day of the ultrasonography, the total number of antral follicles ≥3 mm in diameter (i.e. AFP) was determined by counting the number of follicles ≥3 mm in diameter for each animal in both ovaries.

### Blood collection and AMH assay

Blood samples were collected into tubes containing EDTA by jugular venipuncture on day of ovulation (concurrently with the AFP assessment). The samples were immediately placed in ice, and later centrifuged at 3000 × ***g*** for 15 min for separation of plasma. Plasma samples were frozen at −25°C until later analysis. Plasma AMH was evaluated using an enzyme-linked immunosorbent assay ELISA kit (Ansh Labs, Webster, TX, USA). The sensitivity of the AMH assay was 0.011 ng/ml and intra-assay CV was <5%. All AMH assays were performed at the IgAc (Genese Institute of Scientific Analyses, Sao Paulo, Brazil).

For the purpose of analysing the relationships between AMH concentration and the AFP, heifers within each genetic group were retrospectively classified into one of two AMH categories (low or high) based on the average plasma AMH in each genetic background (the cut-off for low vs high AMH was 0.24 ng/ml for Holstein, 0.60 ng/ml for Gyr and 0.18 ng/ml for Murrah).

### Statistical analysis

All data were analysed with SAS *System for windows* and are presented as the means ± SEM, except for the correlation studies. Analyses of follicular populations were performed with PROC GLIMMIX of SAS 9.3. For the correlation studies, significance was ascertained by Bravais–Pearson *r* critical values performed by PROC CORR of SAS 9.2 and PROC REG to obtain the regression functions. The continuous variables AFP and plasma AMH concentration were analysed and the statistical model only included the effect of the genetic background. To normalize these data, the AFP was transformed to 1/AFP and the AMH concentration was transformed to SQRT. Animals within each genetic background were included as a random effect in the statistical model. To analyse the influence of the AMH categories (Low and High) on the AFP we included the effects of breed (Murrah, Gyr, Holstein), AMH category (Low or High) and their interaction in the statistical model. For all analyses, differences with $p > 0.05$ were considered not significant.

## Results

Despite the high variability in the AFP among individuals within each genetic background, the number of AFP was greater in Gyr (*Bos indicus*) heifers than in Holstein (*Bos taurus*) and Murrah (*Bubalus bubalis*) heifers ($p = 0.01$; Fig. 1). The AFP for each heifer ranged from 22 to 125 for Gyr, from 18 to 110 for Holstein and from 11 to 53 for Murrah heifers.

The plasma AMH concentration profiles followed similar pattern of the follicular population recorded by each breed. Murrah and Holstein presented lower AMH concentration ($p < 0.01$) when compared to Gyr heifers (Fig. 1). Accordingly, there was a positive relationship

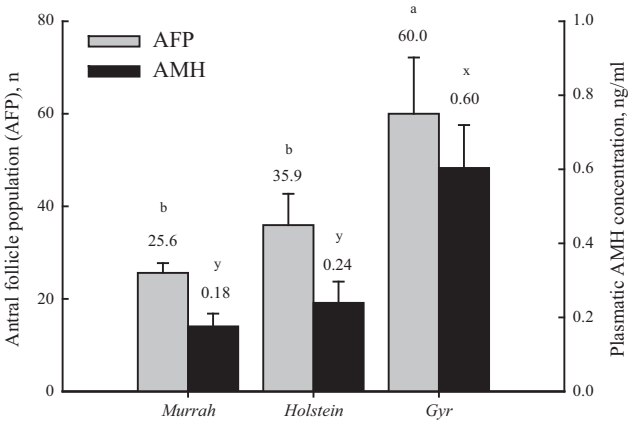


Fig. 1. Number of antral follicle population (AFP) and plasma anti-Mullerian hormone (AMH) concentration in Murrah (*Bubalus bubalis*; n = 13), Holstein (*Bos taurus*; n = 15) and Gyr (*Bos indicus*; n = 10) heifers. Data are presented as the means ± SEM. Different letters within columns of the same colour are significantly different [AFP: a≠b; p = 0.01 and AMH concentration: x≠y; p < 0.001]

between the AFP and plasma AMH concentration for Murrah ($r = 0.62$; $p < 0.01$), Holstein ($r = 0.66$; $p < 0.001$) and Gyr ($r = 0.881$; $p < 0.001$) heifers (Fig. 2). Furthermore, heifers classified with high-plasmatic AMH concentration within each genetic group had greater AFP, without any interaction with genetic background (Table 1).

## Discussion

To our knowledge, this is the first comparative report of plasma AMH concentrations among Murrah (*Bubalus bubalis*), Holstein (*Bos taurus*) and Gyr (*Bos indicus*). The present study provides slight new information regarding the reproductive physiology of these species. The results demonstrate that (ii) Murrah (*Bubalus bubalis*) and Holstein (*Bos taurus*) heifers have lower AFP when compared to Gyr (*Bos indicus*) heifers maintained under the same nutritional and environment conditions; (ii) Murrah (*Bubalus bubalis*) and Holstein (*Bos taurus*) also have lower plasma AMH concentration than Gyr (*Bos indicus*) heifers; and (iii) regardless of the genetic background, the plasma AMH concentrations are positively associated with the AFP.

In agreement with the present results, Gimenes et al. (2009) found that *Bubalus bubalis* heifers (13.1 ± 1.4) had lower number of follicles at the synchronized follicular wave emergence than Nelore *Bos indicus* heifers (29.7 ± 3.1). This lower number of follicles in buffalo could be at least partially explained by the greater amount of atretic follicles observed in the *Bubalus bubalis* ovaries than in bovine ovaries (Mondadori et al. 2007, 2010). These latter authors described differences in the cytoplasmic vesicles quantity, mitochondria shape and inner content, zona pellucida deposition and granulosa cells–oocyte junctions of buffalos when compared to cattle. Maybe, these characteristics can be responsible for some functional differences observed in *Bubalus bubalis* follicular dynamics, when compared to cattle. Furthermore, it

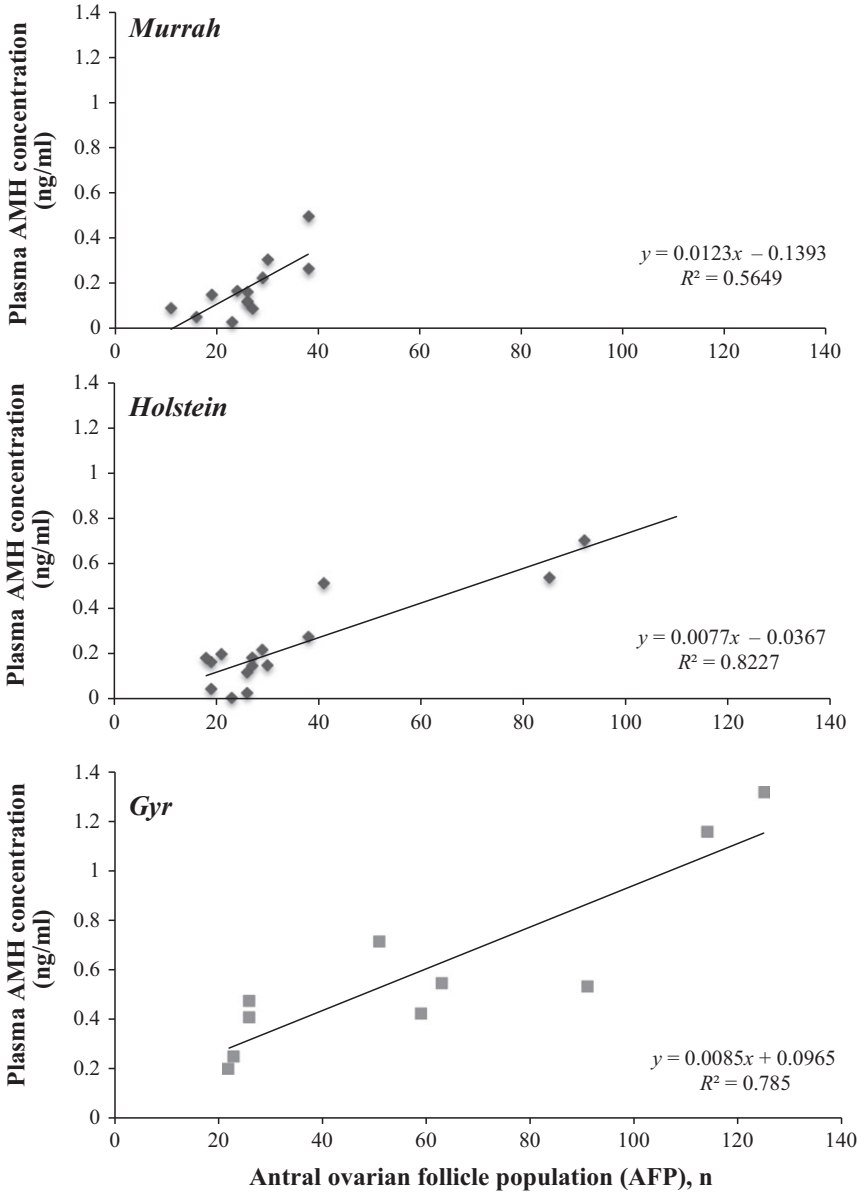


Fig. 2. Relationship between the ovarian antral follicle population (AFP) counted at day of ovulation and the plasma anti-Mullerian hormone (AMH) concentration in Murrah (*Bubalus bubalis*; n = 13), Holstein (*Bos taurus*; n = 15) and Gyr (*Bos indicus*; n = 10) heifers

Table 1. Antral follicular population (AFP) and plasma AMH concentration categories in Murrah (*Bubalus bubalis*), Holstein (*Bos taurus*) and Gyr (*Bos indicus*) heifers

| | Genetic group | | | | | | p values | | |
|---|---|---|---|---|---|---|---|---|---|
| | Murrah (*Bubalus bubalis*) | | Holstein (*Bos taurus*) | | Gyr (*Bos indicus*) | | | | |
| AMH category[1] | Low | High | Low | High | Low | High | Genetic background | AMH category | Group * category |
| No. heifers | 9 | 4 | 11 | 4 | 7 | 3 | – | – | – |
| AFP[2] | 22.0 ± 1.8[b] | 33.8 ± 2.5[b] | 24.1 ± 1.3[b] | 68.5 ± 17.5[b] | 44.3 ± 10.2[a] | 96.7 ± 23.1[a] | 0.01 | <0.0001 | 0.78 |
| Plasmatic AMH (ng/ml) | 0.11 ± 0.02 | 0.32 ± 0.06 | 0.13 ± 0.02 | 0.54 ± 0.11 | 0.41 ± 0.05 | 1.06 ± 0.18 | <0.0001 | 0.001 | 0.26 |

[1]Plasma anti-Mullerian hormone (AMH) categories (Low and High) were retrospectively established according to the means of the AMH concentration within each genetic background (the cut-off for low vs high AMH was 0.24 ng/ml for Holstein, 0.60 ng/ml for Gyr and 0.18 ng/ml for Murrah).
[2]Antral follicle population (AFP) = numbers of antral follicle ≥3 mm counted at the day of ovulation.
Different superscripts within the same row are significantly different (genetic background effect).

has been reported in cattle that the proliferative activity of the granulosa and thecal cells decreases in the atretic follicles (Isobe and Yoshimura 2000). Also, buffalo cows present less proliferative activity of the theca cell when compared to bovine, which could explain the lower follicular growth observed in this species (Feranil et al. 2004).

In spite of the well-document difference in the antral follicle count between *Bos indicus* and *Bos taurus* females (Carvalho et al. 2008; Gimenes et al. 2009; Sartori et al. 2010), a significant individual variability has been reported within each genotype, as previously reported for *Bos taurus* (Burns 2005; Ireland et al. 2008) and *Bos indicus* (Carvalho et al. 2008; Bastos et al. 2010) cattle. For example, Bastos et al. (2010) described large individual variability on the number of antral follicles (2–5 mm) in Nelore (42.7 ± 5.9 [Mean ± SE]; ranging from 25 to 100) and Holstein heifers (19.7 ± 3.2; ranging from 5 to 40) kept under same management. However, in the present data, when heifers were classified according to circulating AMH concentration, animals with high AMH had much greater averages of antral follicles in Holstein (68.5 follicles) and Gyr (97 follicles) heifers. Therefore, it is worth mentioning that the unexpectedly high-ovarian follicular count can be related to individual physiological peculiarities, especially considering the relatively small sample size for each AMH class and within each breed (Holstein [n = 4] and Gyr [n = 3]). This specific finding certainly requires further investigation.

Murrah (*Bubalus bubalis*) and Holstein (*Bos taurus*) heifers in the present study also had lower AMH plasma concentration than Gyr (*Bos indicus*) heifers. The AMH is primarily produced in the granulosa cells of ovarian follicles (Vigier et al. 1984; Rico et al. 2011; Monniaux et al. 2012). Previous reports demonstrated that circulating AMH concentrations are lower for cattle with lower antral follicle count compared to those with high antral follicle count (Ireland et al. 2010). Additionally, buffalo females present lower number of primordial and antral ovarian follicles when compared to cattle (Van Ty et al. 1989; Baruselli et al. 1997; Gimenes et al. 2010; Baldrighi et al. 2013). Furthermore, AMH null mice present increased follicular recruitment; however, it presents enhanced oocyte degeneration and follicular atresia (Visser et al. 2007). Therefore, the lower plasma AMH concentration found in Murrah (*Bubalus bubalis*) and Holstein (*Bos taurus*) may explain the higher rates of follicular atresia resulting in the lower antral follicular populations when compared to Gyr (*Bos indicus*) heifers. However, the present study did not evaluate the atresia rate, and further investigations are certainly required.

Studies showed high correlation between number of follicles on the ovaries and the number of embryo produced after *in vivo* superovulation and *in vitro* OPU/IVF in *Bos indicus* (Pontes et al. 2010, 2011; Guerreiro et al. 2014), *Bos* taurus (Kawamata 1994; Cushman et al. 1999; Singh et al. 2004; Ireland et al. 2007, 2008; Rico et al. 2009; Pontes et al. 2010; Guerreiro et al. 2014) and *Bubalus bubalis* (Romero et al. 1991; Boni et al. 1996; Sá Filho et al. 2009; Galli et al. 2014). Regardless of genetic background, it was possible to select animals with greater AFP when the heifers of the present study were classified according to the plasma AMH levels (high and low). Consequently, this can be a useful marker to select donors for embryo production programs (Rico et al. 2009; Monniaux et al. 2010). However, it is important to emphasize that the cut-off of AMH concentration for selection of high and low AFP animals is specific-related to the plasma AMH levels within each group.

In conclusion, ovarian antral follicular populations are positively correlated with plasma AMH concentrations in Murrah (*Bubalus bubalis*), Holstein (*Bos taurus*) and Gyr (*Bos indicus*) heifers. Moreover, Murrah (*Bubalus bubalis*) and Holstein (*Bos taurus*) heifers have lower plasma AMH concentrations and reduced AFP than Gyr (*Bos indicus*) heifers.

## Acknowledgements

The authors would like to thank the staff at Pirassununga campus – USP (Pirassununga-SP) for allowing the use of their facilities during this study. The authors would also to thank the Santa Eliza Farm, Quebra Cuia Farm and São Caetano Farm for kindly conceding their animals for use in this study. The authors are also grateful to Genese© for the donation of AMH ELISA kits. This research was supported by CNPq (144952/2009-2) and FAPESP (2011/50826-7).

## Author contribution

JM Baldrighi contributed to analysis and interpretation of data, drafting the paper and acquisition of data; MF Sá Filho contributed to research design, drafting the paper and acquisition of data; EOS Batista contributed to analysis and interpretation of data and drafting the paper; RNVR Lopes contributed to acquisition of data; JA Visintin, PS Baruselli, MEOA Assumpção contributed to research design, interpretation of data and revising paper critically.

## Conflict of interest

None of the authors have any conflict of interest to declare.

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**Submitted: 13 Mar 2014; Accepted: 31 Aug 2014**

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