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Anti-Müllerian hormone and ovarian aging in mares

Renata C Uliani¹, Alan J Conley², C Jo Corbin², Aimê M Friso¹, Luciana F S Maciel¹ and Marco A Alvarenga¹

¹Department of Animal Reproduction, School of Veterinary Medicine and Animal Science, São Paulo State University (UNESP), Botucatu, São Paulo, Brazil

²Department of Population Health and Reproduction, School of Veterinary Medicine, University of California Davis, Davis, California, USA

Correspondence should be addressed to R C Uliani or A J Conley: renata.uliani@gmail.com or ajconley@ucdavis.edu

Abstract

Anti-Müllerian hormone (AMH) is used as a marker of follicle population numbers and potential fertility in several species including horses but limited data exist across the lifespan. No one has decreased ovarian reserve experimentally to investigate whether a corresponding, *quantitative* decrease in AMH results. Concentrations of AMH across the lifespan were compiled from 1101 equine females sampled from birth to >33 years of age. Young and old mares (averaging 6 and 19 years) were hemi-ovariectomized and circulating AMH was assessed before and daily thereafter for 15 days. The remaining ovary was removed later and blood was drawn again before and after this second surgery for AMH determination. Polynomial regression analysis and analysis of mares grouped by 5-year intervals of age demonstrated AMH concentrations to be higher in mares aged 5–10 and 10–15 years than 0–5 years of age and lower in mares after 20 years of age. There was high variability in AMH concentrations among neonatal fillies, some of which had concentrations typical of males. Hemi-ovariectomy was followed by a decrease of AMH, almost exactly halving concentrations in intact mares. Concentrations of AMH had returned to intact levels in old mares before complete ovariectomy, as if exhibiting ovarian compensatory hypertrophy, but recovery of AMH was not evident in young mares. AMH may reflect ovarian senescence in mares after 20 years of age but is too variable to do so in the first two decades of life. The ovarian endocrine response to hemi-ovariectomy in mares appears to change with age.

Key Words

- ▶ ovary
- ▶ aging
- ▶ anti-Müllerian hormone
- ▶ ovarian reserve
- ▶ equine

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Introduction

Anti-Müllerian hormone (AMH) has attracted considerable interest in female reproduction as an indicator of reproductive aging and fertility, predicting the size of follicle populations in the ovaries of women and other mammals including ruminants (Monniaux *et al.* 2014, Mossa *et al.* 2017) and horses (Claes *et al.* 2015). Our current understanding of female reproductive longevity assumes that the ovaries contain a finite number of oocytes, comprising the pool of primordial and developing follicles (Gougeon 1996, Monget *et al.* 2012),

which is often referred to as ‘ovarian reserve’. Ovarian reserve declines with age as follicles are lost (Gosden & Faddy 1998) and oocyte depletion or senescence with age heralds the loss of fertility (Wallace & Kelsey 2010). AMH is secreted by follicles, thereby providing an index of existing follicle numbers. It is positively associated with productive herd life (Jimenez-Krassel *et al.* 2015) and is a general index of responsiveness to super-ovulation and perhaps fertility in cattle (Ireland *et al.* 2011). However, because the level of AMH secretion from follicles changes

with their stage of development (Knight & Glister 2006, Monniaux *et al.* 2014, Mossa *et al.* 2017), AMH secretion will be influenced by the follicle population distribution (defined by diameter) as well as the existing numbers within each stage. Assessing the degree to which identifiable follicle populations contribute to circulating AMH is challenging and species are likely to differ in this regard, as they do in other aspects of the regulation of follicular growth (Fortune 1994, Knight & Glister 2006, Monniaux *et al.* 2014). Therefore, the degree to which AMH reflects ovarian reserve and fertility must be defined carefully for each species. Furthermore, while carefully controlled laboratory studies help to establish what can be regarded as normal, these generally utilize very limited numbers of subjects and more extensive, field-based work is needed to assess the practically relevant normal limits.

As with ovarian exhaustion in women (van Disseldorp *et al.* 2008), other primates (Appt *et al.* 2010) and rodent species (Kevenaar *et al.* 2006, Yeh *et al.* 2007), AMH concentrations were shown to decline with age in mares and to correlate positively with antral follicle count (AFC), especially in aging mares over 19 years of age (Claes *et al.* 2015). To date however, few studies have evaluated in a quantitative way how accurately experimentally induced changes in ovarian reserve are predicted by AMH, in part because of the difficulty in counting or estimating total follicle population numbers (Hansen & Soules 2008). Studies in both rodents (Smith *et al.* 1990) and primates (Appt *et al.* 2010) have utilized the ovarian toxicant, 4-vinylcyclohexene diepoxide (VCN) to effectively destroy primordial and other pre-antral follicles (Hoyer & Sipes 1996, Van Kempen *et al.* 2011) and thereby advance ovarian senescence. Those that have also measured AMH have demonstrated a profound reduction in the days and weeks that follow, significantly correlated with AFC (Sahambi *et al.* 2008, Appt *et al.* 2009, Mark-Kappeler *et al.* 2010). How quantitatively this reflects a decrease in follicle numbers is unknown. Recent observations on the response to immuno-contraception in mares indicated that vaccination against porcine zona pellucida also dramatically decreased AMH concentrations (Joone *et al.* 2018). However, studies in reproductively seasonal species demonstrate that AMH is far from a perfect index of fertility and subject to considerable, unexplained individual variability (Roosa *et al.* 2015). Similar observations of poor correlations between AMH and the chances of establishing pregnancy have been reported among both fertile women (Streuli *et al.* 2014) and those seeking fertility services (Casadei *et al.* 2013).

Assessment of follicle populations along with AMH concentrations is unusually difficult in mares given their size, shape and organization of the ovary and the follicles within (Walt *et al.* 1979). AFC can be performed by imaging but only with a resolution limit of 1–2 mm. Unfortunately, the overall size and unusual asymmetry of equine ovaries (Walt *et al.* 1979) makes an accurate, histological assessment of the small follicle populations (most likely to contribute significantly to AMH secretion) problematic. Consequently, only a single published report has detailed follicle numbers that includes pre- as well as post-antral developmental stages in equine ovaries, noting extreme individual variation in both pony and saddle-type mares (Driancourt *et al.* 1982b) at all stages. Anti-Müllerian hormone is thought to be secreted by pre- and early antral follicles (Knight & Glister 2006, Ball *et al.* 2008, Monniaux *et al.* 2014, Mossa *et al.* 2017), far smaller than the smallest follicles that can be counted by non-invasive techniques. Thus, the positive correlations that have been found between AMH and AFC are likely to reflect the close association between peri-antral and antral follicle numbers but, depending on age, perhaps not primordial follicles (Block 1952, Driancourt *et al.* 1982b). In any case, to the best of our knowledge, the impact of experimental reduction of ovarian reserve on AMH concentrations has never been investigated in a large animal species or in a truly quantitative way in any species.

The following studies were undertaken with two principal goals. First, to conduct an extensive field study of the variation of AMH concentrations with age among a large population of mares. We sought to test the hypothesis that, despite a continual loss of follicles with age each breeding season in mares, AMH concentrations do not change predictably until late in their reproductive lives. Second, to validate the measurement of AMH as quantitatively reflecting ovarian follicular reserve in individual mares, we hypothesized that decreasing total follicle numbers would result in a correlated decrease in AMH. To test this hypothesis, we investigated AMH concentrations in young and old mares subjected to hemi-ovariectomy, as the only practical experimental approach to quantifiably manipulate ovarian reserve (decrease it by half) in this species.

Materials and methods

All animal procedures in this experiment were approved by São Paulo State University, according with the Brazilian National Committee of Control in Animal Experimentation – CONCEA (CEUA protocol 27/2016).

Experiment 1: serum concentrations of AMH across the lifespan

A single blood sample was collected randomly without regard for the stage of the estrous cycle, but within a 2-month period (mid-February to mid-April) in the same breeding season, from a total of 1101, registered, non-pregnant Quarter Horse mares ($n=1058$) and newborn filly foals ($n=43$), housed at 23 ranches in Brazil. Mares were in good body condition at the time of sampling. Ages were confirmed by birth registry entries. Ages ranged from birth to >33 years of age (Fig. 1). Blood samples were drawn from the jugular vein, centrifuged (1500g for 10 min) and serum was harvested and stored frozen (-20°C).

Experiment 2: effects of hemi-ovariectomy on AMH concentrations in young and old mares

Six younger mares (6.0 ± 0.8 years of age, range 3–8) and six older mares (18.7 ± 0.5 years of age, range 18–20) underwent unilateral ovariectomy. These were of mixed breed and all in good body condition, with no known history of infertility. All were apparently reproductively sound and cyclic based on a clinical evaluation that included an ultrasound examination of the reproductive tract and ovaries every other day for a complete cycle. Mares received 4 kg of pelleted horse feed, 6 kg of tifton hay and mineral salt and water *ad libitum* for the duration of the treatment protocol and were assigned to the study without regard to stage of the cycle.

Figure 1

Concentrations of anti-Müllerian hormone (AMH, ng/mL) in populations of mares ($N = 1101$) sampled at various ages ranging from birth to >33 years of age. Changes in AMH concentrations with age were analyzed by linear regression analysis performed based on mare ages (0–10, 11–20 and >20 years of age). Linear regression was not significant for ages 0–10 ($P > 0.41$) or 11–20 ($P > 0.48$) years of age, but declined significantly with age in mares >20 years of age ($P < 0.001$).

A blood sample was collected before surgery and every day for 15 days following surgery. Serum was harvested after centrifugation (1500g for 10 min) and stored for analysis (-20°C). These samples were also included in a recently published, unrelated study evaluating inhibin-A and -B concentrations (Conley *et al.* 2017). Unilateral ovariectomy was performed standing through a vertical flank incision of approximately 15 cm in length under sedation (detomidine, $20 \mu\text{g/kg}$) and local anesthesia was induced by lidocaine infiltration. Hemi-ovariectomy was performed at random with respect to cycle stage which was verified by gross examination of ovaries and progesterone concentrations in peripheral blood (Munro & Stabenfeldt 1984) (data not shown). Based on previous studies in pony mares, follicle distribution is equal between left and right ovaries (Driancourt *et al.* 1982a). An incision was made over the lumbar fossa and muscle tissues separated bluntly to gain access to the peritoneal cavity. The ovarian pedicle was clamped and ligated with vicryl and the ovary removed. The skin incision was closed with non-absorbable, braided nylon suture (Braunamid, Aesculap, Center Valley, PA, USA). After surgery, mares were treated with flunixin meglumine (Banamine, 1.1 mg/kg every 12 h for 3 days), ceftiofur (Excede 6.6 mg/kg , repeated 4 days later) and anti-tetanus serum. Mares were examined daily and a jugular blood sample was drawn each day for 2 weeks following unilateral ovariectomy. The mares were evaluated weekly thereafter and, once determined to be in anestrus based on ovarian size and the absence of growing follicles, were again submitted to ovariectomy, to remove the remaining ovary using the same procedure. Blood samples were again drawn immediately before and at 15 days after surgery. The mean interval between the first and second surgery was 111 ± 24 days.

AFCs were conducted on all 12 mares by transrectal ultrasonography (A5 – Sonoscape, China) before hemi-ovariectomy. The follicles of both ovaries were classified according to their size and categorized into groups: group 1, up to 5 mm; group 2, 6–10 mm; group 3, 11–15 mm; group 4, 16–20 mm; group 5, 21–25 mm; group 6, 26–30 mm; group 7, 31–35 mm; group 8, 36–40 mm and group 9, >40 mm. Ovaries obtained after removal of the remaining ovary were submitted to MRI for AFC (VET-MR Grande – Esaote, Italy), essentially as described (Leonhardt *et al.* 2014) using 2D imaging. The images were subsequently evaluated and all detectable follicles present in the ovaries were counted using the same size group classification (Supplementary Table 1, see section on supplementary data given at the end of this article).

Validation of enzyme-linked immunoassay (ELISA) for AMH

Serum AMH concentrations were determined using commercially available ELISA kits (Equine AMH ELISA, Ansh Labs, Webster, TX, USA) according to the manufacturer's instructions. Serial dilutions of a serum pool were shown to vary in parallel to the standard curve (Supplementary Fig. 1). The intra- and inter-assay coefficients of variation were 3.8 and 9.9%, respectively, and the assay sensitivity was 0.07 ng/mL. Spiked-recovery experiments were also conducted. A purified human (Pepin *et al.* 2013, Kano *et al.* 2017) recombinant AMH (rhAMH) was used to estimate the recovery of AMH added to equine serum. This was added to gelding serum at various concentrations extending over the range of the kit calibrators (0–15.6 ng/mL) and was replicated ($n=8$) at a concentration representing the average of measured sample concentrations, ≈ 2 ng/mL. Equine serum quenched the optical density generated in the assay by addition of rhAMH at various concentrations (Supplementary Fig. 2). However, the recovery of AMH calculated from regression analysis across the calibrator range was linear ($y=0.52x$, $R^2=0.99$, Supplementary Fig. 3) and just over 51%. The mean and standard error of the replicates of ≈ 2 ng/mL purified rhAMH in the assay was 0.89 ± 0.04 ng/mL. Additional spiked-recovery experiments were conducted using fluid from an equine granulosa-theca cell tumor (GCT) as a source of highly concentrated, native equine AMH (Ball *et al.* 2008). The fluid measured 18.2 μ g/mL of AMH when diluted 1:500 in assay buffer. A titration of the fluid was linear in assay buffer ($R^2=0.98$) and in equine serum ($R^2=0.99$). Recovery experiments were conducted by adding 1 μ L of GCT fluid to 500 μ L of serum from eight different mares. The average recovery was $88 \pm 6\%$ ($n=8$), but included a recovery from one serum sample that was 54%. If this single point was omitted, the recovery was $93 \pm 4\%$ (range 80–106%, $n=7$). In addition, a subset of samples ($n=43$) were re-assayed after a freeze-thaw cycle. The concentrations determined in each of these assays were highly correlated ($y=1.06x+0.17$, $R^2=0.99$; Supplementary Fig. 4).

Statistical analysis

The distribution of AMH concentrations for mares in Experiment 1 was examined for normality using a Shapiro–Wilk test and subjected to linear regression analysis by time for ages 0–10, 10–20 and >20 years of age. Data were not normally distributed, nor were they

normalized by log transformation and so were also analyzed by non-parametric methods after categorization into age intervals of 5 years and presented as traditional box plots of medians, ranges and 50% CIs. Differences in AMH concentrations among each age range were evaluated using the Kruskal–Wallis test. For Experiment 2, day-by-day changes in AMH after hemi-ovariectomy were analyzed using the GLM procedure (SAS Statistical Software, SAS Institute Inc.) for repeated measures over time with age as a main effect. Differences among means for AMH concentration at sampling before hemi-ovariectomy (pre-hemi), 15 days after hemi-ovariectomy (post-hemi), before (pre-OVX) and after removal of the remaining ovary (post-OVX) were analyzed using a mixed effect analysis of variance ML linear regression (StataCorp, College Station TX) with age and time as categorical main effects. The potential age*time interaction was examined using a global Wald Test after normality was confirmed using a Shapiro–Wilk test. Comparisons among means were made after applying a Bonferroni adjustment. Means $\pm$ standard errors of the means were graphed.

Results

Experiment 1: Serum AMH concentrations varied with age but with considerable variation at each age. For instance, concentrations among fillies on the day of birth ranged from 0.26 ng/mL to 15.47 ng/mL. Overall, linear regression indicated that there was no evidence of declining AMH concentrations until after 20 years of age among these mares (Fig. 1). There was no significant effect of time in mares aged 0–10 years ($P>0.41$) or 10–20 years ($P>0.48$) but a significant decline with time in mares 20 years of age and older ($P<0.001$) by linear regression analysis. After grouping mares by 5-year intervals, AMH concentrations were significantly higher in mares aged 5–10 and 10–15 years of age than those <5 years of age and were significantly lower in mares >20 years of age ($P<0.05$; Fig. 2). Age groups had the following median concentrations of AMH: 0–5 years, 1.8 ng/mL; 5–10 years, 2.4 ng/mL; 10–15 years, 2.5 ng/mL; 15–20 years, 2.2 ng/mL; 20–25 years, 1.8 ng/mL; >25 years, 0.3 ng/mL.

Experiment 2: Young and old mares had similar AMH concentrations before hemi-ovariectomy (1.03 ± 0.08 and 0.96 ± 0.18 ng/mL, $P>0.1$). Concentrations of AMH decreased progressively reaching a nadir about a week after surgery in both young and old mares (Fig. 3). Combining the data from the young and old mares, AMH concentrations decreased by a little more than half from

Figure 2

Non-parametric analysis of concentrations of anti-Müllerian hormone (AMH, ng/mL) in populations of mares, grouped by 5-year age ranges ($N = 1058$; <5 years, $n = 399$; 5–10 years, $n = 272$; 10–15 years, $n = 193$; 15–20 years, $n = 110$; 20–25 years, $n = 64$; >25 years, $n = 20$). Data from day-old fillies ($n = 43$) were omitted from this analysis. Shown are the median concentrations, ranges and 50% confidence intervals. AMH concentrations in intervals marked by different superscript differ, $P < 0.05$.

1.00 ± 0.01 ng/mL before surgery to 0.46 ± 0.01 ng/mL 15 days after surgery ($P < 0.001$). The elimination half-life was estimated based on samples taken from days 0–5 over which time concentrations of AMH declined with a half-life of 3.7 days. Seven mares were in the luteal phase (progesterone >1 ng/mL) when hemi-ovariectomy was performed. Based on the subsequent, rapid disappearance of progesterone, the ovary removed contained the corpus luteum (CL) in two of those mares, neither of which re-ovulated in the 15 days monitored after surgery. Of the five other mares, luteolysis occurred 14, 13, 11 and 11 days after surgery and one did not experience luteolysis during the monitoring period. Thus, none of the seven mares with CL at the time of surgery ovulated in the 15 days monitored after surgery. Six mares did not have functional CL when hemi-ovariectomy was performed. One ovulated

Figure 3

Concentrations of anti-Müllerian hormone (AMH, ng/mL) in young (6.0 ± 0.8 years, dashed line, $n = 6$) and old mares (18.7 ± 0.5 , solid line, $n = 6$) in jugular blood drawn before (day 0) and daily after hemi-ovariectomy. Concentrations of AMH decreased significantly from day 0 (pre-hemi-ovariectomy) to day 15 ($^{\dagger}P < 0.001$) after surgery, but there was no main effect of age or age $\times$ time interaction, and there was no difference in AMH between young and old mares on day 0 ($P > 0.5$).

Figure 4

Concentrations of anti-Müllerian hormone (AMH, ng/mL) in young (6.0 ± 0.8 years, dashed line, $n = 6$) and old mares (18.7 ± 0.5 , dotted line, $n = 6$) in jugular blood drawn before (PRE-HEMI) and 15 days after (POST-HEMI) hemi-ovariectomy, and before (PRE-OVX) and after (POST-OVX) complete ovariectomy when mares were anestrus. † Indicates concentrations of AMH were significantly lower ($P < 0.01$) than before hemi-ovariectomy. AMH concentrations were not different between young and old mares before ovariectomy (PRE-OVX) but the age $\times$ time interaction was significant ($P < 0.02$). Lines are broken to signify that intervals between post-HEMI and pre-OVX varied (111 ± 24 days), but data were true longitudinal samples and analyzed as repeated measures accordingly. Means are represented by solid symbols, and individual values from all mares are depicted as open symbols.

on or before the day of surgery and luteolysis occurred at 13 days post surgery. Four others ovulated 1, 4, 4 and 11 days after surgery. Thus, only mares lacking functional CL at surgery ovulated (4/6 mares) during the course of monitoring after surgery, but with variable delays in days to ovulation. Cycle stage at surgery had no apparent effect on the post-surgical decay of AMH concentrations.

The second surgeries to remove the remaining ovary were done when ultrasound imaging and clinical observations indicated mares had become anestrus. Concentrations of AMH before hemi-ovariectomy (pre-HEMI) and 15 days after hemi-ovariectomy (post-HEMI) were also compared with those at the time of the second surgery (pre-OVX) and after the remaining ovary had been removed (post-OVX; Fig. 4). At the time of the second surgery, AMH concentrations in old mares had recovered to concentrations that were not different from those before hemi-ovariectomy (0.86 ± 0.27 vs 0.86 ± 0.06 ng/mL, respectively). However, AMH concentrations at the time of the second surgery in young mares were still half those seen before hemi-ovariectomy (1.01 ± 0.42 vs 0.50 ± 0.04 ng/mL, $P < 0.01$). The difference in restoration of AMH concentrations in old vs young mares was supported by a significant interaction of age $\times$ time ($P < 0.02$). However, the mean AMH concentrations in old and young mares at the time of the second surgery (before ovariectomy, pre-OVX) were not significantly different ($P > 0.4$) by multiple comparisons analysis (Fig. 4). Still, old mares

appeared to recover AMH production in the months following hemi-ovariectomy, whereas young mares did not. No significant correlations were found between AFC (Supplementary Table 1) and AMH concentrations based on ultrasound examination of ovaries before hemi-ovariectomy or between AMH prior to the second surgery and AFC determined on the second ovary by MRI.

Discussion

We report the most comprehensive survey of AMH concentration ever conducted across the lifespan of female horses. It is the most extensive such study completed in any species, except perhaps human (Hagen *et al.* 2010, Lie Fong *et al.* 2012, Cui *et al.* 2016). The analysis of samples from over 1100 females from birth through their third decade of life provides a unique view of how AMH concentrations change with age in this species. The results of a recent longitudinal study in horses indicated that AMH concentrations were fairly constant during the first 2 years of life and that concentrations at birth were correlated with those at 2 years of age (Scarlet *et al.* 2018). As the current data illustrate, despite the gradual loss of ovarian follicles that occurs inevitably with age in mammals (Gougeon *et al.* 1994, Faddy & Gosden 1995, 1996, Faddy 2000, Bristol-Gould *et al.* 2006, Hansen *et al.* 2008, Coxworth & Hawkes 2010, Monget *et al.* 2012), it is only after 20 years of age that AMH concentrations begin to decline in mares, and thereafter to decline quite rapidly. Although subject to possible sampling bias due to the many fewer samples collected from mares over 20 years of age than from their younger cohorts, these data are consistent with a previous, smaller but more balanced study of AMH in aging mares (Claes *et al.* 2015). It is also consistent with observations in pony mares over 20 years of age which exhibited less follicular activity than did younger mares as they transitioned into the breeding season (Carnevale *et al.* 1997). However, by linear regression analysis there was no decay in AMH concentrations in mares during their first or second decades of life. In fact, when examined by 5-year intervals, there was an increase in AMH in years 5–15 compared to the interval up to 5 years of age. This pattern of AMH concentration over the lifespan is remarkably similar to that seen in women where it also peaks at about 15 years of age and begins to decline around 20 years of age (Hagen *et al.* 2010, Lie Fong *et al.* 2012, Cui *et al.* 2016). Thus, as is the case in women, AMH appears to be a marker of ovarian senescence in aging mares, but may not accurately reflect ovarian

reserve in mares until nearly 20 years of age. Moreover, if the decline in AMH after 20 years of age reflects true ovarian senescence, when it becomes obvious, senescence is not gradual but rather undergoes a rapid acceleration. This has been postulated to occur also in women as they become menopausal (Faddy & Gosden 1996, Hansen *et al.* 2008).

Two other observations in the current data set are notable relative to folliculogenesis in horses and other mammals. First, the increase in AMH from 5 to 15 years of age suggests that either a similar number of follicles are secreting more AMH in mid-reproductive life of mares or there are significantly more follicles present secreting AMH. Consistent with the latter possibility, pony mares 15–19 years of age were found to exhibit greater follicular activity in the late luteal phase than did young mares, 5–7 years of age (Carnevale *et al.* 1993). Studies in horses (Ball *et al.* 2008) and other species indicate that AMH is expressed and secreted by granulosa cells of pre-antral or early antral follicles (Baarends *et al.* 1995, Durlinger *et al.* 1999, Weenen *et al.* 2004, Sahambi *et al.* 2008, Rico *et al.* 2011, Jeppesen *et al.* 2013) but that AMH expression decreases as antral follicles increase in diameter and become more gonadotropin responsive (Fortune 2003, Fortune *et al.* 2010). The selective destruction of follicles at these stages of development in mice provides convincing evidence of this (Sahambi *et al.* 2008). It is not clear what range of follicle populations might be targeted, but vaccination against porcine zona pellucida dramatically decreased AMH in mares (Joone *et al.* 2018). As noted earlier, immunohistochemical analyses of equine ovaries suggest that AMH expression is higher in secondary and small antral than in large antral follicles (Ball *et al.* 2008). However, counting follicle numbers requires careful histomorphometric analysis (Forabosco & Sforza 2007, Hansen *et al.* 2008) that is methodologically challenging even in the smaller size of the human ovary (Hansen & Soules 2008) and is well beyond the scope of the present study in the horse. The second notable observation was the high degree of variation in AMH concentrations in filly foals at birth. This was not observed in a recent study involving a smaller number of filly foals that were included in a longitudinal study (Scarlet *et al.* 2018). Several of the newborn fillies in the current study had AMH concentrations that were in a range expected of colts (Claes *et al.* 2013). Given this variability, it is unclear what extremes of variation in follicle populations might exist on the ovaries of at least some foals at birth. If this variability truly exists in larger populations of neonatal fillies, even if significantly correlated with concentrations at 2 years of

age as previously shown (Scarlet *et al.* 2018), AMH at birth may not be very predictive of concentrations in later life. Regardless, it is clear that AMH concentrations actually increase as mares enter their most fertile years, and AMH only begins to reflect diminishing follicular reserves in mares as they approach their third decade of life.

Despite the increase in AMH concentrations from early-to-mid-reproductive life, halving ovarian reserve in both young and old mares almost exactly halved AMH concentrations. Consistent with the findings of the population-based study, concentrations of AMH were similar in the young and old mares before hemi-ovariectomy. Similarly, the gradual decline post-surgically, consistent with the extended half-life of AMH in horses (Claes *et al.* 2013), was essentially identical in both groups of mares. Surgery was conducted without regard for stage of the cycle because AMH concentrations do not change at different stages of the reproductive cycle (Almeida *et al.* 2011). In the current studies, pre-surgical AFCs by ultrasound of the ovaries were not predictive of AMH concentrations, nor were those of the excised ovary predictive of the subsequent decline in AMH. The random and variable stage of the cycle at the time of examination was likely a significant factor in the variability observed. As noted earlier, this is also consistent with the experimental evidence that follicles, of a size that can be counted non-invasively, likely do not contribute significantly to circulating AMH concentrations (Sahambi *et al.* 2008, Claes *et al.* 2016). Follicles of smaller diameter are more numerous (Driancourt *et al.* 1982a) and therefore contribute most to AFC. Even though losing their ability to secrete AMH, this probably explains the positive correlation between antral follicle count and circulating AMH in published studies that employed larger numbers of mares and more frequently determined AFC (Claes *et al.* 2015). The lack of correlation between AMH and AFC in this study can be attributed to being under-powered with too few mares examined and too few observations on each. To this point, the correlation between AMH concentration and numbers of growing follicles in mice was lost when analysis was conducted on specific days which necessarily limited the number of animals that could be used in that analysis (Sahambi *et al.* 2008). Regardless, hemi-ovariectomy experimentally validated the accuracy of AMH concentrations as an index of total ovarian reserve in individual young and aging mares.

At least two prior studies have employed hemi-ovariectomy to investigate folliculogenesis in mares (Driancourt & Palmer 1984, Sirois *et al.* 1989). However, only one other study in mammals appears to have utilized

hemi-ovariectomy to halve ovarian reserve and examine AMH, a study conducted in cynomolgus macaques (*Macaca fascicularis*) (Appt *et al.* 2010). Concurrent treatment with the follicular toxicant VCN in that study confounded results, likely causing a more dramatic reduction in AMH post-surgically (>60% (Appt *et al.* 2010)) than was found here in horses. Uniquely in the current study however, AMH concentrations were also re-assessed several months later before removal of the second remaining ovary. The results were surprising, difficult to explain based on our current understanding, and raise some interesting questions. By the time of the second surgery, the concentrations of AMH in the older mares had returned to levels seen before hemi-ovariectomy. This suggests that the remaining ovary compensated in some way, restoring the total number of AMH-secreting, presumably pre- and early antral follicles previously distributed between both ovaries. Alternatively, if there was no change in their numbers, then expression and secretion of AMH by those follicles must have increased. More puzzling perhaps, a restoration of AMH concentration was not seen in the younger cohort of mares at the time of the second surgery. However, there was considerable variability in AMH concentrations, especially in the samples collected before the second surgery. While warranting verification in additional studies including a larger number of mares, the current observations suggest that the long-term effects of hemi-ovariectomy may be an interesting way to investigate the effects of aging on reproductive function. In any case, it seems unlikely that under these experimental circumstances (as with the increase seen between 5 and 15 years of age) AMH concentration reflected ovarian follicular reserve accurately.

There is broad agreement that aging changes not only ovarian function but the reproductive axis in general (Brann & Mahesh 2005, Prior 2005, Carnevale 2008, Kermath & Gore 2012, Claes *et al.* 2017), and this might also have been a significant factor in this study. Consistent with involvement of the hypothalamic-pituitary axis in reproductive aging, the rate of double ovulations increases as mares age (Davies Morel & O'Sullivan 2001). Perhaps the accelerated decline in AMH concentrations in mares after 20 years of age indicates that changes in neuroendocrine regulation of follicular growth rate or recruitment in aging mares (Carnevale *et al.* 1993) are somehow amplified or exacerbated by hemi-ovariectomy. Hemi-ovariectomy has been studied in many species including sheep (Duggavathi *et al.* 2008), cattle (Dean & Dailey 2011) and pigs (Coleman *et al.* 1984). Long-term effects on folliculogenesis in sheep indicate that

ovulatory compensation involves an extension of the normal lifespan of ovulatory-sized follicles (Duggavathi *et al.* 2008). However, the relationship between hemiovariectomy and aging appears only to have been studied in rats. Initially, ovulatory compensation is apparently due to a decrease in the rate of follicular atresia in the remaining ovary (Hirshfield 1982), but the ability to compensate is eventually lost with age (Peppler 1971). How this might affect AMH secretion is unknown. The phenomenon of compensatory follicular development is complex, affected by age, and is perhaps not the same across species. Histological studies are in progress in the hope of mapping follicles in the ovaries of the young and old mares from this study to better understand the origins of the noted differences in the recovery of AMH concentrations following hemi-castration.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-18-0391>.

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.

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