DOI: 10.1111/rda.12822

ORIGINAL ARTICLE

WILEY Reproduction in Domestic Animals

Determination of the normal reference interval for anti-Müllerian hormone (AMH) in bitches and use of AMH as a potential predictor of litter size

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Contents

Anti-Müllerian hormone (AMH) is a reliable endocrine marker of ovarian reserve in many species with extensive literature in both humans and cattle. However, there are no known hormonal predictors of ovarian reserve and potential reproductive performance in the bitch. A prospective cohort study was performed involving 155 intact bitches of various ages (range 1.2–7.6 years) and breeds that were presented for routine breeding management over a one-year period. All bitches were artificially inseminated with frozen or fresh semen using the transcervical insemination (TCI) technique. AMH concentrations were measured using a commercially available canine AMH ELISA (Ansh Labs®, Texas, USA), which we validated prior to performing the study. The reference interval (RI) for AMH for all bitches in the study, regardless of body weight, was 2.9–21.1 ng/ml. There was a significant effect of bitch size and age on AMH concentrations. The RI for giant breeds was significantly ($p < .01$) lower (1.75–15.6 ng/ml) than small-sized (5.6–24.2 ng/ml), medium-sized (4.3–23.7 ng/ml) and large-sized (4.3–21.0 ng/ml) bitches. The mean AMH concentration in bitches less than 4 years of age was 12.4 ng/ml, whereas the mean AMH concentration in bitches older than 4 years of age was 10.5 ng/ml ($p < .05$). For each additional year of age above 1 year of age, AMH concentrations fell by 0.5 ng/ml. There was no effect of AMH concentration on the whelping rate. Smaller breeds had smaller litters (and higher AMH concentrations), but within each breed size category, bitches with higher AMH concentrations had significantly larger litter sizes ($p < .01$). For each 1 ng/ml increase in AMH, litter size increased by 0.3 pups/litter. In conclusion, we determined a normal reference interval for AMH in bitches based on body weight using a canine-specific assay. In agreement with findings in humans and other species, we found that there is a decline in AMH concentrations with advancing age in bitches. Finally, the significant positive correlation between AMH concentrations and litter size indicates that AMH may be a useful management tool for the selection of bitches in breeding programmes.

1 | INTRODUCTION

All females are born with a limited number of ovarian germ cells. The ovarian reserve is highly variable between species and, more importantly, amongst individuals. Anti-Müllerian hormone (AMH) is a dimeric glycoprotein secreted by the ovary and, more specifically, the granulosa cells of growing (pre-antral and small antral) follicles from the beginning of folliculogenesis until reproductive senescence/menopause, thereby making it a useful endocrine indicator of ovarian reserve throughout a female's reproductive lifetime (La Marca, Broekmans, Volpe, Fauser, & Macklon, 2009; Visser, de Jong, Laven, & Themmen, 2006). It is with this knowledge that AMH has been used

as a clinical tool for a number of years in the prediction of menopausal transition in women (Broer, Broekmans, Laven, & Fauser, 2014). AMH has also been used as a predictor of ovarian responsiveness to infertility treatments (ovarian stimulation protocols) and pregnancy outcome in women (Broer et al., 2014; Visser et al., 2006). Similarly, in production animals, AMH has been used as an endocrine marker for predicting the pool of ovarian gonadotropin-responsive follicles in the cow (Rico et al., 2009), ewe (Lahoz, Alabart, Monniaux, Mer- Millod, & Folch, 2012) and goat (Monniaux et al., 2011) and as an important clinical tool in the selection of donors suitable for superovulation programmes and production of the maximum number of transferable embryos in dairy cows (Rico et al., 2012). In mares, AMH has also been used to predict the number of follicles available in oocyte aspiration programmes (Vernuft, Lohrke, Tuchscherer, Weitzel, & Viergutz, 2013).

Despite numerous studies in several species, there have been no reports of a correlation between AMH concentrations and ovarian reserve in bitches and the potential fertility and fecundity of an individual bitch. Measurement of AMH concentrations in both bitches and queens has only been reported for the diagnosis of ovarian remnant syndrome, where the detection of AMH is an indicator of the presence or absence of ovarian tissue (Axner & Strom Holst, 2015; Place et al., 2011; Turna Yilmaz, Toydemir, Kirsan, Gunay Ucmak, & Caliskan Karacam, 2015). Apart from the study by Turna Yilmaz et al. (2015), all of these studies have used a human-specific AMH enzyme-linked immunosorbent assay (Gen II ELISA, Beckman Coulter®, Brea, CA, USA) for the determination of AMH, which has not been validated for use in canids.

In many working dog breeding programmes, the selection of the most successful brood bitches can take a number of years as their progeny complete training programmes. In the greyhound industry, the selection of genetically valuable performance bitches for breeding is also delayed, as most of their progeny do not reach the racetrack until 2–3 years of age. Therefore, many breeding programmes rely on an ageing population of bitches and the predicament of breeding less fertile bitches with genetically and economically valuable and often irreplaceable frozen semen. Poor reproductive performance causes a significant negative impact on the entire breeding programme with production targets not being achieved. Using a hormonal marker to predict fecundity would be a powerful management tool when breeding these older bitches.

The aims of this study were (i) to establish a normal reference interval (RI) for AMH in a large number of breeding bitches using a commercially available canine AMH ELISA assay, and (ii) to investigate whether there were any associations between AMH concentrations and reproductive performance in bitches.

2 | MATERIALS AND METHODS

2.1 | Animals

The study population consisted of 155 post-pubertal, intact bitches of different ages (range 1.2–7.6 years), breed and parity (0–2) that were presented for artificial insemination (AI) with fresh (n = 78) or frozen-thawed (n = 77) semen using the transcervical insemination (TCI) technique over a one-year period (2014–2015). The 155 enrolled bitches were randomly selected from a total of 225 bitches presented for AI during the one-year study period. Timing of insemination was dependent mainly on semen type and quality and was determined primarily by measurement of serial blood progesterone concentrations (Wilson & Hollinshead, 2014). AMH concentrations were measured on serum harvested from blood samples that were taken for progesterone testing. All samples were taken during oestrus to avoid any potential cyclical variation in AMH concentrations as demonstrated recently by Nagashima, Hansen, Songasen, Travis, and Place (2016). Blood samples were also taken for AMH determination from three control animals: a 3-year-old castrated male dog, a 5-year-old intact male dog and an 8-year-old neutered bitch as part of establishing and validating the normal reference range of AMH. Bitches were allocated into groups according to body weight: small (<12 kg; n = 25), medium (13–25 kg; n = 14), large (26–40 kg; n = 76) and giant (>40 kg; n = 40) breeds.

Two months after insemination, a questionnaire was sent out to the owners of each bitch enrolled in the study. The compliance rate for return of the completed questionnaire was 100%. Information obtained from the questionnaire enabled the determination of the whelping rate (defined as the proportion of bitches producing at least one pup of those inseminated) and litter size (defined as total number of pups born per litter).

2.2 | Evaluation of AMH and validation of AMH assay

Each blood sample was collected into plain serum tubes. Serum was harvested within 6 hrs of collection of each blood sample and analysed for progesterone concentration. The remainder of the serum for the majority of samples was held at room temperature for no longer than 4 hrs before being frozen in sterile Eppendorf tubes and stored at −20°C prior to analysis for AMH concentration. A proportion (n = 36; 23%) of serum samples were held at 4°C for up to 7 days before being frozen and stored at −20°C due to pre-existing laboratory procedures. It has been shown that human AMH is stable in serum not only at 4°C and −20°C but also at room temperature for up to 14 days (Kumar et al., 2014). As part of our quality control, a pilot study was carried out to determine whether storage temperature and duration had an effect on AMH stability. There was no significant difference in AMH concentrations between samples stored at 4°C prior to freezing and those that were immediately frozen after serum was harvested. All samples were ultimately stored at −20°C in a non-freeze/thaw cycle freezer for a period ranging from 1 to 12 months prior to analysis for AMH concentration. Stored samples were thawed and analysed in one batch for AMH concentration. Each sample was run in duplicate, and the mean concentration of both aliquots was calculated to maximize accuracy. Determination of AMH concentration for each

serum sample was carried out using an enzyme-linked immunosorbent assay (canine AMH ELISA; AnshLabs®, TX, USA) according to the manufacturer's instructions. Briefly, 50 µl of standards, controls and samples was incubated in an AMH antibody-coated microtitration plate. After incubation and washing, biotinylated anti-AMH biotin conjugate was added to each well. After a second incubation and washing step, streptavidin–horseradish peroxidase was added. After a third incubation and washing step, the substrate, tetramethylbenzidine, was added and incubated briefly before adding an acidic stopping solution. The degree of enzymatic turnover of the substrate was determined by dual-wavelength absorbance measurement at 450 and 620 nm. A standard curve was generated using a four-parameter logistic model (online data analysis tool, MyAssays Ltd). The limit of detection of the assay was 0.2 ng/ml; the intra- and interassay coefficients of variation were 3.8% and 7.2%, respectively.

2.3 | Statistical analysis

The relationship of bitch age and size (small, medium, large and giant) on AMH concentrations was determined using multivariable linear (bitch age) or logistic (bitch size) regression analysis. The 95% reference interval (RI) was calculated using Cook's method of outlier detection, and the 90% confidence intervals of the upper and lower limits were established (Friedrichs et al. 2012). Initially, the RI was calculated for all bitches in the sample population. However, there was a significant effect of body weight on AMH; therefore, separate RIs were calculated for each category of body weight (small, medium, large and giant). Reference intervals (RIs) for AMH were determined nonparametrically using the package "reference intervals" in R (Finnegan, 2012).

The reproductive outcomes of interest (dependent variables) were whelping rate and litter size. Multiple logistic regression was used to determine the effects of the independent variables, namely AMH concentration, bitch age, parity, breed, size (small, medium, large and giant), semen type (fresh, frozen) and sperm motility, on the whelping rate. The effects of the same independent variables on litter size were analysed using multivariable linear regression. All analyses were performed using R version 3.1.3 (R Development Core Team, 2014; R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org).

3 | RESULTS

The mean ± standard deviation (*SD*) age of bitches in the study was 3.7 ± 1.5 years (range: 1.2–7.6 years). The mean ± *SD* AMH concentration was 11.6 ± 5.6 ng/ml, and the range was 1.8–33.0 ng/ml. The reference interval for AMH for all bitches in the study, regardless of body weight, was 2.9–21.1 ng/ml. The 90% CI for the lower limit was 1.8–4.6 ng/ml, and the 90% CI for the upper limit was 20.5–22.5 ng/ml. AMH values for the neutered bitch and dog were below the lower limit of the assay (<0.2 ng/ml). The serum AMH concentration in the intact dog was 85.7 ng/ml. AMH values for the bitches included in this study are listed in Table 1. Giant breeds had significantly lower AMH concentrations than small-, medium- and large-sized breeds. There was no difference in mean AMH concentrations amongst small-, medium- and large-sized breeds. However, there was a significant difference in the RI for bitches of different body sizes (Table 1). There was a significant effect of the age of the bitch at the time of insemination on AMH concentrations. The mean AMH concentration in bitches less than 4 years of age was 12.4 ± 5.9 ng/ml, whereas the mean AMH concentration in bitches older than 4 years of age was 10.5 ± 5.2 ng/ml ($p < .05$). For each additional year of age, AMH concentrations fell by 0.5 ng/ml ($p < .05$, regression coefficient −.73; Figure 1).

The mean whelping rate was 74.2% ± 44.1%, and the mean litter size was 5.8 ± 3.4 pups/litter. The only independent variables remaining in the multivariable model of whelping rate were age of the bitch and size of the bitch. The mean whelping rate of bitches less than 4 years of age was 81.1% ± 46.1% compared to 64.6% ± 32.7% in bitches older than 4 years of age ($p < .05$). The mean whelping rates of small, medium, large and giant breeds were 80% ± 43%, 78.6% ± 34.6%, 80.3% ± 42.2% and 57.5% ± 32.5%, respectively. The

TABLE 1 Reference intervals (RI) for AMH in bitches of different body weights

Bitch size	*n*	Reference interval (AMH ng/ml)
Small	25	5.6–24.2[a]
Medium	14	4.3–23.7[a]
Large	76	4.3–21.0[a]
Giant	40	1.75–15.6[b]

[ab]Reference intervals with different superscripts are significantly different ($p < .01$).

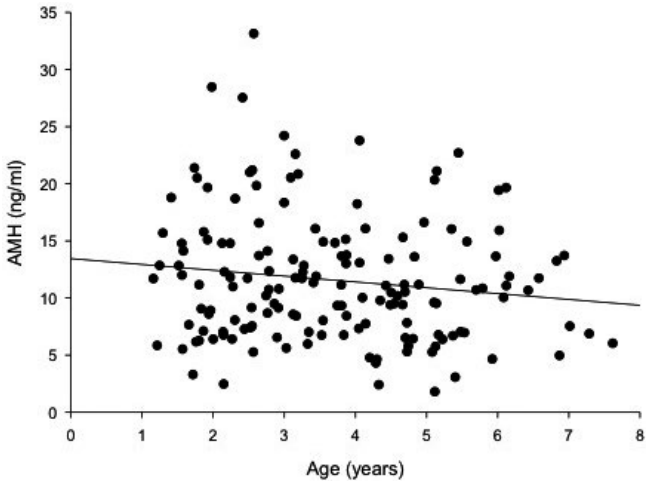

FIGURE 1 Regression graph of anti-Müllerian hormone (AMH) concentrations (ng/ml) in 155 bitches of different ages. For each additional year of age above 1 year of age, AMH concentrations fell by 0.5 ng/ml

whelping rate of giant breeds was significantly lower than small, medium and large breeds ($p < .01$). When controlled for age and breed size, there was no effect of AMH, AI type, semen type, sperm motility or parity on the whelping rate. The mean AMH concentration was 11.9 ± 5.8 ng/ml in bitches that whelped, and 11.0 ± 5.4 ng/ml in bitches that did not whelp ($p = .3$).

The only independent variables remaining in the multivariable model for litter size were AMH concentration and breed size. The mean litter size of small, medium, large and giant breeds was 4.5 ± 2.2, 5.5 ± 2.8, 5.7 ± 2.7 and 6.8 ± 3.2 pups/litter, respectively. In the multivariable model of litter size, the interaction of AMH and breed size was significant. Within each breed size category, bitches with higher AMH levels had larger litter sizes ($p < .01$). For each 1 ng/ml increase in AMH, litter size increased by 0.3 pups/litter.

4 | DISCUSSION

To the author's knowledge, this is the first study to establish the normal reference interval (RI) for AMH in bitches using a canine-specific AMH assay. Furthermore, we determined that the normal RI for AMH in bitches is dependent on body weight.

There has only been one other published report on the use of a canine-specific AMH ELISA assay to measure AMH concentrations in bitches (Turna Yilmaz et al., 2015). In that study, AMH was used as a diagnostic tool for ovarian remnant syndrome (ORS). The mean and range of AMH values that Turna Yilmaz et al. (2015) reported for intact post-pubertal bitches were lower and narrower than what we found in our study. This may be due to the low numbers of bitches in their study ($n = 10$) or variation in the validation of the assay, which they did not describe. The only other two published studies that measured AMH concentrations in bitches (Place et al. 2012; Nagashima et al., 2016) used a human-based AMH ELISA assay (Gen II, Beckman Coulter). The range of AMH values reported by Place et al. (2011) for intact bitches was very narrow, and the concentrations were low (0.1–0.41 ng/ml). The AMH concentration of many of the intact bitches in their study was below the value determined to indicate the presence of ovarian tissue (0.09 ng/ml). The authors attributed this to the pre-pubertal intact bitches having lower AMH concentrations. However, it is most likely due to the low cross-reactivity of the human-based AMH ELISA assay with canine AMH. Turna Yilmaz et al. (2015) did not see an overlap in AMH values between intact and spayed bitches regardless of age. Likewise, in our study, there was a significant difference in AMH concentrations between the intact bitches and the spayed control bitch (AMH was below detectable limits). These findings indicate that the canine-specific AMH ELISA assay has greater specificity and sensitivity than the human-based assays for determination of AMH concentration in canids.

The determination of normal reference intervals for AMH in bitches of different body weights in our study using a readily available commercial canine-specific ELISA should now allow the accurate diagnosis of such clinical conditions as ORS and facilitate universal comparison and analysis of future canine AMH research data.

In agreement with findings in humans (Visser et al., 2006), we found a decline in AMH concentrations with advancing age in bitches. The age-related decline in AMH and the age at which this decline occurs are not well documented in production animals as their lifespan usually ends before reproductive senescence occurs. However, extensive work has been performed on age-related infertility in women as there is a growing worldwide trend for females to reproduce much later in life (Broer et al., 2014). In women, a significant decline in fertility and, correspondingly, AMH concentrations occurs at approximately 35 years of age (Steiner et al., 2011). We found that bitches older than 4 years of age had a lower whelping rate and lower mean AMH concentration than bitches younger than 4 years of age. However, we did not see a corresponding decline in whelping rate at any age point or a correlation between AMH concentration and whelping rate in the bitches in our study. This finding is similar to the many human studies that have concluded that the correlation between AMH and pregnancy outcome is poor (Lin et al., 2013; Smeenk et al., 2007; Tal, Tal, Seifer, & Seifer, 2015; Zarek et al., 2015) and that the most important predictor of pregnancy is age (Revelli et al. 2016). Therefore, owners of breeding bitches and managers of working dog breeding colonies need to place a higher value on the importance of the bitch's age when using genetically valuable and irreplaceable frozen semen. Furthermore, our findings highlight the importance of breeding bitches when they are young (under 4 years of age). This practice would maximize reproductive performance and the overall success of a breeding group or colony.

The positive correlation found in this study between litter size and AMH concentration also has significant practical implications for many canine breeding programmes. This finding is not surprising as AMH has been used for a number of years for the selection of genetically superior dairy heifers and cows to predict those animals that are likely to respond best to superovulation treatments and produce high-quality, transferable embryos (Souza et al., 2015). Use of AMH in this manner has facilitated significant improvement in the efficiency of superovulation and embryo-transfer programmes in dairy cattle in recent years (Rico et al., 2009, 2012; Souza et al., 2015). AMH is specifically expressed by the granulosa cells of small antral growing follicles in females (Visser et al., 2006). It is this exclusive feature of AMH that makes it an endocrine marker of ovarian reserve in females of many species (Monniaux et al., 2013) and a reliable tool for predicting the size of the pool of ovarian gonadotrophin-responsive follicles in the cow (Rico et al., 2012). It is therefore logical that AMH is also a potential predictor of litter size in the bitch. Significant variation in AMH concentration has been documented between individual females in a number of species (cows and goats: Monniaux et al., 2013; humans: La Marca et al., 2009). The practical use of this information differs between production animals and humans. In canine breeding programmes, the inclusion of the measurement of AMH concentration with other breeding management tools may greatly facilitate improved reproductive performance and accelerated genetic gain through the selection of more fecund bitches for insemination with genetically valuable and irreplaceable frozen semen.

Recently, measurement of AMH in post-pubertal dairy heifers has been used to identify individuals with suboptimal fertility

and therefore a shorter productive herd lifespan (Jimenez-Krassel et al., 2015). Elimination of these individuals at an early age will potentially increase the overall reproductive and economic performance of the herd. Similarly, Lahoz et al. (2012) found a correlation between AMH concentration and potential ovarian reserve in pre-pubertal ewe lambs. This means that AMH could be used as a management tool for the selection of replacement ewes with higher predicted fertility. Similar to production animal breeding programmes, it is possible that the measurement of AMH in young post-pubertal bitches identified as potential future breeding stock may facilitate the selection process enabling increased breeding efficiency, accelerated genetic gain and improved pup production numbers. This is particularly so in working dog programmes that can struggle to meet the demand for dogs required for their growing clientele. However, Place et al. (2012) and Nagashima et al. (2016) found pre-pubertal bitches and bitches in an oestrus had lower AMH concentrations than post-pubertal bitches in oestrus. Therefore, further studies are required to determine reference intervals using a canine-specific ELISA AMH assay for pre-pubertal bitches and bitches during other stages of the reproductive cycle.

In conclusion, this is the first study to determine the normal reference interval of AMH in bitches using a canine-specific AMH assay. The clinical applications of these findings are significant including the accurate diagnosis of ovarian remnant syndrome (ORS), determination of the neuter status of shelter animals and, as our study indicates, the prediction of litter size in bitches. Therefore, at a more holistic level, AMH may be included with other canine breeding management tools to facilitate the selection of replacement breeding stock in large-scale working dog programmes as well as the selection of individual bitches for AI with highly valuable frozen semen.

FUNDING

This research was supported by the New Zealand Companion Animal Society.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Vicki Knox for aid in the collection and recording of whelping data and the laboratory staff at NZVP for organising serum samples for analysis.

CONFLICT OF INTEREST

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

AUTHOR CONTRIBUTIONS

FKH was responsible for study design (with contribution from DWH), collection of blood samples from oestral bitches and control animals, insemination of the bitches and collection of whelping information from the owners of the bitches enrolled in the study. CW optimized the AMH assay and completed the ELISAs. DWH carried out collation and statistical analysis of the data. FKH wrote the manuscript with editing contributions from all authors.

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