



**HAL**  
open science

## **Anti-Müllerian hormone as a predictive endocrine marker for embryo production in the goat**

Danielle Monniaux, Gérard Baril, Anne-Lyse Lainé, Peggy Jarrier-Gaillard,  
Noëlle Poulin, Juliette Cognie, Stéphane Fabre

► **To cite this version:**

Danielle Monniaux, Gérard Baril, Anne-Lyse Lainé, Peggy Jarrier-Gaillard, Noëlle Poulin, et al.. Anti-Müllerian hormone as a predictive endocrine marker for embryo production in the goat. *Reproduction*, 2011, 142 (6), pp.845-854. 10.1530/REP-11-0211 . hal-01129488

**HAL Id: hal-01129488**

**<https://hal.science/hal-01129488>**

Submitted on 29 May 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# Anti-Müllerian hormone as a predictive endocrine marker for embryo production in the goat

Danielle Monniaux, Gérard Baril, Anne-Lyse Laine, Peggy Jarrier, Natividad Poulin, Juliette Cognié and Stéphane Fabre

*Physiologie de la Reproduction et des Comportements, UMR 085 INRA-UMR 6175 CNRS-Université de Tours-IFCE, Centre INRA de Tours, 37380 Nouzilly, France*

*Correspondence should be addressed to D Monniaux; Email: dmonniaux@tours.inra.fr*

## Abstract

Recently, we demonstrated the relationship between anti-Müllerian hormone (AMH) circulating concentrations, ovarian follicles, and embryo production in cattle. However, they have not yet been established in a species with a seasonal breeding activity. Thus, goats were subjected to repeated *in vivo* embryo production during the breeding season, at the end of the breeding season, and at the end of the anestrus season. Embryo production after FSH treatment was highly repeatable for each goat. Plasma AMH concentrations, measured before the first FSH treatment, were highly correlated with the number of collected, transferable, and freezable embryos, resulting from the three sessions of embryo production. Plasma AMH concentrations transiently decreased after each exogenous FSH treatment, but they showed little change with season, and no relationship was observed between AMH and endogenous FSH concentrations during seasonal transitions. Follicles of 1–5 mm in diameter were the main target of the FSH treatment and were major contributors to circulating AMH concentrations. Granulosa cell AMH expression decreased as the follicle approached terminal development, while the expression of maturation markers (*CYP19A1* and *FSHR*) increased. In conclusion, circulating AMH concentrations can be predictive of the capacity of a donor goat to produce high or low numbers of high-quality embryos. This prediction could be accurately made from a single blood measurement of AMH during either breeding or anestrus seasons. Variability in the number of gonadotropin-responsive follicles of 1–5 mm in diameter between individuals resulted in the differences in circulating AMH concentrations measured between individuals.

*Reproduction* (2011) **142** 845–854

## Introduction

Multiple ovulation and embryo transfer (MOET) has the potential to improve the number of offspring produced by genetically valuable goats, but the methodology has not become widely used due to its unpredictability (Baldassarre & Karatzas 2004). An average of six to eight transferable embryos per donor can be produced in a successful goat MOET programme; however, a high variability in ovarian responses to gonadotropins is observed between donors (commonly between 0 and 30 transferable embryos per donor), without any variation in the standard operating procedure (Cognie 1999, Cognie *et al.* 2003, 2004, Gibbons *et al.* 2007, Menchaca *et al.* 2010). Laparoscopic ovum pick-up (LOPU) in combination with the *in vitro* production of embryos has also been proposed to improve the number of offspring in small ruminants (Tervit 1996), and this methodology has been considerably developed over recent years in goats (Baldassarre *et al.* 2002). Currently, most protocols combine the stimulation of follicular development by FSH treatment and oocyte recovery by

LOPU, but individual variations in the ovarian response to FSH remain a limitation to the efficiency of oocyte collection (Baldassarre & Karatzas 2004, Gibbons *et al.* 2007).

Variations in ovarian response to FSH reflect the follicular population present during the initiation of treatment in small ruminants (Gonzalez-Bulnes *et al.* 2004, Veiga-Lopez *et al.* 2005), as previously demonstrated in the cow (Monniaux *et al.* 1983, Kawamata 1994, Cushman *et al.* 1999, Singh *et al.* 2004). Despite the development of different strategies for increasing the number of recruitable ovarian follicles at the time of FSH treatment in small ruminants, improvements in terms of embryo production have not been reported (Menchaca *et al.* 2002, Cognie *et al.* 2003, Lopez-Alonso *et al.* 2005, Menchaca *et al.* 2010).

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), is a glycoprotein of 140 kDa belonging to the transforming growth factor- $\beta$  family that is only expressed in the gonads (Cate *et al.* 1986). In female mammals, AMH is specifically

expressed by granulosa cells and it has been found to be a highly reliable endocrine marker of the size of the ovarian pool of growing follicles in humans (Visser & Themmen 2005), mice (Kevenaar *et al.* 2006), and cows (Rico *et al.* 2009). In the cow, recent results from our laboratory indicate that plasma AMH concentrations are characteristic of each animal over a long period of time, and they can be predictive of the number of ovulations and embryos produced in response to ovarian stimulation by FSH (Rico *et al.* 2009, 2011, Monniaux *et al.* 2010). No data are available on circulating AMH concentrations in small ruminants, and it has not been established whether AMH concentrations could be used for the prediction of ovarian responses to gonadotropins and embryo production. Moreover, goats and sheep are seasonal breeders that present a long period of sexual rest under temperate climates: the anestrus season (Chemineau *et al.* 1992). In sheep, both gonadotropin secretion and ovarian antral follicular activity are affected by season (McNatty *et al.* 1984), but how these changes might affect the circulating concentrations of AMH has not been established in small ruminants.

The aims of this study were as follows: i) to establish whether plasma measurements of AMH could be of prognostic value in determining the number of transferable embryos that a given goat can produce after superovulatory treatment in a traditional MOET protocol; ii) to characterize the population of AMH-producing antral follicles in goat ovaries; and iii) to appreciate the effects of season on AMH and FSH concentrations in plasma and their relation with embryo production.

## Results

### Embryo production and its relationship with ovarian follicular population

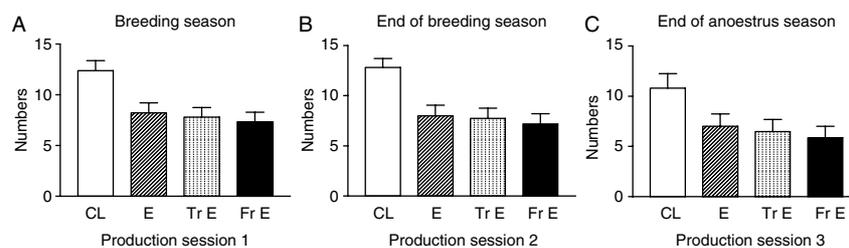
Each of the 15 goats studied was treated for *in vivo* embryo production in January (during the breeding season), April (at the end of the breeding season), and October (at the end of the anestrus season). The number of corpora lutea (CL) counted in the ovaries and the number of embryos recovered after superovulatory

treatment and insemination by hand mating was highly repeatable between the three sessions of embryo production ( $r^2$  varying between 0.67 and 0.68,  $P < 0.001$ , for the number of CL and the number of collected, transferable, or freezable embryos), and there was no significant change in embryo production with the repetition of treatment (Fig. 1). Owing to this high within-animal repeatability of superovulatory responses and embryo production, the results of the three sessions of embryo production were pooled per animal for subsequent analyses. The total number of CL and embryos produced per goat during the three sessions of embryo production was found to be highly variable between the goats (from 12 to 72 CL and from 7 to 56 collected embryos).

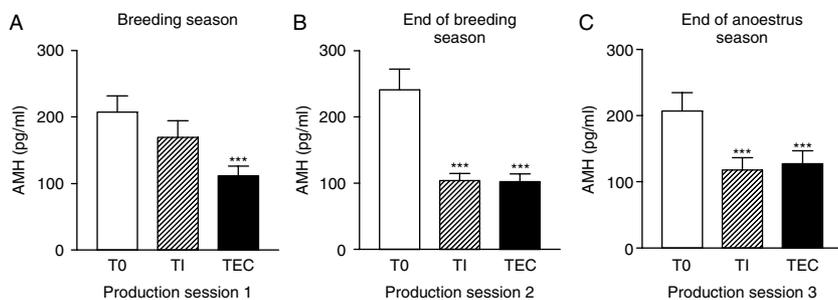
The goats were characterized by their ovarian content in antral follicles larger than 1 mm in diameter, which were recovered by dissection at the time of the third session of embryo collection. Follicles of up to 20 mm in diameter were present in the ovaries. The number of follicles per class of follicular diameter was highly variable between the goats (from 11 to 46 follicles of 1–5 mm in diameter and from 1 to 20 follicles larger than 5 mm in diameter). The number of 1–5 mm diameter follicles was found to be highly correlated with the total number of CL ( $r = 0.72$ ,  $P < 0.01$ ), and the total number of collected ( $r = 0.77$ ,  $P < 0.001$ ), transferable ( $r = 0.78$ ,  $P < 0.001$ ), and freezable embryos ( $r = 0.80$ ,  $P < 0.001$ ) recovered after the three sessions of embryo collection. In contrast, the number of follicles of diameters 5–8 mm and larger than 8 mm was not correlated with the total number of CL and embryos.

### AMH concentrations in the plasma of goats subjected to repeated superovulatory treatment and their relationship with embryo production

The AMH concentrations measured in the plasma of the goats at time before FSH treatment (T0), time at insemination (TI), and time at embryo collection (TEC) for the three sessions of embryo production were found to decrease after each superovulatory treatment (Fig. 2).



**Figure 1** Ovulation and embryo production after repeated superovulatory treatment and insemination by hand mating in goats. Goats ( $n = 15$ ) were subjected to three sessions of *in vivo* embryo production, which were carried out successively in (A) January (during the breeding season, production session 1), (B) April (at the end of the breeding season, production session 2), and (C) October (at the end of the anestrus season, production session 3). The data represent the number of corpora lutea (CL) counted in the ovaries and the number of collected (E), transferable (TrE), and freezable (FrE) embryos recovered by uterine flushing 7 days after estrus detection.



**Figure 2** Anti-Müllerian hormone (AMH) concentrations in the plasma of goats, before and after repeated superovulatory treatment and natural insemination. The data represent AMH concentrations in plasma samples recovered from goats ( $n=15$ ) at each session of embryo production in (A) January (B) April and (C) October, just before the first FSH injection (T0), at the time of insemination (TI), and at the time of embryo collection (TEC). \*\*\* $P < 0.001$  vs T0, within each session of embryo production.

The decrease in AMH concentration was highly significant at TI for the second and third sessions of embryo production and at TEC for all three sessions (all  $P < 0.001$ ). The AMH concentrations were repeatable between the three sessions of embryo collection when measured at T0 ( $r^2 = 0.48$ ,  $P < 0.05$ ), TI ( $r^2 = 0.44$ ,  $P < 0.05$ ), and TEC ( $r^2 = 0.78$ ,  $P < 0.001$ ).

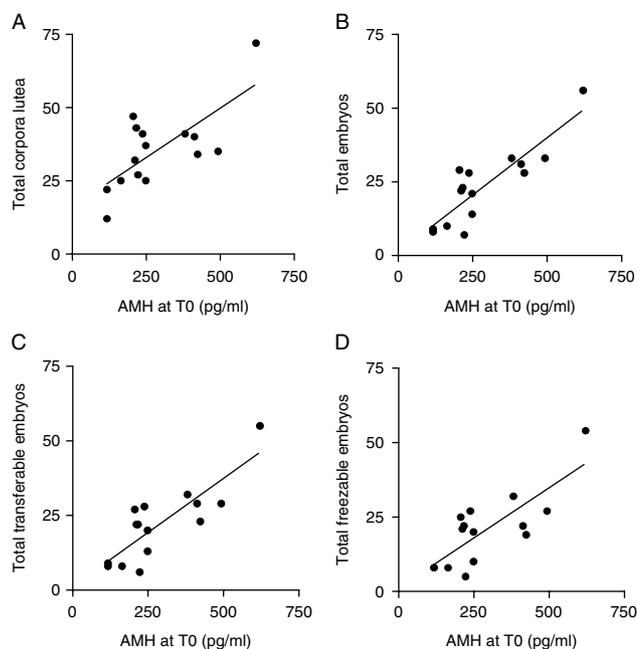
Interestingly, the AMH concentrations measured in the plasma at T0, before the first superovulatory treatment to the goats, were highly correlated with the total number of CL ( $r = 0.71$ ,  $P < 0.01$ ) and the total number of collected ( $r = 0.87$ ,  $P < 0.001$ ), transferable ( $r = 0.83$ ,  $P < 0.001$ ), and freezable embryos ( $r = 0.78$ ,  $P < 0.001$ ), recovered after the three sessions of embryo collection (Fig. 3). Similar relationships were also observed between the AMH concentrations measured at different times of the three sessions of embryo production and the total number of CL and embryos (data not shown).

#### **Intrafollicular AMH concentrations and mRNA expression of granulosa cell markers in goat follicles**

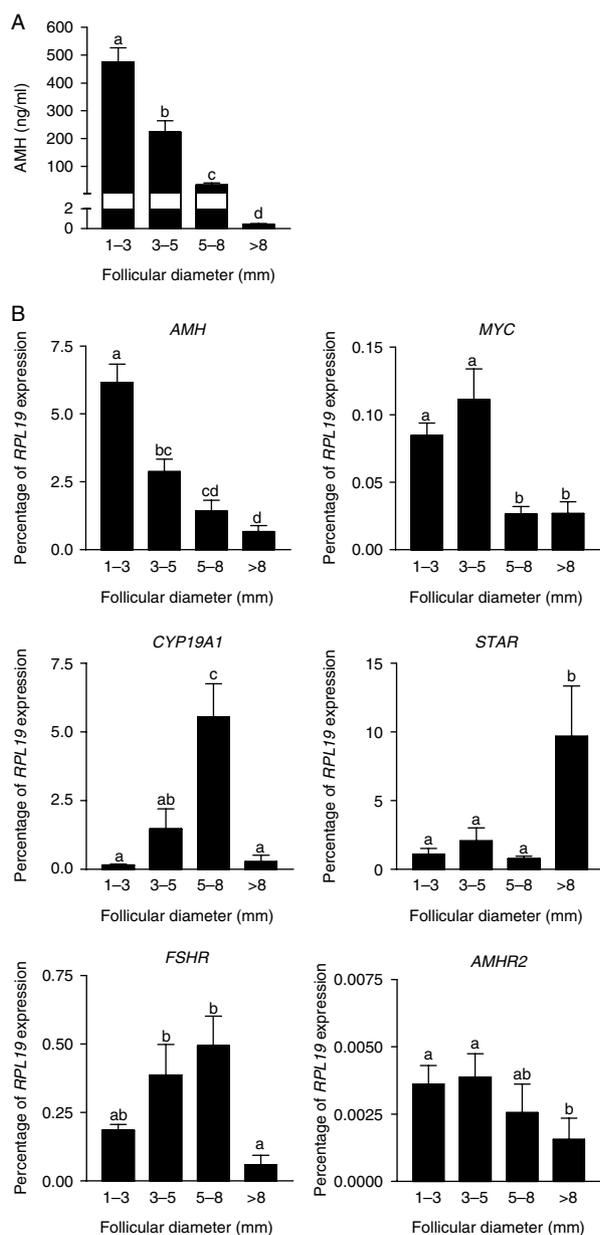
In order to identify the ovarian follicles that contributed most to circulating AMH concentrations in the goats, the follicles recovered at the time of the third session of embryo collection were analyzed for their AMH content. When measured in follicular fluid, AMH concentrations were the highest in 1–3 mm diameter follicles, followed by a twofold decrease in 3–5 mm diameter follicles, and then a more than sixfold decrease when the follicles increased to 8 mm, followed by a further 80-fold decrease in the largest follicles of diameter larger than 8 mm ( $P < 0.001$ , Fig. 4A). The number of 1–5 mm diameter follicles was highly correlated with AMH concentration in plasma at the time of the third session of embryo collection ( $r = 0.89$ ,  $P < 0.001$ ). In contrast, the number of follicles of diameters 5–8 mm and larger than 8 mm was not correlated with the AMH concentrations in plasma ( $r = -0.25$  and  $r = -0.08$ , respectively, both NS).

To further characterize the AMH-producing follicles, the expression of *AMH* and other functional markers was studied in granulosa cells (Fig. 4B). The expression of *AMH* mRNA decreased while the expression of the maturation markers, *CYP19A1* and *FSHR*, increased

when the follicular diameter increased up to 8 mm (all  $P < 0.001$ ). *MYC*, used as a proliferation marker, expression dropped in follicles larger than 5 mm in diameter ( $P < 0.001$ ). Follicles of diameter larger than 8 mm had the lowest *AMH*, *MYC*, *CYP19A1*, and *FSHR* mRNA levels in the granulosa cells, but the highest *STAR* mRNA levels compared with the other follicular size classes. The *AMHR2* marker was also expressed in granulosa cells, without a clear change in mRNA expression levels in follicles up to 8 mm in diameter, but a slight decrease was observed in follicles larger than 8 mm in diameter ( $P < 0.05$ ).



**Figure 3** Relationships between AMH concentrations measured in the plasma at T0, before first superovulatory treatment, and the total results of the three sessions of embryo production. The data represent the relationships between the AMH concentration at T0 and (A) the total number of corpora lutea, (B) the total number of collected embryos, (C) the total number of transferable embryos, and (D) the total number of freezable embryos, all recovered and counted after the three sessions of embryo collection. Each circle represents data from one goat ( $n=15$  goats).



**Figure 4** Follicular markers in goat follicles. The data represent (A) intrafollicular concentrations of AMH and (B) mRNA expression of AMH and different functional gene markers. Follicular fluids and granulosa cells of follicles of 1–20 mm in diameter were recovered from the goats at the time of the third session of embryo collection, classified into four different size classes, and pooled per goat and per follicular class. In the granulosa cells, mRNA accumulation from AMH, MYC, CYP19A1, STAR, FSHR, and AMHR2 genes was studied by RT-qPCR and represented as a percentage of RPL19 expression used as an internal reference. The data are expressed as weighted average AMH concentrations (number of repetitions per follicular class,  $n=13-15$ ) and gene expression (number of repetitions per follicular class,  $n=9-15$ ) per goat. The different letters within each panel indicate significant differences between the follicular size classes ( $P<0.05$ ).

### Effects of season on AMH and FSH concentrations in plasma and their relationship with embryo production

Our results indicate that the measurement of AMH concentrations in plasma can help to predict the capacity of goats to respond to superovulatory treatment and produce high or low number of embryos. With the aim of further defining the best period to measure AMH concentrations in goats, we studied AMH changes in plasma during two periods of seasonal reproductive activity transition.

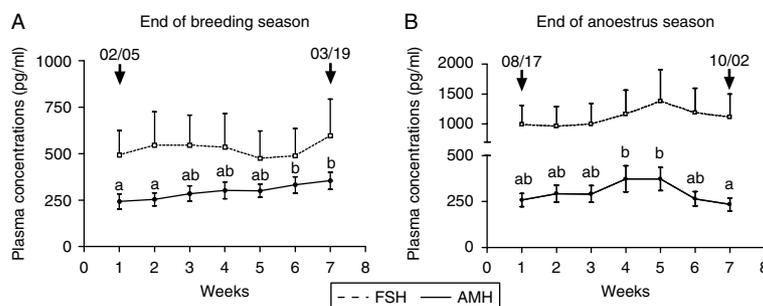
From February to April (Spring), corresponding to the transition between the breeding and the anestrus seasons, five out of the 15 goats studied showed detectable ovulatory activity (progesterone concentrations higher than 1 ng/ml for at least two successive weeks). In Spring, a slight increase ( $\times 1.5$ ) in AMH concentrations was observed ( $P<0.001$ ), whereas the FSH concentrations did not change (Fig. 5A). From August to October (Autumn), which usually corresponds to the transition between the anestrus and the breeding seasons, none of the 15 goats studied showed ovulatory activity. In Autumn, a slight decrease was observed in AMH concentrations during the last 2 weeks ( $P<0.01$ ), whereas the FSH concentrations did not change (Fig. 5B).

The mean AMH concentrations were similar between the two periods studied ( $296.4 \pm 37.3$  vs  $289.9 \pm 40.7$  pg/ml, Spring vs Autumn, NS). In the 15 goats studied, the mean AMH concentrations per animal in Spring and Autumn were highly related ( $r=0.79$ ,  $P<0.001$ , Fig. 6A), indicating that AMH concentrations in the plasma were characteristic of individuals. The mean AMH concentrations per animal in both Spring and Autumn were correlated with the total embryo production of the goats, but the relationships were stronger in Spring compared with Autumn for the total number of collected ( $r=0.72$ ,  $P<0.01$ , in Spring vs  $r=0.52$ ,  $P<0.05$ , in Autumn), transferable ( $r=0.70$ ,  $P<0.01$ , vs  $r=0.55$ ,  $P<0.05$ ), and freezable embryos ( $r=0.65$ ,  $P<0.01$ , vs  $r=0.55$ ,  $P<0.05$ ).

The mean FSH concentrations were twofold higher during Autumn compared with Spring ( $1115 \pm 363.6$  vs  $526.3 \pm 153.0$  pg/ml, Autumn vs Spring,  $P<0.01$ , Fig. 5). In the 15 goats studied, the mean FSH concentrations per animal measured in Spring and Autumn were highly related ( $r=0.93$ ,  $P<0.001$ , Fig. 6B), indicating that FSH concentrations in the plasma were characteristic of individuals, but no relationship was observed between the FSH concentrations per animal and the total embryo production of the goats. Moreover, no relationship was observed between AMH and FSH concentrations in the plasma of the goats.

### Discussion

Our results show, for the first time, that circulating AMH concentrations can be predictive of the ovulatory response to FSH treatment in goats and of the capacity of



**Figure 5** Variations in AMH and FSH concentrations in the plasma of goats during transitions between the breeding and the anestrus seasons. The data represent AMH and FSH concentrations measured in the plasma samples recovered from goats ( $n=15$ ) weekly for 7 weeks, (A) in Spring, at the end of the breeding season (five out of the 15 goats showed natural ovulatory activity during this period), and (B) in Autumn, at the end of the anestrus season (none of the goats had yet recovered natural ovulatory activity during this period). In each panel, the dates of the first and last days of blood sampling (month/day) are indicated above the arrows. For each period, different letters indicate significant differences in AMH concentrations between weeks ( $P<0.05$ ). No difference in FSH concentrations was observed during the seasonal transitions.

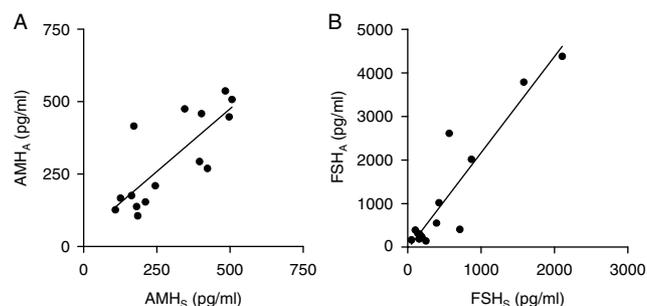
a donor goat to produce high or low number of transferable and freezable embryos. A reliable prediction could be made from the measurement of AMH concentrations in a single blood sample taken from young adult goats before FSH treatment. Moreover, this prediction was valid for a long term, for at least several months after blood sampling, and it could be accurately made during either breeding or anestrus seasons.

From our results, embryo production by MOET was found to be highly repeatable for each goat subjected to FSH treatment and was highly variable between goats. Embryo production in the goat has been reported to be less repeatable by the MOET methodology than by procedures involving LOPU in combination with *in vitro* production (Baldassarre & Karatzas 2004, Pierson *et al.* 2004). A large part of the within-animal variations observed in embryo production by MOET in goats is related to the substantial incidence of premature luteal regression, which is accompanied by a poor embryo recovery rate and the recovery of poor-quality embryos (Cognie *et al.* 2003). In this study, the treatment of animals with a progestagen (fluorogestone acetate (FGA)), a short while after ovulation, most likely led to an increase in the production of transferable embryos and to improve the repeatability by reducing the deleterious effect of the early regression of CL on embryos (Cervantes *et al.* 2007). The observed high within-animal repeatability indicated that embryo production capacity is intrinsically dependent on the donor goat and agrees with data obtained for cows (Tonhati *et al.* 1999, Asada & Terawaki 2002, Benyei *et al.* 2004, Peixoto *et al.* 2004, Monniaux *et al.* 2010) and sheep (Ptak *et al.* 2003).

The ovulation and embryo production rates after FSH treatment were closely related to the number of small antral follicles of 1–5 mm diameter, recovered from goat ovaries at the time of the third session of embryo collection. Variations in ovarian responses to FSH are known to reflect the follicular population present during the initiation of treatment in small ruminants

(Gonzalez-Bulnes *et al.* 2004, Veiga-Lopez *et al.* 2005, Menchaca *et al.* 2010). The population of gonadotropin-responsive follicles is considered to show little numerical change with time, unlike the gonadotropin-dependent follicles that grow through a wave-like pattern (Scaramuzzi *et al.* 2011), and it is suggested that the population of 1–5 mm diameter follicles recovered at the time of embryo collection in goats is representative of this follicular population before treatment. This follicular population would constitute the main target of FSH treatment for ovulation and embryo production in the goat.

Follicles of 1–5 mm in diameter had higher AMH expression in their granulosa cells and higher concentrations of AMH in follicular fluid compared with larger follicles. Moreover, the number of follicles of this size class was highly related to AMH concentrations in plasma. These results strongly suggest that this follicular population is a major contributor to circulating AMH concentrations in goats, but they do not exclude the possibility of contributions of AMH secretions from



**Figure 6** Relationships between mean hormonal concentrations in the plasma of goats, in Spring and Autumn, for AMH and FSH. For each goat, mean concentrations of AMH and FSH were calculated from weekly measurements performed for 7 weeks in each season. The data represent (A) the relationship between AMH in Spring ( $AMH_S$ ) and Autumn ( $AMH_A$ ) and (B) the relationship between FSH in Spring ( $FSH_S$ ) and Autumn ( $FSH_A$ ). Each circle represents data from one goat ( $n=15$  goats).

smaller growing follicles. As the plasma AMH concentration was found to be high in the follicular population that constitutes the main target of FSH treatment, AMH can be considered as an excellent candidate for the endocrine prediction of the capacity of a donor goat to produce high or low number of embryos. It was previously proposed that the response to superovulatory treatment in goats, in terms of the number of embryos, could be predicted from plasma inhibin A levels measured at the start of the superovulatory treatment (Gonzalez-Bulnes *et al.* 2004). In small ruminants, inhibin A is known to be secreted not only by small antral follicles but also mainly by the largest antral follicles (Tsonis *et al.* 1983, Mann *et al.* 1993). From this difference in the pattern of expression, AMH is likely a better endocrine predictive marker of superovulatory responses than inhibin A in small ruminants. In humans, AMH is now considered as the best-known endocrine marker of the ovarian reserve of gonadotropin-responsive follicles (van Rooij *et al.* 2002, Gruijters *et al.* 2003, Visser & Themmen 2005, Visser *et al.* 2006).

The follicular population that produced the highest amounts of AMH comprised a pool of relatively immature follicles. Their granulosa cells expressed high and low levels of *MYC* and *CYP19A1* mRNA, respectively, suggesting that they had a high capacity to proliferate but a low estrogenic activity. In contrast, the follicles of 5–8 mm in diameter expressed low amounts of *AMH* and *MYC* mRNA, but high levels of *FSHR* and *CYP19A1* mRNA in granulosa cells, indicating that they had reached the maturity stage of preovulatory follicles, corresponding to their size (Fernandez-Moro *et al.* 2008). Follicles larger than 8 mm in diameter were characterized by a very low expression of *AMH* and *CYP19A1*, but an enhanced expression of *STAR*, as observed in luteinized cysts in the cow (Monniaux *et al.* 2008). It is suggested that these follicles were remnant of large follicles that did not ovulate but luteinized in response to superovulatory treatment. The expression of *AMHR2*, which encodes the specific AMH type II receptor (di Clemente *et al.* 2003), was detected in the granulosa cells of all follicles, suggesting the existence of a possible autocrine regulation of granulosa cell maturation by AMH in goat antral follicles.

This study is the first report on the influence of season on circulating AMH concentrations in a species that presents a seasonal breeding activity. From our results, the AMH concentrations in the plasma of goats showed little change with season. A small increase and a small decrease in AMH concentrations were observed in Spring and Autumn respectively. It is suggested that these changes in AMH can reflect changes in the number of healthy antral follicles that were found to characterize the transitions between seasons in seasonal breeders (sheep; McNatty *et al.* 1984). In agreement with these observations, the follicular response to FSH treatment was reported to be similar between seasons when using

MOET in sheep (Gonzalez-Bulnes *et al.* 2003) and goats (Pintado *et al.* 1998) and using LOPU in goats (Pierson *et al.* 2004). As a practical consequence, a predictive endocrine test based on the determination of AMH concentrations in plasma could be accurately performed during the breeding or anestrus season in order to select the goats most likely to produce high number of embryos.

No relationship was observed between AMH and FSH concentrations during the seasonal transitions. In particular, the twofold difference in FSH concentrations between Spring and Autumn was not associated with any difference in AMH concentrations in the plasma of the same goats. It should be noted that the FSH assay used in this study did not allow us to evaluate the importance of the different FSH isoforms, which are known to vary with the reproductive state in small ruminants (sheep; Phillips *et al.* 1994, Moore *et al.* 2000). Nevertheless, it seems that for each goat, the amount of AMH would not be directly affected by seasonal changes in circulating FSH concentrations. In contrast, the administration of exogenous FSH to goats for embryo production induced a clear decrease in AMH concentrations, which occurred 3–4 days following each FSH treatment. This decrease could be due to the growth-stimulating effect of FSH on gonadotropin-responsive follicles, leading to a temporary depletion of this follicular population and their development into preovulatory follicles, which are known to produce lower amounts of AMH. Moreover, FSH has been shown to decrease *AMH* expression in the granulosa cells of small antral follicles *in vivo* (rat, Baarends *et al.* 1995) and *in vitro* (human polycystic ovaries, Pellatt *et al.* 2007; cow, Rico *et al.* 2011). Following the acute effect of FSH on both follicular growth and *AMH* expression per cell, the AMH concentrations in the plasma of goats returned to their initial values in <3 weeks. In agreement with our results, in repeated gonadotropin stimulation and LOPU protocols, the goat ovary was shown to recover from LOPU in <5 weeks with no important effects on follicular response or oocyte yield (Pierson *et al.* 2004).

High between-animal variations were observed for FSH concentrations in Spring and Autumn, in agreement with previous observations in sheep (McNatty *et al.* 1984), and the close relationship that was found to exist between seasons for FSH concentrations indicates that FSH concentrations are intrinsically dependent on individuals. As described earlier, AMH concentrations were also intrinsically dependent on individuals, but no relationship was found between FSH and AMH. Overall, these results indicate that each goat could be characterized by its own circulating FSH concentrations, depending on its pituitary sensitivity to the negative feedback of estradiol and inhibin, produced by its ovarian population of gonadotropin-dependent follicles and acting at the pituitary level through a synergistic interaction (Scaramuzzi *et al.* 2011), and by its own

circulating AMH concentrations, reflecting its ovarian population of gonadotropin-responsive follicles. As a consequence, the results reinforce the idea that the population of gonadotropin-responsive and gonadotropin-dependent follicles is regulated by different, and largely independent, mechanisms. An understanding of the mechanisms that determine and/or regulate the size of the pool of gonadotropin-responsive follicles is now of key importance from the perspective of improving embryo production in domestic mammals.

## Materials and Methods

### Animals and experimental design

The experiment was conducted at the Experimental Unit UEPAO in Nouzilly, France (latitude 47°22'N and longitude 00°41'E). Fifteen nulliparous Saanen goats were used for the experiment, which was conducted between January and October 2009. The animals were housed in free stalls and provided with food and water *ad libitum*. All procedures were approved by the agricultural and scientific research agencies (approval number C37-175-2) and were conducted in accordance with the guidelines for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

### Embryo production

Fifteen goats were 10 months old at the beginning of the experimental protocol and they had not received any previous hormonal treatment. They were successively treated for *in vivo* embryo production in January (during the breeding season), April (at the end of the breeding season), and October (at the end of the anestrus season). The intervals between the embryo production sessions were 11 and 28 weeks for the first and the second intervals respectively. In each embryo production session, the goats received an intravaginal sponge impregnated with FGA (Chronogest CR, 20 mg; Intervet Schering-Plough Animal Health, Angers, France) for 11 days and a prostaglandin *i.m.* injection (Cloprostenol, 50 µg; Intervet Schering-Plough Animal Health) that was administered 9 days after the FGA sponge insertion. They were superovulated with a total of 12 mg FSH (Stimufol; Merial, Lyon, France), which was given as twice-daily *i.m.* injections for more than 3 days on a decreasing dose schedule, and the FGA sponges were removed at the time of the fifth injection. The goats were served by the first male at the time of estrus detection and then by a second male 8–16 h later. They were then treated again with FGA intravaginal sponge (20 mg), which was inserted 3 days after estrus detection, in order to reduce the effect of premature luteal regression, which frequently occurs after superovulation in goats, and to improve embryo recovery (Cervantes *et al.* 2007). The number of CL was counted in the ovaries, and the embryos were recovered by flushing the uterine horns 7 days after estrus detection, according to Baril *et al.* (1993). In the first and second sessions of embryo production, the goats were given general anesthesia using an *i.v.* injection of thiopental (Nesdonal, 1 g; Merial), followed by isoflurane gas (2.5–4%; Belamont, Paris, France) administration via tracheal intubation, and CL counting and uterine flushing were performed through

median laparotomy. After surgery, the animals received *i.m.* injections of an antibiotic (oxytetracycline, 1.2 g; Pfizer, Paris, France) and an analgesic (flunixin meglumine, 100 mg; Intervet Schering-Plough Animal Health). In the third session of embryo production, the number of CL was counted and the embryos were recovered postmortem from killed animals.

The embryos collected were counted and their quality was evaluated according to classic morphological criteria (Lindner & Wright 1983, Baril *et al.* 1993, Callesen *et al.* 1995) using the definitions developed by the International Embryo Transfer Society. The embryos that were determined to have a quality of 1–3 (Callesen *et al.* 1995) were defined as transferable (*i.e.* good) embryos. The embryo stages that were between a compacted morula and an expanded blastocyst, and which were determined to have a quality of 1, were defined as freezable (Baril *et al.* 1993).

### Plasma, follicular fluid, and granulosa cell collection

In each of the three sessions of embryo production, blood samples were recovered from the 15 goats just before the first FSH injection (T0), at the time of insemination (TI), and at the time of embryo collection (TEC). Furthermore, blood samples were also recovered weekly in February and March during the 7 weeks between the first and the second sessions of embryo production. The period between February and April corresponds to the transition between the breeding and the anestrus seasons in Spring. Blood samples were also recovered weekly between August and October, a period that usually corresponds to the transition between the anestrus and the breeding seasons in Autumn (Chemineau *et al.* 1992, 2008). After recovery in heparinized tubes, all of the blood samples were immediately centrifuged at 3200 *g* for 10 min at 4 °C in order to recover the plasma, which was stored at –20 °C until the AMH, FSH, and progesterone assays were carried out.

At the time of the third session of embryo collection, the ovaries of the 15 goats were recovered immediately after killing. All follicles larger than 1 mm in diameter were carefully dissected, counted, and individually measured. Then, the follicles were classified according to their size: 1–3, 3–5, 5–8, and >8 mm. Follicular fluids and granulosa cells were recovered from the follicles of both ovaries in each goat, as described previously (Le Bellego *et al.* 2002). For each goat, the follicular fluids were pooled according to the size of the follicles (one to three pools of follicular fluids per follicular size class, when follicles of the size class were present) and stored at –20 °C until the AMH assay was performed. Similarly, the granulosa cell suspensions were also pooled according to the size of the follicles (one to three pools per follicular size class, when follicles of the size class were present), then centrifuged, and the cell pellets were stored at –80 °C until RNA extraction.

### Hormonal assays

#### Anti-Müllerian hormone

The plasma and follicular fluid concentrations of AMH were determined using the active MIS/AMH ELISA kit (Beckman Coulter France, Roissy CDG, France), as described previously

for bovine species (Monniaux *et al.* 2008, Rico *et al.* 2009). The concentrations of AMH were determined in 50 µl samples of undiluted plasma and follicular fluid diluted at 1:5000 for 1–5 mm follicles, 1:1000 for 5–8 mm follicles, and undiluted for follicles larger than 8 mm in diameter. Before the assay, plasma and follicular fluids were thawed in a warm water bath, vortexed, and then centrifuged (3200 g, 10 min, 4 °C) to remove any cell fragments that could interfere with the reagents of the assay. The limit of detection of the assay was found to be 1 pg/well. In order to validate the assay in the caprine species, serial dilutions of different goat plasma and follicular fluids were analyzed using the kit. The goat follicular fluid and plasma dilution curves were linear and parallel to the standard curve (data not shown). The intra-assay and inter-assay coefficients of variation (CV; s.d./mean) were between 8.5 and 10.8% and between 11.3 and 18.4%, respectively, for goat plasma samples containing between 80 and 500 pg/ml AMH.

### FSH

FSH was assayed in the plasma samples recovered weekly in Spring and Autumn. Plasma concentrations of FSH were determined by a double antibody ELISA assay for ovine samples, according to a previously described method (Faure *et al.* 2005) using a monoclonal antibody against the ovine FSH β-subunit (Henderson *et al.* 1995) for coating the microtitration plates and a biotinylated monoclonal antibody against the human α-subunit (Dirnhofer *et al.* 1994) as a second antibody. Purified ovine FSH (NIH RP2) was used as the standard. The FSH concentrations were determined in 50 µl samples of undiluted plasma. All of the samples were analyzed in the same assay. The limit of detection of the assay was found to be 10 pg/well, corresponding to 0.2 ng/ml. In order to validate the assay for the caprine species, serial dilutions of different goat plasma samples were analyzed. The goat plasma dilution curves were linear and parallel to the standard curve (data not shown). The intra-assay CV was found to be between 3.8 and 7.8% for goat plasma samples containing between 3 and 7.5 ng/ml FSH.

### Progesterone

Progesterone was assayed in the plasma samples that were recovered weekly in Spring and Autumn, with the aim of characterizing the goats for the presence or absence of ovulatory activity during these periods. Plasma concentrations of progesterone were determined by ELISA as described previously (Canepa *et al.* 2008). Progesterone was measured on 10 µl undiluted plasma. All of the samples were analyzed in

the same assay. The antiserum cross-reacted with 5α-pregnan-3,20-dione (40%); there were slight reactions with 17-hydroxyprogesterone (1.6%), pregnenolone (0.2%), corticosterone (0.3%), and androstenedione (0.55%); and a lower reaction with the other steroids (<0.1%). The limit of detection of the assay was 4 pg/tube, corresponding to 0.4 ng/ml, and the intra-assay CV was lower than 10%. Goats with progesterone concentrations higher than 1 ng/ml for at least two successive weeks were considered as having ovulatory activity.

### RT and quantitative PCR

Granulosa cell samples were analyzed for the mRNA contents of *AMH*, its receptor *AMHR2*, and the functional markers of granulosa cell proliferation (*MYC*) and differentiation (*FSHR*, *CYP19A1*, and *STAR*). Total RNA was extracted from granulosa cell samples using a Nucleospin RNA II kit (Macherey-Nagel, Hoerd, France) according to the manufacturer's protocol. Total RNA was reverse transcribed using 1 µg RNA, and real-time quantitative PCR (qPCR) reactions were run using SYBR Green Supermix (Bio-Rad) on an iCycler iQ multicolor detection system (Bio-Rad) as described previously (Monniaux *et al.* 2008). The specific primer sequences used for amplification of the different target genes studied and for the internal reference *RPL19* gene encoding a ubiquitous ribosomal protein are given in Table 1. Efficiency curves were generated and amplification efficiencies (*E*) were determined for each primer pair as described previously (Monniaux *et al.* 2008; Table 1). For quantification analysis, the cycle threshold (*C<sub>t</sub>*) of each target gene was compared to that of the *RPL19* internal reference gene according to the ratio

$$R = \left[ \frac{E_{RPL19}^{C_t(RPL19)}}{E_{target}^{C_t(target)}} \right].$$

### Data analysis

All data are presented as mean ± s.e.m., except for the correlation studies. In order to compare repeated measures on the same animals, one-way repeated measures ANOVA followed by a Newman–Keuls multiple comparison test were applied. The repeatability (*r*<sup>2</sup>) of the number of CL or embryos produced after superovulation, or of the AMH concentrations measured in plasma, was calculated as the ratio of the between-animal variance to the sum of the between-animal and residual variances. For analysis of the effects of follicular size on AMH concentrations in follicular fluid, and on mRNA

**Table 1** Quantitative PCR primer sequences and their efficiency.

Gene	Primer sequences (5' → 3')	Efficiency ( <i>E</i> )	
<i>AMH</i>	GTGGTGCTGCTGCTAAAGATG	TCGGACAGGCTGATGAGGAG	1.92
<i>AMHR2</i>	GTGCTTCTCCCAGGCATAC	AATGTGGTCATGCTGTAGGC	1.99
<i>MYC</i>	CTCTGACTCTCTGCTCTC	CTTCTCATCCTCTGTTC	2.10
<i>CYP19A1</i>	TCGTCTGGTCACCCCTTCTG	CGGTCTCTGGTCTCGTCTGG	1.95
<i>FSHR</i>	CAAAGATCCTCTGGTCTGTTT	GTCTCTGGTGAAGATGGCGTAG	2.09
<i>RPL19</i>	AATGCCAATGCCAACTC	CCCTTCGGTACCTATACC	2.09
<i>STAR</i>	ACACCATGTGGAATGTCAGGCT	CACACCTTCAACAAGCAACCC	2.10

gene expression in granulosa cells, ANOVA was performed on the weighted average values per goat and per follicular class, followed by Newman–Keuls multiple comparison tests. When variances were heterogeneous, the data were log-transformed before ANOVA or were analyzed by the non-parametric Kruskal–Wallis test. For the correlation studies, significance was ascertained by Bravais–Pearson  $r$  critical values;  $P < 0.05$  was considered significant for all analyses.

## Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

## Funding

This work was supported by specific funding (Incitement Grant to sustain innovation and research valorization) from the INRA PHASE division.

## Acknowledgements

The authors thank Dr Jean-François Beckers for providing Stimufol, Corinne Laclie and Jessica Roy for doing the FSH and progesterone assays, and the 'ruminant' team of the Experimental Unit UEPAO for animal management and participation in the blood sampling.

## References

- Asada Y & Terawaki Y 2002 Heritability and repeatability of superovulatory responses in Holstein population in Hokkaido, Japan. *Asian-Australasian Journal of Animal Sciences* **15** 944–948.
- Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen AP & Grootegoed JA 1995 Anti-Mullerian hormone and anti-Mullerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. *Endocrinology* **136** 4951–4962. (doi:10.1210/en.136.11.4951)
- Baldassarre H & Karatzas CN 2004 Advanced assisted reproduction technologies (ART) in goats. *Animal Reproduction Science* **82–83** 255–266. (doi:10.1016/j.anireprosci.2004.04.027)
- Baldassarre H, Wang B, Kafidi N, Keefer C, Lazaris A & Karatzas CN 2002 Advances in the production and propagation of transgenic goats using laparoscopic ovum pick-up and *in vitro* embryo production technologies. *Theriogenology* **57** 275–284. (doi:10.1016/S0093-691X(01)00671-9)
- Baril G, Brebion P & Chesné P (Eds) 1993 Manuel de formation pratique pour la transplantation embryonnaire chez la brebis et la chèvre. In *Etude FAO Production et Santé Animales*, no. 115, pp 75–110. Rome: FAO.
- Benyei B, Gaspard A, Komlosi I & Pecsai A 2004 Repeatability and heritability of ovulation number and embryos in dam-daughters pairs in superovulated Holstein-Friesian cows. *Reproduction in Domestic Animals* **39** 99–102. (doi:10.1111/j.1439-0531.2004.00487.x)
- Callesen H, Lovendahl P, Bak A & Greve T 1995 Factors affecting the developmental stage of embryos recovered on day 7 from superovulated dairy cattle. *Journal of Animal Science* **73** 1539–1543.
- Canepa S, Lainé AL, Bluteau A, Fagu C, Flon C & Monniaux D 2008 Validation d'une méthode immunoenzymatique pour le dosage de la progestérone dans le plasma des ovins et des bovins. *Cahier des Techniques de l'INRA* **64** 19–30.
- Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NM, Cheung A, Ninfa EG, Frey AZ, Gash DJ, Chow EP *et al.* 1986 Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the human gene in animal cells. *Cell* **45** 685–698. (doi:10.1016/0092-8674(86)90783-X)
- Cervantes MJ, Juarez ML, Mejia VO, Berruecos VJ, Vera AH & Valencia J. 2007 Use of fluorogestone acetate after breeding to reduce the effect of premature luteal regression in dairy goats when superovulation is induced with FSH. *Animal Reproduction Science* **97** 47–54. (doi:10.1016/j.anireprosci.2006.01.008)
- Chemineau P, Daveau A, Maurice F & Delgadillo JA 1992 Seasonality of oestrus and ovulation is not deeply modified by submitting Alpine goats to a tropical photoperiod. *Small Ruminant Research* **8** 299–312. (doi:10.1016/0921-4488(92)90211-L)
- Chemineau P, Guillaume D, Migaud M, Thierry JC, Pellicer-Rubio MT & Malpoux B 2008 Seasonality of reproduction in mammals: intimate regulatory mechanisms and practical implications. *Reproduction in Domestic Animals* **43** (Suppl 2) 40–47. (doi:10.1111/j.1439-0531.2008.01141.x)
- di Clemente N, Josso N, Gouedard L & Belville C 2003 Components of the anti-Mullerian hormone signaling pathway in gonads. *Molecular and Cellular Endocrinology* **211** 9–14. (doi:10.1016/j.mce.2003.09.005)
- Cognie Y 1999 State of the art in sheep-goat embryo transfer. *Theriogenology* **51** 105–116. (doi:10.1016/S0093-691X(98)00235-0)
- Cognie Y, Baril G, Poulin N & Mermillod P 2003 Current status of embryo technologies in sheep and goat. *Theriogenology* **59** 171–188. (doi:10.1016/S0093-691X(02)01270-0)
- Cognie Y, Poulin N, Locatelli Y & Mermillod P 2004 State-of-the-art production, conservation and transfer of *in-vitro*-produced embryos in small ruminants. *Reproduction, Fertility, and Development* **16** 437–445. (doi:10.1071/RD04029)
- Cushman RA, DeSouza JC, Hedgpeth VS & Britt JH 1999 Superovulatory response of one ovary is related to the micro- and macroscopic population of follicles in the contralateral ovary of the Cow. *Biology of Reproduction* **60** 349–354. (doi:10.1095/biolreprod60.2.349)
- Dirnhofer S, Madersbacher S, Bidart JM, Ten Kortenaar PB, Spottl G, Mann K, Wick G & Berger P 1994 The molecular basis for epitopes on the free beta-subunit of human chorionic gonadotrophin (hCG), its carboxyl-terminal peptide and the hCG beta-core fragment. *Journal of Endocrinology* **141** 153–162. (doi:10.1677/joe.0.1410153)
- Faure MO, Nicol L, Fabre S, Fontaine J, Mohoric N, McNeilly A & Taragnat C 2005 BMP-4 inhibits follicle-stimulating hormone secretion in ewe pituitary. *Journal of Endocrinology* **186** 109–121. (doi:10.1677/joe.1.05988)
- Fernandez-Moro D, Veiga-Lopez A, Ariznavarreta C, Tresguerres JA, Encinas T & Gonzalez-Bulnes A 2008 Preovulatory follicle development in goats following oestrous synchronization with progestagens or prostaglandins. *Reproduction in Domestic Animals* **43** 9–14. (doi:10.1111/j.1439-0531.2008.01248.x)
- Gibbons A, Pereyra Bonnet F, Cueto MI, Catala M, Salamone DF & Gonzalez-Bulnes A 2007 Procedure for maximizing oocyte harvest for *in vitro* embryo production in small ruminants. *Reproduction in Domestic Animals* **42** 423–426. (doi:10.1111/j.1439-0531.2006.00802.x)
- Gonzalez-Bulnes A, Garcia-Garcia RM, Santiago-Moreno J, Dominguez V, Lopez-Sebastian A & Cocero MJ 2003 Reproductive season affects inhibitory effects from large follicles on the response to superovulatory FSH treatments in ewes. *Theriogenology* **60** 281–288. (doi:10.1016/S0093-691X(02)01367-5)
- Gonzalez-Bulnes A, Garcia-Garcia RM, Carrizosa JA, Urrutia B, Souza CJ, Cocero MJ, Lopez-Sebastian A & McNeilly AS 2004 Plasma inhibin A determination at start superovulatory FSH treatments is predictive for embryo outcome in goats. *Domestic Animal Endocrinology* **26** 259–266. (doi:10.1016/j.domaniend.2003.11.004)
- Grujters MJ, Visser JA, Durlinger AL & Themmen AP 2003 Anti-Mullerian hormone and its role in ovarian function. *Molecular and Cellular Endocrinology* **211** 85–90. (doi:10.1016/j.mce.2003.09.024)
- Henderson KM, Camberis M & Hardie AH 1995 Generation of monoclonal antibody to ovine FSH and its application in immunoneutralization and enzymeimmunoassay. *Journal of Reproduction and Fertility. Supplement* **49** 511–515.
- Kawamata M 1994 Relationships between the number of small follicles prior to superovulatory treatment and superovulatory response in Holstein cows. *Journal of Veterinary Medical Science* **56** 965–967. (doi:10.1292/jvms.56.965)

- Kevenaar ME, Meerasahib MF, Kramer P, van de Lang-Born BM, de Jong FH, Groomer NP, Themmen AP & Visser JA** 2006 Serum anti-Müllerian hormone levels reflect the size of the primordial follicle pool in mice. *Endocrinology* **147** 3228–3234. (doi:10.1210/en.2005-1588)
- Le Bellego F, Pisselet C, Huet C, Monget P & Monniaux D** 2002 Laminin- $\alpha$ 6 $\beta$ 1 integrin interaction enhances survival and proliferation and modulates steroidogenesis of ovine granulosa cells. *Journal of Endocrinology* **172** 45–59. (doi:10.1677/joe.0.1720045)
- Lindner GM & Wright RW Jr** 1983 Bovine embryo morphology and evaluation. *Theriogenology* **20** 407–416. (doi:10.1016/0093-691X(83)90201-7)
- Lopez-Alonso C, Encinas T, Veiga-Lopez A, Garcia-Garcia RM, Cocero MJ, Ros JM, McNeilly AS & Gonzalez-Bulnes A** 2005 Follicular growth, endocrine response and embryo yields in sheep superovulated with FSH after pretreatment with a single short-acting dose of GnRH antagonist. *Theriogenology* **64** 1833–1843. (doi:10.1016/j.theriogenology.2005.04.021)
- Mann GE, Campbell BK, McNeilly AS & Baird DT** 1993 Follicular development and ovarian hormone secretion following passive immunization of ewes against inhibin or oestradiol. *Journal of Endocrinology* **136** 225–233. (doi:10.1677/joe.0.1360225)
- McNatty KP, Hudson NL, Henderson KM, Lun S, Heath DA, Gibb M, Ball K, McDiarmid JM & Thurley DC** 1984 Changes in gonadotrophin secretion and ovarian antral follicular activity in seasonally breeding sheep throughout the year. *Journal of Reproduction and Fertility* **70** 309–321. (doi:10.1530/jrf.0.0700309)
- Menchaca A, Pinczak A & Rubianes E** 2002 Follicular recruitment and ovulatory response to FSH treatment initiated on day 0 or day 3 postovulation in goats. *Theriogenology* **58** 1713–1721. (doi:10.1016/S0093-691X(02)01084-1)
- Menchaca A, Vilarino M, Crispo M, de Castro T & Rubianes E** 2010 New approaches to superovulation and embryo transfer in small ruminants. *Reproduction, Fertility, and Development* **22** 113–118. (doi:10.1071/RD09222)
- Monniaux D, Chupin D & Saumande J** 1983 Superovulatory responses of cattle. *Theriogenology* **19** 55–81. (doi:10.1016/0093-691X(83)90124-3)
- Monniaux D, di Clemente N, Touzé JL, Belville C, Rico C, Bontoux M, Picard JY & Fabre S** 2008 Intrafollicular steroids and anti-Müllerian hormone during normal and cystic ovarian follicular development in the cow. *Biology of Reproduction* **79** 387–396. (doi:10.1095/biolreprod.107.065847)
- Monniaux D, Barbey S, Rico C, Fabre S, Gallard Y & Larroque H** 2010 Anti-Müllerian hormone: a predictive marker of embryo production in cattle? *Reproduction, Fertility, and Development* **22** 1083–1091. (doi:10.1071/RD09279)
- Moore LG, Ng Chie W, Hudson NL & McNatty KP** 2000 Isoforms and half-life of FSH from sheep with different reproductive states. *Journal of Endocrinology* **165** 185–192. (doi:10.1677/joe.0.1650185)
- Peixoto MG, Pereira CS, Bergmann JAG, Penna VM & Fonseca CG** 2004 Genetic parameters of multiple ovulation traits in Nellore females. *Theriogenology* **62** 1459–1464. (doi:10.1016/j.theriogenology.2004.02.019)
- Pellatt L, Hanna L, Brincat M, Galea R, Brain H, Whitehead S & Mason H** 2007 Granulosa cell production of anti-Müllerian hormone is increased in polycystic ovaries. *Journal of Clinical Endocrinology and Metabolism* **92** 240–245. (doi:10.1210/jc.2006-1582)
- Phillips DJ, Hudson NL, Gentle LR & McNatty KP** 1994 Bioactive follicle-stimulating hormone concentrations in plasma during the estrous cycle of the ewe. *Biology of Reproduction* **51** 1292–1298. (doi:10.1095/biolreprod51.6.1292)
- Pierson J, Wang B, Neveu N, Sneek L, Cote F, Karatzas CN & Baldassarre H** 2004 Effects of repetition, interval between treatments and season on the results from laparoscopic ovum pick-up in goats. *Reproduction, Fertility, and Development* **16** 795–799. (doi:10.1071/RD04066)
- Pintado B, Gutierrez-Adan A & Perez Llano B** 1998 Superovulatory response of Murciana goats to treatments based on PMSG/anti-PMSG or combined FSH/PMSG administration. *Theriogenology* **50** 357–364. (doi:10.1016/S0093-691X(98)00145-9)
- Ptak G, Tischner M, Bernabo N & Loi P** 2003 Donor-dependent developmental competence of oocytes from lambs subjected to repeated hormonal stimulation. *Biology of Reproduction* **69** 278–285. (doi:10.1095/biolreprod.102.011312)
- Rico C, Fabre S, Médigue C, di Clemente N, Clément F, Bontoux M, Touzé JL, Dupont M, Briant E, Rémy B *et al.*** 2009 Anti-Müllerian hormone is an endocrine marker of ovarian gonadotropin-responsive follicles and can help to predict superovulatory responses in the cow. *Biology of Reproduction* **80** 50–59. (doi:10.1095/biolreprod.108.072157)
- Rico C, Médigue C, Fabre S, Jarrier P, Bontoux M, Clément F & Monniaux D** 2011 Regulation of anti-Müllerian hormone production in the cow: a multiscale study at endocrine, ovarian, follicular, and granulosa cell levels. *Biology of Reproduction* **84** 560–571. (doi:10.1095/biolreprod.110.088187)
- van Rooij IA, Broekmans FJ, te Velde ER, Fauser BC, Bancsi LF, de Jong FH & Themmen AP** 2002 Serum anti-Müllerian hormone levels: a novel measure of ovarian reserve. *Human Reproduction* **17** 3065–3071. (doi:10.1093/humrep/17.12.3065)
- Scaramuzzi RJ, Baird DT, Campbell BK, Driancourt MA, Dupont J, Fortune JE, Gilchrist RB, Martin GB, McNatty KP, McNeilly AS *et al.*** 2011 Regulation of folliculogenesis and the determination of ovulation rate in ruminants. *Reproduction, Fertility, and Development* **23** 444–467. (doi:10.1071/RD09161)
- Singh J, Dominguez M, Jaiswal R & Adams GP** 2004 A simple ultrasound test to predict the superstimulatory response in cattle. *Theriogenology* **62** 227–243. (doi:10.1016/j.theriogenology.2003.09.020)
- Tervit HR** 1996 Laparoscopy/laparotomy oocyte recovery and juvenile breeding. *Animal Reproduction Science* **42** 227–238. (doi:10.1016/0378-4320(96)01533-3)
- Tonhati H, Lobo RB & Oliveira HN** 1999 Repeatability and heritability of response to superovulation in Holstein cows. *Theriogenology* **51** 1151–1156. (doi:10.1016/S0093-691X(99)80018-1)
- Tsonis CG, Quigg H, Lee VW, Leversha L, Trounson AO & Findlay JK** 1983 Inhibin in individual ovine follicles in relation to diameter and atresia. *Journal of Reproduction and Fertility* **67** 83–90. (doi:10.1530/jrf.0.0670083)
- Veiga-Lopez A, Gonzalez-Bulnes A, Garcia-Garcia RM, Dominguez V & Cocero MJ** 2005 The effects of previous ovarian status on ovulation rate and early embryo development in response to superovulatory FSH treatments in sheep. *Theriogenology* **63** 1973–1983. (doi:10.1016/j.theriogenology.2004.09.055)
- Visser JA & Themmen AP** 2005 Anti-Müllerian hormone and folliculogenesis. *Molecular and Cellular Endocrinology* **234** 81–86. (doi:10.1016/j.mce.2004.09.008)
- Visser JA, de Jong FH, Laven JS & Themmen AP** 2006 Anti-Müllerian hormone: a new marker for ovarian function. *Reproduction* **131** 1–9. (doi:10.1530/rep.1.00529)

---

Received 11 June 2011

First decision 20 July 2011

Revised manuscript received 1 September 2011

Accepted 19 September 2011