Understanding anti-Müllerian hormone and its value as a phenotypic marker of fertility within commercial sheep production

by

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BSc. Honours (University of Adelaide)

A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

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The University of Adelaide

2022

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List of abbreviations

analysis of variance (ANOVA)

anti-Müllerian hormone (AMH)

antral follicle count (AFC)

artificial insemination (AI)

assisted reproductive technologies (ARTs)

basic fibroblast growth factor (bFGF)

bone morphogenetic protein (BMP 15)

bone morphogenetic protein 4 (BMP-4)

bovine serum albumin (BSA)

carbon dioxide (CO_2)

control internal drug release (CIDR)

corpus luteum (CL)

cumulus oocyte complexes (COCs)

enzyme-linked immunosorbent assay (ELISA)

equine chorionic gonadotrophin (eCG)

follicle stimulating hormone (FSH)

germinal vesicle breakdown (GVBD)

gonadotrophin releasing hormone (GnRH)

growth differentiation factor 9 (GDF-9)

heat inactivated oestrous sheep serum (SS)

hypothalamic-pituitary-gonadal (HPG)

in vitro culture (IVC)

in vitro production (IVP)

in vitro fertilisation (IVF)

juvenile *in vitro* fertilisation and embryo transfer (JIVET)

keratinocyte growth factor (KGF)

kisspeptin and neurokinin B (NKB)

kisspeptin, neurokinin B and dynorphin neurons (KNDy neurons)

kit ligand (KL)

laparoscopic ovum pick-up (OPU)

leukemia inhibitory factor (LIF)

luteinising hormone (LH)

mature *in vitro* fertilisation and embryo transfer (MIVET)

Meat & Livestock Australia (MLA)

multiple ovulation and embryo transfer (MOET)

nitrogen (N_2)

oxygen (O_2)

prostaglandin F2alpha ($PGF_{2\alpha}$)

standard error of the mean (SEM)

synthetic oviductal fluid medium (SOF)

tumour necrosis factor alpha (TNFα)

transforming growth factor beta (TGF-β)

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Abstract

Anti-Müllerian hormone (AMH) as a phenotypic marker of fertility has recognised potential in humans, cattle, goats and sheep due to its strong positive relationship to the antral follicle pool and increasing evidence of its potential to select the individuals most likely to respond within an assisted reproductive technology. However, the literature regarding AMH as a marker of fertility in sheep is extremely limited. Therefore, the purpose of this thesis was to better understand changes in AMH throughout life and evaluate its potential as a phenotypic marker of fertility within the Australian sheep industry. The results from this thesis have identified pre-pubertal differences in AMH concentration between two predominant breeds (Merino and Suffolk) that are no longer apparent in adulthood. Furthermore, we identified two distinct pre-pubertal AMH profiles for both the Merino and the Suffolk, with peaks in AMH concentration corresponding to that of previous literature. However, the profile identified in the Merino showed a sharp, distinctive decline in AMH concentrations in ewe lambs at four-weeks of age, and this appears to influence blastocyst development within a hormone-unstimulated *in vitro* embryo production system. This suggests that in the Merino, AMH sampling at four-weeks of age may assist in the selection of more fertile animals. In chapter two, we conclude for the first time that AMH concentration across a natural oestrous cycle in the Merino is highly repeatable. This chapter also determined a strong negative correlation between AMH concentration at the time of progesterone pessary insertion and the onset of oestrous and the day of ovulation following pessary removal, suggesting AMH concentration may be used to improve the success of artificial insemination and embryo transfer programs through optimisation of transfer timing to ovulation. Following on from this finding, the results from chapter three have identified AMH concentrations from ewe lambs as young as 7-days old have the potential to predict the response to, and outcomes of FSH stimulation within a juvenile *in vitro* fertilisation and embryo transfer (JIVET) program at 7-weeks of age. Selecting ewe lambs

at 7-days of age with an AMH concentration ranging between 1.03 and 4.78 ng/ml should yield the most “A” grade cumulus-oocytes complexes (COCs) and subsequently the greatest percentage of embryos, with there being a clear positive correlation between AMH at 7-days of age and the total number of COCs recovered, and blastocysts produced. In the final chapter we have reiterated the potential of using a range of AMH concentrations to identify animals with increased fertility within a natural mating program. Ewes within a flock with AMH concentrations ranging between 0.40 and 3.66 ng/ml at 292-days of age birthed up to 30 more lambs in comparison to those outside this range whilst ewes with an AMH concentration less than 0.64 ng/ml at 82-days of age scanned more fetuses than those above this value. This thesis has increased the understanding of AMH concentrations and changes in both pre-pubertal lambs and mature ewe oestrous cycles and how these relate to differences with ovarian phenotype. The results within this thesis demonstrate that in the Australian sheep industry fertility outcomes within both an assisted reproductive technology and natural mating system can be improved by selecting animals that lie within a clearly defined range of AMH concentration.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Signed: __   _____________

Jamee Elise Daly

Date: 19/05/2022

Acknowledgements

First, I would like to sincerely thank and acknowledge the help and support of my principal supervisor Associate Professor William van Wettere. I have valued the support, advice and opportunities that have been provided throughout my PhD. The combination of professionalism and personable supervision has helped shaped me into the researcher I am.

I would also like to thank Dr's Jen Kelly, Simon Walker and Karen Kind for their continued insight and support. Without this guidance and access to their knowledge I would not have had the opportunity to complete this thesis. Thanks also go to each of my fellow PhD students, particularly Megan. I could not have completed the trial work without your help. Across five years I have developed some of the closest friendships I have ever had, and I value the growth and support that these have provided me with.

To my family, the ongoing support and encouragement to complete this thesis has not gone unnoticed. To my mum and dad, thank you most for prioritising my studies and for helping to shape me into the person I am today. If it weren't for you both I would not have had the strength and determination needed to finalise this. To my siblings; Renee, Adam and Krysta thank you for being there in whatever capacity I needed.

Finally, to my husband, Brandon. There are not enough words to truly represent how much I appreciate, and need your unconditional support in life. Your support is unwavering and the trust and love I know you have in me, and what I do, has pushed me to do everything I can to make you proud. Thank you for truly having your own language of love and encouragement, I could not do any of this without you.

Chapter One: Introduction

The Australian sheep industry is the world's largest exporter of sheep meat, despite only accounting for 7% of the world's domestic sheep population (Australia 2021). To match increasing consumer demand for sheep meat, there is a need for the Australian sheep industry to develop tools to increase the reproductive efficiency and productivity of its breeding flock. Phenotypic markers of fertility account for genetic and environmental impacts on the reproductive potential of an individual animal, which makes them a promising tool for increasing the efficiency of the Australian breeding flock. Anti-Müllerian hormone (AMH) has recognised potential as a phenotypic marker of fertility in humans, cattle, goats and importantly sheep due to its strong positive relationship to the antral follicle pool (van Rooij *et al.* 2002; Torres-Rovira *et al.* 2014; Fleming *et al.* 2015). In women and cattle, the use of AMH to select females most likely to respond within a hormonal stimulation protocol has produced positive outcomes (Rico *et al.* 2009; Monniaux *et al.* 2010; Guerreiro *et al.* 2014; Ribeiro *et al.* 2014; Souza *et al.* 2015; Batista *et al.* 2016; Ghanem *et al.* 2016; Jamil *et al.* 2016). However, literature describing the suitability and benefits of using AMH as a phenotypic marker of fertility in ewes is limited.

Significant breed differences in AMH concentrations exist in cattle (Batista *et al.* 2014). Suffolk and Merino ewes serve two different purposes (meat and wool respectively) within the Australian sheep industry therefore it is important to understand if differences in AMH exist between sheep breeds. Additionally, different profiles of pre-pubertal AMH concentration have been identified within Sarda ewe lambs (Torres-Rovira *et al.* 2016). Therefore, the aim of chapter three was to understand pre-pubertal patterns of AMH and if they differ between breeds, to establish optimal age for prediction of fertility within either an assisted reproductive technology (ART) or natural mating protocol.

Similarly, the assessment of AMH changes in the mature ewe, across an oestrous cycle, will allow for optimisation of timing for fertility predictions. AMH concentration is highly reproducible in cattle (Rico *et al.* 2009; Monniaux *et al.* 2013) with a single time point sample randomly selected within one oestrous cycle strongly correlated (r=0.97) across multiple cycles (Ireland *et al.* 2011). In ewes, one study has investigated changes of AMH within an oestrous cycle (Waheeb 2017), and this was over nine days. In this study, AMH concentration was measured 2 days prior to synchronisation of ovarian activity, at the time of synchronisation, and then daily for 7 days. Results from this study showed AMH concentration had an inverse relationship to oestrogen, and a similar daily pattern to that of progesterone, although the strength in declines between AMH and progesterone differed (Waheeb 2017). Therefore, the main objective in chapter four was to determine changes and repeatability of AMH across 14 days of a Merino oestrous cycle, and investigate the relationship between progesterone and AMH. As such, a secondary aim of this chapter was to determine whether AMH concentration could predict the outcomes of synchronisation with progesterone, given 1) it's use in standard oestrous synchronisation protocol in Australia and 2) a positive relationship has been identified between these two hormones.

A limited number of studies in sheep have shown AMH as a promising marker of fertility. In pre-pubertal sheep, there is a correlation between AMH and AFC (Torres-Rovira *et al.* 2014) and in both Sarda (Torres-Rovira *et al.* 2016) and Rasa Aragonesa ewes (Lahoz *et al.* 2012), ewe lambs with high, pre-pubertal AMH concentrations were more fertile at their first mating. Within these studies, AMH concentration was also correlated to the response to hormonal stimulation, prior to the assessment of fertility. Therefore, in chapter five the ability of AMH to predict oocyte and embryo quality and outcomes of assisted reproductive technologies (ARTs) (juvenile or mature *in vitro* fertilisation and embryo transfer programs) was quantified. In chapter six AMH concentrations in hormone-unstimulated Merino ewe lambs were evaluated to determine if it could predict fertility when ewe lambs were naturally mated. The emphasis of a natural mating is to ensure that the results from this chapter are industry applicable by replicating most commonly used mating practices within the Australian sheep industry.

Thesis format

This thesis presents the results of four experiments which focus on either understanding anti-Müllerian hormone (AMH) changes within sheep or the potential of AMH as a phenotypic marker of fertility. Chapter one provides a background on the need to increase reproduction within the Australian sheep industry and the potential to use AMH concentrations to increase reproductive efficiency in ewes. It also outlines the aims and format of this thesis.

Chapter two presents a review of the literature. This review outlines the stages of reproductive development in sheep, and where AMH plays a role, to determine areas in which improvements to reproduction can be made. This review also investigates the potential of AMH as a phenotypic marker of fertility within the Australian sheep industry through summarising existing literature and identifying knowledge gaps for investigation.

Chapter three compares AMH concentration in two predominant Australian sheep industry breeds, Merino and Suffolk, 1) as both juvenile and mature animals and 2) in pre-pubertal ovarian development. Further to this, this chapter aimed to identify clear profiles of AMH concentration within a pre-pubertal Merino or Suffolk ewe lamb.

Chapter four titled: ‘AMH concentration changes throughout the oestrous cycle in the Australian Merino’ aimed to determine the repeatability of AMH throughout an oestrous cycle and identify relationships to ovarian and follicle characteristics and dynamics. In addition, the relationship between AMH and progesterone was briefly investigated.

Chapter five titled: ‘AMH as a phenotypic marker in an assisted reproductive technology’ discusses how AMH concentrations at different ages and at key points within an assisted reproductive technology can be used to predict reproductive outcomes. Specifically, can juvenile or mature *in vitro* fertilisation and embryo development be optimised using AMH concentration as a selection tool for donor animals?

Chapter six ‘AMH concentration as a phenotypic marker of fertility within a natural mating system’ investigates AMH concentration at different ages, at times aligned within the management of a natural mating system, to predict the fertility of ewes at their first mating. Additionally, this chapter aimed to identify external factors that may affect AMH concentration.

Chapter seven is a short communication accepted for publication with major revisions. It includes data generated as part of a pilot study prior to the work completed in Chapter six. Entitled ‘Circulating anti-Mullerian hormone (AMH) from 5-month-old Australian Merino ewe lambs predicts birthing rates at their first service’ this short communication presents initially findings on the validity of using AMH as a marker of fertility with the Australian sheep industry.

Chapter eight is a concluding chapter that draws on the conclusions of each of the previous chapters to determine synergies between them to optimise the use of AMH within the Australian sheep industry. The results from each of these chapters will determine whether AMH concentration is of value as a phenotypic marker of fertility within the Australian sheep industry.

Project Aims

This thesis attempts to answer fundamental questions around the endocrinology of AMH in sheep. Additionally, this thesis aims to determine for two predominant Australian sheep industry breeds (Merino and Suffolk) the reliability of AMH concentration as a predictor of the fertility of ewes and ewe lambs, within both natural mating systems and assisted reproductive technologies. Based on gaps identified within the literature, this thesis was designed to address the following;

1. Is there a difference in AMH concentration between two of the most predominant breeds of sheep in the Australian industry, Merino and Suffolk? Specifically, is the development of the antral follicle pool different between or within these breeds prior to puberty and do differences also present in adulthood?
2. How does AMH concentration change post-pubertally throughout the oestrous cycle. Is it a repeatable measure with minimal within animal variation?
3. Does AMH concentration determined at a young age (7-days old) have a relationship to oocyte and embryo quality following an *in vitro* fertilisation and embryo transfer program for either juvenile or mature animals?
4. Can a single sample of AMH taken at routine husbandry time points (lamb marking/ weaning) be used to select the most fertile animals for inclusion in the breeding flock?

Chapter Two: Review of the literature

Towards improving the outcomes of reproduction within the Australian sheep industry using anti-Mullerian hormone as a phenotypic marker of fertility

Introduction

Over the next five years, global sheep meat consumption is forecast to increase by 4.8% (Australia 2021). Currently, Australia is the largest exporter of sheepmeat, despite having only 7% of the world's domestic sheep population (Australia 2021). Self-replacing wool and sheep meat sectors make up 79% of the Australian sheep production industry, with the productivity and profitability of these enterprises reliant on the reproductive efficiency of its breeding flock.

High reproductive efficiency 1) ensures adequate supply of replacement breeding animals, 2) increases the rate of genetic improvement and 3) increases the number of animals that can be sold (Hinch and Brien 2014). Current strategies to increase the productivity of the Australian sheep flock include: genetic selection through breeding programmes such as MERINOSELECT and LAMBPLAN, adoption of younger ages at first mating (ewe lamb mating) (Armstrong *et al.* 1997), the retention of female lambs to increase flock size and the use of assisted reproductive technologies (ARTs) (Smith 1986; Horton 1996; Morton 2008; Granleese *et al.* 2015). Whilst each of these strategies has merit they also have considerable limitations, primarily because genetic selection does not account for the impact of external factors on reproduction. The retention of more female lambs is difficult due to ongoing drought conditions and their impact on feed availability, which also increases the need to maximise reproductive efficiency of each breeding ewe. The success of ewe lamb matings is also highly variable due to ongoing drought conditions, particularly for the Merino breed which dominates the Australian sheep flock. Furthermore, there is large variation in the outcome of ARTs and they are expensive which therefore limits their widespread adoption by industry.

The Australian sheep industry has limited means with which to identify reproductively efficient ewe lambs, with best practice genetic selection based on parentage being utilised to improve reproductive efficiency, among other parameters (Rowe 2010). The focus of most genetic selection is the improvement of traits such as growth rate and carcass weight (Rowe 2010), which can be to the detriment of reproduction outcomes. Ewe lambs with inherently poor fertility and fecundity may be selected for use in conventional mating regimes and ART protocols, which can significantly impair efficiency and, thus, reduce producer profit margins.

The ability to identify ewe lambs with increased fertility and fecundity is complicated by the profound impact that exogenous factors (eg nutrition, climate) have on reproductive development and, thus, outcomes. During gestation, development of the conceptus is sensitive to changes in nutrient supply, resulting in phenotypic variation which affected both post-natal growth and reproductive potential (Walkom et al., 2016). In cattle, it is well established that conditions experienced *in utero* alter size of the ovarian reserve (i.e. number of primordial and pre-antral follicles present on the ovary) and size of the antral follicle pool (Rae et al., 2002; Da Silva et al., 2003) and consequently affect fertility and fecundity. These changes in the ovary cannot be detected using genotype, and the use of ultrasound to determine the size of the antral follicle pool is not physically feasible in young ewes. Therefore, identification of phenotypic marker/s which correlate with ovarian status in ewe lambs and, therefore reproductive potential, would allow producers to identify, and select animals within a genetically similar population with higher reproductive output for inclusion in a breeding flock (Walkom *et al.* 2016). To be commercially relevant, such phenotypic marker/s should accurately identify ewes that produce a high number of good quality oocytes and embryos when used as donors for ARTs, or are more likely to become pregnant when used as recipients. In addition, the phenotypic marker/s should allow more fertile and fecund replacement ewes to be selected for inclusion in natural mating protocols, thereby improving overall flock reproductive efficiency weaning rates beyond the current concentration of 81 lambs born per 100 ewes presented to the ram (Kleemann and Walker 2005).

This review will, therefore, focus on three main areas: 1) reproductive efficiency of the Australian sheep breeding flock, and the factors affecting it, 2) current understanding of reproductive development of the female sheep, with particular focus on control of ovarian follicle development and 3) potential phenotypic marker/s of fertility, fecundity and lifetime reproductive performance in female sheep.

Reproductive efficiency of the Australian sheep flock

The Merino is the most utilised sheep breed in Australia, making up 75% of the 42 million ewes within the national flock (42 million ewes) and 60% of the 21 million lambs weaned annually (Snowder and Fogarty 2009; Rowe 2010). Merino ewes are most commonly joined for the first time at approximately 18 months of age. Joining of ewes is usually timed so that lambing occurs approximately one month prior to peak pasture production, with the majority of ewes in Australia joined between mid-December and May. As the Merino is not a strictly seasonal breeder, joining and pregnancy rates during summer / early autumn are higher compared with other, more seasonal British breeds. Rams are typically run with ewes for two 17-day cycles, or approximately 5 weeks.

Reproductive efficiency of a sheep flock can be defined as the number of lambs born per ewe per year (Martinez *et al.* 2015) and as such is a critical component to the success of the industry. The proportion of lambs marked per mated ewe is typically lower for Merinos compared with other breeds. Geenty *et al.* (2014) compared the reproductive output of 3832 Merino and 796 Cross-bred ewes artificially inseminated over four years and across eight locations. Based on these data, it was concluded that conception rates were typically lower, and the number of lambs born and weaned per mated ewe were lower for Merino compared with cross-bred ewes: 1.45 versus 1.67 and 82% versus 102%, respectively (Geenty *et al.* 2014). Importantly, it was concluded that fertility following artificial insemination (AI) was comparable with outcomes of natural mating systems (Geenty *et al.* 2014). Commercial data from South Australian Commercial Merino flocks (Kleemann and Walker 2005) reported pregnancy rates and weaning rates of 90% and 81%, respectively. The reduced fecundity of Merino ewes may reflect the intense selection for fine wool production, as singleton lambs produce finer wool than twin lambs (Kelly *et al.* 2007). The increased number of lambs born per ewe for the South African meat Merino compared with the commercial wool Merino (92% versus 81%), supports the impact of wool characteristics on ewe fertility (Fourie and Cloete 1993).

Timing of reproductive losses

Understanding the timing of, and reasons for, the loss of potential lambs between joining and weaning is integral to the development of strategies to increase the number of lambs weaned by each ewe. Reproductive inefficiencies, such as pregnancy failure and loss of potential lambs between joining and weaning, are caused by a multitude of factors, but can be roughly divided into pre-natal and post-natal reproductive failure. Within the Australian flock, pre-natal reproductive failure (i.e. mated ewes which fail to give birth to a lamb, or more than one lamb) accounted for 44% of potential lamb losses (Kleemann and Walker 2005; Hinch and Brien 2014; Paganoni *et al.* 2014). The death of lambs between birth and weaning (post-natal reproductive losses) accounted for 55% of potential lamb losses (Kleemann and Walker 2005; Hinch and Brien 2014), but the causes of this are beyond the scope of this review and, therefore, will not be discussed. In South Australian flocks, 42% of reproductive inefficiency was due to loss of potential embryos between ovulation and weaning (Kleemann and Walker 2005), with the majority of losses (53%) occurring during the first 17-days post-mating (Paganoni *et al.* 2014) (figure 1). This suggests that reducing early embryo loss has the potential to significantly increase the number of lambs born, and weaned per mated ewe.

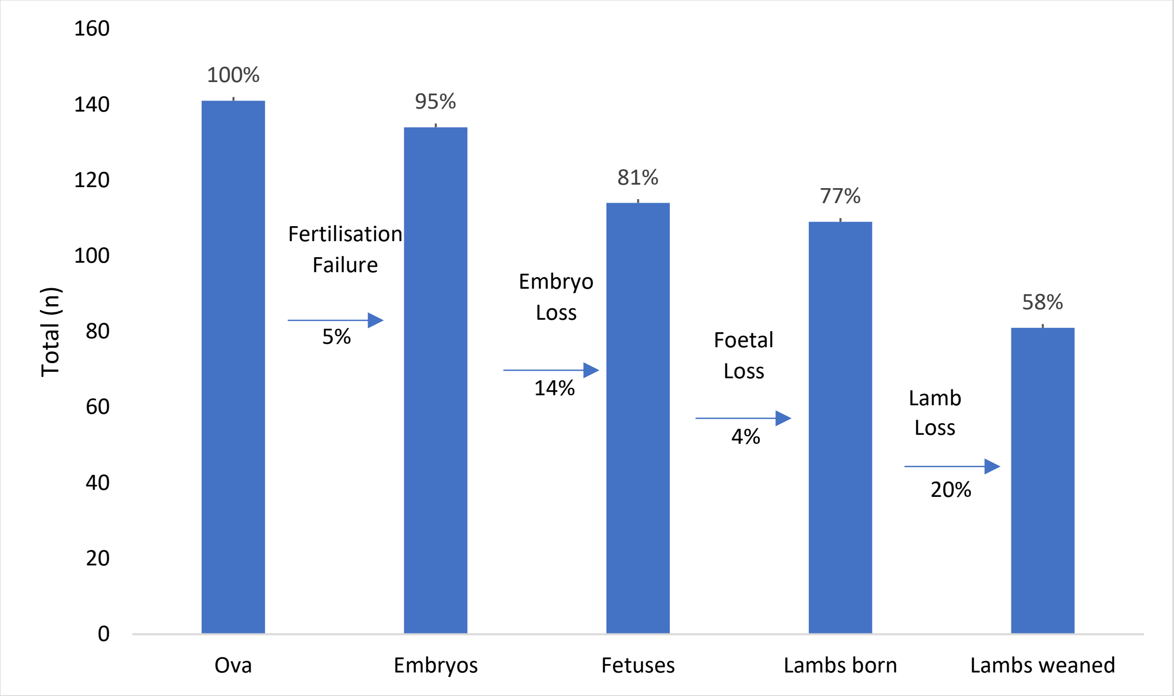

Figure 1: Reproductive wastage in South Australian Merino sheep. Adapted from Kleemann and Walker, 2005.

Causes of reproductive losses

The fertility and fecundity of ewes is significantly affected by genotype, nutrition and climate, due in part to alterations in ovarian function (Stevenson and Call 1983), ovulation rate (Aungier *et al.* 2014) and embryo quality (Edwards *et al.* 2016). Not only is the fertility of breeding females affected by these factors, but climatic (Rhind *et al.* 2001) and nutritional variation during pregnancy also affect ovarian development of the progeny produced. As reviewed by (Rhind *et al.* 2001), a variety of environmental factors, including thermal stress, exposure to endocrine disruptors, overexposure to glucocorticoids and steroids, as well as altered maternal nutrition affect fetal programming of the reproductive axis, which subsequently alters reproductive productivity in adult life. Birth type, and sex of the co-twin further influences reproductive development of the progeny *in utero* (Kelly *et al.* 2017). The number of follicles, cumulus-oocyte complexes (COCs) recovered and blastocyst development were greater in twin ewe lambs compared with singletons (Kelly *et al.* 2017). As a result of these factors, phenotypic variation in reproductive development and potential is inevitable within genotypes and within litters and, therefore, reproductive potential cannot be predicted based solely on genotypic markers. Ultimately, nutrition is the biggest limiting factor for commercial enterprises because the environment plays a crucial role in feed quality and availability and insufficient feed has critical consequences on reproductive development (Table 1). Therefore, the development of phenotypic markers of reproductive performance will increase the accuracy with which ewe fertility and fecundity can be predicted as it will account for environmental conditions the animal has previously been exposed to.

Table 1: Comparison of studies implementing nutritional challenges during pregnancy and the subsequent effects on ovarian development

Study	Breed	Nutritional Challenge	Timing	Reproductive Parameter
(Rae *et al.* 2002)	Mature Scottish Blackface ewes	50% maintenance requirement	Days 0- 95	Reduced number of ovulations
(Rae *et al.* 2001)	Mature Scottish Blackface ewes	50% maintenance requirement	Days 0- 95	Fewer germ cells
				Delayed germ cell maturation
				Delayed onset of meiosis
(Kotsampasi *et al.* 2009)	Chois ewe	50% maintenance requirement	Days 33-100	Delayed pubertal onset
			Days 33-100	Reduced corpora lutea
			Days 0 -33	Increased number small follicles
(Long *et al.* 2010)	Rambouillet x Columbia ewe	50% maintenance requirement	Day 28- 79	Reduced fertility in ovine progeny

(Gunn *et al.* 1995)	Scottish Blackface ewe	Low concentration nutrition	Day 0 -100	Increased embryo and foetal losses
(Da Silva *et al.* 2003)	Dorset horn x mule	75 g/day weight gain	Days 0- 100	Fewer primordial follicles Fewer total follicles

Assisted reproductive technologies

Assisted reproductive technologies (ART's) are a means of improving the performance of the Australian sheep flock (both Merino and meat breeds), as they enable rapid genetic improvement. ARTs include artificial insemination, *in vivo* and *in vitro* embryo transfer (Armstrong *et al.* 1997; Amiridis and Cseh 2012). Multiple ovulation and embryo transfer (MOET) involves stimulating donor ewes to super-ovulate using equine chorionic gonadotrophin (eCG) or follicle stimulating hormone (FSH) (Boni *et al.* 2008; Kaya *et al.* 2018). The ewes are artificially inseminated using semen collected from superior, elite males, and the resulting embryos collected and transferred to recipient ewes. This method is therefore restricted in terms of male genetics being utilised (Amiridis and Cseh 2012). To allow for more genetic manipulation, mature *in vitro* fertilisation and embryo transfer (MIVET) was developed to generate embryos *in vitro.* By doing so, different cohorts of oocytes from the same donor can be fertilised with different sire genetics prior to transfer into recipient ewes, thus enabling widespread transfer of genetics (Morton 2008). Both MOET and MIVET offer a generation interval of approximately 12 months (Amiridis and Cseh 2012). The use of juvenile animals can further reduce the generation interval (Armstrong 2001; Morton 2008; Granleese *et al.* 2015). As such, juvenile *in vitro* fertilisation and embryo transfer (JIVET) protocols have been developed to enable oocytes to be harvested, and embryos and lambs produced, from juvenile ewe lambs (Earl *et al.* 1995; Armstrong 2001). This can decrease the generation interval by up to 6 months and further improve the rate of genetic gain (Armstrong 2001; Morton 2008). Specifically, collection of oocytes from 3- to 4-week old lambs results in the most rapid rate of genetic gain, and enables high concentrations of selection intensity in female lambs (Morton 2008; Granleese *et al.* 2015).

Value and limitations of assisted reproductive technologies

The value of using ARTs, together with the implementation of either natural mating or AI, has been investigated within Australian farming systems (Granleese *et al.* 2017). Utilising MOET with either natural mating or AI yielded an extra 39% genetic gain. The use of JIVET on top of this strategy increased genetic gain by an additional 1% (Granleese *et al.* 2017). An increase in genetic gain of 9-34% is achievable when the above mentioned strategies are utilised alongside genomic selection (Granleese *et al.* 2017). Although there are clear benefits for the application of ART's within the sheep industry, adoption of MOET, MIVET and JIVET is limited by unpredictable variation between individuals in their response to ovarian stimulation protocols as well as the number and quality of *in vivo* or *in vitro* embryos produced (Loi *et al.* 1998; Ptak *et al.* 2003; Morton *et al.* 2005; Amiridis and Cseh 2012).

Using JIVET as an example, Kelly *et al.* (2005) reported the mean number of oocytes collected from juvenile lambs undergoing JIVET across five generations varied from 66 to 165. Similarly, Gou et al. (2009) reported large variation in cleavage rate (50.7 to 71.3%) and the number of transferred embryos developing into fetuses (11 to 45.9%).

Underlying causes of variability in ARTs

As previously discussed, there is a multitude of factors responsible for the variation in donor and recipient performance in ARTs (Daly *et al.* 2020). These factors can be described as endogenous factors (genetics, follicular status) (Amiridis and Cseh 2012) and exogenous factors relating to nutrition and season, as well specifics of the super ovulatory treatment (hormone injected, dose rate and timing of the dose). Additionally, and discussed in detail later, phenotype also plays a role in individual variation. These factors affect the ovarian and intra-follicular environment, affecting oocyte maturation and, thus, the production of developmentally competent oocytes and embryos.

Reproductive losses are greater in cattle used as embryo recipients of *in vitro* produced embryos rather than those undergoing artificial insemination (Peterson and Lee 2003), and in ewes pregnancy rates of 75% were achieved in naturally mated ewes compared with 58% when used as embryo recipients of *in vitro* produced embryos (Thompson *et al.* 1995). In addition only 42% of *in vitro* embryo production (IVP) embryos survived to day 60 in recipient ewes (Thompson *et al.* 1995). Similarly, when embryos from 6 – 8-week-old ewe lambs were transferred into recipients, pregnancy rates were only 54% after 60 days, with fewer than 40% of recipients carrying their conceptus to term (Gou *et al.* 2009). These losses occur due to the death of embryos following their transfer into recipients (Armstrong *et al.* 1997; Ptak *et al.* 1999; Morton 2008), and generally occur before or around the time of maternal recognition of pregnancy (de Brun *et al.* 2016). This suggests that either poor quality embryos are being produced as a result of ovarian stimulation or recipient animals are not able to provide an optimal environment for pregnancy establishment (Daly *et al.* 2020). The use of younger animals is also challenging due to oocytes collected from juvenile animals being less developmentally competent compared with their adult counterparts.

The quality of the oocytes produced by donor ewes is a key contributing factor to the success of ARTs (de Souza-Fabjan *et al.* 2014). Developmentally competent oocytes are paramount to the success *in vivo* or *in vitro* fertilisation, and subsequent *in vitro* embryo culture (de Souza-Fabjan *et al.* 2014). Similarly, the quality of the recipient used to receive the embryo contributes to the success of transfer (Broadbent *et al.* 1991). The stage of embryo development and the synchronisation of the recipient is critical, asynchrony by as little as 2 days has negative consequences for the development of the embryo, which significantly impacts pregnancy rates (Geisert *et al.* 1991). Similarly, circulating concentrations of progesterone in the recipient also affect embryo survival (Geisert *et al.* 1991; Clemente *et al.* 2009; Lonergan and Forde 2015), further demonstrating the importance of the uterine environment. It is evident that the success of ARTs depends on oocyte developmental competence and the uterine environment, with appropriate management of both donors and recipients vital to overcoming variation in the success of ART (Thompson *et al.* 1995). Identifying phenotypic marker/s of ovarian function, which accurately predict the quality of the follicle-oocyte complexes present as well as the status of the ovarian-uterine environment at the start of ART and mating protocols could improve the overall success of these programs.

Phenotypic Markers of ovarian function

Proper development of the ovarian environment allows for successful ovulation and therefore pregnancy establishment and maintenance. As discussed, all of these factors are influenced by pre and neo-natal challenges, particularly nutritional challenges (Rhind *et al.* 2001) resulting in significant variation in individuals. Genomic selection is not sufficient to identify reproductively viable animals, as this method does not account for external challenges. Current, persistent variation in the response of individual to ovarian stimulation protocols is impeding the adoption of ARTs within the Australian sheep industry, and as such there is a need to better identify ewe lambs for breeding purposes. Therefore, a reliable phenotypic marker that accurately predicts which animals are likely to establish and maintain pregnancy, as well as those likely to produce numerous high-quality eggs and embryos would be of enormous benefit. Phenotypic markers should be easy to measure and be both predictive and representative of ovarian and uterine potential of any individual. A single phenotypic marker would be ideal, thus enabling the selection of animals to be used for natural mating and AI programs, as well as the selection of donors and recipients in more advanced reproductive technologies.

Antral Follicle Count

The number of antral follicles present on the surface of the ovary, antral follicle count (AFC), is a reliable phenotypic marker of fertility in animals. AFC is a mildly invasive method of assessment, as it utilises transvaginal ultrasound probes to visualise the number of antral follicles present on the ovary (Fleming *et al.* 2015). The accuracy of this method is variable as it depends on both the probe used and the experience of the technician performing the procedure (Fleming *et al.* 2015). Even so, in cattle, AFC has proven to be an accurate marker of fertility outcomes for AI and other more advanced embryo technologies (Ireland et al., 2008; Silva-Santos et al., 2014a; dos Santos et al., 2016). Ward *et al.* (2006) determined that animals with very high AFC produce more embryos after superovulation, but that the proportion of high-quality embryos was lower than obtained from cows with a low AFC. Although literature regarding the relationship between AFC and fertility is limited in sheep, AFC has been shown to predict the quality of oocytes, evaluated by *in vitro* developmental competence, collected from 40 - day old, Sarda ewe lambs (Torres-Rovira et al., 2014).

Repeatability and variation

In both *Bos indicus* and *Bos taurus* cows, individual variation in the number of pre antral and antral follicles is high (Erickson 1966; Burns *et al.* 2005; Silva-Santos *et al.* 2011; Silva-Santos *et al.* 2014b). There was an approximate seven-fold variation in the maximal number of follicles 3mm or greater between cattle in the same herd (Burns *et al.* 2005). Even so, an extensive study by Burns *et al.* (2005) assessed the repeatability of AFC within individual cows across a variety of parameters (Table 2). AFC repeatability was extremely high at 0.95 when determined at approximate wave emergence within the same oestrous cycle or during consecutive oestrous cycles of an individual (Burns *et al.* 2005). When extended over two seasons AFC had a repeatability of 0.65 in Holstein-Friesian dairy cows (Mossa *et al.* 2012). Even when AFC was determined at an unknown stage of follicle growth repeatability was still moderate (0.37; (Gobikrushanth *et al.* 2017). Cruz *et al.* (2018) concluded that a single measurement of AFC, at an unknown stage of the oestrous cycle, could be used to select more fertile replacement heifers. AFC is highly repeatable within individuals, thereby making it an excellent phenotypic marker for predicting reproductive potential.

Table 2: Repeatability of maximal number of antral follicles (>3mm in diameter) during follicular waves in cattle from Burns et al., 2005

	No. of animals	No. of follicle waves	antral follicles per follicular wave	Repeatability
Season				
Spring	5	18	8–39	0.86
Summer	30	96	11–54	0.95
Fall	9	24	12–38	0.94
No. of follicular waves per oestrus cycle				
Two	28	86	8–54	0.93
Three	7	28	11–44	0.95
Lactational status				
Lactating	20	58	12–44	0.94
Non-lactating	24	80	8–54	0.93
Heifers (10-13 months old)	17	56	8–42	0.92
Cows (3 – 7 years old)	27	82	11–54	0.96
Overall	44	138	8–54	0.95

Relationship with ovarian characteristics and fertility

In cows, AFC is positively correlated with the size of the ovarian reserve (Ireland *et al.* 2008; Silva-Santos *et al.* 2014a) and correlates to oocytes and embryos quality following *in vitro* maturation (Silva-Santos *et al.* 2014a; dos Santos *et al.* 2016). Numerous studies have identified that AFC can be used as a marker for embryo production in cattle (Table 3).

Table 3: Relationship between high or low AFC and parameters of ovarian reserve and quality in cattle

Breed	Parameter	High AFC	Low AFC
Holstein[1]		≥25	≤15
	Peak AFC/wave	39.61 ± 2.3^{a}	11.95 ± 1.2^{b}
	Healthy follicle	29056 ± 4564^{a}	6016 ± 1685^{b}
	Polyovular	66080 ± 53965^{a}	352 ± 217^{b}
Nelore[2]		≥57	≤21
	Primordial	31639 ± 22398	18766 ± 18493
	Primary	12714 ± 14341	9116 ± 9359
	Secondary	3996 ± 3288	5155 ±6794
	Total	48349 ± 30149^{a}	33037 ± 31710^{b}
Aberdeen Angus[2]		≥45	≤13
	Primordial	19 777 ± 25412	11273 ± 20713
	Primary	11337 ± 11568	15617 ± 1383
	Secondary	4174 ± 2893	3591 ± 3200
	Total	35288 ± 3839^{a}	30481 ± 43360^{b}
Nelore cattle[3]		≥40	≤7
	Total oocytes recovered	1109^{a}	101^{c}
	Viable oocytes (%)	80.07^{a}	71.29^{b}
	Blastocyst rate (%)	41.97^{a}	13.04^{c}
	Proportion vitrifiable	81.23^{a}	58.33^{c}
Bradford[4]		≥40	≤10
	Total oocytes recovered	738^{a}	116^{b}
	Viable oocytes/ OPU	21.65 ± 10.5^{a}	3.2 ± 2.44^{b}
	Blastocyst rate (%)	16.53	9.45
	Proportion vitrifiable	67.21^{a}	36.36^{b}

[1]Ireland *et al.*, 2008 [2]Silva-Santos *et al.*, 2014a [3]Dos Santos *et al.*, 2016 [4]Silva-Santos *et al.*, 2014b

There is inconsistency in the literature in regard to the association between AFC and pregnancy rate, which may limit the effectiveness of AFC as a marker of on-farm fertility. In two–year-old Bradford heifers, puberty occurred later in heifers with a low (418.7 ± 9.0 days) compared with medium (379.2 ± 8.4 days) or high (383.4 ± 8.7 days) AFC, with growth and development also reduced in low AFC heifers (Cruz *et al.* 2018). This resulted in reduced insemination rates of Bradford heifers in the low AFC group (low= 2/16; med= 12/17 and high =7/17) (Cruz *et al.* 2018). It is therefore possible, that AFC may allow the selection of more fertile Bradford heifers as early as weaning (Cruz *et al.* 2018). Additionally, pregnancy rates to first AI, after 6 weeks of AI and overall were 22%, 14% and 12% higher for cows with more 2-3 mm antral follicles (Martinez *et al.* 2016). To add to the ambiguity around the relationship between AFC and pregnancy rates, de Moraes *et al.* (2019) reported higher pregnancy rates in cows with a low AFC (57.7%) compared with those with a medium (47.9%) or high (49.7%) AFC. Together, these data imply that: 1) additional factors likely play a role in pregnancy rates; 2) there may be an optimal range for AFC for improved pregnancy rates; and 3) there are differing relationships to oocyte and embryo quality.

There are limited studies relating AFC to reproductive function in sheep, and those available generally assess the response within an assisted reproductive protocol. Importantly, Veiga-Lopez *et al.* (2005) demonstrated a positive correlation between the number of 2-3 mm follicles present coincident with the first FSH injection and ovulation rate. Similarly, Gonzalez-Bulnes *et al.* (2000) determined the number of small follicles present on the ovary (AFC) at the beginning of gonadotrophin treatment was related to the ovulatory response. Together these studies suggest the presence of dominant follicles on the ovary at the time of treatment affects the outcome. An extensive study by Mossa *et al.* (2007) found that following superovulation, ewes with few follicles (<8 follicles on a given day) had fewer corpora lutea, oocytes and embryos as well as fewer good quality embryos. However, proportionally there was no relationship between follicle number and embryo quality (Mossa *et al.* 2007), suggesting that AFC predicts the ovarian response to exogenous gonadotrophins not embryo quality.

Relationship between Antral follicle count and AMH

AFC has been used as a key marker of ovarian reserve, with the repeatability and reliability of the results indicating applicability as a phenotypic marker (Bancsi *et al.* 2002). Many studies to date have compared AMH and AFC as markers of ovarian reserve, with recent studies concluding that AMH is a more reliable and accurate predictive marker (reviewed by Fleming *et al.* (2015). When comparing the ability of AMH and FSH to accurately represent the AFC for a particular woman undergoing IVF, AMH was by far superior (Jamil *et al.* 2016). Therefore, a single measure of AMH may be best suited for the prediction of reproductive viability in sheep, as AMH is representative of AFC as well as other reproductive parameters (van Rooij *et al.* 2002; Jamil *et al.* 2016). Across multiple species, the link between AFC and AMH has been extensively studied, and with good reason. (Ireland *et al.* 2008) determined a strong correlation (0.8-0.9) between AMH and both ovarian size (ovary weight, height and length) and the total number of morphologically healthy oocytes in the ovary. Furthermore, Ireland *et al.* (2011) determined in cattle, that like AFC, a single measurement of AMH taken on any day of an oestrous cycle can reliably predict the number of healthy follicles and oocytes present in an ovary, and potentially the future fertility of young adult cattle (Ireland *et al.* 2011). A positive correlation between AFC and AMH has been observed in numerous bovid genotypes; Holstein (r=0.79, r=0.66; (Rico *et al.* 2009; Baldrighi *et al.* 2014), Murrah (r=0.62; (Baldrighi *et al.* 2014) and Zebu (r=0.54) (Stojsin-Carter *et al.* 2016). Importantly, plasma AMH concentrations correlated positively with AFC in pre-pubertal sheep (Torres-Rovira *et al.* 2014), with pregnancy rates higher following first mating in ewe lambs with higher AFC and AMH prior to puberty (Torres-Rovira *et al.* 2016). Moving forward we suggest AMH as the preferred phenotypic marker of ovarian reserve and reproductive potential Discussed in detail below, we believe AMH has a greater potential as a marker of fertility, because it reflects antral follicle count and has major actions within multiple stages of reproductive function in the ewe.

Development of the hypothalamic-pituitary- gonadal axis and reproductive function in the ewe

Understanding reproductive function in the ewe is fundamental to the selection of an appropriate phenotypic marker of fertility. This section will begin with an overview on the mechanisms which regulate reproductive function, before focussing on the stages of reproductive function where AMH has strong actions. By doing so we can demonstrate the appropriateness of utilising AMH as a phenotypic marker of reproductive function in the Australian sheep industry.

Reproductive function in the ewe is controlled through communication and feedback within the hypothalamic-pituitary-gonadal (HPG) axis (Figure 2). Gonadotrophin releasing hormone (GnRH) is produced in the hypothalamus and is responsible for the release of the gonadotrophic hormones, Luteinising Hormone (LH) and FSH from the anterior pituitary gland (Clarke and Cummins 1984). Gonadotrophin hormone secretion is driven by the pulsatile release of GnRH from the hypothalamus (Edwards and Juengel 2017). The main characteristic of the ewe reproductive cycle is the changes in pulse amplitude and frequency of GnRH which are primarily controlled through inhibitory and stimulatory effects of ovarian secreted steroids and peptides (Karsch *et al.* 1987). LH and FSH together with steroids (oestradiol and progesterone) and growth factors of ovarian origin are responsible for the control of follicle growth and ovulation (Edwards and Juengel 2017). Specifically, oestrogen alters GnRH and LH secretion in two ways; low concentrations of oestrogen, in conjunction with progesterone inhibit GnRH and LH secretion, but as oestrogen concentrations increase they stimulate GnRH and LH secretion (Karsch *et al.* 1987; Clarke 1995; Karsch and Evans 1996). These positive and negative feedback mechanisms modulate the growth and development of ovarian follicles, discussed in detail later. Below we discuss the establishment of the HPG axis through pre-pubertal changes and pubertal onset.

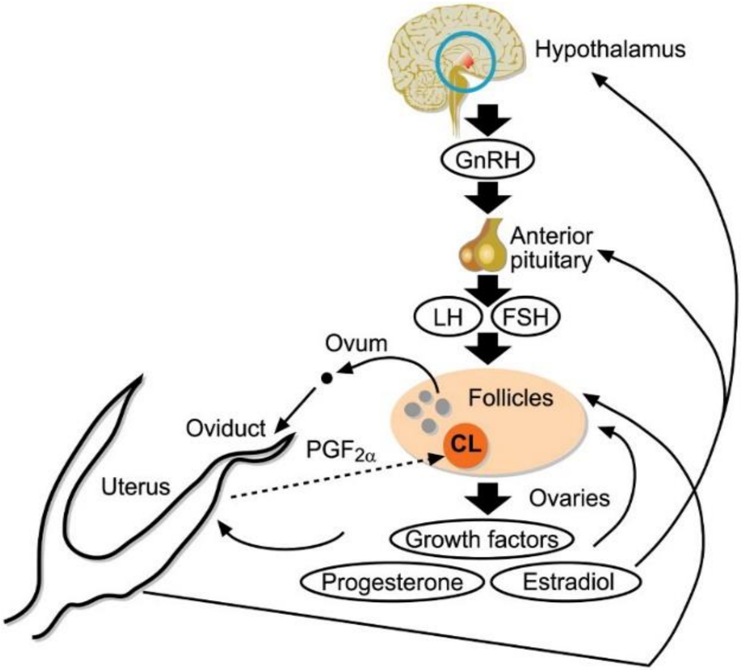

Figure 2: Schematic outlining the relationship between the hypothalamus, pituitary gland and ovaries and the effects on the reproductive tract from Edwards and Juengel 2016

Pre-pubertal ovarian development

Pre- pubertal endocrine changes

The ovine hypothalamus is sensitive to the effects of steroids as early as mid- gestation (Jackson *et al.* 2013), suggesting early development of the HPG axis. Early post- natal steroid production completes the actions of prenatal steroids to differentially organise the

GnRH surge mechanism (Jackson *et al.* 2013). These changes facilitate the growth and development of the antral follicle pool. In Merino lambs, prior to the pubertal increase, intra-ovarian progesterone and oestradiol concentrations peak at birth. Progesterone concentrations rise from birth to two weeks of age before declining and fluctuating minimally until 10 weeks of age (Tassell *et al.* 1978). Oestradiol was also highest at birth, with concentrations then nearly undetectable concentrations until weeks 6 and 8, before dropping significantly again at 10 weeks of age (Tassell *et al.* 1978).

Pre-pubertal gonadotrophin patterns

In the ewe lamb, post- birth, pre-pubertal changes in gonadotrophin secretion likely differ between breeds. In the Merino, FSH and LH both fluctuate between birth and 10 weeks of age (Tassell *et al.* 1978). Specifically, FSH gradually increased up until 8 weeks of age, after which a slight decrease occurred (Tassell *et al.* 1978). This pattern was shared in Ouled Djellell ewe lambs, with a spike in FSH occurring at 10 weeks of age (Mahdi and Khallili 2008), followed by another small peak at 18-weeks and then relatively stable production (Mahdi and Khallili 2008). LH changes with greater fluctuation early in neonatal life of medium wool Merinos; however, the patterns of increase are similar to that of FSH (Tassell *et al.* 1978). Ouled Djellell ewe lambs showed similar, moderate changes in LH until 8 weeks after which an increase in circulating concentrations occurred (Mahdi and Khallili 2008). A moderate peak in LH occurred at 18-weeks, followed by a decline until a pre-ovulatory LH surge occurs at approximately 26 weeks of age (Mahdi and Khallili 2008). In the days preceding the first LH surge both the frequency and amplitude of LH pulses increased and fluctuated between 0.5 and 5.0 ng/ml from 18 days prior to this first LH surge in a Suffolk ewe lamb (Ryan *et al.* 1991). Coinciding with the LH surge a distinct peak in FSH occurred. However, unlike LH, little fluctuation in amplitude was seen in the 18 days prior to this surge, the change in frequency of LH pulsing was characteristic of the transition from pre-pubertal to mature ovarian cyclicity (Ryan *et al.* 1991).

Pre-pubertal Ovarian Steroid patterns

Concentrations of progesterone and oestradiol are almost undetectable in the pre-pubertal ewe lamb. This remains the case for progesterone until after the first LH surge, it takes until a second GnRH and LH surge for progesterone to achieve an amplitude similar to that observed in the mature ewe (Ryan *et al.* 1991). Extending from Tassell *et al.* (1978), oestradiol concentrations are undetectable in the ewe until as late as 16 days prior to the first ovulatory LH surge (Ryan *et al.* 1991). This first and only sustained rise in oestradiol is followed closely by the initial pre-ovulatory surge in both LH and FSH (Ryan *et al.* 1991).

Pre-pubertal follicle patterns

Unlike other domestic species, the ovaries of ewe lambs can contain antral follicles at birth (Kennedy *et al.* 1974; Tassell *et al.* 1978; Mahdi and Khallili 2008). However, as differences in the number of follicles containing oocytes has been reported between Blackface and Welsh ewe lambs at birth (Land 1970) it is likely that breed differences in pre-pubertal follicle patterns exist. Post-birth changes in ovarian follicle size and number coincide with changes in ovarian steroid and pituitary gland gonadotrophin secretion. Maximum antral follicle diameter was greater at birth than at 1 week of age, which is consistent with higher oestradiol concentrations found in both Merino and Ouled Djellell ewe lamb ovaries at birth (Tassell *et al.* 1978; Mahdi and Khallili 2008). Up until 16 weeks of age, the diameter of the largest antral follicle fluctuated between 3 and 4 mm, after which maximum follicle diameters increased and peaked coincident with the pre-pubertal LH surge (Mahdi and Khallili 2008). The total number of antral follicles increased significantly between birth and 10 weeks of age, after which the number of follicles dropped. Between 18 and 26 weeks the size of the antral follicle pool remained relatively unchanged, with small fluctuations in the number of 1 – 2 mm follicles occurring. Coincident with this, the number of 2 – 3 mm follicles peaked at 18-weeks of age, fluctuated until 24 and then peaked again at 26, coinciding with the initiation of the first ovulatory surge (figure 3) (Mahdi and Khallili 2008).

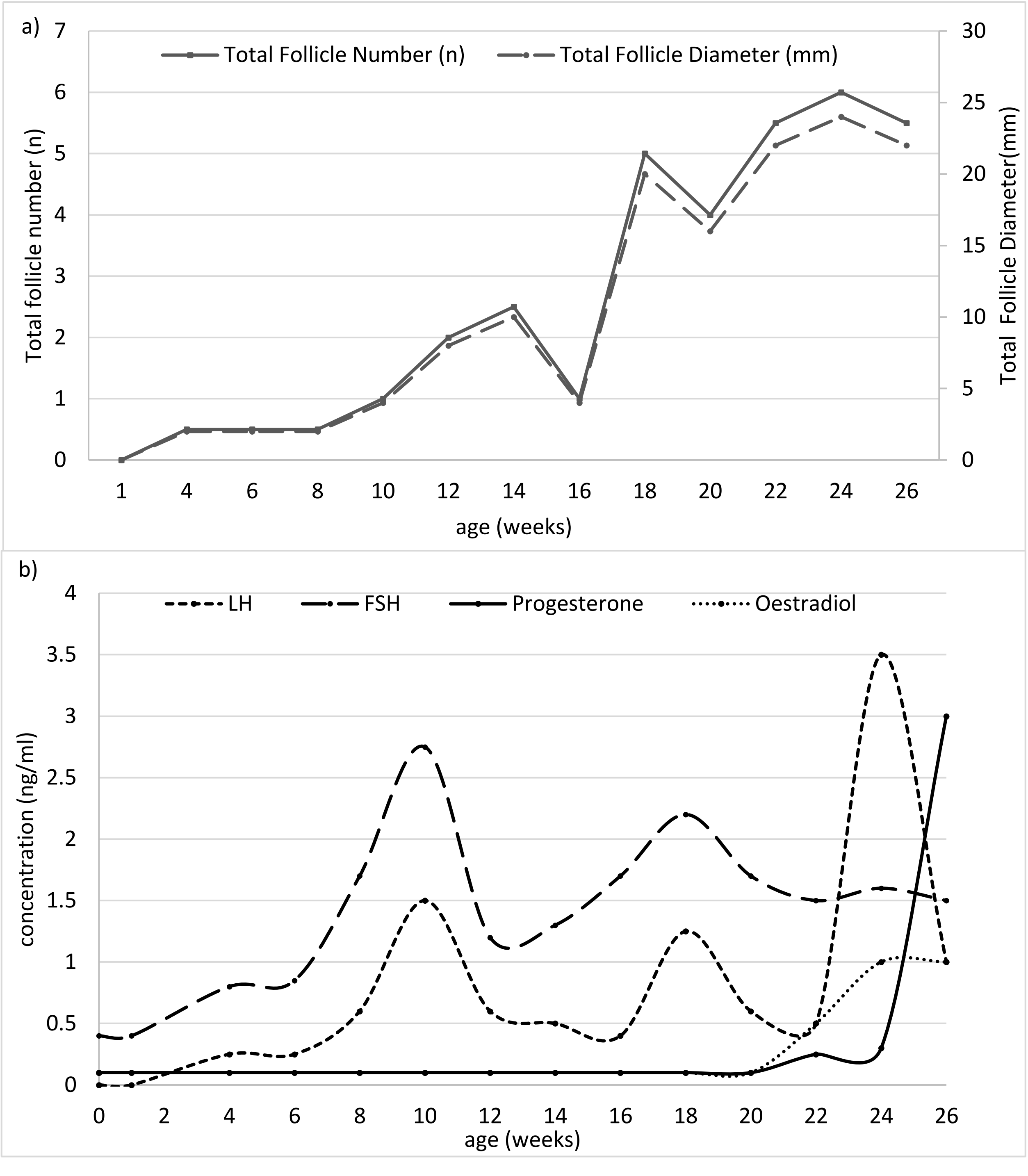

Figure 3: (a) Mean number of follicles >3mm and diameter of follicles and (b) Mean circulating progesterone, oestradiol, luteinising hormone (LH) and follicle stimulating hormone (FSH) from birth to 26 weeks of age in 10 Ouled Djellel ewe lambs. Modified from Mahdi and Khallili 2008. First ovulation occurred at 24 weeks of age

Regulation of GnRH

Considerable evidence is now available supporting the hypothesis that a group of neurons, kisspeptin, neurokinin B and dynorphin (KNDy) neurons, is responsible for synchronising the pulsatile secretion of GnRH in sheep (Nestor *et al.* 2018). Essentially, the KNDy neuron group consists of two stimulatory peptides; kisspeptin and neurokinin B (NKB) and one inhibitory peptide; dynorphin (Nestor *et al.* 2018). Kisspeptin plays a stimulatory role in the initiation of this increase in GnRH pulsing, and Redmond et al. (2011) reported an increase in the frequency and amplitude of LH pulsing 15 minutes after 28-week old ewe lambs received an injection of 20 ug of kisspeptin. Therefore, as summarized by (Nestor *et al.* 2018), kisspeptin was the driver of the GnRH secretion during a pulse, and NKB and dynorphin acted within this network to initiate and terminate each pulse. At the beginning of a pulse, initial release of NKB initiated a positive feedback loop, rapidly increasing KNDy neural activity, kisspeptin release and therefore the GnRH secretion (Nestor *et al.* 2018). Within minutes, dynorphin release held KNDy activity, for approximately 5 minutes before terminating kisspeptin release, and the GnRH pulse. Whilst the initiation of reproductive cyclic activity was controlled through KDNy neural networks, ongoing regulation of the tonic and surge action of GnRH and LH was through feedback actions of ovarian steroid hormones, oestrogen and progesterone. These steroid hormones also act to regulate KNDy neurons. Oestrogen acts throughout the follicular phase decreasing kisspeptin release, while progesterone acts throughout the luteal phase to inhibit the pulse frequency through stimulation of dynorphin (Nestor *et al.* 2018).

Pubertal Onset

The onset of puberty is stimulated by an increased frequency of LH pulsing which is stimulated by an increased release of GnRH from hypothalamic neurons (Redmond *et al.* 2011). The pulsatile release of GnRH from the hypothalamus, culminates in the release of LH from the pituitary gland (Clarke 1995) and once the initial LH surge has occurred is controlled through steroid production from the ovary. Oestradiol enhanced GnRH neuron responses to kisspeptin, through increasing ovarian sensitivity to kisspeptin (Pielecka-Fortuna *et al.* 2008). This feedback mechanism initiates the pre-ovulatory GnRH/LH surge (Jackson *et al.* 2013). Using incremental oestradiol implants from the late luteal phase through to the peak of the follicular phase, Karsch and Evans (1996) demonstrated a progressive decline in GnRH as oestradiol concentrations increased prior to the GnRH surge (Karsch and Evans 1996). However, once oestradiol concentrations reach a critical concentration it stimulated increased pituitary gland sensitivity to GnRH, by increasing GnRH receptors in addition to increasing the hypothalamic secretion of GnRH (Turzillo and Nett 1999). Throughout the luteal phase and early follicular phase, negative feedback actions of oestradiol and progesterone restrict the LH surge (Clarke 1995). As the corpus luteum regresses, so does the production of progesterone which reduces the negative feedback on GnRH receptor expression (Turzillo and Nett 1999), this causes the frequency of GnRH pulses to increase initially (Karsch and Evans 1996). As the oestrous cycle transitions into the early follicular phase, oestradiol concentrations rise, increasing GnRH frequency, whilst decreasing amplitude (Karsch and Evans 1996). During the late follicular phase, the continued growth of pre-ovulatory follicles causes significant increases in oestrogen concentrations (Clarke 1995). Oestrogen at these high concentrations exerts positive feedback on the pituitary gland, stimulating an increase in LH production (Clarke 1995).

Anti-Mullerian Hormone

The ovarian environment is governed by complex interactions within the hypothalamic-pituitary gonadal axis, culminating in the onset of regimented, sequential oestrous cycles once puberty is attained, as described above. The hormones, growth factors and gonadotrophin within the hypothalamic-pituitary-gonadal (HPG) axis are tightly regulated throughout the oestrous cycle, and AMH is one hormone involved closely in this regulation at multiple levels. This section of the review will detail the establishment of the ovarian follicle pool, as well as the oestrous cycle and how AMH acts within these levels. By doing so, we can establish the value of AMH as a phenotypic marker of fertility. Additionally, AMH has been shown to reliably predict fertility in a number of production settings in cattle. Limited evidence has been provided for its use in sheep as a phenotypic marker of fertility, and will be discussed in this review.

Anti-Mullerian hormone (AMH) is a 140-kDa glycoprotein dimer of the transforming growth factor (TGF)-β family, and is only expressed in the gonads (Monniaux *et al.* 2013). AMH is a highly conserved molecule composed of identical 70-kDa monomers and signals through heteromeric receptor complexes with type I and II receptor serine/threonine kinase (Campbell *et al.* 2012). Upon ligand binding to the type II receptor, the type I receptor is recruited which forms a heterotetramerix receptor complex, activating the type I receptor through transphosphorylation which causes downstream signalling via Smad proteins (Durlinger *et al.* 2002b).

AMH role in ovarian development

There are three key stages of ovarian development as illustrated in Figure 4 (Juengel et al., 2002); days 17 – 40, days 40- 74 and days 75- 100 of fetal life and AMH plays a strong role in two of these.

Days 17-40

- migration of germ cells
- Gonadal sexual differentiation as determined by anti-Müllerian hormone
- germ cell nests
- ovigerous cord becomes apprent

Days 40-74

- maximum number of germ cells
- increasing from 50 000 to 800 000
- first primordial follicles apprent

Days 75-100

- Most germ cells lost by atresia
- first primary (growing) follicles observed potentially regulated by anti-Müllerian hormone

Figure 4: AMH role within key stages of fetal ovarian development adapted from McNatty et al.,1995

The first identified function of AMH was during sexual differentiation of the fetus (Rey *et al.* 2003). Migration of germ cells begins between days 17 and 22 of gestation, with gonadal sexual differentiation completed by day 32 (McNatty *et al.* 1995). Specifically, AMH is produced by the testes of a growing male fetus, and is responsible for the regression of the Müllerian ducts (Rey *et al.* 2003). Mammalian sex determination and differentiation involves development of the gonads and the reproductive tract in contrasting ways (Munsterberg and Lovellbadge 1991). While the genital ridge develops as a result of a testes-determining gene on the Y chromosome and the absence of the Y generates an ovary, the reproductive tracts include both Wolffian and Müllerian ducts prior to sex determination (Munsterberg and Lovellbadge 1991). For female sex differentiation, the Wolffian duct system degenerates (Munsterberg and Lovellbadge 1991) and the Müllerian ducts give rise to the fallopian tubes, uterus and the upper third of the vagina in the growing female foetus (Munsterberg and Lovellbadge 1991; Rey *et al.* 2003).

Development of the mammalian ovary has previously been described in detail by (Smith *et al.* 2014). In the ewe lamb, development of the ovary and the establishment of the primordial follicle pool is completed prior to birth. From day 75 the initial stages of follicle formation begin. Oogonia in the germ cell nests enter meiosis to become oocytes that arrest at diplotene of prophase I (Nilsson *et al.* 2011). Each follicle contains a single oocyte surrounded by granulosa and theca cells and the first formation of a follicle is termed, primordial follicle, and the formation of these follicles is called follicle assembly (Nilsson *et al.* 2011). This stage is critical for the formation of the ovarian follicle pool or reserve, with disruptions during this period likely to reduce the size of the ovarian reserve. The development of the ovarian follicle pool is complete after 100 days gestation in sheep (Smith *et al.* 2014).

There are a variety of extracellular signalling molecules that regulate the development of the ovary, and particularly follicle assembly (Skinner 2005). Changes in progesterone and oestrogen in the developing ovary help regulate the timing of the assembly process, with both of these steroid hormones inhibiting follicle assembly (Kezele and Skinner 2003). *In vitro* culture of 0 day old rat ovaries showed a spontaneous development of primordial to primary follicles; however, 4 day old rat ovaries required stimulus (Kezele and Skinner 2003). Both oestrogen and progesterone at the same concentration significantly inhibited the transition from primordial to primary follicle in 4 day old rat ovaries, but did not affect the 0 day old ovaries (Kezele and Skinner 2003). Kit ligand (KL), tumour necrosis factor alpha (TNFα), basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), bone morphogenix protein 4 (BMP-4), keratinocyte growth factor (KGF), insulin and anti-Müllerian hormone (AMH) are thought to mediate cell-cell interactions in the primordial follicle (Skinner 2005). KL, bFGF, LIF, KGF and BMP-4 play key roles in mediating interactions between pre-cursor theca cells, granulosa cells and the oocyte, for a review see (Skinner 2005). Furthermore, the presence of AMH during follicle assembly is interesting as it is not expressed by the primordial follicle, or the oocyte (Nilsson *et al.* 2011).

Confocal fluorescent microscopy showed AMH localised to stromal cells surrounding oocyte nests (Nilsson *et al.* 2011), and AMH has the capacity to prevent primordial follicle development (Durlinger *et al.* 2002a; Visser and Themmen 2005). AMH was upregulated in day 0 rat ovaries during primordial follicle assembly (Kezele *et al.* 2005) and was the only hormone to have a negative effect on primordial follicle assembly (Nilsson *et al.* 2011).

AMH role within the oestrous cycle

Sheep have a 17 day oestrous cycle, during which follicles grow and develop in a sequential, cyclic manner (Driancourt *et al.* 1985). Day 0 is deemed ovulation, and is characterized by a 12 day luteal phase (days 2 -13) followed by 5 day follicular phase (days 14-1) which culminates after ovulation (Figure 5). There is a set sequence of events; recruitment, selection and dominance and a dependence on both FSH and LH to control these events (Driancourt 2001), with regulation of gonadotrophins through feedback mechanisms from progesterone, oestrogen and inhibin. This governed and sequential growth and development of follicles ultimately controls ovulation, and therefore reproductive potential. Therefore, understanding the mechanisms that control follicle growth and development provide a means to predict fertility of individuals. Outlined below AMH acts within the oestrous cycle at two different stages, making it a fundamental regulator of follicle development.   Across all species there appears to be a highly conserved series of events for follicle development from the primordial stage through to ovulation (Driancourt 2001). (Scaramuzzi *et al.* 1993).

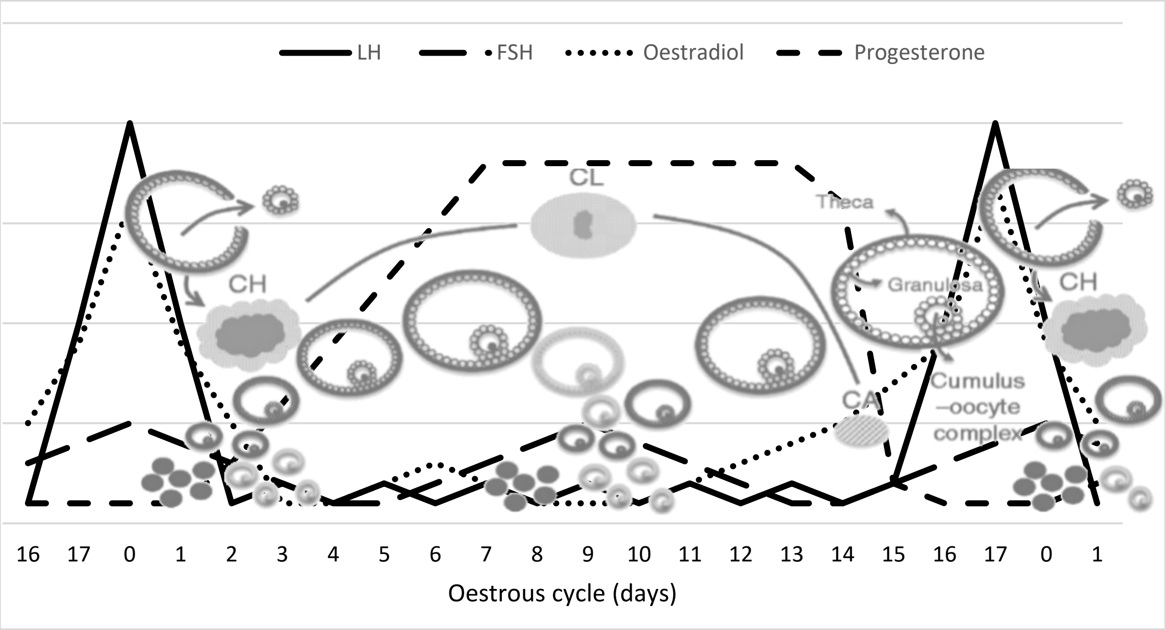

Figure 5: Changes in progesterone, oestradiol, lutenising hormone and follicle stimulating hormone throughout the oestrous cycle in the ewe and accompanying changes in follicle development. Adapted from (Donadeu *et al.* 2012)

In sheep, at recruitment, a cohort of follicles begins transitioning from the antral follicle pool to enter a stage of gonadotrophin dependent growth (Driancourt 2001). From here, follicles switch from being sustained predominantly by FSH, to relying solely on LH for growth (Driancourt 2001). Across most species the transition during selection to the dominant follicle is often the follicle that first acquires LH receptors (Driancourt 2001). During the dominant stage, the preovulatory follicle grows and matures; this also causes the remaining follicles to regress via atresia (Driancourt 2001).

AMH plays an individual regulatory role in follicle recruitment, as a paracrine regulator of primordial follicle activation (Durlinger *et al.* 2002a; Pankhurst 2017; Umer *et al.* 2019). Importantly, it inhibits primordial follicle growth from the ovarian follicle pool by avoiding premature exhaustion of the ovarian reserve (Umer *et al.* 2019). To date, AMH is the only negative regulatory factor for transition from the primordial to primary follicle stage (Visser and Themmen 2014), which prevents premature exhaustion of the ovarian reserve AMH also plays a regulatory role in the selection of dominant follicles (Umer *et al.* 2019); modifying pre-antral/ small antral follicle growth by decreasing their responsiveness to FSH and also dictating the recruitment of the large pre-antral and small antral follicle growth onto the pre-ovulatory stage (Durlinger *et al.* 2002b). Specifically, the response to FSH treatment was highest in small pre-antral follicles with high levels of AMH expression, indicating AMH as a regulator of follicle response(Campbell *et al.* 2012).

The adult ewe ovary contains anywhere from 12,000-86,000 primordial follicles (Driancourt *et al.* 1985). In the adult, non-pregnant ewe between 100 and 400 growing follicles will be present at any time, with between 10 and 40 visible on the surface of the ovary (Driancourt *et al.* 1985). In sheep, the growth of follicles to a diameter of 2 – 2.5mm takes approximately six months (Souza *et al.* 1997), and occurs independently of gonadotrophins (Webb *et al.* 1999). Patterns of follicle growth in the adult ewe occur in three distinct waves throughout the luteal phase (Souza *et al.* 1998).

Across all species expression of AMH is restricted to granulosa cells in females (Rico *et al.* 2009). The pre-granulosa cells of primordial follicles do not produce AMH, but once recruited into the growing pool these cells immediately start to express AMH (Durlinger *et al.* 2002a). AMH was first expressed in the mouse ovary when primary follicles began to appear (day 3-4 old ovary) (Durlinger *et al.* 2002a); AMH was down-regulated in the transition between primordial and primary follicles (Kezele *et al.* 2005), with expression next seen in the granulosa cells of the recruited primordial follicles (Visser and Themmen 2005). AMH was then continually expressed within the follicle until they reached the size where they were FSH independent (Durlinger *et al.* 2002b). With maximal levels of AMH found in the granulosa cells of pre antral and small antral follicles in cattle, sheep and goats (Juengel *et al.* 2021) Due to the release of AMH from the granulosa cells of antral follicles the concentration of AMH in the blood serum was proportional to the number of developing follicles present in the ovary (Dewailly *et al.* 2014). In sheep, western blot analysis determined that AMH was present in the follicular fluid and granulosa cells of small (<2.5 mm), gonadotrophin responsive antral follicles as well as medium (2.3 – 3.5 mm) and large (3.5 to 8 mm) gonadotrophin-dependent antral follicles (Campbell *et al.* 2012) (figure 6). Peak AMH concentrations were found in primordial, primary and secondary follicles (Umer *et al.* 2019) and were maintained in pre-antral and early antral follicles, decreasing as follicle size increased from 2mm (Campbell *et al.* 2012; Veiga-Lopez *et al.* 2012). In mice, cattle and goats, AMH was also localised to granulosa cells of pre-antral and small antral follicles (Durlinger *et al.* 2002b; Monniaux *et al.* 2011; Ilha *et al.* 2016).

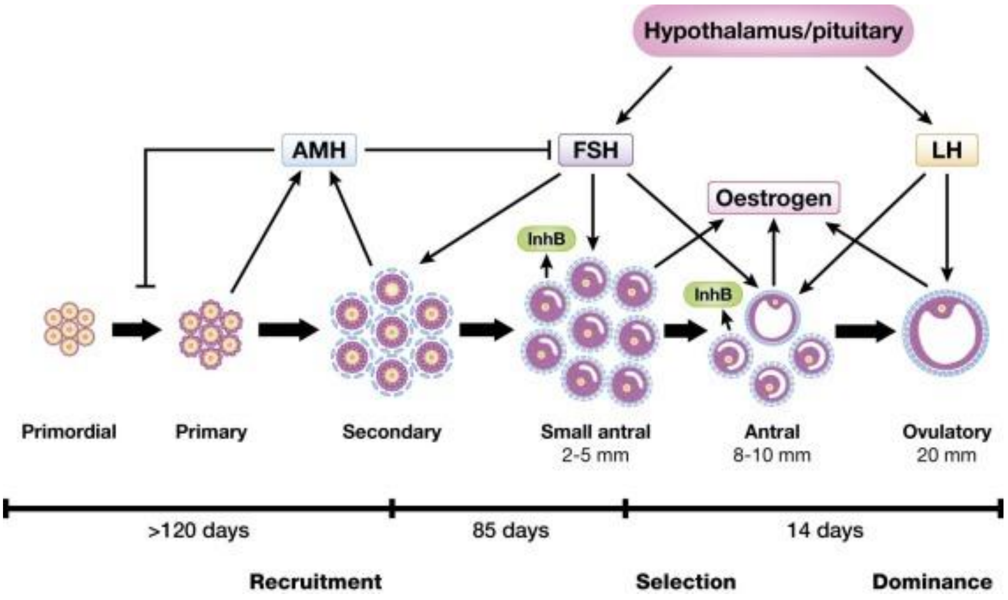

Figure 6: Role of AMH during folliculogenesis. Taken from Fleming et al., 2015

Gonadotrophin regulation of follicle development and relationship to AMH

Gonadotrophins are crucial for the control of follicle growth and recruitment (Skinner 2005; Webb *et al.* 2007; Bartlewski *et al.* 2011). Fundamentally, the sequential need for gonadotrophins, FSH for the recruitment of follicles and LH for the final, pre-ovulatory stages of growth, governs this growth and development (Driancourt 2001). During the luteal phase, LH pulse amplitude and frequency is characteristically low, therefore FSH governs the growth of follicle waves, as seen in peaks between days 1 - 2, 5 - 8 and 11 - 12 of the luteal phase (Souza *et al.* 1998). During the follicular phase, FSH accelerates the rate of pre-antral follicle development, whilst LH has no action at this early stage of development (Ciftci 2014). Granulosa cells on developing secondary follicles (2mm in size) have FSH receptors (McNatty *et al.* 2000), whilst LH receptors were not detected until an antrum formed within the follicle at around 4 mm (Ciftci 2014). FSH primes follicles by increasing sensitivity to LH (Driancourt *et al.* 1985). FSH stimulates the growth and mitosis of fully differentiated granulosa cells of large pre- ovulatory follicles to acquire LH receptors (Baird *et al.* 1991). The development of receptors for LH on granulosa cells of antral follicles increases the sensitivity of the follicle to LH (Driancourt *et al.* 1985). The acquisition of LH receptors on granulosa cells is crucial for the development of the pre-ovulatory follicle, as subsequent growth of the dominant follicle is independent of FSH and relies on oestradiol and its effect on LH (Bartlewski *et al.* 2011). Selection of dominant follicles for further development is controlled through a reduction in FSH (Driancourt 2001). Regulation of the FSH sensitivity of these follicles is controlled by AMH (Visser and Themmen 2005). In mice, *in vivo* and *in vitro* studies suggest that follicles are significantly more sensitive to FSH in the absence of AMH (Visser and Themmen 2005), with preantral and small antral follicle growth also inhibited by AMH, as it reduced their sensitivity to AMH (Durlinger *et al.* 2002b). Therefore, it is clear that there are two key regulatory processes during follicle development, initial follicle recruitment, and also selection of the dominant follicle, with AMH having inhibitory effects at both levels.

Steroid regulation of follicle development and relationship to AMH

Androgens, oestrogens and progestins also regulate follicle growth, through negative feedback effects on pituitary gonadotrophin secretion, affecting GnRH and FSH, and direct modulations of follicular function (Erickson and Danforth 1995; Eppig 2001; Bartlewski *et al.* 2011). Secretion of GnRH is inhibited by ovarian feedback exerted through interactions between estradiol and progesterone acting on the hypothalamus (Scaramuzzi *et al.* 2011). Progesterone is the key steroid governing the luteal phase, and remains in low concentrations in the periphery until day four of the follicular phase. AMH is positively correlated (r=0.88) to progesterone throughout this time, with both progressively declining to lowest values (<1ng/ml for both) at ovulation (Waheeb 2017). Progesterone begins to increase until day three of the luteal phase and remains stable at approximately 20 nmol/ml for the entirety of the luteal phase (Souza *et al.* 1998). Through this period progesterone concentrations were much greater in comparison to AMH concentration (16 ng/ml vs 10 ng/ml respectively) (figure 7) (Waheeb 2017). During the late luteal phase progesterone produced by the corpus luteum inhibits LH release, decreasing pulse frequency and preventing stimulation of oestrogen production (Baird and McNeilly 1981). Unsurprisingly, AMH was negatively correlated to oestradiol (r=-0.74) whereby AMH peaks (11 ng/ml) as oestradiol reaches basal concentrations (1 ng/ml)(figure 7) (Waheeb 2017). Oestradiol and inhibin control FSH secretion through negative feedback at the pituitary level (Scaramuzzi *et al.* 2011). Oestradiol controls the follicular phase, and the daily fluctuations in follicle growth whilst inhibin plays a stronger role in negative feedback of FSH, and only increases as a result of the increase in the number of large antral follicles (Baird *et al.* 1991) with the peak oestradiol around ovulation, when AMH and progesterone are at lowest concentrations (Waheeb 2017). Oestradiol and inhibin produced by the granulosa cells of large antral follicles inhibit FSH production late in the follicular phase of growth (Baird *et al.* 1991; Hunter *et al.* 2004). This concurrent pattern of AMH and progesterone, which can be extrapolated to different timings of peak CL function and small antral follicle development raises remarkable questions into the relationship between the CL, AMH and follicular waves

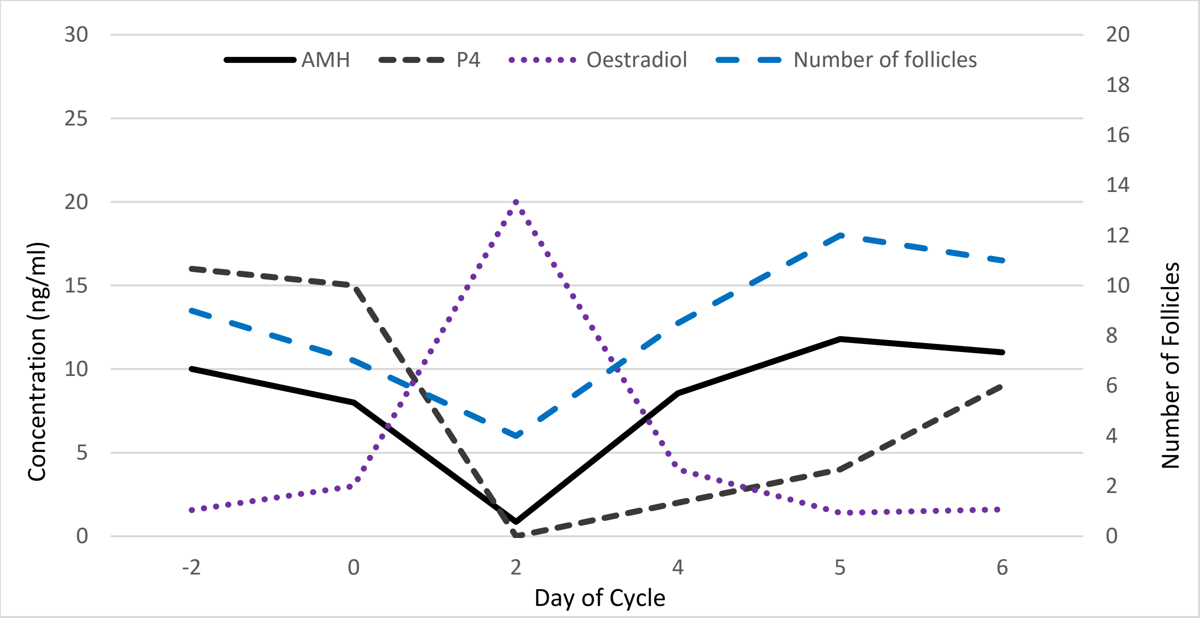

Figure 7: Concentrations of AMH, Progesterone and Oestradiol (ng/ml) and number of small/ medium antral follicles (n) from 2 days prior to PGF2α injection through the follicular phase to day 6 of the early luteal phase. Adapted from (Waheeb 2017)

Oocyte regulation of follicle development and relationship to AMH

The oocytes themselves also play a crucial role in the development of ovarian follicles through complex interactions between regulatory factors governing both oocyte and follicle development (Eppig 2001). Studies over the last decade have concluded a functional control from the oocyte over granulosa cells in committed follicles during transitions from the gonadotrophin responsive and gonadotrophin-dependent stages (Scaramuzzi *et al.* 2011). The assembly of primordial follicles relies on the oocytes to segregate and associate with squamous granulosa cells (Skinner 2005). In addition, factors and signals secreted from granulosa cells and cumulus cell differentiation enable the development of the oocytes (Eppig 2001). Conversely, oocytes are able to regulate progesterone production as well as play key roles in controlling the development and function of granulosa cells (Eppig 2001). Initiation of primordial follicle growth occurs after oocytes enter a prolonged diplotene stage of meiosis, with an oocyte specific gene, *Figla*, required for initial formation of primary follicles (Eppig 2001). Once the oocyte completes its growth phase, it was the greatest capacity to regulate cell survival, growth, survival and regulation of expansion and metabolism (Gilchrist *et al.* 2008).

Beyond this primary stage of growth, there are multiple oocyte-secretory factors interacting to promote the ongoing growth and development of the follicles, oocytes and surrounding granulosa cells(Scaramuzzi *et al.* 2011; Juengel *et al.* 2021). Primordial and committed follicles have multiple receptors and ligands for growth factors of the transforming growth factor (TGF)-β superfamily, including AMH, as well oestradiol and FSH (Scaramuzzi *et al.* 2011; Juengel *et al.* 2021)

Growth differentiation factor 9 (GDF-9) is a paracrine regulator of follicle development (Gilchrist *et al.* 2008), and in cattle and sheep exclusively expressed by the oocyte (Scaramuzzi *et al.* 2011). GDF-9 affects both granulosa and theca cell function and critical for oocyte developmental competency in ruminants (Gilchrist *et al.* 2008). Development of follicles beyond the primary stage was unachievable in GDF-9 null mice (Dong *et al.* 1996), demonstrating the important role GDF-9 plays in early follicle development, within the primordial oocyte. Similar actions by AMH are evident in bovine ovaries, whereby AMH inhibits follicle activation and early follicular growth (Fortune 2003), however relationships between GDF9 and AMH remain to be investigated sufficiently. Bone morphogenetic protein (BMP 15) is an oocyte-secreted factor that follows an expression pattern similar to that of GDF-9. In sheep BMP-15 plays a strong role in the prolificacy of some breeds. Highly prolific sheep breeds are heterogygous to a FecX gene, often having increased ovulation rates (Nagdy *et al.* 2018). This FecX gene is homologous to BMP 15, and is a candidate marker for reproduction in sheep as sheep homozygous for this gene are sterile (Eppig 2001). BMP15 is involved in the regulation of the feedback mechanisms between the ovary and pituitary, whereby reduced expression of BMP15 increased gonadotrophin signalling and increased follicular development (Eppig 2001). Together, GDF-9 and BMP15 are crucial for early follicular development and ovulation in sheep (Eppig 2001), both acting in a concentration-dependent manner on adjacent cumulus and granulosa cells (Scaramuzzi *et al.* 2011). Such that, reduced concentrations of BMP15 or GDF9 slightly alter the responsiveness of granulosa cells to FSH or LH while the absence prevents normal follicular growth beyond the primordial or primary stages (Scaramuzzi *et al.* 2011).

Importantly, oocyte-derived BMP15 upregulates *AMH* gene expression in ruminants (Pierre *et al.* 2016; Poole *et al.* 2016). AMH and BMPs within the granulosa cells have a positive effect on each other; as the temporal sequence of *BMP15* expression in oocytes parallels *AMH*, therefore it is possible both AMH and BMP's negatively affect FSH receptors (Juengel *et al.* 2021). AMH reaches highest levels in granulosa cells of gonadotropin-responsive follicles, diminishing in gonadotropin-dependent follicles and throughout terminal follicular development in cattle (Rico *et al.* 2009). In rat a pig granulosa cells reduce FSH-stimulated aromatase and the expression of LH receptors (di Clemente 1994) further supporting a relationship between AMH and FSH responsiveness of follicles.

It becomes clear that the ability of the ovary to release an oocyte depends on development of the granulosa and theca cells as the follicle develops, and this is the major determining factor of reproductive potential. Constant communication between the hypothalamic-pituitary gonadal axis, the growing follicles, as well as the cumulus cells and enclosed oocyte, drives this development of an oocyte capable of being fertilised and developing into a viable offspring (Eppig 2001). As outlined above AMH plays a crucial role across interactions between gonadotrophins, metabolic hormones and growth factors controlling follicle development, ultimately affecting the number of follicles that ovulate, the developmental competence of the oocytes that are ovulated (Webb *et al.* 2007), and therefore fertility.

AMH and our understanding from cattle

AMH repeatability and variation

In cows, AMH concentrations are highly reproducible (Rico *et al.* 2009 ; Monniaux *et al.* 2013). In young adult beef heifers, a single time point measurement of AMH was strongly, positively correlated (r=0.97) with average AMH at a range of time points at different days over multiple oestrous cycles (Ireland *et al.* 2011; El-Sheikh Ali *et al.* 2013). Additionally, AMH remains static during the oestrous cycle of dairy cows (Rico *et al.* 2009; El-Sheikh Ali *et al.* 2013; Souza *et al.* 2015) demonstrating limited variation within animals.

In comparison to cattle, the repeatability of AMH in sheep is much less clear. In pre-pubertal Rasa Aragonasa ewe lambs repeatability of AMH levels was low; most likely due to between animal variation in the timing of peak AMH (Lahoz *et al.* 2014). The timing of the peak varied across 3, 4.5 and 6 months of age (Lahoz *et al.* 2014). Further, AMH concentrations were significantly different at 1.6 months, 6.4 and 16 months of age in Sarda ewe lambs (Torres-Rovira *et al.* 2016) and no correlations between pre-pubertal and adult AMH concentrations have been identified in Rasa Aragonasa ewes (Lahoz *et al.* 2014). This limits the application of this one time point measure beyond the first mating, and suggests that a sample prior to each mating period may be necessary as AMH concentration is not repeatable across age. Additionally, determining the optimum time to test pre-pubertal animals is challenging and further investigation in this area is warranted, especially given the increased ovarian activity in ewe lambs at 6 weeks of age (Paramio and Izquierdo 2014). Interestingly, within animal variability in AMH was higher in Rasa Aragonesa ewe lambs which exhibited an AMH peak at 4.5 months of age, suggesting that establishing the pre-pubertal profile of AMH levels may be crucial to identifying appropriate timing and concentration of AMH to use as a fertility marker. One study in Sarda ewe lambs has determined how AMH concentrations change over the first 9 weeks of age (Torres-Rovira *et al.* 2016) with all ewe lambs exhibiting one of two distinct AMH profiles. Essentially, AMH concentrations were either very low, with little variability during the first 9 weeks of age, or increased significantly to peak between five and six weeks of age (Torres-Rovira *et al.* 2016), supporting the increased ovarian activity seen in ewe lambs (Paramio and Izquierdo 2014). AMH concentrations at 1 week of age did not differ between the ewe lambs, suggesting the development of the antral follicle pool and AMH expression is delayed initially post birth. Unsurprisingly, between the two cohorts, those exhibiting the increase in AMH were also categorised as having high AFC at 50 days of age.

Data describing the variation in AMH concentrations between animals is also limited, however AMH ranged from 0.04 – 0.3 ng/ml in Katahdin ewes, with concentrations included across all ewe age groups (<1, 1-2, 2-3 and > 4 years of age) (Acharya *et al.* 2016). To date, a single study has been conducted, using Barki sheep, to determine the variability in AMH during a luteal and follicular phase (Waheeb 2017), with AMH levels varying between 0.8 and 12 ng/ml during these phases (Figure 8). There was a progressive decline in AMH between the late luteal through to early follicular phase, with lowest concentration of 0.86 ng/ml (Waheeb 2017). AMH levels peaked two days later during the early luteal phase (11.8 ng/ml), and then declining again thereafter (Waheeb 2017). These changes in AMH reflecting changes in the number of small to medium antral follicles (Waheeb 2017).

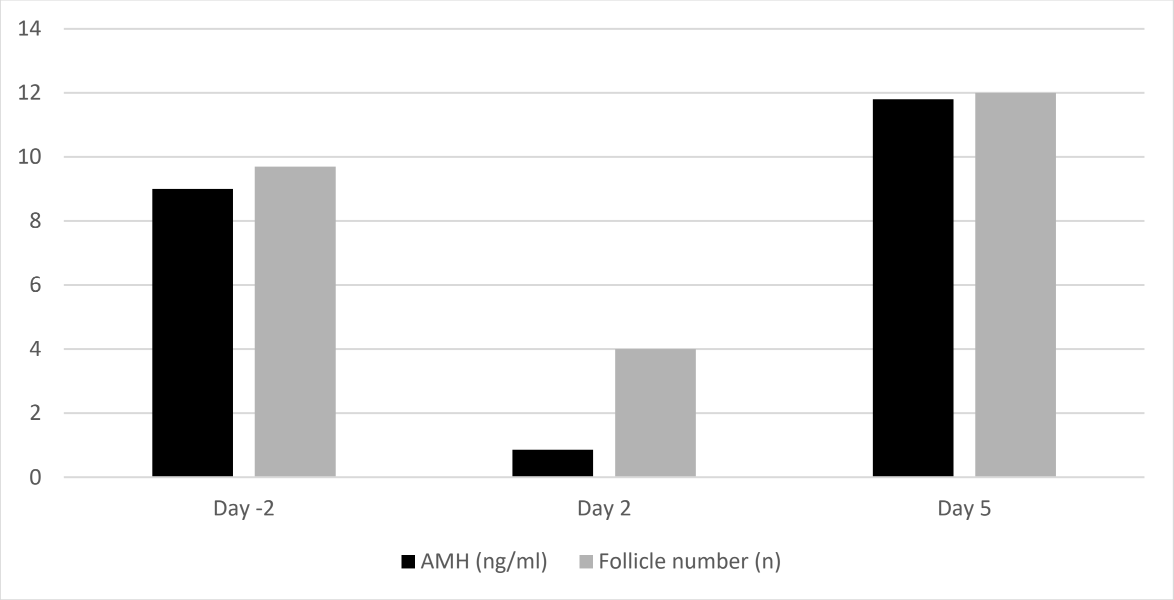

Figure 8: AMH Concentrations and Follicle numbers across the oestrous cycle in Barki sheep (n=10). Adapted from Waheeb et al., 2017. Day 0= ovulation.

AMH and embryo technologies

To date, the majority of literature has assessed links between AMH and reproductive parameters in cows. This literature demonstrates strong, positive correlations between AMH and AFC, the number of COC's collected and the number of transferrable embryos obtained following a superovulation protocol and when measured at time of laporoscopic ovum pick-up (OPU) (Batista *et al.* 2016) (Table 4). The evidence that AMH concentrations can be used to aid selection of suitable donors for inclusion in reproductive technologies is extensive and convincing. Plasma AMH predicted the number of large follicles produced in response to super ovulation stimulation protocols (Rico *et al.* 2009; Souza *et al.* 2015), as well as the number of collected and transferable embryos produced per cow following superovulation (Monniaux *et al.* 2010). Importantly, not only did AMH predict the capacity of an individual cow selected for embryo production to respond to gonadotrophin treatment, the highly repeatable values obtained for each individual animal in conjunction with repeatable individual responses following repeated OPU protocols (Rico *et al.* 2012) suggested a cut-off value of AMH could potentially be determined to eliminate low responding animals from the OPU program.

Table 4: Relationship between AMH in cattle and the outcomes of assisted reproductive technologies

Study	Treatment	AMH predictive of
Rico et al., 2009	Superovulation protocol	Number of large follicles produced Number of large follicles at oestrous Number of CL during luteal phase post treatment
Souza et al., 2015	Superovulation protocol	Number of large follicles produced Number of CL at flush Number of COCs collected Number of transferrable embryos
Monniaux et al., 2010	Superovulation protocol	Number of collected and transferrable embryos
Ghanem et al., 2016	Superovulation protocol	Antral Follicle Count (AFC) Number of small and medium follicles Number of follicles at oestrous Number of CL during luteal phase of treatment Number COC's recovered Quality of recovered COC's Number of blastocysts produced
Batista et al., 2016	Laproscopic ovum pick-up	Antral Follicle Count (AFC) Number of oocytes collected Number of Blastocysts produced
Ribeiro et al., 2014	Natural service and AI	Pregnancy rate Pregnancy Loss between d 30-65
Guerreiro et al., 2014		Aspirated Follicle Number of in vitro produced embryos Number of COCs

Based on the literature, AMH appears to be a useful strategy to identify suitable donors for inclusion in embryo biotechnologies in sheep (Table 5). This may facilitate the application of biotechnologies in the sheep industry by improving embryo production per animal and, therefore, reducing the costs associated with unresponsive animals (Lahoz *et al.* 2014). In sheep, circulating AMH was strongly positively correlated with AFC and particularly the development of larger follicles (>3mm in diameter) in response to FSH stimulation in pre-pubertal Sarda ewe lambs (Torres-Rovira *et al.* 2014). Specifically, when ewe lambs were classified into low, medium or high AFC groupings based on the number of 2 mm follicles present on their ovary, AMH concentrations were found to match the AFC categories (Torres-Rovira *et al.* 2014). Low AFC, and in conjunction low AMH, were predictive of poor *in vitro* oocyte developmental competence. Similarly, correlations between plasma AMH and number of punctured follicles (≥3mm) ($r=0.75$, $P<0.001$), recovered COC's ($r= 0.41$, $P<0.05$) and the number of COC's suitable for IVM ($r=0.39$, $P<0.05$) demonstrate that AMH is a strong predictor of the ovarian response to FSH stimulation (Lahoz *et al.* 2014). All ewes were reared under the same conditions to limit the additional factors, such as season and uterine condition, and there was no association between fertility and age or live weight. Therefore, AMH was correlated directly to follicle population suggesting AMH may be used a fertility marker for lifelong performance in sheep without the confounding factors affecting women and other species.

Selecting ewes based on AMH may also be an accurate way to select those animals with a higher potential embryo yield during MOET (Pinto *et al.* 2018) (Table 5). In Santa Ines ewes, AMH concentration pre eCG selection test, or before hormonal treatment, were weakly correlated to structures recovered (included all unfertilised oocytes, degenerated and viable embryos) and unrelated to CL count or viable embryo numbers (Pinto *et al.* 2018). However, there was a strong, positive correlation between AMH coincident with the timing of the first FSH dose and the CL count, recovered structures and number of viable embryos obtained (Pinto *et al.* 2018).

Table 5: Differences in AMH concentration and outcomes of in vitro embryo production from 40 day old abattoir derived ewe lamb ovaries. Adpated from Torres-Roviera et al., 2014

	High AFC	**Medium AFC**	**Low AFC**
Number of follicles	≥30	16 – 29	≤15
AMH concentration	3.03 ± 0.22 ng/ml[a]	0.36 ± 0.13 ng/ml[b]	0.08 ± 0.01 ng/ml[c]
AMH concentration post FSH	2.84 ± 0.13 ng/ml[a]	0.42 ± 0.13 ng/ml[b]	0.08 ± 0.01 ng/ml[c]
Proportion of ovaries (%)	56[a] (8.5)	222[b] (33.7)	380c (57.8)
IVM oocytes (per ovary; mean ± SEM)	1832[a] (36.4 ± 2.9)	1928[b] (9.9 ± 2.7)	343[c] (1.1 ±2.8)
Day 8 vitrified embryos	99[a] (10± 2)	104[a] (9.7 ±1.9)	10[b] (4.1 ± 1.9)
Total Embryo produced	207[a] (17.4 ± 2.8)	189[a] (16.6 ± 2.6)	27[b] (8.3 ± 2.7)

AMH as a predictor of pregnancy outcomes

Based on the available literature, it remains equivocal whether AMH levels can predict the ability of cows to become pregnant, and further investigation is warranted. Concentrations of AMH were 50 pg/ml higher in cattle that were pregnant at the end of the breeding season, and 51 pg / ml higher in cows which became pregnant following a spontaneous oestrous cycle. Pregnancy rates were similar for cows in either intermediate (141-450 pg/mL) or high (451-3198 pg/mL) AMH groups and both were higher than the low AMH group, indicating AMH levels greater than 141 pg/mL are adequate for successful pregnancy (Ribeiro *et al.* 2014). This also indicates that an optimal range for AMH concentrations likely exists. In this same study the incidence of pregnancy loss was higher in cows with low (10-140 pg/mL AMH) compared with intermediate or high AMH levels (Ribeiro *et al.* 2014).

However, a more recent study using 108 surrogate Korean Hanwoo cows for OPU derived, *in vitro* produced embryos demonstrated that AMH did not predict the pregnancy outcome (Ghanem *et al.* 2016). The percentage of non-pregnant cows was lowest for animals with intermediate compared with low or high AMH concentrations (49.3 % vs 63.6 (High AMH) and 64.3 (Low AMH)), with a higher percentage of intermediate AMH cows delivering (50.7 % vs 36.4% (High AMH) and 35.7% (Low AMH)) ((Ghanem *et al.* 2016). These data suggest that further research is needed to determine the true value of AMH, and whether there is an optimal range or value, as an indicator of pregnancy outcomes.

The ability for AMH to predict the likelihood of a heifer surviving in the herd following birth of her first calf, as well as how long they remain within the herd (herd longevity) has also been investigated (Jimenez-Krassel *et al.* 2015). Productive herd life is an important determinant of dairy herd productivity, and prolonging herd life by 172 days is worth an additional $200 (Jimenez-Krassel *et al.* 2015). This study found that heifers with the lowest survival rate after the birth of their first calf had the lowest circulating AMH concentration when compared with other heifers of the same age (Jimenez-Krassel *et al.* 2015). Multinomial logistic analysis, suggested that poor reproductive performance, whether that be age at first AI/conception/calving or conception rates or calving internal is a more likely reason for the reduced herd longevity and survival rate.

In women, there are conflicting results in the few studies that have investigated the potential of AMH to identify pregnancy outcomes. Following extensive meta-analysis of data from 6306 women, Iliodromiti *et al.* (2014) concluded AMH had some association with live birth in women undergoing IVF; however, the predictive accuracy was poor. (Kato *et al.* 2015) also determined that serum AMH levels of less than 0.7 ng/ml were negatively correlated to pregnancy, but only in women aged 38 and older. Similarly, three studies indicated that AMH can predict the chances of live-birth in women with a diminished ovarian reserve (Gleicher *et al.* 2010) or those undergoing assisted reproductive technology (Lukaszuk *et al.* 2014). Similarly, Nelson *et al.* (2015) and Khader *et al.* (2013) determined that when combined with clinical characteristics AMH can accurately identify the likelihood of live birth with a low prediction error in women. These data demonstrate that the accuracy with which AMH predicts pregnancy outcomes is highest in women undergoing assisted reproductive technologies.

AMH research in sheep

To date, limited research has assessed the validity of using AMH as a phenotypic marker of reproductive output in sheep (Table 6). The available literature focuses on two sheep breeds; Rasa Aragonesa and Sarda, and the studies are broad extending into ewe lambs, ewes and ewes with prolific genes. Plasma AMH was detected in 93% of 3.6 month old lambs, demonstrating its practical application for selecting young animals for breeding (Lahoz *et al.* 2012). AMH concentration was significantly correlated with the lamb's ability to conceive at first mating, suggesting it could be a useful tool for producers to select animals into a breeding program (Lahoz *et al.* 2012; Torres-Rovira *et al.* 2016). In both Sarda (Torres-Rovira *et al.* 2016) and Rasa Aragonesa (Lahoz *et al.* 2012), ewe lambs with high AMH concentrations were more fertile at first lambing. Further, animals with a high AMH were 82 days younger at first lambing, and had a resulting 0.17 extra lambings, and 0.38 extra lambs per ewe per year (Lahoz *et al.* 2016). In adult Rasa Aragonesa ewes, AMH concentrations were positively correlated with their ability to become pregnant at their first and second mating (Spearman’s rho = 0.34 and 0.33, respectively), and overall, with AMH unaffected by the age or weight of the ewe (Lahoz *et al.* 2012). AMH was highest in those ewes that became pregnant following their first mating, and lowest in those that did not become pregnant at all. Ewes that only became pregnant in their second mating, were also higher than those that did not become pregnant at all, but lower than those that did in their first mating. (Lahoz *et al.* 2012). In apparent contrast, Acharya et al. (2016) divided Katahdin ewes into four groups based on AMH concentrations quartiles, and found no relationship with the number of lambs born. However, the age distribution of ewes within the quartiles may have affected these results, with 36% of the ewes in the lowest quartile less than 2 years old compared with only 15% of the ewe older than 4 years of age.

Table 6: Summary of the relationship between AMH, when used quantitatively, divided into categories or in conjunction with AFC as a predictor of outcomes of ARTs and pregnancy outcomes in different breeds of sheep at different ages.

Reference	**Breed**	**Age**	**System**	**Treatment**	**AMH categories**	**AMH timing**	**Outcomes with AMH**
Lahoz et al., 2012	Rasa Aragonesa	3.6 mnths	hormonal stimulation for inducing ovulation natural mating at 10 and 14 months if not pregnant at first mating	600 IU eCG	Quantitative	3.6 mnths	Greater fertility at first mating and second mating
Lahoz et al., 2014	Rasa Aragonesa	19 months old	Hormonal stimulation for laproscopic ovum pick-up over four consecutive sessions	No FSH first two sessions 8 mg (60 hours), 8 mg (48 hours), 6 mg (36 hours), 6 mg (24 hours), and 4mg (12 hours) last two sessions	Quantitative	Beginning of FSH treatment	More punctured follicles
Lahoz et al., 2015	Rasa Aragonesa	Adult	Multiple ovulation and embryo transfer	FSH	Quantitative	4 days prior to and at time of FSH injection	More CL
Lahoz et al., 2016	Rasa Aragonesa	3 mnths	eCG and natural mating	eCG stimulation pre-pubertally	HIGH vs LOW	At time of eCG stimulation	82 days younger at first lambing, 0.17 extra lambings

							0.38 extra lambs per year
Acharya et al., 2016	Katahdin	0.7-7 yrs	Natural mating	N/A	Quantitative, or 4 quartiles	Prior to mating	No relationship
Torres-Rovira et al., 2014	Sarda	40 days	FSH ovarian reserve test (EFORT)	60mg FSH	HIGH, MED, LOW AFC *Positively correlated with AMH at these times*	40 days	Greatest number of 2mm follicles More COCs collected Greater fertility at first mating
Torres-Rovira et al., 2016	Sarda	50, 195 and 496 days old	FSH ovarian reserve test (EFORT)	105 IU FSH (50 and 195) 175 IU FSH (496)	HIGH, MED, LOW AFC *Positively correlated with AMH at these times*	50 days of age	Greater response to FSH Higher pregnancy rates at first mating

Conclusion

Whilst the relationship between oocyte or embryo quality and AFC in sheep warrants further investigation as AFC in pre-pubertal sheep was predictive of oocyte quality in an *in vitro* production system, when categorised as having low medium or high AFC (Torres-Rovira *et al.* 2014), AMH may be a more accurate measure of reproductive potential. The combination of reproductive inefficiencies in mature ewes and the industry desire to mate juvenile animals (ewe lambs) and utilise assisted reproductive technologies demonstrate the need for an encompassing method of selection for breeding animals. Additionally, both the intra-uterine and the early life environment affect reproductive development and results in a multifactorial problem when trying to select animals for reproductive performance. AMH as discussed in detail, plays an inhibitory role in follicle recruitment and therefore controls the ovarian reserve of a female, with circulating concentrations reflective of the size of the gonadotrophin responsive follicles present on the ovary. In addition, AMH also regulates follicle development within the later stages of the oestrous cycle. This makes AMH an excellent potential phenotypic maker for selection of reproductive potential in sheep.

Ultimately, it is evident that AMH has the potential to advance the selection of breeding ewes. There is an abundance of convincing data in cattle supporting the suggestion that a single measure of AMH early in life will allow selection of more fertile animals for inclusion into the breeding herd (Cruz *et al.* 2018). In sheep, intensive studies describing the changes in AMH during the first 5 months of life have yet to be conducted; however, two European studies have identified blood samples taken from ewe lambs within this period correlate to their fertility at first mating (Lahoz *et al.* 2012; Torres-Rovira *et al.* 2016). However, this was conflicted by evidence that AMH concentrations pre-pubertally and in adulthood do not correlate. Additionally, there is evidence that a single time point within the oestrous cycle does not affect how accurately AMH predicted fertility in cattle (Ireland *et al.* 2011).

However, the changes in AMH which occur during the ovine oestrous cycle have yet to be determined. Therefore, it cannot be assumed that a single time point measure of AMH taken randomly during the oestrous cycle will reliably predict fertility outcomes. Furthermore, cattle literature has also identified that breed affects AMH concentrations and therefore research must be undertaken to determine whether this is the case in sheep. Finally, for both sheep and cattle it is clear that AMH is a strong marker for selection into *in vitro* embryo technologies (Rico *et al.* 2009; Monniaux *et al.* 2010; Lahoz *et al.* 2014; Torres-Rovira *et al.* 2016); however, the correlation between AMH and oocyte and embryo quality is yet to be established for either species. A thorough investigation of these key areas in the sheep will provide evidence as to whether AMH can be used as a phenotypic marker for the selection of animals for breeding.

Chapter Three: AMH concentration post- natal changes in the South Australian Merino and Suffolk ewes and ewe lambs

Abstract

Early life concentrations of anti-Müllerian hormone (AMH) may be a suitable phenotypic, predictive marker for fertility in ewes. However, concentrations may differ between breeds, and AMH profiles have not been established for breeds utilised in Australia. This study used oestrous synchronised mature Merino (n= 32) and Suffolk ewes (n= 50) and their female offspring, Merino (n= 22) and Suffolk (n= 20), to investigate breed differences in AMH in pre-pubertal and mature ewes. AMH was measured pre- and post- oestrus synchronisation in mature ewes and throughout the pre-pubertal period (birth to 18 weeks of age) in ewe lambs. The relationships between AMH and measures of fertility and reproductive potential were determined in mature and pre-pubertal ewes. Therefore, this study had four key objectives. 1) determine whether breed differences in AMH concentrations exist in mature or pre-pubertal Suffolk and Merino ewes, 2) determine whether AMH concentrations differed within breed for ewes that did not give birth, or had a single lamb or twins, 3) identify how AMH concentrations change prior to puberty in Suffolk and Merino ewe lambs and 4) determine whether pre-pubertal AMH patterns in either breed were related to differences in ovarian phenotype and embryo development at slaughter at18-weeks of age.

Pre-pregnancy AMH concentrations were highest in Suffolk ewes that were not pregnant (4.94 ± 0.99 ng/ml), compared with having a single or a twin (2.59 ± 0.35 vs 3.51 ± 0.48 ng/ml, respectively). Conversely, pre-pregnancy AMH concentrations were lowest in Merino ewes that were not pregnant (2.92 ± 0.59 ng/ml), and were numerically higher in twin compared with singleton bearing ewes (3.72 ± 0.84 vs 3.24 ±0.61 ng/ml). Pre-pregnancy AMH concentration for not pregnant Suffolk ewes were even higher than those Merino ewes that birthed twins, possibly indicating differences in antral follicle populations at time of conception between breeds. AMH concentrations were unaffected by oestrus

synchronisation, and did not differ between mature Merino and Suffolk ewes pre (3.26 ± 0.38 vs 3.69 ± 0.40 ng/ml) or post (3.65 ± 0.37 vs 3.80 ± 0.38 ng/ml) synchronisation.

AMH concentrations were upto 4-fold higher in Merino compared with Suffolk ewe lambs ($P<0.01$) at 2 weeks (13.1 ± 2.57 vs 2.8 ± 0.93 ng/ml), 6 weeks (26.1 ± 2.46 vs 11.1 ± 1.55 ng/ml), 10 weeks (10.8 ± 1.37 vs 5.6 ± 0.92 ng/ml) and 18-weeks (1.55 ± 0.21 vs 0.81 ± 0.08 ng/ml). Both the Suffolk and Merino ewe lambs had distinct differences in AMH concentration across the first 18 weeks of life, and within each breed, two distinct AMH profiles were evident. In 13 Suffolk lambs AMH concentration peaked between 6 and 8 weeks of age and decreased thereafter (PEAK), with no distinct peak observed in the remaining 7 animals (NO). Average AMH concentration differed between these cohorts of Suffolk lambs (NO; 1.5 ± 0.22 vs PEAK; 6.0 ± 0.39 ng/ml, $P< 0.05$). In contrast, an AMH peak was observed in all Merino lambs at 6 weeks of age. However, in 14 lambs this peak was preceded by a trough at 4 weeks of age (DROP; AMH = 3.8 ± 2.76 ng/ml), with no trough observed in the remaining 8 animals (PEAK; AMH = 19.4 ± 3.76 ng/ml) ($P< 0.05$). At 18-weeks of age, Suffolk ewe lambs had more surface follicles greater than 5mm (0.65 ± 0.18 vs 0.18 ± 0.11, $P<0.001$) and secondary follicles (7411 ± 2241 vs 2550 ± 640, $P< 0.001$) in comparison with Merino ewe lambs, indicating a greater reproductive potential otherwise no differences in ovarian phenotype were identified. Significant differences in AMH concentration exist between pre-pubertal Suffolk and Merion ewe lambs, differences not identified in the mature ewe. Within each breed two distinct profiles exist, however there is no evidence of a relationship between these profiles and observed ovarian phenotype at 18 weeks of age.

Introduction

Anti-Müllerian hormone (AMH) has the potential to be an accurate phenotypic marker of fertility in juvenile and mature ewes. AMH is a glycoprotein of the transforming growth factor family and in mice, cows, ewes and women, is expressed exclusively by the granulosa cells of growing antral follicles (Rico *et al.,* 2009). To date, AMH has been extensively studied as a marker with which to select more fertile animals for inclusion in a variety of reproductive technologies, and has proven to be an accurate predictive marker of *in vivo* embryo production in cattle (Ireland *et al.,* 2008, Monniaux et al., 2010), goats (Monniaux et al., 2011), sheep (Lahoz et al., 2014) and humans (Moro et al., 2016). Much of the work in livestock species has been completed in cattle, and demonstrates that AMH is strongly correlated to the number of large antral follicles, cumulus-oocyte-complexes (COCs) and transferable embryos obtained following protocols for superovulation (Rico et al., 2009; Monniaux et al., 2010; Souza et al., 2015; Batista et al., 2016). In dairy cows, AMH also has positive associations to pregnancy rates (Ribeiro *et al.* 2014), with high AMH associated with longer productive herd life, increased survival rate following birth of the first calf and lower cull rates (when culled for poor reproduction) (Jimenez-Krassel *et al.* 2015). Interestingly, it appears that AMH concentrations differ significantly between breeds of cattle, with circulating AMH higher in Jersey cows, compared with both crossbreds and pure Holsteins (Ribeiro *et al.* 2014).

However, limited work has been conducted in sheep. Similar to cattle, AMH in Sarda ewe lambs has been positively correlated with the number of total antral follicles (Torres-Rovira *et al.* 2014), ovarian response to FSH stimulation, and pregnancy rates at first breeding (Torres-Rovira *et al.* 2016). Further, Rasa Aragonesa ewe lambs with higher AMH concentrations were younger at first mating, which resulted in an extra 0.17 extra lambings, or 0.38 extra lambs, per ewe per year (Lahoz *et al.* 2012). The use of AMH for selection of ewes for embryo technologies is also in its infancy; however, AMH was positively correlated with superovulation response and *in vivo* embryo production response in donor Santa Ines ewes (Pinto *et al.* 2018).

At a fundamental concentration, understanding how AMH expression fluctuates between birth and puberty in ewe lambs is required to assess physiological changes and to determine the age at which AMH could be used as a phenotypic selection marker of fertility. In Rasa Aragonesa ewe lambs, AMH tended to increase from 3 to 4.5 months and then decline by 6 months of age to concentrations similar to that of the adult animal; however, there was no relationship between pre-pubertal and adult concentrations (Lahoz *et al.* 2014). It is possible that early follicular development, and AMH production, progress differently within cohorts of ewe lambs. In Sarda ewe lambs, there appear to be two distinct patterns of AMH concentration between birth and 9 weeks of age (Torres Rovira et al., 2016). Ewe lambs categorised as having high antral follicle count (AFC) (≥30 follicles) at 50 days of age had high circulating concentrations of AMH (approximately 1 ng/ml), peaking around 5 weeks of age, and low circulating follicle stimulating hormone (FSH). Ewe lambs with a low AFC phenotype (≤15 follicles) had significantly higher FSH concentrations that peaked at 3-4 weeks of age and constantly low circulating AMH (≤0.1 ng/ml) (Torres-Rovira et al., 2016).

The Merino is a specialised wool breed in Australia, and accounts for 74% of the national total breeding stock, including pure breed lamb production (70%), crossbred lamb production (27%) and other purposes (3%) (Innovation 2021). The Suffolk is a meat breed of sheep, and is utilised as one of many crossbreeds in Australia (Fogarty and Mulholland 2014), within composite, second cross, pure meat and dual-purpose flocks. These account for a further 15% of the national breeding stock (Innovation 2021). The effects of the environment *in utero* and during the post-natal period on reproductive development demonstrate the need to identify a baseline profile for AMH circulation from birth through to puberty in order to identify animals with the potential for high reproductive efficiency. Limited data in sheep (Lahoz *et al.* 2012) and cattle (Ribeiro *et al.* 2014; Batista *et al.* 2016; Hirayama *et al.* 2017) support the existence of within and between breed differences in post-natal AMH.

The objective of this experiment was, therefore, four-fold. 1) determine whether differences in AMH concentrations exist between two of predominant sheep breeds, Merino and Suffolk, in pre-pubertal and mature animals 2) determine whether AMH concentrations before or after oestrus synchronisation in mature Suffolk and Merino ewes predict AI outcome, 3) identify pre-pubertal AMH patterns in Merino and Suffolk ewe lambs and 4) investigate whether the pre-pubertal AMH patterns within breed relate to ovarian phenotype or ovarian development.

Materials and Methods

Experimental design and animal management

All procedures were conducted according to the guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes. The work was approved by the University of Adelaide Animal Ethics Committee (Approval number S-2017-068) and conducted at the Roseworthy campus, University of Adelaide, Roseworthy, South Australia (34.5274^{o} S, 138.6881^{o} E). This study utilised 50 Suffolk and 32 Merino ewes mated in late Summer 2018 and their ewe lambs (Suffolk (n=20), Merino (n=22)). All ewes were managed under standard husbandry practice at Roseworthy campus. They were provided feed and water *ad libitum* and reared under the same conditions in separate paddocks according to breed.

50 Suffolk and 32 Merino mature multiple-parous ewes aged between 4 and 7 years underwent a routine oestrus synchronisation protocol using intravaginal progesterone pessaries (0.3 mg progesterone, CIDR, Pharmacia and Upjohn, New South Wales, Australia) for 12 days in Summer (Southern Hemisphere), 2018. All ewes were at an unknown stage of the estrous cycle at time of pessary insertion. A jugular blood sample was collected at time of pessary insertion (day 1) and removal (day 12). All ewes then underwent artificial insemination (AI) 48 hours after pessary removal (day 14). Pregnancy scanning was performed approximately two months post AI to determine whether the ewes were dry or pregnant, as well as determining litter size. All ewe lambs were born within a two- week period, with monitoring for parturition every 12 hours to allow for determination of litter size. All lambs remained with their mothers until 18 weeks of age when they were slaughtered under standard abattoir practice and their ovaries were collected for *in vitro* embryo production and histology.

Blood sampling and AMH measurement

To create individual pre-pubertal AMH profiles for all 42 lambs, blood samples were collected every 14 days starting at two weeks of age and ending at 18 weeks of age. All samples were collected at the same time of day, and within the same period of time. Blood samples were collected (within 2 hours) via jugular venepuncture into 9 ml lithium heparin vacuum tubes (vacuette, Southern Cross Science Pty. Ltd., Edwardstown, South Australia, Australia) and stored on ice. Samples were then centrifuged at 1500 rpm for 15 minutes, and plasma was removed and stored at -20°C until assayed for AMH. Plasma AMH concentrations was measured in 50 µl samples in duplicate for each week for all ewe lambs using the AMH (Ovine) ELISA following manufacturer’s instructions (AL-155, Ansh Labs, Webster, Tx; MDC: 0.025ng/ml) (7.94%, 2.66% intra and inter-assay coefficients of variation, respectively). Variation determined over 14 assay plates, including all re-analysed samples (80 samples per plate), all samples from the same collection day were run on the same plate. All mature ewe samples were analysed over 3 plates, the remaining 11 were used to analyse lamb samples, on average 2 sample collections from each lamb were run on a single plate. MDC calculated by the interpolation mean plus two standard deviations of 24 replicates of calibrator A (0 ng/ml) and low calibrator (0.37 ng/ml). Upper limit of standard curve at absorbance of 3.42 (20 ng/ml) measured at 450 nm.

In vitro maturation, fertilisation and culture

At slaughter one ovary from each lamb was snap frozen in liquid nitrogen, whilst the other was placed in PBS warmed to 33°C. Ovaries were transported back to the lab, and frozen ovaries were transferred to -80°C freezers for storage. The ovary in warmed PBS was weighed, and all surface follicles were measured using electronic callipers.

Procedures for in vitro embryo production were similar to those previously outlined (Walker *et al.*, 1996). Briefly, follicles ≥2 mm were aspirated into aspiration medium containing HEPES-buffered TCM 199 (Sigma Chemical Co., St Louis, MO, USA) containing 2% (v/v) heat-inactivated estrous sheep serum (SS), 100 IU/mL heparin (Pharmacia and Upjohn, Bentley, Western Australia), 100 μg/mL streptomycin sulphate (CSL Limited, Parkville, Victoria, Australia) and 100 U/mL penicillin G (CSL Limited) for cumulus- oocyte complex (COC) retrieval. COCs (within lamb) were then rinsed three times in aspiration medium and twice in sodium bicarbonate-buffered TCM 199 supplemented with 20% (v/v) sheep serum (SS), 5 µg mL^{-1} FSH (Folltropin; Vetoquinol Inc., Ontario, Canada), 0.1 IU mL^{-1} human chorionic gonadotrophin (hcg; Chorulon, Intervet; Bendigo, VIC, AUS), 1 µg mL^{-1} oestradiol- 17β and 100 μM cysteamine; maturation medium). Maturation took place in 4 well dishes (Nunc; Thermo-Fischer Scientific; Massachusetts, USA: Maximum 20 COCs per well) containing 600 µL IVM medium covered with 300 µL mineral oil. COCs were matured at 38.8°C in a humidified atmosphere of 5% CO_2 in air.

After a 24-h maturation period, excess cumulus cells were removed (leaving corona radiata intact) by gently pipetting in aspiration medium supplemented with 400 IU mL^{-1} hyaluronidase followed by three washes in synthetic oviduct fluid (SOF) supplemented with 2% (v/v) SS (fertilisation (IVF) medium). Oocytes (within lamb) were placed in culture wells containing 450 µL IVF medium covered with 300 µL mineral oil. Motile spermatozoa were obtained using a 'swim-up' procedure. Frozen-thawed semen (150 µL), pooled from two rams of proven fertility, was layered under 1 ml of IVF medium in a 14 ml Falcon tube (BD Biosciences; North Ryde, NSW, AUS). After 20 min the concentration of spermatozoa in the upper fraction was determined and ~0.5 x 10^6 spermatozoa were added to each well. The COCs and spermatozoa were co-incubated at 38.8°C in a humidified atmosphere of 5% CO_2 in air.

After approximately 24 h, remnant cumulus cells were removed by gentle pipetting and presumptive zygotes washed three times in SOF containing 8 mg mL^{-1} bovine serum albumin (BSA, Fraction V; Invitrogen; Thermo-Fischer Scientific) and amino acids at sheep oviduct concentrations (Walker *et al.*, 1996); (*in vitro* culture (IVC) medium). Presumptive zygotes were cultured in wells containing 600 µL IVC medium covered with 300 µL mineral oil at 38.8°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Oocytes that failed to divide were removed 24 h later and cleavage rate recorded. Embryo development was recorded on Day 7 (Day 0 = day of IVF) and presented as number of blastocysts produced from oocytes recovered.

Ovarian Histology

Following aspiration, the ovary was trimmed of fat, weighed and fixed in 5% paraformaldehyde (Thermo-Fischer Scientific) at 4°C for 24 h. Histology was only used to assess the primordial follicle pool within the ovary with total surface antral follicle counts performed prior to aspiration. Following three washes in phosphate buffered saline (PBS; Thermo-Fischer Scientific) ovaries were transferred to 70% ethanol (Thermo- Fischer Scientific). Ovaries were then dehydrated in a series of ethanol concentrations, cleared in xylene, and embedded in paraffin. Ovarian sectioning was performed as 5µm sections every 20 µm. The number of follicles per ovary were expressed as an average per unit area (mm^2). The number of follicles across 5 images per slide was counted and divided by the area of the image (mm^2) for all slides per ovary and multiplied by 20 to account for every 20th section. This deduced the number of follicles per ovary per mm^2. This value was then multiplied by the volume of the ovary to estimate total follicle number across the whole ovary. The volume of the ovary was determined using the volume of an ellipsoid or sphere based on the shape and measurement of the ovarian section. Follicular development stages were determined microscopically. Follicles were classified as primordial, secondary, tertiary or pre-antral according to the classification by (Bartlewski *et al.* 2011). All sections were stained using hematoxylin and eosin (H+E stain).

Statistics

One-way ANOVA was used to determine differences in number of lambs born and pregnancy rate between breed in mature ewes, as well as in AMH concentration between and within breed for pre and post oestrous synchronisation. In ewe lambs, one-way ANOVA were used to determine differences in ovarian characteristics (Total number of follicles <1mm, between 1-3 mm, between 3-5 mm and >5 mm, total number of follicles, average size of the smallest and largest follicle, total number of primordial, primary and secondary follicles) or *in vitro* development (oocyte recovery, cleavage and embryo development rates) between breeds (Merino and Suffolk). For each breed two distinct patterns of AMH were found, so comparisons were drawn within breed between ewes that did or did not experience a clear peak in AMH concentrations over the18 week period. For within breed comparison of AMH Merino ewe lambs were classified at 4-weeks of age as having either a PEAK (n= 14) or DROP (n= 8) in AMH concentration. For within breed comparison Suffolk ewe lambs were classified as having a PEAK in AMH concentration at 6-weeks of age (n= 14) or NO peak in AMH concentration at any age (n= 6). Comparisons of ovarian phenotype and embryo development within breed were determined using ANOVA. Pearsons' correlations (Pearson's) were used to determine the relationship between ovarian characteristics (Total number of follicles < 1mm, between 1-3 mm, between 3-5 mm and >5 mm, total number of follicles, average size of the smallest and largest follicle, total number of primordial, primary and secondary follicles) or *in vitro* development (oocyte recovery, cleavage and embryo development rates) and plasma AHM concentrations. This was performed for each week for all animals and within breed AMH classification. All dataanalysed using SPSS Version 26 (IBM Corp.).

Results

Effects of breed on mature circulating AMH concentrations

There were no differences between Merino and Suffolk mature ewes for pre or post oestrous synchronisation AMH concentration, average AMH concentration or average number of lambs born (Table 1). Pre and post oestrous synchronisation AMH concentrations were positively correlated in Merino ($P < 0.001$, $r= 0.585$) and Suffolk ($P< 0.001$, $r= 0.540$) ewes. There was no relationship between pre-oestrous synchronisation AMH concentrations or post-oestrous synchronisation AMH concentrations and pregnancy status or number of lambs born for Merino ewes (Table 2). AMH concentration was significantly (P<0.05) higher pre-oestrous synchronisation for dry ewes compared with those Suffolk ewes that birthed a lamb (Table 2). AMH concentration tended to be higher pre-oestrous synchronisation for ewes that did not birth a lamb, compared with those that birthed a single but not those that birthed twins (Table 2; P=0.058). AMH concentration was not different between those Suffolk ewes that birthed a single or twin (Table 2). For either breed, no correlation was found between maternal (pre or post synchronisation prior to AI) and progeny AMH (at any age from 2-to 18- weeks) or the primordial or antral follicle pool of the progeny.

Effects of breed on pre- pubertal circulating AMH concentrations

On average, over the 18- week period, AMH was higher (P<0.001) in Merino compared with Suffolk ewe lambs (Table 3). AMH concentration was significantly (P<0.05) higher in Merino ewe lambs at 2 weeks, 6 weeks, 8 weeks, 10 weeks, and 18 weeks of age compared with Suffolk ewe lambs (Table 3).

There were no differences in the number of surface follicles less than 1 mm, between 1 and 3 mm or between 3 and 5 mm or total follicles at 18-weeks of age between the Merino and Suffolk ewe lambs (Table 4). Merino ewe lambs had fewer follicles > 5 mm compared with Suffolk ewe lambs (P < 0.05; Table 4).

The total number of primordial and primary follicles was similar for Merino and Suffolk ewe lambs (Table 4). Merino ewe lambs had fewer secondary follicles compared with Suffolk ewe lambs (P < 0.05; Table 4). There were no differences in oocyte recovery, cleavage or blastocyst rate between Merino and Suffolk ewe lambs (Table 4).

AMH profiles, comparison of profiles and correlations with ovarian and in vitro characteristics in Merino ewe lambs

AMH concentration was significantly ($P<0.05$) greater in PEAK Merino ewe lambs at 4 weeks of age compared with DROP Merino ewe lambs (Figure 1). AMH concentration did not differ between PEAK and DROP Merino ewe lambs at any age after 4-weeks of age (Figure 1), but tended to be higher in DROP Merino ewe lambs at 18-weeks of age ($P= 0.062$).

There was no difference in total number of follicles and number of surface antral follicles < 1 mm, between 1 and 3 mm, between 3 and 5 mm or > 5 mm was similar for DROP and PEAK Merino ewe lambs (Table 5). Merino ewe lambs with a DROP in AMH at 4-weeks of age had significantly ($P<0.05$) fewer primary and secondary follicles compared to those that had a peak in AMH at 4-weeks of age (Table 5). There was no significant difference in the number of primordial follicles (Table 5), or COCs recovered or cleavage rate between Merino lambs with either a PEAK or DROP in AMH (Table 5). Merino ewe lambs with a DROP in AMH tended to have lower blastocyst development rate compared to those with a PEAK in AMH ($P= 0.056$; Table 5).

For all Merino ewe lambs there was a positive correlation ($P < 0.05$; $r= 0.453$) between AMH concentration at 6-weeks of age and the number of surface follicles < 1 mm at 18-weeks of age. AMH concentration at 4-weeks of age was positively correlated ($P < 0.01$; $r= 0.701$) to the blastocyst development rate across all Merino ewe lambs. AMH concentration at 8-weeks of age in Merino ewe lambs was positively correlated to the number of surface follicles between 1 and 3 mm ($P < 0.05$; $r= 0.767$) as well as the total number of surface follicles ($P < 0.01$; $r= 0.930$). AMH concentration at 10-weeks of age was negatively correlated to the number of surface follicles between 3 and 5 mm at 18-weeks of age ($P < 0.05$; r= -0.426).

AMH profiles, comparison of profiles and correlations with ovarian and in vitro characteristics in Suffolk ewe lambs

AMH concentrations were significantly ($P < 0.05$) higher in PEAK Suffolk ewe lambs (n = 14) compared with NO Peak Suffolk ewe lambs (n = 6) at 2-, 4-, 6-, 8- and 10-weeks of age (Figure 2;). There was no difference in AMH concentration between PEAK and NO Peak Suffolk ewe lambs at 12-, 14-, 16- or 18-weeks of age (Figure 2).

No significant differences were found in total number of surface follicles or number of surface follicles in any size category at 18-weeks of age between Suffolk ewe lambs that did or did not have a peak in AMH at 6-weeks of age (Table 6). The number of primordial, primary or secondary follicles did not differ between Suffolk ewe lambs regardless of an AMH peak. There were no differences between the number of COCs recovered or the cleavage and blastocyst development rates (Table 6) between Suffolk ewe lambs that did or did not have a Peak in AMH at 6-weeks of age.

For all Suffolk ewe lambs AMH concentration at 18 weeks of age was positively correlated to the total number of primary follicles at 18-weeks of age ($P < 0.01$; r= 0.643). At 4-weeks of age AMH concentration was negatively correlated to the number of total follicles >5 mm ($P < 0.01$; r= -0.581) and positively correlated to the total number of primordial ($P < 0.05$; r= 0.570) and primary follicles at 18-weeks of age ($P < 0.05$; r= 0.547). At 6- and 8-weeks of age AMH concentration was correlated to the total number of primordial follicles at 18-weeks of age (r=0.439 and 0.551, respectively; $P < 0.05$). At 10-weeks of age AMH concentration was negatively correlated to the number of follicles between 3 and 5 mm at 18-weeks of age ($P < 0.05$, r= -0.489). At 12-weeks of age, AMH in Suffolk ewe lambs was positively correlated to the number of follicles between 1 and 3 mm ($P < 0.01$, $r = 0.639$), > 5 mm ($P < 0.05$, $r = 0.506$) and the total number of surface follicles ($P < 0.05$; $r = 0.559$) at 18-weeks of age. At 16-weeks of age, AMH was negatively correlated to the number of surface follicles between 3 and 5 mm ($P<0.05$; -0.475). At 18-weeks of age AMH concentration in Suffolk ewe lambs was positively correlated to the number of surface follicles between 1 and 3 mm ($P < 0.05$, $r = 0.496$), the total number of surface follicles ($P < 0.05$, $r = 0.556$) and negatively correlated to the total number of primordial follicles at 18-weeks of age ($P < 0.05$; $r = -0.506$).

Discussion

The current data demonstrate that AMH concentrations are consistently lower in Suffolk compared with Merino ewe lambs during the first 18 weeks of age; however, this difference was not observed in adulthood. There were also significant between breed differences in ovarian phenotype at 18- weeks of age, with the ovaries of Suffolk ewe lambs containing more secondary and large antral follicles than those of Merino ewe lambs. The inclusion of more Suffolk ewes in the study was due to a higher conception rate for this breed. This is unsurprising given British breeds are often more prolific than the Merino, with a study demonstrating crossbred ewes are used to improve the fertility of the Merino (Borys and Osikowski 1998). Together this suggests that the greater antral follicle pool in the Suffolk prior to puberty may reflect a difference in the maturation rate of the ovary, as Suffolk’s had a faster post-natal growth rate. The inclusion of more animals in the assessment of pre- and post-synchronisation AMH concentrations may strengthen these relationships. These differences in pre-pubertal AMH between breeds confirm the need for breed specific cut-off values for selection or rejection of high or low fertility ewe lambs, respectively, within the Australian sheep industry. Importantly, within each breed there were also two distinct profiles of AMH which most likely reflect differences in post-natal growth and development of the antral follicle pool (Torres-Rovira et al., 2016). In Suffolk ewe lambs the development of the antral follicle pool appears to differ during the first 10 weeks of life, creating two distinct cohorts, those with or without a peak in AMH at 6-weeks of age. However, this appears to result in only minor differences in their ovarian phenotype at 18-weeks of age. Conversely, in the Merino, differences in antral follicle pool development based on AMH concentrations appear to be restricted to the first 6 weeks of life. Merino ewe lambs exhibited either a clear reduction or increase in AMH, and thus AFC, between 2- and 4 weeks of age, with those animals in which AMH increased possessing more primary follicles and producing better quality oocytes at 18-weeks of age. It is, therefore, suggested that the optimal time to detect divergence in post-natal development of the antral follicle pool is between 2- and 6-weeks of age in Merino ewe lambs and between 4- and 8-weeks in Suffolk ewe lambs, with 6 weeks being optimal. This is an important finding, as previous work (Torres-Rovira et al., 2016) suggests that differences in post-natal ovarian phenotype are maintained through to sexual maturity, with the potential to predict fertility. In addition, results in the current study support the idea that there may be breed differences in the way in which AMH is regulated and expressed. AMH concentrations in the Merino ewes were not different when comparing those that did or did not birth a lamb, and there was no significant difference in AMH concentration between those that were dry, had a single or those that had a twin. AMH concentrations were higher in Merino ewes that birthed twins. Conversely in Suffolk ewes, AMH concentration was significantly higher in dry ewes; however, AMH concentrations were not different between ewes that gave birth to a singleton or twins.

AMH concentration as an indicator for antral follicle growth in sheep

Based on previous evidence in Sarda ewe lambs (Torres-Rovira et al., 2016), it can be assumed that AMH concentrations in Suffolk and Merino ewes and ewe lambs are indicative of the growth and development of the antral follicle pool. Ewe lambs with high AFC at 50-days of age had higher AMH concentrations during the first 9 weeks of life, compared with ewe lambs identified as having low AFC (Torres-Rovira *et al.* 2016). Studies in young, Murrah, Holstein and Gyr heifers support this, with circulating concentrations of AMH strongly correlated to the total number of morphologically healthy follicles present in the ovary (Ireland *et al.* 2008; Baldrighi *et al.* 2014). Furthermore, overall average circulating AMH also correlated to the average peak AFC during two or three waves of an oestrous cycle (Ireland *et al.* 2008). A recent review suggests the pattern of AMH expression is likely controlled through signalling from both the oocytes and theca cells, and bone morphogenetic proteins (BMPs) in the primary follicle (Juengel *et al.* 2021). *BMP15* expression within the oocyte mirrors that of *AMH* gene expression, upregulating the expression in ruminants (Pierre *et al.* 2016). BMP2, BMP4 and BMP6 produced by granulosa or theca cells stimulate *AMH* expression and potentially sustain AMH production during follicle development (Estienne *et al.* 2015). In the developing antral follicle steroid hormones and FSH may affect *AMH* expression across different species; however their effects differ significantly (Juengel *et al.* 2021). The rate of AMH expression affects the rate of development of follicle transition from primary to pre- antral (Monniaux *et al.* 2012). Ultimately, the regulation of AMH through factors produced by the oocyte and the surrounding granulosa and theca cells demonstrate the relationship between AMH and the antral follicle pool. (Scaramuzzi *et al.* 2011) found the population of small antral follicles remained relatively stable across the life of an individual, much like that of AMH (Monniaux *et al.* 2011; Evans *et al.* 2012). Recently, AMH has been shown to be synthesised and secreted by epithelial cells in the oviduct and the endometrium (Nasrin Ferdousy *et al.* 2020), which may influence changes in AMH through different stages of reproductive life, including puberty (Juengel *et al.* 2021). Therefore, the results contained within this study are restricted to that of the pre-pubertal animal and conclusions into post-pubertal AMH and follicle development cannot be drawn.

Differences in AMH concentration between Australian Merino and Suffolk ewes

As expected, in the current study circulating concentrations of AMH differed between the two breeds (Merino and Suffolk) in the pre-pubertal period, although this was not significant in the mature animals. This is similar to a study between two Moroccan sheep breeds (Timahdite and D'man) where significant differences were seen in the development of the ovarian follicle pool (Jorio *et al.* 1991). The number of antral follicles was significantly greater in Timahdite compared with D'man, whilst the number of growing or pre-antral follicles was similar (Jorio *et al.* 1991). Suffolk ewes are a predominant breed of sheep used for meat in Australia, whereas Merino ewes are used for their production of wool. From five successive matings, pregnancy rates, mean litter size and the number of lambs born per year were lower in Merino compared with Merino cross Suffolk ewes (Evans and Robinson 1980). Similarly, fertility was greater in dual purpose Merino ewes bred for meat production compared with those bred solely for their wool characteristics (Fourie and Cloete 1993). Within Merino, there is a negative correlation between clean fleece weight and the number of lambs weaned per ewe joined (-0.26 ± 0.05) (Safari *et al.* 2007), demonstrating the negative impact that selection for wool characteristics has on reproduction. Comparisons between beef and dairy cattle breeds further supports the impact that production characteristics have on AMH, with circulating AMH concentrations typically higher in beef compared with dairy cows (Baldrighi *et al.* 2014; Batista *et al.* 2014; Pfeiffer *et al.* 2014; Ribeiro *et al.* 2014). Interestingly, increased emphasis on selection for milk production also appears to affect AMH, with concentrations higher in Jersey compared with Holstein cows (Ribeiro *et al.* 2014). Within populations of ewes bred for wool, the variation may be reduced as a result of the intense genetic selection, and less so in animals bred as dual-purpose. Torres-Rovira *et al.* (2014) determined a low variation in AMH concentrations for Sarda ewe lambs, when broken down into high, medium or low AFC groups indicating a strong relationship between follicle sizes and AMH. Sarda ewes are most commonly used for milk production. Therefore, in the current study the differences in AMH concentrations between breeds is unsurprising, the correlations to fertility and reproductive potential are more so and therefore expanded below.

AMH concentrations were significantly higher in dry Suffolk ewes (4.94 ng/ml) compared with Suffolk ewes that were pregnant and either dry or pregnant Merino ewes. The increased concentration of AMH is likely reflective of the size of the follicles within the growing antral follicle pool at the time of synchronisation, and these animals may have significantly increased the variation across the cohort. It is possible that extremely high concentrations of AMH are not actually indicative of increased fertility, particularly when breed differences are taken into consideration. In cows undergoing a superovulation and embryo transfer program, animals which circulating AMH ranged between 100 and 200 pg/ml produced more transferable embryos compared with animals with < 100 pg/ml of AMH (Monniaux *et al.* 2010). Furthermore, there was no difference in mean or maximal number of transferable embryos between animals that had >200 pg/ml AMH (Monniaux *et al.* 2010). In women circulating concentrations of AMH were up to three times higher in women with polycystic ovary syndrome compared with controls (Mulders 2004), and may indicate ovarian hyper stimulation syndrome in those undergoing IVF (Nakhuda *et al.* 2006). Whilst, an underlying ovarian dysfunction is unlikely in these animals, the response to synchronisation in the Suffolk ewes that were dry may have been dampened by the stage of antral follicle development at time of synchronisation. AMH concentrations are greatest in follicles of 2 mm in size (Campbell *et al.* 2012), and decrease as the follicles grow in size. These extremely high concentrations of AMH in the dry Suffolk ewe indicate a stage of the follicular wave in which there are a significant number of 2 mm follicles on the surface of the ovary, and perhaps this stage of the cycle limits the effects of progesterone synchronisation.

AMH concentration and relationship with litter size

In the Merino ewe, it is evident that dry animals have the lowest concentrations of AMH, and this increases as litter size does. This trend is similar to our previous work (Daly *et al.*, In press; Chapter seven). The average concentration for twin-bearing Merino ewes is similar to that of Suffolk twin-bearing ewes and much lower than the dry Suffolk ewes, again suggesting that there may be an optimal range for the selection of ewes into synchronisation protocols for mating. AMH concentrations taken pre- and post-oestrous synchronisation in mature ewes showed limited relationships to pregnancy status or number of live born lambs. In the mature Suffolk ewe, AMH concentrations were significantly greater pre-synchronisation in ewes that did not conceive and this warrants further investigation. In mature Merino ewes, AMH concentration was much lower for not pregnant ewe's pre-synchronisation, compared with Merino ewes that were pregnant. This suggests two areas of future work 1) investigation into breed cut off values and 2) closer assessment of the ovarian follicle pool at time of synchronisation. It appears from the current work that AMH concentrations in the Suffolk have the potential to predict fertility within a natural mating protocol, when taken close to the time of mating.

Differences in post-natal AMH profile between Australian Merino and Suffolk ewe lambs

Early in the post- natal period, AMH concentrations were higher in Merino compared with Suffolk ewe lamb. It is hypothesised that the peak seen in Merino ewe lambs may be specific to this breed, and that Suffolk ewe lambs may be much more variable throughout the pre-pubertal period as they share a similar pattern of development to Sarda ewe lambs. Flock variation must also be considered, it is possible that the genetics of the flock and environmental conditions may have influenced these findings, and it is recommended that additional flocks in different locations for both Merino and Suffolk ewes have AMH profiling completed to confirm this. Lahoz *et al.* (2014) found large variation in peak AMH concentrations from 3- through to 6-months of age, resulting in low repeatability in Rasa Aragonesa ewe lambs. Rasa Aragonesa ewes are a dual-purpose animal, mainly raised for meat, much like the Suffolk in Australia, therefore it is possible that in the Suffolk ewe lamb, a peak in AMH may yet occur, and this unaccounted variation between individuals may explain the limited relationships found in Suffolk ewe lambs and embryo development. AMH concentrations are positively correlated with fertility in cattle (Ribeiro *et al.* 2014; Jimenez-Krassel *et al.* 2015) and sheep (Lahoz *et al.* 2012; Lahoz *et al.* 2016) and in the current study, the Merino experienced what was likely a peak in antral follicles or drop in antral follicles at four weeks of age, with AMH concentrations correlated with differences in embryo development. Therefore, in agreement with Torres-Rovira *et al.* (2014), it is likely that animals could be classified as either high or low AFC based on AMH concentrations, and this could be more predictive of fertility outcomes.

The pattern of follicle development in the current study is consistent with earlier work in Merino (Kennedy 1974), Timahdite and D'man (Jorio *et al.* 1991) ewe lambs. The latter study showed an initial decrease in growing and pre-antral follicles from birth to 15-days of age, with no evidence of antral follicles. The number of growing follicles then reached a dramatic peak at 4-weeks of age before a modest decline at 8-weeks of age. There was a further, significant drop in follicle numbers at 12-weeks of age, and by 16-weeks of age these numbers had started to increase which is consistent with the Merino (Kennedy 1974). Pre- antral follicles displayed a similar pattern, aside from a relatively small change in numbers from 15-days to 4-weeks of age. At 4-weeks of age, both pre-antral and antral follicle numbers peaked. Similarly, to growing follicles, pre-antral follicles reached lowest value at 12-weeks of age and began to increase, whilst antral follicles also reached lowest values at 12-weeks, this was unchanged at 16-weeks of age (Jorio *et al.* 1991). Interestingly, during this time the size distribution of normal follicles differed significantly (P<0.05) between breeds until 12- to 16-weeks of age where distribution was similar (Jorio *et al.* 1991). Given the relationship between follicle number, size and the regulation of AMH by granulosa and theca cells discussed above, breed differences in the expression of AMH may be apparent. Discussed in detail below are differences in post-natal development of the follicle pool between and within breeds. Consideration into the relationship between the distribution of follicles may have had an impact the circulating concentrations of AMH.

AMH profile in the Suffolk from birth to 18 weeks of age

In the current study, the profile of antral follicle development in the Suffolk is consistent with previous observations in Sarda ewe lambs from Sardinia, Italy (Torres-Rovira *et al.* 2016). Similar to one cohort of Sarda ewe lambs (Torres-Rovira *et al.* 2016), one cohort of Suffolk ewe lambs in the current study exhibited no discernible peak in AMH over the 18-week period. However the other cohort of Sarda ewe lambs, showed a peak in AMH at 6-weeks of age, which was preceded by a steady increase in AMH from birth followed by a decline until 9-weeks of age (Torres-Rovira *et al.* 2016) and this profile was also observed in another cohort of Suffolk ewe lambs in the current study. The limitation in comparison is in the timing of sampling, Sarda ewe lamb sampling ceased at 9-weeks of age compared with 18-weeks of age in the current study. Interestingly, the differences dissipated after 12 weeks of age in the current study. However, in Rasa Aragonesa ewes, AMH concentrations were higher at 18- compared with 12- weeks of age (Lahoz *et al.* 2014), which may reflect differences in the utilisation of the breed, discussed below. The only difference in ovarian phenotype was a tendency for increased primordial follicle numbers in Suffolk ewe lambs exhibiting a peak in AMH concentration, and therefore antral follicle population, pre-pubertally at 18-weeks of age likely due to the fact that by 12 weeks of age AMH concentrations were similar. As reviewed by (Scaramuzzi *et al.* 2011) it is possible that these early differences in AMH are caused by differences in growth and metabolic rate of these lambs. There is the potential that this is a genetic effect, or an environmental maternal affect either *in utero* nutrition or through milk production.

AMH profile in the Australian Merino from birth to 18 weeks of age

The AMH profiles of the Merino ewe lambs were unexpected (Figure 1). All Merino ewe lambs exhibited a peak in AMH concentration at 6-weeks of age. What is interesting is that it appears that Merino ewes may experience one of two significant changes in antral follicle populations from 2- to 4-weeks of age, creating two different cohorts within the sample population. Based on changes in AMH, we hypothesize the size of the antral follicle pool increased steadily from 2-weeks of age until six-weeks of age in one cohort of ewe lambs, a pattern of antral follicle development is consistent with one cohort of both Suffolk and Sarda ewe lambs. Conversely, the second cohort, those with a higher concentration of AMH at 2-weeks of age, exhibited a significant decline in AMH and, therefore potentially antral follicles, at 4-weeks of age, which differs markedly to both the Suffolk ewe lambs in the current study and Sarda ewe lambs (Torres-Rovira et al., 2016). For all Merino ewe lambs in the current study, there was a subsequent decline in AMH concentration after 6-weeks of age, with no further differences in AMH concentration observed from this point onwards. A peak in AMH at 4-weeks of age was associated with the presence of significantly more primary and secondary follicles, and a numerical, but not significant, increase in the number of primordial follicles present on the ovary at 18-weeks of age. This finding agrees with Torres-Rovira *et al.* (2014), that Sarda ewe lambs with a high AFC possessed more primary follicles, and numerically a greater number of primordial follicles.

Correlations between AMH, the primordial and antral follicle pool and embryo development in the Suffolk

In Suffolk ewe lambs, AMH concentrations between 2-and 8-weeks of age were strongly correlated with the size of the pre-antral follicle pool, whereas between 10- and 18-weeks of age AMH concentration was strongly correlated with the antral follicle pool. AMH concentrations at 2- and 4-weeks of age were reflective of the number of primary follicles at 18-weeks of age, with AMH concentrations at 4-, 6- and 8-weeks of age reflective of the number of primordial follicles present at 18-weeks of age. In sheep, the primordial follicle pool is established prior to birth (McNatty *et al.* 1995) and AMH is only produced once primordial follicles are recruited and transition to the primary follicle stage of development begins (McGee and Hsueh 2000). The relationship between AMH concentrations during the first two months of life and the number of primordial and primary follicles is indicative of significant recruitment of primordial follicles into the growing pool during the first month, with stabilisation of the pre-pubertal preantral follicle pool occurring from 4-weeks of age. It is well characterised that AMH production is highest in small antral follicles and decreases with increasing follicle size (Campbell *et al.* 2012; Veiga-Lopez *et al.* 2012; Fleming *et al.* 2015). In the current study, high AMH concentrations at 12- and 16-weeks of age reflected low numbers of 3-5mm follicles, and at 12- and 18-weeks of age high AMH reflected high numbers of follicles between 1-3mm. This is unsurprising given (Monniaux *et al.* 2012) determined intrafollicular AMH concentrations were higher in 1-3 mm follicles compared with 3-5 mm follicles in Romanov sheep. Taken with the fact that AMH concentrations at 12- and 18-weeks of age correlated to the total number of surface follicles, the current data suggest that there is little change in the size and dynamics of the antral follicle population from 12-weeks of age in the Suffolk. In Suffolk ewe lambs there were two significantly different developmental patterns of AMH, and the timing of these were mirrored by significant correlations to primordial follicle pool development across this time period which indicate a relationship between the timing of primordial follicle recruitment, AMH and the antral follicle pool. Interestingly, these differences dissipate from 12-weeks of age, resulting in only minor differences in ovarian phenotype, and embryo development at 18-weeks of age.

Correlations between AMH, the primordial and antral follicle pool and embryo development in the Australian Merino

In Merino ewe lambs, AMH concentrations at 4-weeks of age could be used as a marker of fertility, with higher concentrations of AMH at 4-weeks of age associated with a higher rate of blastocyst development following *in vitro* oocyte maturation and fertilisation. This is an important finding, as it suggests that positive or negative proliferation of the ovarian follicle pool at 4-weeks of age translates into differences in oocyte quality within pre-pubertal Merino ewe lambs. This agrees with previous evidence in Rasa Aragonesa ewes (Lahoz *et al.* 2016), that animals with high AMH produced an additional 0.38 lambs per year. Similarly, when ewes were classified into groups based on AFC (Torres-Rovira *et al.* 2014; Torres-Rovira *et al.* 2016), those with high AFC had higher AMH and were more fertile at their first mating. In the current study in all the Merino ewe lambs, there were no discernible relationships between AMH at any age and the size of the pre-antral follicle pool, and limited correlations with the size of the surface antral follicle pool. There was a positive correlation between the number of small surface antral follicles (< 1 mm) and AMH at 6-weeks of age, and between the number of 1 – 3 mm follicles and AMH at 8-weeks of age. In contrast, high AMH at 10-weeks of age was associated with the presence of fewer 3 to 5 mm surface antral follicles at 18-weeks of age. Similar to the Suffolk ewe lambs, the negative relationship between larger antral follicles and AMH at 10-weeks of age could suggest the presence of an increased number of smaller follicles on the surface of the follicle. The positive relationship between AMH at 8-weeks of age and the number of 1-3 mm follicles may also reflect a stabilisation of the antral follicle pool from this time in Merino ewe lambs. The reasons for a lack of relationship to the ovarian follicle pool remain unclear. It is likely that the development of the pre-antral follicle pool is incomplete in the prepubertal animal. In the current study all animals were prepubertal and, therefore, AMH profiling for both breeds is indicative of follicular activity prior to puberty. Merino ewe lambs reach puberty across a wide range of ages (283-480 days of age) with seasonality and growth rates impacting this age (Hawker and Kennedy 1978). The ewes in the current study were significantly younger (130 days of age) which, together with the absence of corpora lutea, confirms their pre-pubertal status. In ewe lambs, the dynamics of the antral follicle pool remain relatively unchanged between 18- and 26-weeks of age, reflecting a stable pattern of FSH and LH release (Mahdi and Khallili 2008). The profiles of AMH observed in both Suffolk and Merino ewe lambs in this study suggests the antral follicle pool is also relatively stable from 10- to 18-weeks of age. In both cattle and sheep, where peak AMH concentrations are seen between 2- and 3-months of age, this peak is preceded by a rise in plasma FSH concentration (Torres-Rovira *et al.* 2014; El-Sheikh Ali *et al.* 2017) which would likely drive the increase in antral follicles (Rawlings *et al.* 2003) and therefore rise in AMH. As puberty approaches, the actions of FSH are reversed, in adult female cattle and sheep AMH concentrations decrease to a relatively stable base adult concentration, while FSH concentrations continue to increase (Torres-Rovira *et al.* 2014; El-Sheikh Ali *et al.* 2017). Therefore, there are differences in the feedback from FSH receptors dependant on the age of the animal. Extending on this, breed differences in the expression AMH have not been investigated, but given the relationship between FSH and AMH, both pre-pubertal and in adulthood, it is reasonable to suggest that notable differences in the expression off FSH receptors and FSH between breed, may also impact AMH concentrations. Serum FSH concentrations are slightly higher in Chinese Merino compared with Chinese Hu sheep (Li *et al.* 2019), and the expression of FSH receptors during the anoestrous period are significantly higher in Chinese Merino ovaries (Li *et al.* 2019). Given this strong seasonal effect on the expression of FSH receptors (Li *et al.* 2019), it is possible that this may influence follicle development, and consequently AMH concentration in the Suffolk in the current study. This may explain the significantly higher concentrations of AMH in dry Suffolk ewes and strengthen the need for breed specific ranges or cut-off values of AMH, perhaps between season.

Conclusion

The results from the current study suggest significant differences in the pre-pubertal development of the ovarian reserve between Australian Merino and Suffolk ewe lambs. However, this development does not result in major breed differences in ovarian phenotype at 18-weeks of age. Similarly, in the mature Merino and Suffolk ewe there were no differences in AMH concentration pre- or post-oestrous synchronisation. Similar to Sarda ewe lambs, prepubertal AMH concentrations in the Suffolk appear to conform to one of two clearly defined profiles. These differing cohorts seem to reflect differences in the development of the primordial follicle pool. Additionally, AMH concentrations determined closer to the time of slaughter, and therefore closer to the stage of development of the ovary, are more informative of phenotype suggesting that AMH samples may need to be collected just prior to mating or assisted reproductive technology in the Suffolk. In contrast, there are significant differences in the AMH profiles of South Australian Merino ewe lambs. However, this does not reflect any changes in the development of the primordial or antral follicle pool. Perhaps the most substantial conclusion from this work is the clear difference in profile of AMH at 4-weeks of age in the Merino ewe lamb, and the correlation at this time to *in vitro* embryo development. Taken together, this data suggests that AMH concentrations determined when a South Australian Merino lamb is 4 weeks old would offer the most informative prediction of oocyte quality. In conclusion, the results from the current study show different patterns of ovarian development, within breeds, and timing of sample collection may be informative of the development of the ovarian phenotype. For the Merino, AMH concentrations taken at 4-weeks of age may be used to predict animals with improved oocyte quality; however, in the Suffolk an AMH concentration determined closer to the time of the intervention may be required. In adulthood, AMH concentration is unaffected by oestrous synchronisation in either breed, and there are no differences in AMH between breed either pre- or post-synchronisation. The relationship between AMH and fertility differs significantly between breeds; where in the Merino increases in AMH are associated with a greater pregnancy rate and number of live lambs born, significantly more Suffolk ewes with high AMH concentrations were dry. This raises interesting questions into the expression of AMH, comparison between breed and perhaps across seasons. Future work in this area would involve: 1) extensive blood sampling of animals at ages where significant differences in AMH are seen and 2) mating the animals to assess the validity of using AMH concentration as a marker of fertility in the South Australian Merino and Suffolk ewe.

List of tables

Table 1: Pre and Post oestrous synchronisation AMH concentrations, average AMH concentration and number of lambs born for Merino and Suffolk ewes

	Merino	Suffolk	Significance
Pre[1] AMH (ng/ml)	3.26 ± 0.38	3.69 ± 0.40	0.468
Post[1] AMH (ng/ml)	3.65 ± 0.37	3.80 ± 0.38	0.791
Average AMH (ng/ml)	3.46 ± 0.34	3.74 ± 0.35	0.585
Number lambs born	0.69 ± 0.08	0.68 ± 0.07	0.994

[1] oestrous synchronisation using progesterone pessary

Table 2: Relationship between Pre and Post oestrous synchronisation AMH concentrations (ng/ml) and pregnancy scanning rates and number of lambs born for Merino and Suffolk ewes

	Merino			Suffolk		
	n	Pre^1 AMH	Post^1 AMH	*n*	Pre^1 AMH	Post^1 AMH
Pregnancy Status						
Dry	*10*	2.92 ± 0.59	3.43 ± 0.63	*16*	4.94 ± 0.99^a	4.36 ± 0.91
Pregnant	*22*	3.42 ± 0.49	3.75 ± 0.46	*34*	3.10 ± 0.32^b	3.53 ± 0.36
Number lambs born						
0	*10*	2.92 ± 0.59	3.43 ± 0.63	*16*	4.94 ± 0.99^a	4.36 ± 0.91^
1	*14*	3.24 ± 0.61	3.81 ± 0.64	*15*	2.59 ± 0.35^b	3.50 ± 0.76
2	*8*	3.72 ± 0.84	3.63 ± 0.66	*19*	3.51 ± 0.48^{ab}	3.55 ± 0.28

[1] oestrous synchronisation using progesterone pessary

Table 3: plasma AMH concentrations from 2 through to 18 weeks of age for Merino and Suffolk ewe lambs.

AMH (ng/ml)	Merino	Suffolk	Significance
Week 2	13.07 ± 2.57	3.69 ± 0.93	0.002
Week 4	8.52 ± 2.22	8.08 ± 1.77	0.880
Week 6	26.12 ± 2.46	11.12 ± 1.55	<0.001
Week 8	16.67 ± 1.67	5.77 ± 1.19	<0.001
Week 10	10.80 ± 1.37	5.61 ± 0.92	0.004
Week 12	4.33 ± 0.81	2.84 ± 0.51	0.142
Week 14	3.32 ± 0.63	2.66 ± 0.43	0.397
Week 16	1.60 ± 0.25	1.33 ± 0.18	0.385
Week 18	1.55 ± 0.21	0.81 ± 0.08	0.004
Average	9.05 ± 0.77	4.59 ± 0.55	<0.001

Table 4: Comparison of ovarian phenotype and embryo development parameters between Merino and Suffolk ewe lambs at 18-weeks of age.

	Merino	Suffolk	Significance
Total surface antral follicles <1mm	0.91 ± 0.31	1.40 ± 0.43	0.354
Total surface antral follicles 1-3 mm	6.05 ± 0.67	5.30 ± 0.77	0.468
Total surface antral follicles 3-5 mm	0.91 ± 0.23	0.75 ± 0.19	0.598
Total surface antral follicles >5 mm	0.18 ± 0.11	0.65 ± 0.18	0.029
Total surface antral follicles	8.05 ± 0.66	8.10 ± 0.79	0.950
Total primordial follicles	144 208 ± 44 398	148 579 ± 33 314	0.945
Total primary follicles	12 581 ± 4239	23 419 ± 7746	0.221
Total Secondary follicles	2550 ± 640	7411 ± 2241	0.040
Number of COCs recovered	2.05 ± 0.41	2.59 ± 0.45	0.381
Cleavage rate	0.54 + 0.37	0.62 ± 0.10	0.561
Blastocyst development rate (%)	0.11 ± 0.26	0.15 ± 0.23	0.628

Table 5: Comparison between Merino ewe lambs with either a PEAK or DROP AMH profile over 18 weeks and ovarian phenotype and embryo development parameters

	Drop (n=8)	Peak (n=14)	Significance
Total surface antral follicles <1 mm	1.00 ± 0.47	0.75 ± 0.25	0.706
Total surface antral follicles 1-3 mm	6.07 ± 0.93	6.00 ±0.93	0.961
Total surface antral follicles 3-5 mm	0.86 ± 0.29	1.00 ± 0.38	0.770
Total surface antral follicles >5 mm	0.21 ± 0.15	0.13 ± 0.13	0.698
Total surface antral follicles	8.14 ± 0.94	7.88 ± 0.85	0.851
Total primordial follicles	132 615 ± 46 087	163 045 ± 93 727	0.748
Total primary follicles	8107 ± 1427	19 852 ± 10 812	0.185
Total Secondary follicles	2256 ± 509	3028 ± 1517	0.572
Number of COCs recovered	2.08 ± 0.56	2.00 ± 0.62	0.925
Cleavage rate	0.53 ± 0.11	0.55 ± 0.14	0.924
Blastocyst development rate	0.03 ± 0.03	0.26 ± 0.14	0.056

Table 6: Comparison between Suffolk ewe lambs with a PEAK or NO peak AMH profile over 18 weeks and ovarian phenotype and embryo development parameters

	Peak (n=14)	No Peak (n=6)	Significance
Total surface antral follicles <1mm	1.07 ± 0.34	2.17 ± 1.22	0.256
Total surface antral follicles 1-3mm	6.07 ± 0.96	3.50 ± 1.06	0.130
Total surface antral follicles 3-5mm	0.64 ± 0.20	1.00 ± 0.41	0.404
Total surface antral follicles >5mm	0.50 ± 0.20	1.00 ± 0.37	0.216
Total surface antral follicles	8.29 ± 0.99	7.67 ± 1.38	0.729
Total primordial follicles	197 302 ± 58 799	34 891 ± 14 385	0.093
Total primary follicles	29 797 ± 10 661	8538 ± 2699	0.217
Total Secondary follicles	8577 ± 2699	4691 ± 3141	0.442
Number of COCs recovered	2.83 ± 0.59	2.00 ± 0.55	0.412
Cleavage rate	0.53 ± 0.12	0.70 ± 0.20	0.609
Blastocyst development rate	0.18 ± 0.07	0.10 ± 0.10	0.548

List of figures

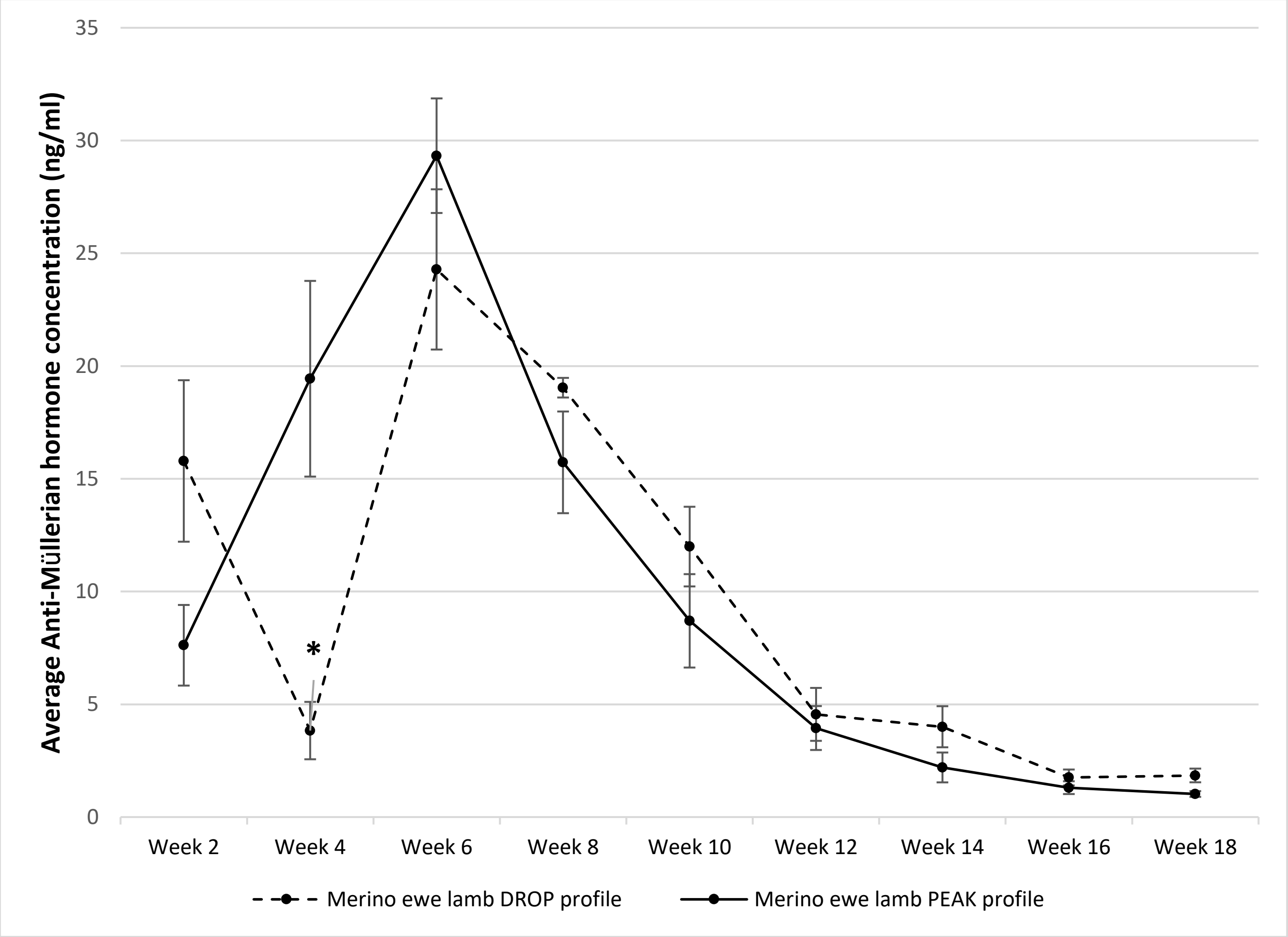

Figure 1: Average AMH profiles for Merino ewe lambs (n=22) identified as having either a peak AMH (n= 14) concentration at 4 weeks of age, or having a significant drop in AMH (n= 8) at 4 weeks of age. *indicate significant difference between PEAK and DROP Merino within that time point

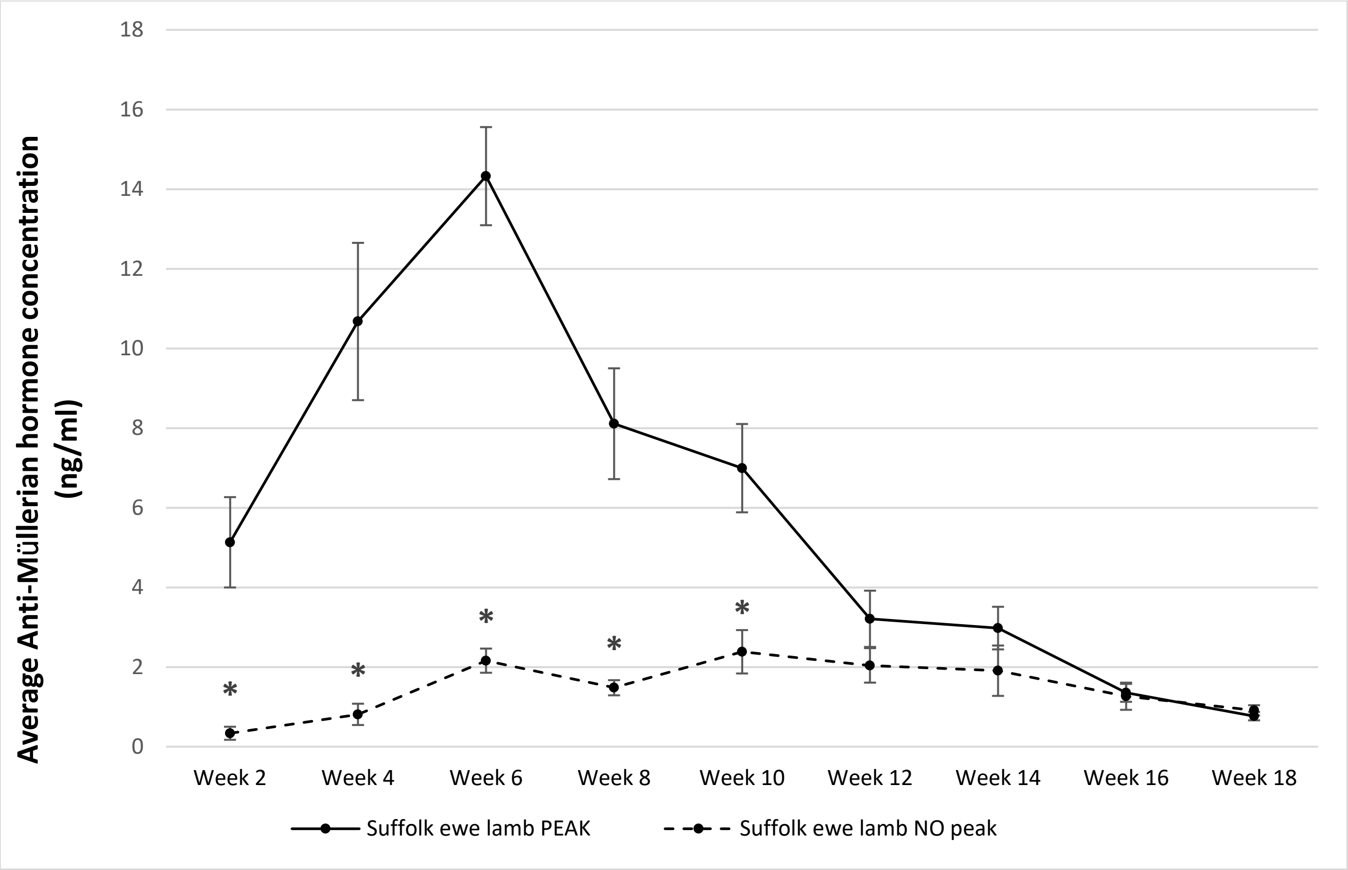

Figure 2: Average AMH profiles for Suffolk ewe lambs (n=20) identified as having either a PEAK AMH concentration between weeks 4 and 8 of age (n=14), or having NO Peak AMH throughout the 18 week period (n = 6). **indicate significant difference between PEAK and NO Peak Suffolk within that time point*

Chapter Four: AMH concentration changes throughout the oestrous cycle in the Australian Merino

Abstract

Anti-Müllerian hormone (AMH) plays an inherent role at key stages of the reproductive cycle by regulating primordial follicle activation and selection of dominant follicles for ovulation. AMH is therefore an accurate phenotypic marker of ovarian reserve and the antral follicle pool with proven effectiveness as a marker of response to hormonal stimulation in sheep. Literature regarding repeatability in sheep is limited and conflicting with one study suggesting a low repeatability over the first 6 months of life, but high between laparoscopic ovum pick up (LOPU) sessions. Additionally, only one study over 9 days of an induced oestrous cycle has mapped AMH concentration. Therefore, using ten naturally cycling and ten progesterone-pessary treated animals, the aim of this study was to determine if AMH is repeatable and how AMH changes across the oestrous cycle in Merino ewes. AMH concentration was highly repeatable across 14 days of the oestrous cycle in naturally cycling, and from days 3 to 14 in progesterone treated ewes. AMH concentration reflected the changes in follicle development throughout the oestrous cycle, and were less variable in those treated with a progesterone pessary. Interestingly, a negative correlation between AMH on the day of pessary insertion and both onset of oestrous and day of ovulation ($P<0.05$) suggests AMH concentration may be used to reduce variation within an assisted reproductive technology (ART). The relationship between AMH concentration and progesterone is important and complex, and this is the first study to identify the relationship between these two hormones across and oestrous cycle. In conclusion, this is the first study to thoroughly track the changes in AMH throughout an oestrus cycle in sheep and in relation to progesterone. We found AMH to be highly repeatable within a natural sheep oestrous cycle, and that it has potential to reduce the variation in an ART through predicting the onset of oestrous and ovulation.

Introduction

Anti-Müllerian hormone (AMH) is a highly conserved glycoprotein of the transforming growth factor (TGF-β) family (Monniaux *et al.* 2013). The inherent role of AMH is in the sexual differentiation of the fetus (Rey *et al.* 2003). In all species, AMH has a fundamental role of controlling the oocyte reserve in the mature female (Umer *et al.* 2019). Specifically, AMH is a paracrine regulator of primordial follicle activation in mice (Durlinger *et al.* 2002a) preventing exhaustion of the immature follicle reserve and playing a regulatory role in the selection of dominant follicles (Umer *et al.* 2019). Therefore, AMH has been considered a phenotypic marker of follicle reserves in cattle (Ireland *et al.* 2008; Monniaux *et al.* 2010; Mossa *et al.* 2017; Cardoso *et al.* 2018), goats (Monniaux *et al.* 2011), and sheep (Campbell *et al.* 2012; Lahoz *et al.* 2012). More recently, AMH has proven to be an effective marker of fertility in cattle (Ribeiro *et al.* 2014; Jimenez-Krassel *et al.* 2015; Batista *et al.* 2016). Although AMH is a potential marker of fertility in sheep, concentrations during the oestrous cycle in the ewe are not well documented.

In the dairy cow, AMH remains static throughout the oestrous cycle (Rico *et al.* 2009; El-Sheikh Ali *et al.* 2013; Souza *et al.* 2015). Further, a single measurement of AMH in beef heifers is positively correlated (r=0.97) with average AMH concentrations across a variety of time points over multiple days and oestrous cycles (Ireland *et al.* 2011; El-Sheikh Ali *et al.* 2013). Extensive work demonstrates that for Holstein dairy cows, the profile of AMH during the natural oestrous cycle changes similarly for all animals (Rico *et al.* 2011). Essentially, there is a rapid decrease in AMH concentrations after oestrus, reaching minimal values between days 4 and 8 of the cycle followed by a slow increase until the next oestrus (Rico *et al.* 2011). Given that healthy follicles between 1 and 5 mm in diameter exhibited the greatest granulosa cell expression of AMH, this pattern within the oestrous cycle is not surprising (Rico *et al.* 2011). In sheep, it appears that AMH concentrations have a low repeatability within animal across six months. However, this assessment (Lahoz *et al.* 2014) is based on three samples at 3, 4.5 and 6 months of age and is likely to be confounded by AMH peaks occurring over different times and days. However, the repeatability estimate was stronger in lambs in which concentrations peaked at 4.5 months of age. Promisingly, AMH concentration was repeatable when compared over either 2 ($r=0.70$, $p<0.001$) or 4 ($r=0.57$, $p<0.001$) successive laparoscopic ovum pick-up sessions, each 7 days apart (Lahoz *et al.* 2014). Therefore, the major aim of this study was to characterise AMH changes during the oestrous cycle as well as determining the repeatability of AMH concentrations within the cycle.

To date, only one study has assessed AMH concentrations across 12 days of an oestrous cycle induced by prostaglandin F2alpha (PGF2α) treatment. Starting two days prior to treatment, AMH concentration in Barki sheep rapidly declined reaching minimal concentrations two days post-injection, coinciding with the lowest concentration for progesterone and the peak oestrogen concentration (Waheeb 2017). Whilst the peak in progesterone was not identified within the sampling period, AMH concentrations peaked five days post-ovulation, when oestrogen concentrations reached their lowest concentration and progesterone was still rising. The results indicate an inverse relationship between AMH concentration and oestrogen concentrations but the nature of the relationship with progesterone remains unclear. The relationship between AMH and progesterone concentrations is of interest because intravaginal progesterone pessaries are used widely in controlled breeding programs (Wildeus 2000) Progesterone- based oestrous synchronisation protocols effectively manipulate the growing follicle pool in the sheep (Rosasco *et al.* 2019) but, from an applied perspective, there remains significant variation in the timing of oestrous onset among individuals. Given AMH is considered a strong phenotypic marker of ovarian follicle reserves, understanding the relationship between progesterone concentrations and

AMH will allow an investigation of the potential value of using AMH concentration as a predictor of ovulation, particularly in progesterone-treated animals.

Materials and Methods

All procedures were conducted according to the guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes and the work approved by Primary Industries and Regions South Australia (PIRSA) (Approval numbers 14/17 and 19/20). The work was conducted using 20 South Australian Merino ewes between five and six years of age located at the Turretfield Research Centre, Rosedale, South Australia (34°38’S, 138° 44’E). All ewes were provided feed and water *ad libitum* and managed under standard husbandry practices.

Experimental design and animal management

In Autumn (Southern Hemisphere) 2019, Ten control ewes were identified as being in oestrus (Day 0) using testosterone-treated wethers (Ropel, Jurox Animal Health, Rutherford, New South Wales, Australia) fitted with harnesses/crayons. Another 10 ewes were randomly selected and received intravaginal progesterone pessaries (0.3 mg progesterone, CIDR, Pharmacia and Upjohn, New South Wales, Australia) for 14 days (Day 1 = day of pessary insertion). On day 14, pessaries were removed and each ewe received 400 i.u. equine chorionic gonadotrophin (eCG) (i.m.; Novormon, Syntex, Buenos Aries, Argentina or Pregnecol, Bioniche Inc., Belleville, Ontario, Canada). Ewes were exposed to testosterone - treated wethers fitted with harnesses/crayons at pessary removal. To determine time of onset of oestrus, ewes were observed every three hours from 21 – 48h after pessary removal and then daily until 96h.

Daily trans-rectal ultrasound observations were made from Day 1 and continued until ovulation was detected or until Day 18. Daily follicle maps (see below) were created for each ewe and data on follicle numbers and size as well as follicle wave formation extracted. The day of emergence of the ovulatory follicle was determined by retrospective inspection of the follicle map once ovulation had occurred.

Ultrasonography

Ovarian morphology was examined by trans-rectal ultrasonography using a real time 7.5mHz linear-array transducer (Mindray Z6 Vet Portable Scanner, Bellshill, Scotland). Sheep were examined in a recumbent position in a sheep conveyor handler (Peak Hill Industries, Peak Hill, New South Wales, Australia) using procedures similar to those outlined by Ginther et al. (1995). Multiple five-second video images of each ovary were recorded and subsequently examined to identify follicle location and to measure follicle diameter. Each measurement was a mean of the two longest dimensions taken at approximate right angles to each other.

All follicles were counted and measured on a daily basis. Small, medium and large follicles were defined as having mean diameters of ≤2mm, 2.1 – 3.7mm and ≥3.8mm respectively. Any follicle ≥3.8mm in diameter was deemed to be potentially ovulatory given observations that follicles of this size (based on their last measurement) were capable of ovulating.

Blood sampling and AMH measurement

All samples were collected at the same time of day, and within the same period of time. All samples were collected in 9 ml lithium heparin vacuum tubes (BD vacutainer, Oakville, ON, Canada) and stored on ice until centrifuged at 1500 rpm for 15 minutes. Serum was subsequently stored at -20°C until assayed for AMH and progesterone concentrations.

Hormone assays

The plasma AMH concentrations were measured in 50 µl duplicate samples according to the manufacturer's specifications using the AMH (Ovine) ELISA kit (AL-155, Ansh Labs, Webster, Tx) (6.08%, 0.44% intra and inter assay coefficient of variation, respectively). Variation determined over 8 assay plates, including all reanalysed samples (80 samples per plate), all samples from the same collection day were run on the same plate. On average 2 days of the oestrous cycle from each ewe were run on a single plate. MDC calculated by the interpolation mean plus two standard deviations of 24 replicates of calibrator A (0 ng/ml) and low calibrator (0.37 ng/ml). Upper limit of standard curve at absorbance of 2.91 (20 ng/ml) measured at 450 nm. Progesterone was extracted from plasma samples as per the Arbor Assays steroid liquid sample extraction protocol (Appendix A). Concentrations of progesterone were determined using Arbor Assay ELISA kits (Arbor Assays, Ann Arbor, MI, USA) (6.5%, 9.2% intra and inter assay variation, respectively) according to manufacturer's instructions.

Statistics

Differences between progesterone-treated and control ewe follicle parameters and AMH concentrations were determine using ANOVA (SPSS Version 26(IMB Corp.)). Repeated Measures analysis was used to determine significant differences in AMH concentration for each group. Correlations between AMH concentrations across each day of the oestrous cycle and the timing of oestrus, day of CL regression, ovulation number, time of emergence and age of the pre-ovulatory follicle, number of follicle waves and follicle size were analysed using SPSS Version 26.

Results

AMH profiles in progesterone-treated and naturally cycling ewes

There were no significant differences in AMH concentrations on any day of the oestrous cycle between naturally cycling and progesterone- treated ewes. Variation on each day was reduced in progesterone-treated ewes (Figure 1). Whilst AMH concentration were highly repeatable, with minimal variation, across all days of the oestrous cycle in naturally cycling ewes, AMH concentrations were only similar from days 3 to 14 in the progesterone-treated ewes (Figure 1).

As shown in Table 1, in progesterone-treated ewes luteal regression ($P< 0.01$), timing of oestrus ($P< 0.001$) and ovulation ($P=0.059$) occurred earlier compared with naturally cycling ewes. Age of the pre- ovulatory follicle was longer in progesterone- treated animals ($P<0.01$). There were no differences in the number of ovulatory follicles or ovulations, the time of emergence of the first follicle wave or the number of follicle waves (Table 1). Naturally cycling ewes had a significantly higher mean number of follicles ≥ 3mm (Table 1; $P < 0.05$) but there was no difference in the number of follicles within other size categories (Table 1).

Correlation of AMH concentration and progesterone concentrations in natural cycling ewes

Progesterone concentration was lowest on Day 2 and highest on Day 13, with a noticeable peak on Day 4, and drop at Day 11 (Figure 2). AMH concentration on all days of the cycle, except Day 4, was strongly and positively correlated with progesterone concentration on Day 4 (Table 3, $P<0.05$, $P<0.01$, *Appendix three*). AMH concentration on all days expect Days 2, 3 and 4 was strongly and positively correlated with progesterone concentration on Day 12 (Table 3, $P<0.05$, $P<0.01$, *Appendix three*).

Correlation of AMH concentrations and parameters of the oestrous cycle

In naturally cycling ewes, the number of follicles ≤ 3mm was positively correlated with AMH concentrations on Day 5 of the oestrous cycle (Figure 3, Table 2; $P<0.05$). Otherwise, there were no significant correlations between AMH concentrations and parameters of the oestrous cycle. In progesterone-treated ewes, the mean AMH concentration on Day 1 (prior to pessary insertion) was strongly and negatively correlated with the time of onset of oestrus (Figure 4, Table 4; $P < 0.05$) and the day of ovulation (Figure 5, Table 4; $P < 0.05$). As AMH increased from 2 to 4 and then 6 ng/ml, onset of oestrous occurred sooner at 27, 24.3 and 21 hours respectively, while each 2 ng/ml increase also reduced the timing of ovulation by 12 hours (day 17, 16.5 and 16 respectively). AMH concentration on Day 2 was correlated with the number of follicles ≤ 3mm prior to pessary insertion (Table 4; $P < 0.05$).

Discussion

It is concluded that AMH concentrations throughout a single ovine oestrous cycle are highly repeatable for individual ewes. Variation in AMH concentrations are significantly reduced in animals treated with a progesterone pessary. Importantly, AMH concentrations in naturally cycling animals were unrelated to parameters of the oestrous cycle with the exception of Day 5 where there was a significant ($P<0.05$) correlation with the number of small follicles. Interestingly, ewes with high AMH concentrations on the day of pessary insertion (Day 1) displayed oestrus earlier than those with lower AMH concentrations. Not surprisingly, these ewes subsequently ovulated earlier, suggesting that AMH concentration prior to pessary treatment is a predictor of the timing of oestrus in ewes prepared for fixed-time insemination. Furthermore, in these animals, AMH concentration on the day of pessary removal (Day 14) was correlated with daily concentrations of AMH from two days after pessary insertion, suggesting that progesterone stabilises the size of the follicle pool. AMH and progesterone concentrations displayed similar profiles across the oestrous cycle, with a noticeable change in this relationship after Day 5. AMH concentrations were relatively high compared with progesterone concentrations prior to a peak in both being reached on Day 4. Thereafter, the relative increase in progesterone concentration was more substantial than the increase in AMH concentration. Strong correlations between AMH concentrations across the entire cycle and progesterone on Days 4 and 12 indicate potentially important points of regulation. It is hypothesised that this mirrors a change in the regulatory factors produced by granulosa cells as well as other important oocyte-secreted factors.

Repeatability of AMH within ewes

The current data demonstrates that AMH concentration is highly repeatable within animal for both progesterone-treated and naturally cycling ewes over a single oestrous cycle. In naturally cycling ewes, there was no significant difference in AMH concentrations over 14 days. AMH concentrations in dairy cattle remain static throughout the oestrous cycle (Rico *et al.* 2009; El-Sheikh Ali *et al.* 2013; Souza *et al.* 2015) and, in adult beef heifers, a single AMH measure was positively correlated with all other measures taken at different times of the cycle (Ireland *et al.* 2011). However, to date, repeatability of AMH concentrations in sheep has remained unclear. In Rasa Aragonesa ewes, the timing of AMH peaks was highly variable, limiting repeatability (Lahoz *et al.* 2014). AMH concentrations at 1.6, 6.4 and 16 months of age in Sarda lambs showed significant variation, as did weekly sampling over 9 weeks as there was significant variation depending on the presence of either low or high number of antral follicles. Otherwise, variability within each of these cohorts was low, suggesting a high repeatability of AMH over the first nine weeks of life (Torres-Rovira *et al.* 2016). Interestingly, in the current study, AMH concentrations across all days of the oestrous cycle in progesterone-treated ewes were correlated with progesterone concentrations on all days except Days 1 and 2, suggesting that progesterone can alter the development of the ovarian follicle pool. The progesterone pessary elicited a gradual increase in progesterone concentration until a peak occurred four days after insertion and it is possible that a threshold concentration is required to effect changes in the pool of growing follicles.

AMH concentrations throughout the oestrous cycle

In naturally cycling ewes, concentrations were lowest on Day 6 and highest on Day 12. In progesterone-treated animals, AMH profiles were similar but less variable and were lowest on Day 7 and highest on Day 11. Similarly, to Barki ewes, in which ovarian activity was stimulated using PGF2α, AMH concentrations were lowest 48 hours after the onset of a new oestrous cycle (Waheeb 2017). However, in the current study AMH concentrations did not peak until day 14, whereas in the Barki ewes a peak in concentration was seen on day 5 (Waheeb 2017). Another clear difference between these two studies, is the range of AMH. In the current study, AMH in Merino ewes ranged from 1- 8 ng/ml, which is lower than was reported for Barki ewes (1 - 11 ng/ml). This may reflect breed differences in AMH concentration, as evident in the previous chapter, or the effects of endogenous stimulation on ovarian activity. Explained in detail below, the relationship between progesterone and AMH is complex; however, it does appear similar to Barki ewes, whereby AMH concentrations share a similar pattern to that of progesterone, just with more subtle fluctuations (Waheeb 2017).

Relationship between AMH and follicle dynamics

In this study, the pattern of AMH concentration across the oestrous cycle reflects changes in antral follicle development and activity. The total number of follicles was lowest on Day 5 for both groups of ewes, one and two days prior to their lowest AMH concentrations. Not surprisingly, in naturally cycling ewes the peak in the number of small follicles occurred on

Day 1 whilst the highest number of large follicles (≥3mm) occurred on Day 12, at both these times AMH concentrations was also high. This pattern of follicle growth is similar in both cattle and sheep where the rises in FSH and oestrogen concentrations and the selection of the dominant follicle occur in a comparable manner (Clarke 1995). The rise in oestrogen concentrations stimulates an increase in LH release (Clarke 1995), which is necessary for previously FSH-dependent follicles to maintain growth and development (Hunter *et al.* 2004). AMH is implicated in this process (Umer *et al.* 2019) as it decreases the responsiveness of other smaller follicles to FSH (Durlinger *et al.* 2002b). In the current study, the number of small follicles peaked again on Day 11, suggesting that follicles may become gonadotrophin dependent at this time, inhibiting the development of smaller follicles and allowing for the progression of the ovulatory follicle, aligning with the peak in AMH concentration and number of large follicles on Day 12. Together, these data demonstrate the relationship that exists between AMH and the antral follicle pool. In progesterone-treated animals, a different relationship was found. It appears the progesterone pessary caused a delay in the peak in AMH concentration by 24 hours, and the number of small follicles by 48 hours. In addition, AMH concentration reached minimal values three days later than in the naturally cycling animals. The peak in the number of follicles ≥3mm was observed five days earlier than in the naturally cycling animals, again suggesting a suppression of AMH concentration by the progesterone pessary.

AMH concentration is now widely accepted as a measure of the size of the growing antral follicle pool (Campbell *et al.* 2012; Veiga-Lopez *et al.* 2012; Umer *et al.* 2019) and this is supported by the current data. AMH concentration was correlated with the number of follicles ≤ 3mm on Day 5 for both the progesterone-treated and naturally cycling ewes. In the progesterone-treated ewes, the total number of follicles was lowest on Day 5 and, for naturally cycling counterparts, the number of follicles ≤3mm was also lowest on this day.

AMH concentration is maintained in small antral follicles (Campbell *et al.* 2012; Veiga-Lopez *et al.* 2012) and these follicles generally vary between 2 and 3mm in size (Campbell *et al.* 2012). AMH concentrations are highest in primordial, primary and secondary follicles (Umer *et al.* 2019) with concentrations maintained within pre-antral and early antral follicles, and then decreasing as the size of the follicle increases (Campbell *et al.* 2012; Veiga-Lopez *et al.* 2012) with AMH concentration declining significantly once follicles grow beyond 3 mm (Campbell *et al.* 2012). Our previous work (Seccafien *et al.* 2019) has found an increase in follicular fluid AMH as the number of ≤2mm follicles increase, therefore suggesting AMH production is optimised in follicles that are about 2 mm in size. The correlation between AMH concentration and the number of follicles ≤3mm observed in the current study support this finding.

AMH as a marker of the onset of oestrus and ovulation

It is clear from this study that progesterone treatment affects follicular dynamics and that this also affects AMH concentrations. One important finding from this study is that AMH concentrations in progesterone-treated ewes appear to influence the timing of ovulation, with AMH concentrations prior to pessary insertion correlating significantly and negatively with the onset of oestrus and day of ovulation. On average AMH concentrations of 2 ng/ml resulted in an oestrous onset of 27 hours, and ovulation on day 17. As AMH increased to 4 ng/ml onset of oestrous averaged 24.3 hours and day of ovulation 16.5, and a further increase in AMH by 2 ng/ml, saw a further reduction in oestrous onset (21 hours) and half day earlier ovulation (day 16). There was also a positive relationship between the number of follicles ≤3mm and AMH concentration the day after pessary insertion (Day 2). This suggests that the nature of the follicle pool at the time of pessary insertion may impact how progesterone affects follicle development. Furthermore, the correlation between AMH concentration and the number of small follicles 24 hours after pessary insertion, indicates that the impact of exogenous progesterone is quickly established. AMH inhibits FSH-dependent growth of antral follicles (Durlinger *et al.* 2002b) and it has been hypothesised that increased follicular concentrations of AMH found in persistent follicles may prevent the development of other follicles (Díaz *et al.* 2018). AMH regulates primordial follicle activation (Durlinger *et al.* 2002a), thereby controlling the recruitment of a pool of follicles from which one or more pre-ovulatory follicles are selected (Hunter *et al.* 2004). Furthermore, AMH also affects selection of dominant follicles (Umer *et al.* 2019), by decreasing the responsiveness of pre-antral and small antral follicles to FSH, aiding in the selection of a dominant follicle (Durlinger *et al.* 2002b). As indicated in the previous chapter, it is evident that AMH concentration is an accurate predictor of the response of follicles to hormonal stimulation. Therefore, it is not surprising that within the progesterone treatment, the data indicate that AMH may be involved in the control of ovulation as well as determining the outcome of hormonal stimulation. It is hypothesised that the increased AMH concentration leads to an earlier ovulation as a result of smaller follicles being able to respond more rapidly to the decline in progesterone and rise in gonadotrophins at time of pessary removal.

Relationship between progesterone and AMH

In the current study, it is not surprising that AMH concentrations appear reduced and less variable in ewes treated with progesterone pessaries. In heifers, progesterone treatment resulted in smaller maximal diameter of the dominant follicle and had a suppressive effect on follicle development (Adams *et al.* 1992). The role of progesterone treatment is to control physiological aspects of the oestrous cycle by increasing the exposure of the ewe to this hormone (Wildeus 2000). AMH modifies pre-antral and small antral follicle growth by decreasing the responsiveness to FSH and dictates the recruitment of the large pre-antral and small antral follicles (Durlinger *et al.* 2002b). Essentially, in gonadotrophin- dependant small follicles, AMH supresses the action of FSH and, in the larger growing follicles, reduces the sensitivity of the follicles to FSH (Umer *et al.* 2019). Additionally, high progesterone concentrations can delay FSH secretion in heifers (Adams *et al.* 1992) and is the key regulator of FSH concentrations in ewes (Baby and Bartlewski 2011). FSH inhibits growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) induced AMH expression, and reduces AMH mRNA in mice ovaries (Roy *et al.* 2018) further explaining the reduced variation seen in progesterone-treated ewes. As explained above, differences between ewes with high and low AMH concentrations in the total number of follicles suggests a suppressive effect of progesterone on AMH concentrations. To best understand the physiological relationship between AMH, progesterone and follicle dynamics, naturally cycling animals were used. The difficulty in drawing conclusions from pessary-treated animals is that responses are the result of both exogenous and endogenous progesterone concentrations. Luteal regression occurs at different times throughout pessary treatment resulting in progesterone profiles which differ between sheep. This may explain why limited correlations were found between AMH and progesterone concentrations in the treated ewes. This study has determined that AMH concentrations are highly repeatable within an oestrous cycle, which indicates that minor changes in concentration may reflect more significant changes within the ovary. The days of particular interest are those when AMH and progesterone concentrations are correlated namely Days 4 and 12, and less so Days 5 and 7 (Appendix three).

Across an oestrous cycle, the pattern of AMH and progesterone concentration is similar; however, variation in AMH concentrations is more moderate. AMH concentration was highest on Day 1, and lowest on Day 3, with progesterone concentrations also declining rapidly between Days 1 and 3, in both natural cycling and progesterone- treated ewes, suggesting a positive relationship between these two hormones. There was an unexpected peak in progesterone concentration on Day 4, a pattern shared with AMH. Prior to this peak, progesterone concentrations were relatively lower than AMH concentrations but, after Day 5, the reverse occurred. Together, these data suggest that, in a natural oestrous cycle, progesterone may suppress AMH prior to Day 4, after which the CL and granulosa cells may drive AMH concentrations. AMH is produced exclusively by the granulosa cells of small growing follicles (Campbell *et al.* 2012), whilst luteinised granulosa cells are responsible for the production of progesterone (Songsasen *et al.* 2011). It is therefore possible that, as the CL changes in age, the action of progesterone is altered thus affecting the growth of follicles. This would most likely occur on days when the AMH concentration is correlated with the progesterone concentration.

Alternatively, AMH may play a regulatory role within the luteal phase. Anomalies in the progesterone profile align closely with the days when AMH concentrations are positively correlated with progesterone concentrations. On Day 4, progesterone concentration correlated significantly with AMH concentration across all days except Day 4 whilst on Day 11, AMH concentrations increased mildly whereas progesterone showed a decline. Within 24 hours, the progesterone concentration was strongly correlated with AMH concentration and this continued up to the peak in progesterone on Day 13. Within the transforming growth factor family, TGFβ1 attenuates the expression of the progesterone receptor (Kane *et al.* 2008), GDF-9 stimulates progesterone synthesis (Elvin *et al.* 2000) while BMP-15 suppresses progesterone production (Chang *et al.* 2013). Taken together, it is possible that these factors together with AMH are responsible for the dynamic changes in progesterone concentrations at key stages within the oestrous cycle, leading to the correlations that are outlined above.

On Day 1, follicles ≤3mm were at their highest number whilst follicles ≥3mm were at their lowest number. Progesterone concentrations peaked on Day 13 coincident with the next highest value of AMH and a peak in the number of follicles ≥ 3mm. The relationship between small antral follicle number and AMH is well established thus explaining the high number of follicles ≤3mm at the beginning of a new cycle. The next peak in AMH coincides with the highest number of large follicles (≥3mm) and this is surprising given AMH is not expressed in large antral follicles. As mentioned above, progesterone treatment resulted in a smaller maximal diameter of the dominant follicle and this is in keeping with the suppressive effect of progesterone on follicle development (Adams *et al.* 1992). For the first five days of the natural cycle, progesterone concentrations were lower than those of AMH and, on Day 5, there was a correlation between AMH and number of follicles ≤3mm, further supporting the idea that progesterone may influence the size of growing follicle pool. Additionally, aside from days 2, 3 and 4, AMH concentration on all days of the oestrous cycle were correlated with progesterone concentrations on Day 12. This is important as AMH was positively correlated to follicles ≤3mm at day 5, and progesterone reached its lowest concentration at day 11, further suggesting a strong regulatory role of AMH at around day 12.

In summary, this is the first study to measure AMH concentrations during a natural oestrous cycle and, therefore, demonstrate that AMH concentration is highly repeatable for individual ewes, and reiterate the positive relationship between AMH and the antral follicle pool. The relationship between AMH and progesterone is complex and there are different perspectives on the interactions between AMH, the size of the antral follicle pool and progesterone concentration. Further investigation is warranted to better understand the role of AMH and other transforming growth factor family members on progesterone production and control of the antral follicle pool. In this study, the action of the progesterone pessary appears to inhibit the production of AMH resulting in changes in the proliferation of small follicles and the growth of large follicles. The strong negative correlation identified between AMH on

Day 0 and the onset of oestrus and time of ovulation suggests that AMH concentration may be a potential marker of these parameters in controlled breeding programs.

List of tables

Table 1: Comparison of follicular measurements throughout the oestrous cycle in control ewes (n=9) and ewes treated with intravaginal progesterone pessaries (n=10) over 14 days

	Control	Treated[1]	Sig.
Onset of oestrus (h)*	50.4 ± 5.29	23.4 ± 0.88	0.003
CL regression (day**)	15.5 ± 0.34	8.22 ± 1.64	0.002
Number of CL	1.7 ± 0.15	1.2 ± 0.2	0.66
Day of ovulation**	17.6 ± 0.31	16.5 ± 0.17	0.059
Number of ovulated follicles	1.5 ± 0.17	1.4 ± 0.16	0.548
Number ovulated follicles	2.8 ± 0.42	2 ± 0.33	0.171
Emergence of the first ovulatory follicle (h)	11.6 ± 0.5	6.1 ± 0.91	0.084
age of the first ovulatory follicle (h)	6.0 ± 0.47	10.5 ± 0.91	0.02
Number of follicle waves	4.8 ± 0.58	4.1 ± 0.55	0.878
Number follicles ≤3mm pre[1]	13.2± 1.70	10.0± 1.08	0.129
Number follicles ≥3mm pre[1]	2.2 ± 0.66	2.2 ± 0.39	1.0
Number follicles ≤3mm post[1]	9.0 ± 0.89	8.2 ± 0.88	0.532
Number follicles ≥3mm post[1]	4.3 ± 0.45	2.5 ± 0.37	0.006

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[1] Treatment with intravaginal progesterone pessary

*Relative to day 14/day of pessary removal

**Relative to day of pessary insertion (treated ewes) or day after onset of oestrus in control ewes

Table 2: Correlations(R^2) between AMH concentration and parameters of the oestrous cycle and follicular development in control ewes (n=9) from day 1 to 14 _*indicate significance $P < 0.05$._

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Oestrus (hr)[1]	-0.13	-0.17	-0.11	-0.14	-0.22	-0.20	-0.18	-0.18	-0.18	-0.06	-0.29	-0.24	-0.20	-0.23
Number of CL	0.18	0.05	0.05	-0.29	0.17	0.22	0.17	0.21	0.18	0.16	0.16	0.13	0.21	0.18
CL regression	0.15	0.22	0.29	0.28	0.12	0.09	0.12	0.13	0.13	0.23	0.00	0.08	0.11	0.10
Day of ovulation	0.09	0.10	0.17	0.18	0.02	0.02	0.06	0.07	0.08	0.20	-0.06	0.01	0.07	0.05
Number of ovulated follicles	-0.39	-0.23	-0.30	0.05	-0.38	-0.38	-0.37	-0.43	-0.41	-0.35	-0.38	-0.42	-0.35	-0.37
Emergence of first ovulatory follicle	-0.01	-0.01	0.01	-0.22	-0.08	-0.06	-0.05	-0.03	-0.06	-0.04	-0.13	-0.09	-0.14	-0.12
Age of first ovulatory follicle	0.10	0.10	0.14	0.38	0.10	0.08	0.10	0.09	0.13	0.21	0.08	0.10	0.20	0.17
Number follicles ≤3mm pre	0.51	0.60	0.63	0.51	0.68*	0.59	0.55	0.59	0.59	0.50	0.56	0.61	0.65	0.65
Number follicles ≥3mm pre	-0.12	-0.22	-0.24	-0.33	-0.12	-0.08	-0.10	-0.07	-0.06	-0.14	-0.01	-0.03	-0.13	-0.07
Number follicles ≤3mm post	0.14	-0.06	-0.09	-0.48	0.05	0.13	0.10	0.13	0.07	-0.02	0.15	0.06	0.05	0.05
Number follicles ≥3mm post	0.42	0.32	0.42	0.27	0.57	0.51	0.46	0.51	0.56	0.51	0.48	0.56	0.58	0.57

[1] onset of oestrus after pessary removal

Table 3: Correlations (R^2) between AMH and progesterone concentration in control ewes (n=9) from day 1 to 14 _*indicate significance P < 0.05**indicate significance P<0.01_

		AMH concentration (ng/ml)													
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Progesterone concentration (ng/ml)	Day 1	0.07	0.17	0.03	-0.09	-0.04	0.05	0.08	0.13	0.11	0.08	0.09	-0.01	0.09	0.14
	Day 2	0.00	-0.04	-0.08	-0.42	0.05	0.11	0.05	0.14	0.17	0.09	0.08	0.06	0.12	0.18
	Day 3	0.42	0.60	0.64	0.54	0.61	0.51	0.47	0.44	0.45	0.41	0.41	0.52	0.44	0.43
	Day 4	0.92**	0.74*	0.78*	0.56	0.85**	0.90**	0.90**	0.87**	0.87**	0.92**	0.89**	0.89**	0.87**	0.83**
	Day 5	0.61	0.49	0.46	0.29	0.59	0.64	0.63	0.67*	0.68*	0.63	0.71*	0.64	0.72*	0.72*
	Day 6	0.25	0.16	0.15	-0.12	0.37	0.38	0.30	0.31	0.32	0.20	0.37	0.35	0.32	0.33
	Day 7	0.74*	0.59	0.56	0.22	0.69*	0.76*	0.74*	0.76*	0.74*	0.66	0.81**	0.74*	0.72*	0.73*
	Day 8	-0.15	-0.24	-0.32	-0.30	-0.15	-0.08	-0.12	-0.13	-0.10	-0.13	-0.03	-0.12	-0.07	-0.07
	Day 9	0.05	-0.19	-0.25	-0.29	-0.09	0.03	0.03	0.04	0.04	0.01	0.15	0.02	0.04	0.04
	Day 10	-0.22	-0.40	-0.39	-0.47	-0.19	-0.14	-0.20	-0.20	-0.17	-0.18	-0.14	-0.19	-0.14	-0.16
	Day 11	-0.25	-0.42	-0.38	-0.34	-0.30	-0.26	-0.25	-0.18	-0.13	-0.11	-0.20	-0.21	-0.13	-0.11
	Day 12	0.83**	0.66	0.64	0.43	0.74*	0.83**	0.84**	0.83**	0.84**	0.85**	0.88**	0.83**	0.82**	0.82**
	Day 13	-0.03	0.01	-0.07	-0.06	-0.08	-0.01	0.00	0.05	0.07	0.08	0.02	-0.06	0.10	0.12
	Day 14	0.34	0.38	0.43	0.37	0.37	0.35	0.35	0.33	0.35	0.44	0.25	0.31	0.37	0.33

Table 4: Correlations (R^2) between AMH and progesterone concentrations throughout the oestrous cycle in progesterone treated ewes (n=10) from day 1 to 14 **indicate significance P < 0.05, **indicate significance P<0.01*

		AMH concentration (ng/ml)													
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Progesterone concentration (ng/ml)	Day 1	0.54	0.27	0.49	0.42	0.61	0.38	0.41	0.28	0.14	0.25	0.25	0.10	0.59	0.34
	Day 2	-0.37	0.22	-0.30	-0.14	0.00	-0.49	-0.24	-0.09	-0.26	-0.36	-0.27	-0.53	-0.44	-0.43
	Day 3	0.50	0.53	0.43	0.32	0.09	0.40	0.33	0.06	-0.06	0.08	0.17	0.04	-0.01	0.05
	Day 4	0.22	0.37	0.43	0.25	0.65*	0.18	0.23	0.63	0.48	0.36	0.29	0.16	0.27	0.30
	Day 5	0.52	0.42	0.67*	0.59	0.79**	0.58	0.42	0.57	0.45	0.46	0.39	0.50	0.64*	0.50
	Day 6	0.30	0.10	0.33	0.38	0.60	0.49	0.20	0.52	0.49	0.43	0.50	0.51	0.54	0.34
	Day 7	0.27	0.13	0.39	0.29	0.51	0.33	0.19	0.79**	0.69*	0.66*	0.71*	0.48	0.56	0.60
	Day 8	0.30	0.41	0.51	0.51	0.49	0.39	0.16	0.66*	0.46	0.47	0.60	0.42	0.48	0.53
	Day 9	-0.03	-0.39	0.01	-0.08	-0.11	0.10	-0.32	0.30	0.35	0.39	0.40	0.33	0.22	0.18
	Day 10	-0.02	-0.15	0.30	0.14	0.10	0.16	-0.34	0.44	0.40	0.40	0.31	0.44	0.28	0.31
	Day 11	0.28	0.05	0.47	0.32	0.07	0.37	0.00	0.45	0.38	0.42	0.54	0.44	0.42	0.57
	Day 12	0.07	-0.38	-0.02	-0.25	-0.26	0.07	-0.21	0.27	0.36	0.42	0.43	0.19	0.03	0.09
	Day 13	-0.08	-0.50	-0.41	-0.30	-0.40	-0.03	-0.18	-0.22	-0.05	0.05	0.15	0.03	-0.05	-0.20
	Day 14	0.00	0.39	0.40	0.30	0.05	0.06	-0.22	-0.16	-0.31	-0.33	-0.42	-0.07	-0.19	-0.15

Table 5: Correlations (R^2) between AMH concentration and parameters of the oestrous cycle and follicular development in progesterone treated ewes (n=10) from day 1 to 14 **indicate significance* $P < 0.05$

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Oestrus (hr)[1]	-0.65*	-0.49	-0.51	-0.37	-0.33	-0.48	-0.63	-0.04	0.02	-0.11	-0.10	-0.07	-0.23	-0.20
CL regression (day)	0.16	0.13	0.01	0.16	0.41	0.18	0.28	0.53	0.46	0.39	0.62	0.21	0.33	0.28
Number of CL	0.60	0.34	0.52	0.27	0.44	0.36	0.52	0.55	0.41	0.40	0.61	0.05	0.38	0.48
Day of ovulation	-0.72*	-0.40	-0.53	-0.27	-0.50	-0.45	-0.62	-0.35	-0.25	-0.31	-0.38	0.00	-0.30	-0.24
Number of ovulated follicles	0.35	-0.13	0.12	-0.06	-0.37	-0.05	0.00	-0.53	-0.58	-0.26	-0.28	-0.45	-0.04	-0.19
Emergence of first ovulatory follicle	-0.17	0.18	0.02	0.06	0.18	-0.30	0.01	0.07	-0.07	-0.16	-0.16	-0.27	0.01	0.10
Age of first pre- ovulatory follicle	0.02	-0.30	-0.14	-0.15	-0.34	0.17	-0.15	-0.19	-0.02	0.07	0.04	0.21	-0.09	-0.16
Number follicles ≤3mm pre	0.08	0.75*	0.24	0.57	0.48	0.45	0.40	0.15	0.09	-0.20	0.08	0.33	0.00	0.12
Number follicles ≥3mm pre	0.10	0.02	0.14	0.01	0.17	0.26	0.28	0.47	0.59	0.53	0.41	0.50	0.23	0.44
Number follicles ≤3mm post	0.17	0.59	0.38	0.35	0.53	0.36	0.28	0.43	0.35	0.03	0.21	0.22	-0.03	0.11
Number follicles ≥3mm post	0.64	0.07	0.28	0.32	0.12	0.30	0.43	0.22	0.32	0.94	0.56	0.55	0.93	0.77

[1] onset of oestrus after pessary removal

List of figures

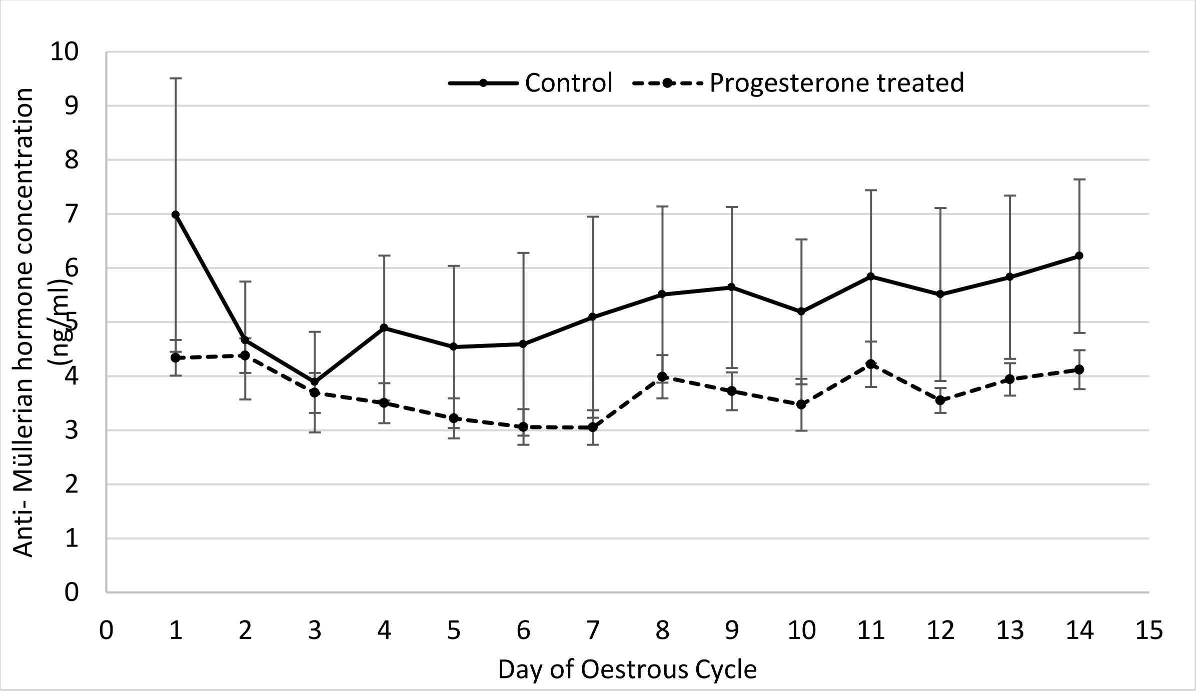

Figure 1: Mean daily concentrations (±SEM) of AMH for control (n=9) and progesterone treated (n=10) ewes across the first 14 days of the oestrous cycle.

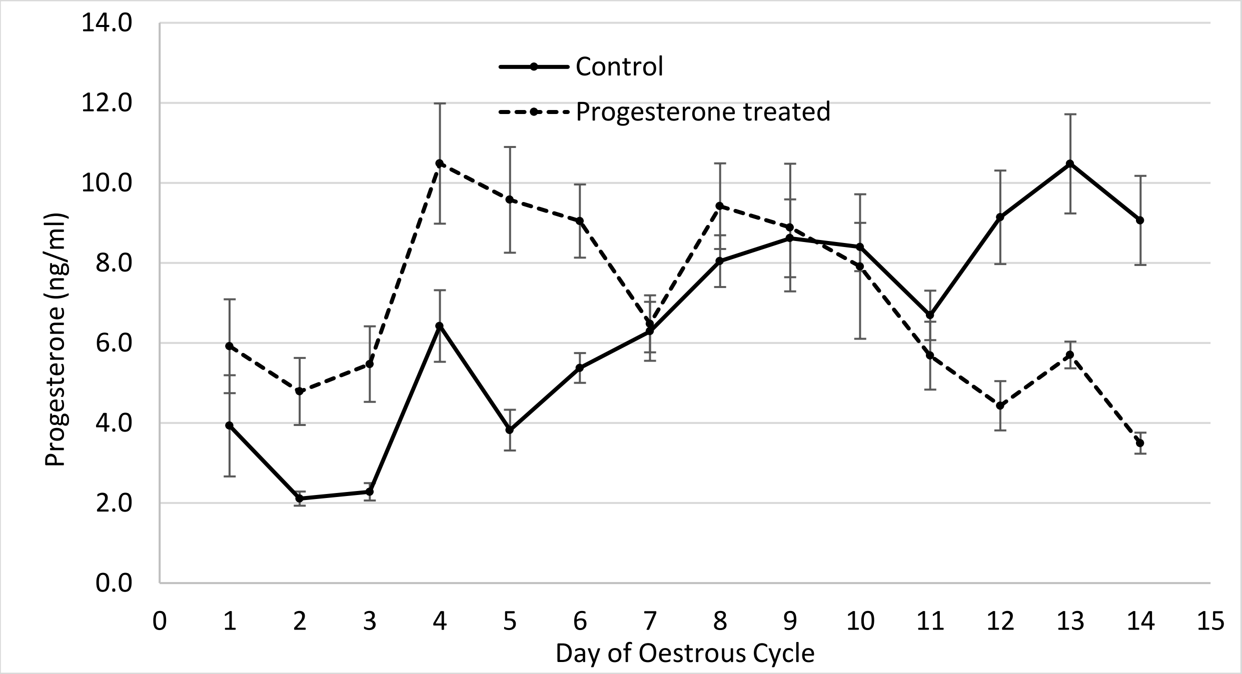

Figure 2: Mean daily concentrations (±SEM) of progesterone for control (n=9) and progesterone treated (n=10) ewes across the first 14 days of the oestrous cycle.

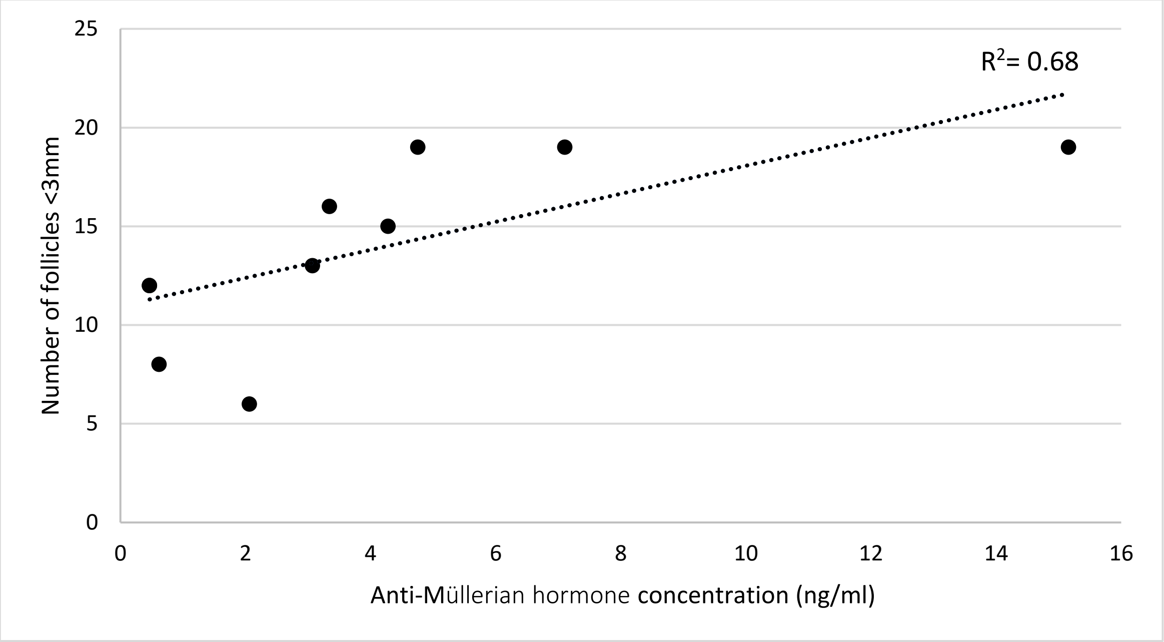

Figure 3: Correlation between Day 5 AMH concentration and the number of follicles ≤ 3mm in control animals (n=9)

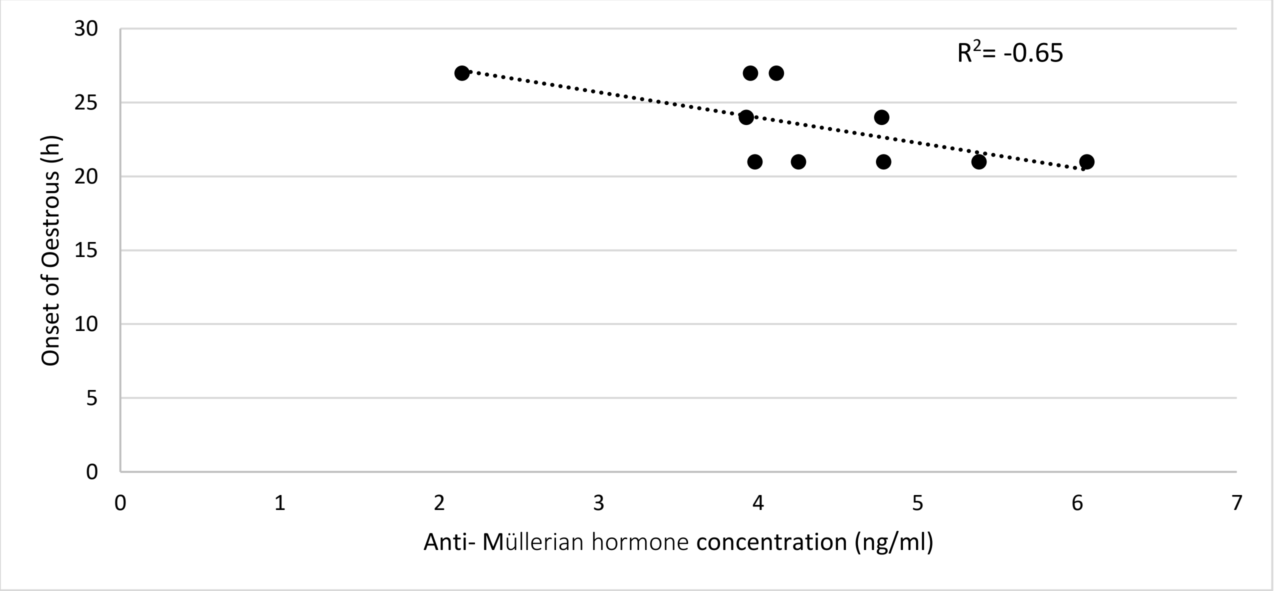

Figure 4: Correlation between Day 1 (day of pessary insertion) and the onset of oestrous in progesterone treated animals (n=10)

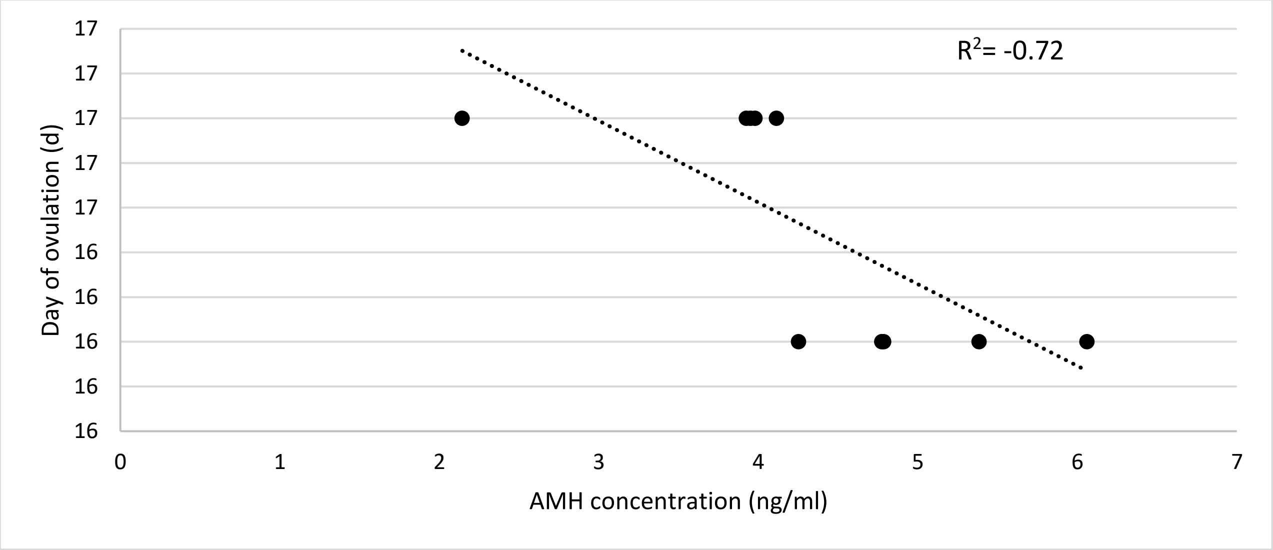

Figure 5: Correlation between Day 1 (day of pessary insertion) and the day of ovulation in progesterone treated animals (n=10)

Chapter Five: AMH as a phenotypic marker in an assisted reproductive technology

Abstract

The use of AMH as a means to select animals for use in assisted reproductive technologies has proven beneficial in sheep This study had three aims; 1) to determine whether AMH concentration from 7 day old lambs could be used as an endocrine marker to improve the success of juvenile *in vitro* fertilisation and embryo transfer (JIVET) 2)establish if AMH is able to select mature animals most likely to respond in this assisted reproductive technology and 3)compare the differences between AMH concentration, FSH stimulation and *in vitro* fertilisation and embryo development outcomes from juvenile and mature animals. . Compared with lambs with an AMH concentration < 1.031 ng/ml, ewe lambs at 7-days of age with an AMH concentration ranging between 1.031 – 4.78 ng.ml yielded the most A grade oocytes (25± 5.8 vs 5.8 ± 3.0) and increased the number of blastocysts produced from cleaved (43.3 ± 7.7 vs 6.3 ± 6.3) or recovered oocytes (34.4± 7.8 vs 1.5 ± 1.5) . Alternatively, selecting ewe lambs with a pre-FSH stimulation AMH at 7-weeks of age > 31.14 ng/ml could be used to predict the follicular response to FSH stimulation, as these ewe lambs produced the greatest number of COCs (94.8 ± 16.4 vs 70.8 ± 8.5 or 16.3 ± 16.4). Additionally, stimulation of ewes at 14-months of age was much less successful in comparison with juveniles at 7-weeks of age; however, the number of A grade oocytes was similar (25.8± 3.87 vs 27.2 ± 4.36) and b grade oocytes was greater (49.1± 4.10 vs 31.9 ± 3.62), suggesting a greater quality recovered.

In addition, AMH concentration at 7-days, 7-weeks or 12-months of age was not predictive of any significant outcomes from those stimulated at 14-months of age. Interestingly, AMH concentration determined at 5-months of age in the mother of the ewes stimulated at 14-months of age was correlated to the number of blastocysts produced by their progeny. Similar to data presented in chapter three, these data suggest a peak in AMH concentration occurs in lambs around 6-weeks of age, and extends the data presented in chapter one by indicating that AMH concentration at 7-days of age is similar to that in the mature animal at 12- or 14-months of age. Together this information highlights that AMH concentration in week old lambs can be used to select animals for inclusion in a JIVET program, and stimulation of ewe lambs at 7-weeks of age yields a much greater response in comparison with mature animals. The additional cost incurred with using AMH concentration to select animals suitable for use in a JIVET program is justified given the high costs associated with these programs.

Introduction

Anti-Müllerian hormone (AMH) is a glycoprotein produced by granulosa cells and regulates folliculogenesis by inhibiting recruitment of follicles from the resting pool (Dewailly and Laven 2019). AMH is considered an accurate marker of ovarian reserve across multiple species (Campbell *et al.* 2012; Fleming *et al.* 2015; Umer *et al.* 2019). In both sheep (Lahoz *et al.* 2014; Lahoz *et al.* 2016; Torres-Rovira *et al.* 2016) and cattle (Rico *et al.* 2009; Monniaux *et al.* 2010; Souza *et al.* 2015; Ghanem *et al.* 2016), circulating concentrations of AMH predict the follicular response to FSH stimulation. Recently, McGrice *et al*. (2020) demonstrated a positive correlation between circulating AMH concentrations in three- and five-week old lambs and both surface antral follicle number and efficiency of blastocyst production following *in vitro* maturation and fertilisation of aspirated oocytes. This further supports evidence that AMH concentration is a predictive indicator of the quality of *in vivo* produced embryos in cattle (Rico *et al.* 2009; Monniaux *et al.* 2010) and goats (Monniaux et al. 2011).

Assisted reproductive technologies (ARTs), such as *in vitro* embryo production of sheep embryos (IVP) require oocyte collection and maturation (IVM), *in vitro* oocyte fertilisation (IVF) and the culture of presumptive zygotes (IVC). The resultant embryos can then be either frozen or transferred into synchronised recipients (Amiridis and Cseh 2012). Further, to increase animal numbers and rapidly reduce the generation interval, a procedure, referred to as JIVET (juvenile *in vitro* fertilisation embryo transfer) (Earl *et al*. 1994), allows oocytes harvested from 4- to 8-week old lambs to undergo IVP procedures. The reported resultant offspring rates from these JIVET procedures range from nine-to-fourteen lambs per donor lamb (Kelly *et al*. 2005).

However, in sheep, variation in the number of embryos, and therefore progeny produced by donor animals, currently limits commercial adoption of ARTs (Daly *et al.* 2020). The ability to select donors for inclusion in ARTs, through the use of predictive markers of embryo number and quality, would facilitate more rapid adoption of ARTs by the Australian, and global, sheep industries (Daly *et al.* 2020). There is limited use of IVP by the sheep industry, and it is often reserved for obtaining financially affordable embryos of high genetic merit, or for research focussed on developmental biology (Amiridis and Cseh 2012). Factors attributed to the low commercial adoption of IVP technology (mature and juvenile *in vitro* fertilisation and embryo transfer) are the large variation in the follicular response to hormone treatment in sheep and the variation between donors in the number of embryos produced (Cox and Alfaro 2007; Amiridis and Cseh 2012). In a study by Kelly *et al.* (2005), the number of cumulus-oocyte complexes (COCs) harvested from individual lambs ranged from 0 to 435. Another factor limiting the efficiency of IVP technology is the significant influence oocyte quality has on the number of embryos obtained through the IVP process (Cox and Alfaro 2007; Paramio and Izquierdo 2014). It is well documented that oocytes obtained from juvenile lambs have reduced developmental competence compared with their adult counterparts (Obrien *et al.* 1996; Ledda *et al.* 1997; Obrien *et al.* 1997) and this contributes to the large variation in blastocyst development rates reported between juvenile donors. The age of the donor lambs has a marked impact on follicular response. When the same cohort of lambs (between 1- and 7-months of age) was used for three successive stimulations, 22%, 47% and 74% (respectively) of lambs failed to respond (Ptak *et al.* 2003) indicating an initial unresponsiveness to treatment in some lambs followed by an increasing refractoriness. Several other factors influence the follicular response to treatment and oocyte quality including genotype and live-weight (Kennedy *et al*. 1974; Tassell *et al*. 1978; Sonjaya and Driancourt 1987; Jorio *et al*. 1991, 1997; Ptak *et al*. 2003; Rawlings *et al.* 2003; Morton *et al*. 2007; Valasi *et al*. 2007), maternal nutrition during pregnancy (Kelly *et al*. 2005) and sex of co-twin (Kelly *et al*. 2017). The identification of a reliable, non-invasive phenotypic marker for follicular response and/or oocyte quality, that considers these endogenous and exogenous effects, would reduce the variation in lamb response to ovarian stimulation. Therefore, to assess the efficacy of AMH as a marker of fertility, a number of parameters need investigation: 1) youngest age at which circulating AMH could be used to accurately predict the suitability of ewe lambs for inclusion in a JIVET or mature *in vitro* fertilisation and embryo transfer (MIVET) procedure, 2) relationship between maternal and progeny AMH concentrations on reproductive outcomes and 3) effect hormone stimulation (follicle stimulating hormone (FSH)) has on AMH concentrations and subsequent relationship between early life AMH and reproductive outcomes at different ages .

Therefore, the current study aimed to determine whether, 1) circulating concentrations of AMH in lambs at 7-days of age would accurately predict the number and quality of embryos obtained following IVP of oocytes collected from both juvenile and mature animals, 2) AMH concentrations in the dam are related to the following attributes of their progeny; AMH concentrations, follicle responsiveness to FSH and the number of embryos produced following IVP and 3) FSH hormone stimulation affects AMH concentration and its ability to predict *in vitro* embryo production parameters in both juvenile and adult ewes. The ability to identify suitable donor ewe lambs and ewes as early as possible in their life based on the

AMH concentrations of their dam, would be a valuable strategy to improve the outcomes of JIVET, MIVET and other superovulation and embryo collection programs.

Methods and Methods

All procedures were conducted according to the guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes with approval from the Animal Ethics Committees of both the University of Adelaide (Approval number S-2017-068) and Primary Industries and Regions South Australia (Approval numbers 15/18 and 07/20). All animals were located at Turretfield Research Centre, Rosedale, South Australia (34°38’S, 138° 44’E) and managed under standard husbandry practices.

Experimental Design

All lambs were born from ewes in which AMH concentrations were previously measured at 5-months of age. Observations were made on 35 lambs, 28 of which were born in the autumn and 7 in spring. Ten lambs from autumn and all 7 lambs from spring were used in a JIVET study at 6 - 8 weeks of age and the remaining 18 animals from autumn were used in a MIVET (mature *in vitro* fertilisation and embryo transfer) study at 14-months of age. Blood samples (5 ml per animal) were collected via jugular venepuncture at 7-days of age (n = 35), prior to (PRE) and after (POST) FSH stimulation at 6 - 8 weeks of age (n = 17), 12-months of age (n=28), and PRE and POST FSH stimulation at 14-months of age (n = 18).

Blood sampling and AMH measurement

Plasma AMH concentration was measured in 50 µl plasma samples in duplicate for each of the time points sampled using an Ovine AMH ELISA kit (AL—155 Ansh Labs, Webster, TX; MDC:0.025ng/ml) (7.55%, 5.67% intra and inter-assay coefficient of variation, respectively) according to manufacturer’s instructions. Variation determined over 5 assay plates, including all re-analysed samples (80 samples per plate), all samples from one animal were run on the same plate. MDC calculated by the interpolation mean plus two standard deviations of 24 replicates of calibrator A (0 ng/ml) and low calibrator (0.37 ng/ml). Upper limit of standard curve at absorbance of 3.15 (20 ng/ml) measured at 450 nm.

Stimulation protocol and oocyte collection

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich, (St Louis, MO, USA). All procedures for lambs used in JIVET or ewes used in MIVET were similar, as outlined below.

Lambs and ewes were weighed and subsequently treated with 4 x 2 mL injections (i.m.) of FSH (160mg NIF-FSH-P1, Folltropin; Vetoquinol Inc., Ontario, Canada) given at approximately 12 h intervals. In addition, each lamb received (i.m.) 500 IU eCG (Pregnecol; Vetoquinol) at the time of the last FSH treatment. Oocytes were collected approximately 48 h after the time of the first FSH treatment (Kelly *et al* 2005a). Ovaries were exposed by para-ventral laparotomy, which was performed under general anaesthesia induced by xylazine (Ilium; Troy Laboratories; Glendenning NSW, AUS.; 20 mg mL^{-1}, 0.83 mL kg^{-1}) and ketamine (Parnell Laboratories; Alexandria, NSW, AUS; 100 mg mL^{-1}, 0.67–0.83 mL kg^{-1}). COCs were aspirated from antral follicles ≥2 mm in diameter using a 10 mL syringe and a 20 g needle. The syringe contained approximately 1 mL HEPES-buffered TCM 199 supplemented with 2% (v/v) heat inactivated oestrous sheep serum (SS) and 100 IU mL^{-1} heparin (Pharmacia and Upjohn; Michigan, USA), 100 µg/mL streptomycin sulphate (CSL Limited, Parkville, Victoria, Australia) and 100 U/mL penicillin G (CSL Limited) (aspiration medium). COCs were recovered from the aspirate using a stereomicroscope (x40 magnification) and placed into maturation medium (sodium bicarbonate-buffered TCM199) supplemented with 20% (v/v) SS, 5 mg mL^{-1} FSH, 0.1 IU mL^{-1} hCG (Chorulon, Intervet;

Bendigo, VIC, AUS), 1 mg mL^{-1} oestradiol-17β and 100 mM cysteamine. Recovered COCs were classified as follows: A grade - compact cumulus (4 to 5 layers) with homogeneous ooplasm, B grade - 1-2 layers of compact cumulus and coarse homogeneous ooplasm, C grade – less compact uneven layers of cumulus cells, with irregular ooplasm and/or dark clusters (De Loos *et al.* 1989; Fouladi-Nashta *et al.* 2007). Atretic and completely denuded oocytes (D grade) were included in the total COCs recovered count and then discarded from the study.

In Vitro Maturation, Fertilisation and Culture

Procedures were similar to those outlined previously (Walker *et al*. 1996). Briefly, COCs (within each lamb) were rinsed three times in aspiration medium and then twice in maturation medium. Maturation took place in 4-well Nunc dishes (Thermo-Fischer Scientific; Massachusetts, USA) containing 600 µl maturation medium covered with 300 µl mineral oil (maximum 30 COCs per well). COCs were matured at 38.8°C in a humidified atmosphere of 5% CO_2 in air. After a 24 h maturation period, excess cumulus cells were removed by gently pipetting in aspiration medium supplemented with 400 IU mL^{-1} hyaluronidase, followed by three rinses in synthetic oviductal fluid medium (SOF) supplemented with 2% (v/v) SS (IVF medium). Ova were placed in culture wells containing 450 µl IVF medium covered with 300 µl mineral oil. Frozen–thawed semen (150 µL), pooled from two rams of proven fertility, was layered under 1 mL IVF medium in a 14-mL Falcon tube (BD Biosciences; North Ryde, NSW, AUS). After 20 min, the concentration of spermatozoa in the upper fraction was determined and approximately 0.5 x 10^6 motile spermatozoa were added to each well. The COCs and spermatozoa were co-incubated at 38.8° C in a humidified atmosphere of 5% CO_2 in air. After approximately 24 h, remnant cumulus cells were removed by gentle pipetting and presumptive zygotes were washed three times in SOF containing 8 mg mL^{-1} BSA (Fraction V; Invitrogen; Thermo-Fischer

Scientific) and amino acids at oviduct fluid concentrations (IVC medium) (Walker *et al.* 1996). Presumptive zygotes were cultured in wells containing 600 µL IVC medium covered with 300 µL mineral oil at 38.8°C in a humidified atmosphere of 5% CO_2: 5% O_2 : 90% N_2. Presumptive zygotes that failed to divide were removed 24 h later, and the cleavage rate was recorded. The stages of embryo development were recorded on Day 6, 7 and 8 (Day 0 = day of IVF). All embryos that had not progressed to a blastocyst were recorded as such. These were embryos that had arrested development at either the 8-16 cell, morula or compact morula stage.

Statistics

Correlations (kendall tau) were assessed between plasma AMH concentrations at all ages and oocyte parameters (COCs recovered, number of A, B, C and D grade oocytes), embryo developmental rates (cleavage and blastocyst production rates). One-way ANOVA was used to determine differences in outcomes between JIVET and MIVET ewes, and Fisher's LSD post hoc test was used for between group comparisons. As per McGrice *et al.* (2020) quartiles were calculated for lamb AMH concentration at 7-days of age. PRE and POST FSH stimulation and JIVET lambs were divided into low (lowest quartile), medium (middle two quartiles) and high (upper quartile) categories according to plasma AMH concentrations at each of these stages (Table 1). Lambs with AMH concentrations below the measurable range were included in the low-AMH group for analyses. One- way ANOVA was used to determine differences in oocyte parameters (COCs recovered and oocyte grade), cleavage rate and blastocyst development rates. All analysis was completed using SPSS Version 26 (IBM Corp.)

Results

Effect of age and FSH stimulation on circulating AMH concentrations

Aside from 6 ewe lambs, AMH concentration was measurable at 7-days of age, and AMH was detected at all other time points for all lambs. At 7-days of age, concentrations were lower (P < 0.001) than at 7-weeks of age (PRE and POST FSH stimulation); but were similar to those obtained at 12-months of age (Table 2) At 7-weeks of age, AMH concentrations were higher (P < 0.001) PRE compared with POST FSH stimulation No differences in AMH concentrations were detected for PRE and POST FSH stimulation at 14-months of age. At 7-weeks of age, PRE and POST FSH stimulation AMH concentrations were higher than both 12-months of age and PRE and POST stimulation at 14-months of age (Table 2).

Regardless of age, there was a significant positive correlation (r=0.727, P<0.001, n=35) between AMH concentrations PRE and POST FSH stimulation. When FSH stimulation occurred at 7-weeks of age, there were significant positive correlations (r=0.536 and r=0.761, respectively; P < 0.05; n = 17) between AMH at 7-days of age and AMH both PRE and POST FSH stimulation. However, AMH at 7-days of age was not correlated with AMH at 12- or 14-months of age. If lambs were stimulated at 14-months of age, AMH determined at 12-months of age was significantly positively correlated (r= 0.857 and 0.622, respectively; P < 0.01; n = 18) with AMH concentrations PRE and POST FSH stimulation.

The total number of COCs recovered from ewe lambs at 7- weeks of age was higher (P<0.01) than those recovered from ewes at 14- months of age, as was the total number of blastocysts produced (P< 0.01; Table 3). However, cleavage rate (P< 0.05), blastocysts from cleaved and blastocysts from total number of oocytes recovered (P<0.01; Table 3) was significantly greater in the ewe lambs at 14- months of age (Table 3). The numbers of 8-16 cell embryos were also higher (P<0.01) in 7-weeks old ewe lambs compared with 14-month old ewes

(Table 3). There were more B, C and D grade oocytes as a proportion of recovered oocytes from JIVET ewe lambs ($P<0.01$). The total number of embryos was higher for 7-week old ewe lambs compared with 14-month old ewes ($P= 0.053$). However, the proportion of blastocysts that developed post IVF on days 6, 7 or 8 were not different in 7-week old lambs compared with 14- month old ewes (Table 3).

Circulating AMH concentrations and oocyte and embryo production in ewe lambs stimulated with FSH at 7 weeks of age

There was a significant ($P < 0.05$) positive correlation between AMH concentrations at 7-days of age and total COCs recovered (Figure 2a), the number of B grade oocytes recovered, cleavage rate (Table 4) and the total number of blastocysts produced (Figure 2b). AMH concentration PRE FSH-stimulation at 7- weeks of age was significantly ($P < 0.05$) positively correlated with total COCs collected (Figure 3a) and the number of A (Figure 3b), B and C grade oocytes (Table 4). There were significant ($P < 0.05$), positive correlations between AMH concentrations POST FSH stimulation and the number of COCs (Figure 4a), the number of A and B grade oocytes (Figure 4b and Table 4, respectively), and cleavage rate (Table 4). AMH POST FSH stimulation positively correlated to the number of blastocysts (Table 4 and Figure 4c), as well as the rate of development defined by the number of embryos developed by day 7, and tended to correlate to blastocysts developed by day 6 (Table 4).

When grouped according to AMH concentrations at 7-days of age or PRE FSH-stimulation, lambs with medium AMH concentrations produced the highest number of A grade oocytes ($P<0.05$; Table 5). When grouped according to AMH concentration at 7-days of age, ewe lambs with AMH concentrations in the low group (<1.03 ng/ml) produced less blastocysts from cleaved or total number of oocytes recovered in comparison with the medium (1.03-

4.77 ng/ml) or high (>4.78 ng/ml) groups ($P<0.05$; Table 5). Ewe lambs with high AMH concentration PRE FSH-stimulation (31.15 ng/ml) at 7-weeks of age produced the greatest number of COCs, B and C grade oocytes ($P<0.05$; Table 5). The number of D grade oocytes was similar between lambs with medium or high AMH concentration PRE-FSH stimulation, and lowest in the low AMH group ($P<0.05$; Table 5).

When grouped according to AMH concentration POST-FSH stimulation, ewe lambs with high AMH (>18.26 ng/ml) produced significantly more B grade oocytes and blastocysts in comparison with the low AMH group (<4.86 ng/ml), but did not differ from the medium AMH group ($P<0.05$; Table 5). Ewe lambs with medium AMH concentrations produced more A grade oocytes than low AMH concentration lambs, but did not differ from the high AMH concentration group ($P<0.05$; Table 5).

Circulating AMH concentrations and oocyte and embryo production in ewes stimulated with FSH at 14-months of age

AMH concentrations at 7-days of age ($r = 0.49$, $P < 0.01$) and prior to FSH stimulation at 14-months of age ($r = 0.40$, $P < 0.05$) were significantly correlated with the number of B grade oocytes recovered per ewe at 14-months of age. There were no significant correlations between AMH concentration PRE or POST FSH stimulation at 14-months of age and any other oocyte or embryo parameter. There were no correlations between oocyte and embryo parameters and AMH concentrations at 12-months of age.

Circulating AMH concentrations in the dam and concentrations of AMH, oocyte and embryo production in offspring

Dam AMH concentration was not significantly correlated with their progeny AMH concentrations at 7-days or 7-weeks of age (pre- and post-FSH) and 12- or 14-months of age (pre- and post-FSH stimulation).

Dam AMH concentration was positively correlated to the total number of blastocysts produced from offspring at 14-months of age ($P<0.05$; Figure 5).

Discussion

The current data provides novel insights into the relationship between AMH concentrations in Merino ewe lambs and the number and quality of oocytes and embryos produced in response FSH stimulation at 7-weeks and 14-months of age. Specifically, high AMH concentrations at 7-days of age can be used to identify Merino ewe lambs which will produce more oocytes and blastocysts when used as donors for JIVET, but not MIVET programs. An additional difference between juvenile and mature ewe lambs was the increased ovarian response to FSH observed in juvenile animals, resulting in more COCs recovered and a consequentially higher number of blastocysts. AMH concentrations in 7-day old Merino ewe lambs were correlated with AMH concentrations pre and post FSH stimulation at 7-weeks of age but not 14-months of age, while at 12-months of age AMH was correlated to pre- and post- FSH stimulation concentrations at 14-months age. AMH concentrations in ewe lambs at 7-days of age, prior to and after FSH stimulation, predicted the ovarian response and outcomes of *in vitro* maturation and fertilisation. Given the highly predictive results of AMH in ewe lambs in the current study, AMH concentrations were divided into ranges, to determine potential cut off values with which to select donors for inclusion in *in vitro* fertilisation and embryo transfer programs (JIVET). The results indicate that ewe lambs with mid-range AMH concentrations at 7- days of age (1.03 – 4.77 ng/ml) appear to be superior donors, suggesting those with low (<1.03 ng/ml) or high (>4.78 ng/ml) concentrations of AMH should be excluded from FSH stimulation protocols in order to achieve optimum efficiency within a JIVET program. Although AMH concentrations in mature ewes two months prior to (12-months of age), or at time of stimulation (14-months of age), appeared to have only minimal benefit for predicting response to ovarian stimulation, the dam's AMH concentration at 5-months of age was predictive of the reproductive outcomes of an *in vitro* embryo production program undertaken in their progeny at 14-months of age, but not 7-weeks of age.

Further support of post-natal AMH and follicle profile in juvenile Merino ewe lambs

In ewes, antral follicles first appear on the ovary on day 135 of gestation, with a large number of antral follicles present at time of birth (Kennedy 1974; Tassell *et al.* 1978). There is a peak in antral follicle numbers in ewe lambs between 4- and 8- weeks of age (Kennedy 1974; Tassell *et al.* 1978), before a subsequent decline from 12-weeks that stabilises at approximately 33-weeks of age (Tassell *et al.* 1978). This pattern of post-natal antral follicle development supports the current data, with AMH concentrations significantly higher at 7-weeks of age compared with 7-days and 12- and 14-months of age. This change in AMH with lamb age is also consistent with previous reports that AMH is higher at 7- weeks of age compared with one week of age, and is lower at 6.5- and 17.5-months of age (Torres-Rovira *et al*., 2016) and that AMH peaks at 6-weeks of age in Suffolk ewe lambs (chapter three), and 4-weeks of age in Merino ewe lambs (chapter three).

AMH from 7-day old lambs and pre FSH-stimulation is an effective indicator of stimulation response

Merino ewe lambs with higher AMH concentration at 7-days of age produced more COCs and blastocysts when stimulated at 7-weeks of age. The current data demonstrate that plasma AMH at both 7-days of age and prior to FSH stimulation can predict oocyte recovery number and blastocyst production rates for 7-week old lambs, and therefore can be used as a reliable means to select suitable donor ewe lambs for inclusion in JIVET programs. This finding is supported by previous evidence from our group that selection of donor ewes for inclusion in JIVET programs can be based on AMH concentrations at 3- and 5-weeks of age

(McGrice, 2020). The advantage of determining AMH concentrations at 7-days of age is the allowance of additional time for sample analysis and allocation and proper management of both donor and recipient animals prior to undergoing *in vitro* assisted reproduction technologies.

AMH concentration post stimulation most informative, but least industry applicable

In the current study, ewe lambs with high AMH after FSH stimulation produced the most COCs, as well as more A grade oocytes and blastocysts, indicating a positive relationship between AMH and oocyte quality. This builds on previous evidence (McGrice *et al.* 2020) that Merino ewe lambs with HIGH AMH at either 3- or 5-weeks of age produced more blastocysts in total. In women, AMH concentrations prior to and after FSH stimulation were 1.77 and 2.05 times higher, respectively, in women that became pregnant following IVF (Eldar-Geva *et al.* 2005). AMH did not differ between the follicular and luteal phases in these women, and both basal and post-stimulation AMH were strongly, positively correlated with both antral follicle count and the number of oocytes collected. Similarly, the positive relationship between AMH and blastocyst production following IVF in ewe lambs was strongest when AMH was measured after FSH stimulation. However, the relationship between AMH and the number of COCs recovered was similar regardless of whether AMH was measured before or after FSH stimulation. This finding suggests that ewe lamb AMH concentrations prior to FSH stimulation reflect the size of the FSH sensitive antral follicle pool and thereby the number of COCs which can be recovered. Post FSH stimulation AMH concentrations are reflective of the developmental competence of the follicles. Selecting donor ewe lambs based on AMH concentrations following FSH treatment is unlikely to be adopted, given the significant financial cost associated with the hormonal stimulation protocol. Alternatively, depending on the value of the animals and availability of recipients, this additional measure of COC and subsequent embryo quality, may prove beneficial.

Benefit of categorising ewe lambs based on AMH concentration at 7-days of age

Interestingly, for AMH at both 7-days and 7-weeks of age, high AMH (>4.8, >31.1 ng/ml respectively) did not result in the best *in vitro* embryo development. With this in mind, using the AMH assays employed in the current study, Merino ewe lambs with AMH concentrations ranging from 1.0 to 4.8 at 7-days of age, or 10.0 to 31.1 ng/ml at 7-weeks of age prior to FSH stimulation, were more likely to perform well in an *in vitro* fertilisation and embryo transfer program. The major purpose of utilising juvenile animals for oocyte collection and embryo production is to rapidly multiply up animal numbers and enhance the rate of genetic gain. Given the high costs associated with JIVET programs, optimisation of animal selection for these programs that improves overall blastocyst production and viable embryo transfer rates is required before this technology will be commercially adopted by industry. Ewe lambs with AMH concentrations ranging between 1.0 and 4.8 ng/ml at 7-days of age produced the highest proportion of high quality (A grade) oocytes, and the highest proportions of blastocysts from cleaved and total number of oocytes recovered. Similarly, ewe lambs with AMH concentrations ranging from 10.0 to 31.1 ng/ml at 7-weeks of age without FSH stimulation produced the most A grade oocytes, even though the number of COCs recovered was highest for the high AMH group. Similarly, in cows undergoing a superovulation and embryo transfer program, animals in which circulating AMH ranged between 100 and 200 pg/ml produced more transferable embryos compared with animals with less than 100 pg/ml of AMH (Monniaux *et al.* 2010). Furthermore, there was no difference in mean or maximal number of transferable embryos in animals that had >200 pg/ml AMH (Monniaux *et al.* 2010). It is therefore suggested that, there is an optimal range of AMH within which to select ewe lambs for inclusion in JIVET programs, with studies in women demonstrating that very high AMH concentrations are indicative of reproductive dysfunction. Circulating concentrations of AMH were two to three times higher in women with polycystic ovary syndrome compared with controls (Mulders 2004), and were also higher in women with no menstruation compared with those with irregular menstruation (La Marca *et al.* 2004). Further, higher concentrations of AMH may also be indicative of ovarian hyper stimulation syndrome in women undergoing IVF (Nakhuda *et al.* 2006). Combined, these data suggest that AMH concentrations above a certain point may not reflect continued improvement in ovarian function; however, these ranges are yet to be determined for sheep. Within a population of sheep ovarian dysfunction is rare, therefore determining ranges of AMH that reflect ovarian disorders is unlikely. However, much like the cattle data it is likely that an optimal range exists for IVP outcomes.

AMH concentration at 7 days of age is not useful for predicting outcomes as mature ewes

The current data indicate that measuring AMH in 7-day old lambs does not predict their suitability for inclusion in an *in vitro* fertilisation and embryo transfer program at 14-months of age. It is therefore possible, that early post-natal AMH concentrations do not predict ovarian function in adulthood. In the current study, AMH was only correlated between Day 7 and 7-weeks of age, and between 12- and 14-months of age, further suggesting pre-pubertal AMH may not reflect post pubertal AMH and that differences in ovarian follicle dynamics and intra-follicular function may exist between pre- pubertal and mature ewes. In partial support of this, Lahoz *et al.,* (2014) reported low repeatability within animal, in AMH concentrations between 3- and 6-months of age, reflecting differences between animals in the age at which AMH concentrations peaked. However, Torres-Rovira *et al.* (2016) provided evidence that ewe lambs with high post-natal AMH and antral follicle counts (AFC) also had higher AMH concentrations and AFC post-puberty, a relationship which is not supported by the current data. Within animal repeatability in post-pubertal AMH was high in the current study, and similar to cattle in which AMH is highly repeatable throughout the oestrous cycle (Ireland *et al.* 2011; El-Sheikh Ali *et al.* 2013), indicating post-pubertal AMH appears to be stable in the ewe.

The lack of a relationship between AMH in juvenile lambs and 12- to 14-month-old ewes is consistent with the outcomes from previous studies (Lahoz *et al.*, 2014). A potential reason for this is, AMH is not constant early in life compared with adulthood (Rico *et al.* 2009). Concentrations of AMH peak at 3, 4.5 or 6 months of age in different ewe lambs (Lahoz *et al.* 2014), with significant differences in concentration also observed in four-week-old Merino lambs, and during the first 12 weeks of life in Suffolk ewe lambs (chapter three). Together, these data suggest that the inability of pre-pubertal AMH to predict AMH in adulthood may be due to the large variation observed in the post-natal and pre-pubertal periods. In addition, the relationship between FSH and AMH may differ before and after puberty. Anti-Müllerian hormone reduces the sensitivity of follicles to FSH in human granulosa cells (Pellatt *et al.* 2011) and similar inverse relationships have been observed in ewe lambs (Torres-Rovira *et al.* 2016) and cows (Monniaux *et al.* 2013). Torres-Rovira (2016) suggested that FSH may not be responsible for the increased ovarian activity observed in ewe lambs between 4- and 8-weeks of age. In the current study, the response to FSH stimulation was lower in 14-month old lambs compared with 7-week old ewe lambs, resulting in fewer oocytes recovered and embryos produced. It is likely that the actions of FSH and AMH differ in post-pubertal ewes, limiting the value of early life AMH as a predictive marker of the ovarian response of mature ewes to FSH stimulation. Furthermore, Torres-Rovira *et al.,* (2016) reported significantly higher follicle populations in pre-pubertal animals compared with peri-pubertal (195 days) and post-pubertal (496 days) animals, resulting in a greater variation across the pre-pubertal animals. This observation may also affect the responsiveness and outcomes in adult animals and enforce pre-pubertal AMH as an unreliable marker of reproductive outcomes in mature ewes undergoing IVP programs.

Possible relationship between dam AMH and progeny fertility

To the best of our knowledge, the current study is the first to demonstrate that blastocyst development rates for ewes at 14-months of age was positively correlated with the AMH concentrations of their dams at 5-months of age. It is possible that AMH concentrations in the dam may demonstrate a relationship between ovarian development in the dam and their offspring. This is particularly useful given the limited predictability and usefulness of AMH as a selection tool in adult ewes in a MIVET program. In women, studies have reported an association between age at menopause in mothers and daughters, with one study reporting a 44% heritability (van Asselt *et al.* 2004), suggesting a relationship between ovarian reserve across the generations. AMH is a marker of ovarian reserve, and given the time of sampling in the dam, it is possible that this reflected the ovarian follicle pool prior to pregnancy and therefore warrants further investigation. Generally speaking, reproductive traits are much less heritable than other performance traits such as wool or meat traits, even so relationships are evident (Safari *et al.* 2005). Heritability of lambs weaned per ewe joined was as low as 0.07 ± 0.01, and lambs born per ewe lambing was stronger at 0.13 ± 0.01 (Safari *et al.* 2005). In another study using crossbred ewes similar ranges were seen; litter size (0.19 ± 0.04) and fetal number (0.18 ± 0.04) were moderately heritable, whilst fertility (0.11 ± 0.04) and pregnancy rate (0.13 ± 0.04) were less so (Afolayan *et al.* 2008).

There is huge variability in response to hormonal stimulation and therefore embryo outcomes in sheep (Ptak *et al.* 2003; Morton 2008). Using AMH to select donor animals is one way to reduce the variation. The results from the current study demonstrate that for these Merino ewe lambs circulating AMH concentrations at 7-days of age between 1.031 – 4.78 ng/ml could be used to identify the best performing Merino ewe lambs for use in a JIVET program at 7-weeks of age. Breed differences may exist, therefore replication across breeds as well as multiple cohorts of Merino ewes are needed to confirm the accuracy of this range of AMH. The significantly higher concentrations of AMH at 7-weeks of age, support the increased ovarian function in pre-pubertal lambs and high responsiveness to FSH stimulation. As a result, these ewe lambs produced greater numbers of viable embryos in comparison with ewe lambs with AMH concentrations outside of this medium range.

Understanding differences in response to FSH stimulation in juvenile and mature animals for in vitro fertilisation and embryo development

In sheep, *in vitro* oocyte recovery procedures from juvenile animals are most commonly performed around 6- to 8-weeks of age to optimise the recovery of oocytes and embryos from a naturally increasing antral follicle pool (Kennedy 1974; Tassell *et al.* 1978; Paramio and Izquierdo 2014). This is evident in the current study given the significantly greater AMH concentrations in the juvenile animals; specifically circulating AMH pre- FSH stimulation is significantly higher in lambs at 7-weeks of age compared with mature 14-month old ewes. The results from the present study indicate that a significantly greater number of COCs were recovered, and blastocysts produced following FSH stimulation in juvenile animals, in comparison with mature animals. Therefore, consistent with the available literature (Paramio and Izquierdo 2014), the current data demonstrated that stimulation of the ewe lambs results in a significantly greater response. Unsurprisingly, the percentages of D grade oocytes, cleavage rate and the number of embryos arresting prior to developing into blastocysts in juvenile ewe lambs were greater in comparison with mature ewes. These findings support previous evidence that oocyte developmental competence is reduced in juvenile or pre-pubertal ewes (Armstrong 2001). Additional studies have reported differences in mitochondria and vesicles present (Reader *et al.* 2015) and in the energy metabolism and ultrastructure (Obrien *et al.* 1996; Ledda *et al.* 1997) of juvenile and mature ewe oocytes all of which likely contributed to the poorer oocyte developmental competence observed in the current study. Interestingly, there was no difference in the proportion of A grade oocytes from COCs recovered or subsequent blastocysts development rates between the juvenile and mature animals suggesting that the quality of a proportion of the oocytes is similar. Ledda *et al.* (1997) obtained similar pregnancy rates from embryos transferred from juvenile and adult oocytes, supporting the idea that juvenile and adult oocytes can produce similar outcomes. However, despite this, it is noteworthy that the total number of blastocysts obtained per donor ewe was approximately 4-fold higher in juvenile compared with mature animals. As such the use of AMH as a marker for selection into JIVET procedures would be of value, as it could indicate those animals that not only produce greater numbers of COCs, but are also likely to produce more embryos and therefore offspring.

Conclusion

In conclusion, AMH concentrations from lambs as young as 7 days of age could be used to select animals for use in juvenile *in vitro* fertilisation and embryo transfer programs as this timing allows producers to effectively manage ewes and lambs. AMH concentration post FSH stimulation provided most significant correlations to oocyte developmental competence but would only prove beneficial if there are constraints in the number of available recipient animals. Unsurprisingly, juvenile animals were more responsive to FSH stimulation. However, this did not translate into better quality oocytes or increased embryo development rates. This initial work demonstrates a relationship between the post-pubertal ovarian follicle pool across generations, and indicates more work is required to quantify and strengthen the relationship between AMH dam and maternal ovarian reserve and that of mature progeny.

List of tables

Table 1. AMH concentrations of lambs classified as having low, medium or high values at 7-days of age and immediately before and after FSH stimulation 7-weeks of age.

	AMH concentration (ng/ ml)		
	Low	Medium	High
n	4	9	4
Day 7	≤1.03	1.031 – 4.78	>4.78
Pre-FSH stimulation	≤10.0	10.0 – 31.14	>31.14
Post-FSH stimulation	≤4.86	4.86 – 18.25	>18.25

Table 2. AMH concentration (mean ±SEM) obtained for Merino lambs and ewes at 7-days, 7-weeks (pre- and post-FSH stimulation), 12-months and 14-months (pre- and post-FSH stimulation) of age. ^{ab} *Within column, different superscripts indicate significant differences; P < 0.05s*

Age	**n**	**Treatment**	**Mean ±SEM (ng/ml)**	**Range (ng/ml)**
7 days	35	-	5.6 ± 1.66^{a}	0.00 – 48.8
7 weeks	17	Pre-FSH	22.4 ± 3.98^{b}	3.6 – 67.5
7 weeks	17	Post-FSH	12.9 ± 2.21^{b}	1.4 – 36.6
Twelve months	28	-	5.2 ± 3.98^{a}	0.2 – 79.4
Fourteen months	18	Pre-FSH	2.0 ± 0.49^{a}	0.2 – 9.1
Fourteen months	18	Post-FSH	2.1 ± 0.43^{a}	0.2 – 6.6

Table 3: Mean differences between COC recovery and quality and embryo production and quality for 7- week old lambs and 14- month old ewes following an *in vitro* fertilisation and embryo production program.

	7- week old lambs (n=17)	14- month old ewes (n=18)
	Mean ± SEM	*Mean ± SEM*
COCs recovered	63.6 ± 9.06*	9.7 ± 0.85
A grade oocytes (%) [1]	25.8 ± 3.87	27.2 ± 4.36
B grade oocytes (%) [2]	31.9 ± 3.62*	49.1 ± 4.10
C grade oocytes (%) [3]	34.2 ± 2.36*	21.7 ± 2.77
D grade oocytes (%) [4]	7.7 ± 1.37*	2.2 ± 1.01
Cleavage rate[5]	76.8 ± 6.90*	92.5 ± 3.17
8-16 cell embryos	23.4 ± 7.55*	0.94 ± 0.22
Total number of blastocysts produced	17.6 ± 4.94*	5.7 ± 0.87
Total blastocysts from cleaved (%) [6]	32.7 ± 6.1*	60.3 ± 7.38
Efficiency: Total blastocysts from COCs recovered (%) [7]	25.9 ± 5.74*	53.8 ± 6.90

*indicate significance concentration <0.05. Data presented as mean ± S.E.M

[1] A grade oocytes- a grade oocytes as a percentage of oocytes recovered

[2] B grade oocytes- b grade oocytes as a percentage of oocytes recovered

[3] C grade oocytes- c grade oocytes as a percentage of oocytes recovered

[4] D grade oocytes- a grade oocytes as a percentage of oocytes recovered

[5] cleavage rate - oocytes cleaved as a percentage of oocytes

[6] Blastocysts from cleaved- blastocysts produced as a percentage of oocytes cleaved

[7] Efficiency- blastocysts produced as a percentage of total oocytes recovered

Table 4. Correlation coefficients between AMH concentrations at 7-days and 7-weeks of age and (1) the quantity and quality of oocyte-cumulus complexes (COCs) harvested at 7-weeks of age and (2) subsequent blastocyst production rates following *in vitro* maturation and fertilisation.

	Total COCs	A grade	B grade	C grade	D grade/ atretic	Cleavage[1]	Total Blast[2]	Day 6[3]	Day 7[4]	Day 8[5]	Blastocysts/ cleaved[6]	Efficiency [7]
7-day old lambs												
	0.35*	0.19	0.49**	0.29	0.30	0.39*	0.43*	0.11	0.47*	0.29	0.18	0.26
7-week old lambs												
Pre-FSH	0.63*	0.42*	0.64**	0.55**	0.41*	0.11	0.29	0.07	0.27	0.22	0.08	0.07
Post-FSH	0.44*	0.41*	0.47**	0.27	0.20	0.39*	0.54**	0.36^	0.39*	-0.04	0.30	0.35

**$p<0.05$, ***$p <0.01$, ^*trend*, $p= <0.1$

[1] cleavage- oocytes cleaved as a percentage of oocytes exposed to spermatozoa
[2] Total blast- total number of blastocysts from all stages of blastocyst development
[3] Day 6 – the total number of embryos that had developed on day 6 culture
[4] Day 7- total number of embryos that had developed on day 7 culture
[5] Day 8 - the total number of embryos that had developed on day 8 culture
[6] Blast cleaved - the percentage of blastocysts produced from oocytes cleaved
[7] Efficiency - the percentage of blastocysts produced from oocytes recovered

Table 5. The relationship between AMH concentration (low, medium and high) at 7-days of age and 7-weeks of age (pre- and post-FSH stimulation) on parameters of oocyte recovery and outcomes of *in vitro* maturation and fertilisation (mean ± SEM).

^{ab}*Different superscripts within rows indicate significant differences (P<0.05)*

		n	COCs	A grade	B grade	C grade	D grade	Blastocysts[1]	Blast/cleaved	Efficiency[2]
AMH 7 days										
Low	*<1.03 ng/ml*	4	31.8±15.57	5.8 ± 3.0^{a}	9.5± 5.6	13.3±6.2	2.5±1.0	0.3±0.3	6.3±6.3^{a}	1.5±1.5^{a}
Medium	*1.03-4.77 ng/ml*	9	73.9±13.12	25.0 ± 5.8^{b}	20.7±4.3	24.6±4.9	3.7±0.7	22.3±7.3	43.3±7.7^{b}	34.4±7.8^{b}
High	*>4.78 ng/ml*	4	72.3±12.1	16.0±4.4^{ab}	29.3±8.7	24.5± 7.9	4.3±1.0	24.5±10.3	35.3±11.7^{ab}	31.4±10.6^{ab}
AMH PRE stimulation										
Low	*<10.0 ng/ml*	4	16.3±7.5^{a}	3.0±2.0^{a}	5.0±2.3^{a}	6.3±2.8^{a}	1.8±0.8^{a}	3.3±2.3	31. 8±13.8	20.7±11.4
Medium	*10.01-31.14 ng/ml*	9	70.8±8.5^{b}	25.8± 4.4^{b}	17.9± 3.4^{b}	23.3± 3.1^{b}	3.6± 0.6^{ab}	26.3± 8.2	36.9±9.7	32.5± 8.9
High	*>31.15 ng/ml*	4	94.8±16.4^{c}	17.0±8.7^{ab}	40.0±1.5^{c}	34.3±8.4^{c}	5.3±1.1^{b}	12.5±4.8	24.1±7.7	16.4±8.5
AMH POST STIMULATION										
Low	*<4.86ng/ml*	4	33.3±18.2	4.0± 1.8^{a}	11.8±8.4^{a}	14.5±7.8	2.5±0.7	1.8±0.9^{a}	24.9±14.9	15.1±11.7
Medium	*4.87–18.25 ng/ml*	9	67.7±12.9	23.0±4.7^{b}	17.7±3.9^{ab}	22.8±5.0	4.4±0.9	16.0±5.7^{ab}	29.4±7.5	22.8±6.9
High	*>18.26 ng/ml*	4	84.8±7.7	22.3±9.8^{ab}	33.8±6.0^{b}	27.3±6.6	3.5±0.9	37.0±12.2^{b}	47.5±13.4	43.6±13.0

[1] Total number of blastocysts include all stages (early blastocyst, blastocyst, expanded blastocyst and hatching blastocyst)

[2] Efficiency: Blastocysts/ total oocytes (%)

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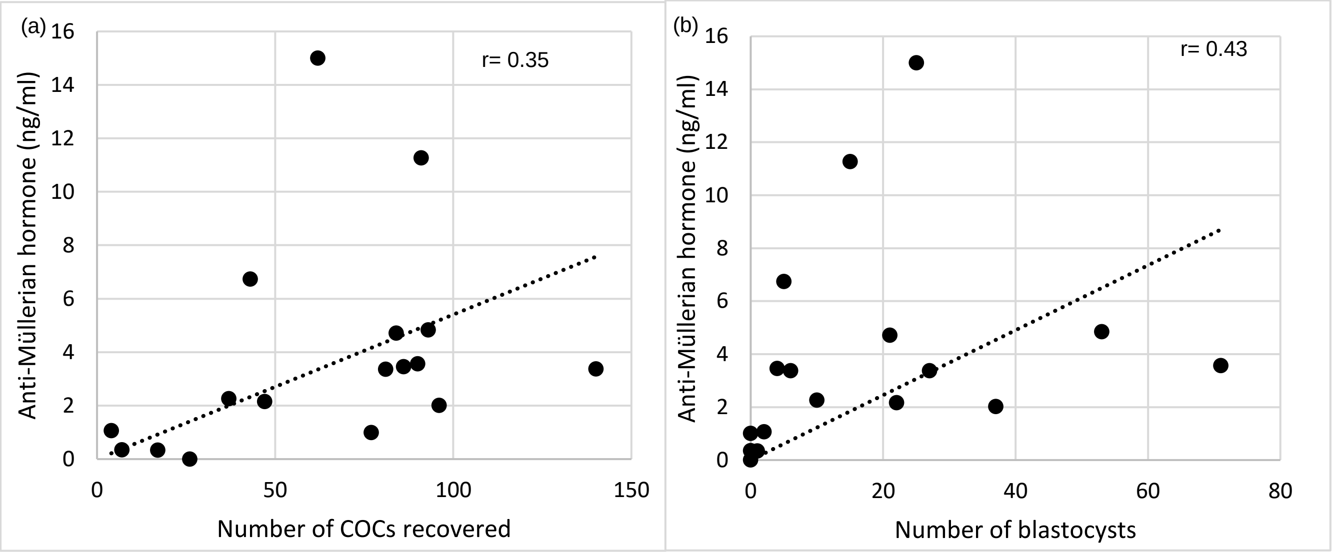

Figure 1: Correlation between 7-day old ewe lamb plasma AMH concentration and (a) total COCs recovered and (b) total blastocysts produced following the *in vitro* maturation and fertilisation of COCs collected at 7- weeks of age.

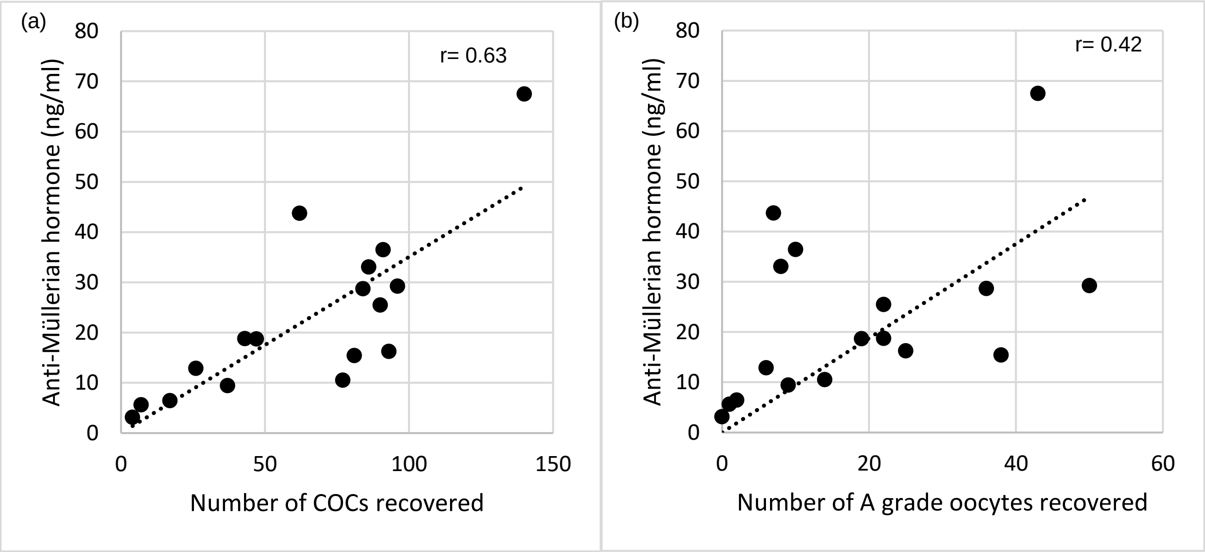

Figure 2: Correlation between lamb plasma AMH concentration before FSH stimulation at 7- weeks of age and (a) total COCs recovered, (b) A grade COCs recovered

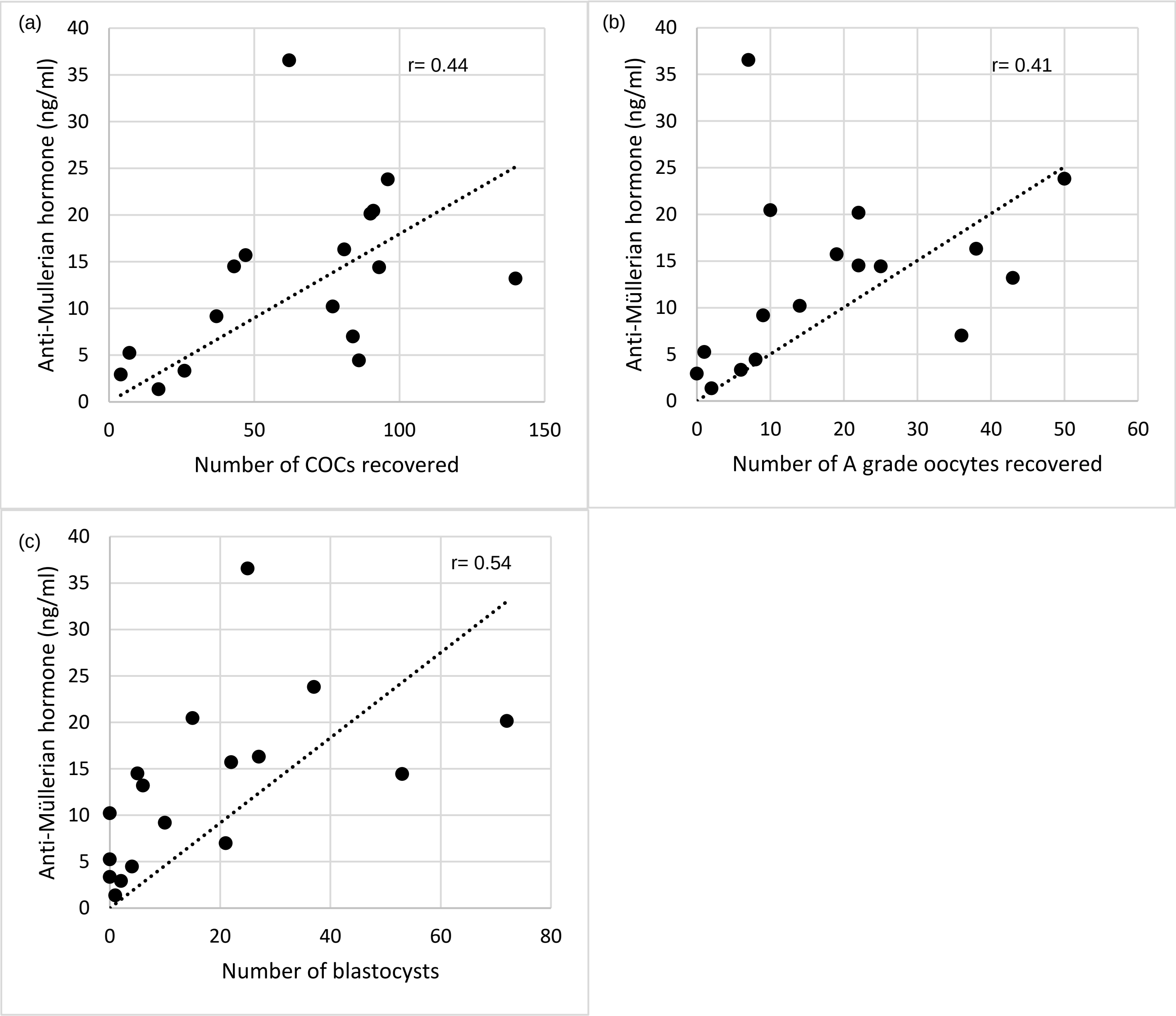

Figure 3. Correlation between lamb plasma AMH concentration after FSH stimulation at 7- weeks of age and (a) total COCs recovered, (b) A grade COCs recovered and (c) blastocysts produced

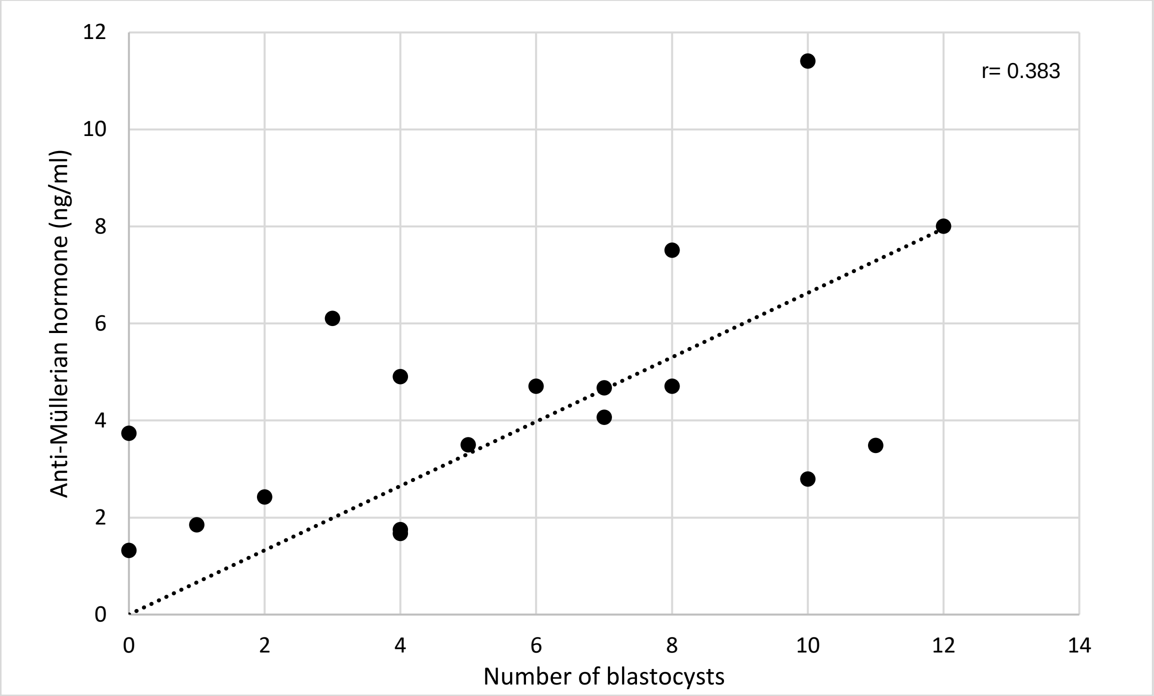

Figure 4: Correlation between maternal AMH concentration and total blastocysts produced following *in vitro* maturation and fertilisation of oocytes recovered from ewes at 14-months of age.

Chapter six: AMH concentration as a phenotypic marker of fertility within a natural mating system

Abstract

Anti-Müllerian hormone (AMH) is an accurate phenotypic marker of ovarian reserve, antral follicle count, embryo production and response to hormone stimulation. However, only two studies in sheep have associated pre-pubertal AMH concentration with the fertility of the ewe at first mating. Therefore, to determine if AMH concentration could be used to predict the fertility of Merino ewes within a natural mating system, 309 ewes across two Australian commercial flocks were used. AMH samples were collected from ewes at 44- and 292-days of age (n = 216; Armidale, NSW) or 82- and 324-days of age (n = 293; Tullamore, NSW) and were correlated to the number of fetuses present at ultrasound scanning and number of lambs born. Ewes with an AMH concentration ranging from 0.40 to 3.66 ng/ml at 292-days of age gave birth to 24 and 30 more lambs, respectively, compared with those with AMH below or above this range. Similarly, the number of pregnant ewes and number of fetuses present at pregnancy scanning were higher for ewe lambs with an AMH concentration < 0.64 ng/ml at 82 days. A number of external factors were also observed to affect AMH concentrations in ewe lambs including sire, birth type, rear type and sex of the co-twin. Interestingly, small negative correlations were also identified between AMH concentration at 44-days of age and weaning weight, and with pre-mating weight at both 82- and 324-days of age, indicating further investigation is warranted. Although a number of external factors were found to impact AMH, the key finding from this study was the identification of a range of AMH concentrations at specific ages that are predictive of ewe fertility within a natural mating system.

Introduction

Anti-Müllerian hormone (AMH) has the potential to be an effective phenotypic marker of ewe and ewe lamb fertility. A highly conserved glycoprotein of the transforming growth factor (TGF-β) family, AMH is expressed exclusively by the granulosa cells of growing antral follicles in female women, mice, cattle and sheep (Rico *et al.,* 2009). This makes AMH an accurate reflective marker of ovarian reserve, and it is currently the most reliable method of assessing ovarian reserve and therefore reproductive potential (Fleming et al., 2015). To date, AMH has been extensively studied as a way to select suitable animals for use in a variety of assisted reproductive technologies. Based on this body of research, it is evident that AMH is an accurate, predictive marker of *in vivo* embryo production in cattle (Ireland *et al.,* 2008, Monniaux et al., 2010), goats (Monniaux et al., 2011), sheep (Lahoz et al., 20914) and humans (Moro et al., 2016). However, limited research has been conducted on the potential of AMH as a marker of fertility within the Australian sheep industry.

In 2019, Australia was the largest global exporter of lamb and domestic consumption also increased, resulting in the production of 680,000 tonnes of lamb meat (MLA and AWI Wool and Sheepmeat Survey, 2019). The most effective way to meet consumer demand for lamb meat, and ensure sustainability and productivity of the Australian sheep flock is to increase the number of lambs weaned per mated ewe. Two thirds of the Australian breeding flock are Merino or Merino cross ewes, and yet Merino ewes rear 13% fewer lambs compared with other breeds (MLA and AWI Wool and Sheepmeat Survey, 2019). Intensive genetic selection for superfine Merino wool has reduced liveweight by 4% in some genotypes, which also restricts fertility and fecundity (Snowder and Fogarty, 2009). In the Merino, loss of potential lambs (reproductive wastage) between mating and lambing is the primary cause of their lower fertility and fecundity (Kleemann and Walker 2005). Up to 20% of losses were caused by partial or multiple ovulation failure, and up to 13% by ewes mating but not lambing (Kleemann and Walker 2005). It is, therefore, evident that improvements to the reproductive performance of the Merino will be highly beneficial to the Australian sheep industry. Increasing genetic selection for reproduction is not the most efficient means to improve reproductive performance as reproductive traits have a low heritability (Lalit *et al.* 2017) .

Additionally, exogenous and endogenous factors, including environment temperature and maternal nutrition and health, play a crucial role in pre- and neo- natal development of the reproductive tract. Ewe undernutrition can delay fetal ovarian development (Rae et al., 2001) which reduces early life fertility of ewe lambs (Long et al., 2010) as well as their fertility as adults (Gunn et al., 1995). Equally, development of the ovarian follicle pool is disrupted in ewe lambs born to ewes that are over fed during pregnancy (Da Silva et al., 2003). Birth type and sex of co-twin also influence the reproductive development of ewe lambs (Kelly et al., 2017). Taken together, these data demonstrate that a multi-facetted approach is needed to improve the reproductive efficiency of the Australian sheep flock. Phenotypic markers consider any external factors and stressors imposed on an individual, thereby reflecting the internal environment of any given animal at the time of selection.

Therefore, the primary aim of this study was to determine if AMH concentrations at key stages of reproductive development and coincident with routine key husbandry procedures, can predict the fertility of Australian Merinos within a natural mating system. A secondary aim was to determine whether AMH concentration was affected by the sire, birth/ rear type and sex of the co-twin, as well as ewe weight at weaning or prior to mating

Materials and Methods

Experimental design and animal management

All procedures were conducted according to the guidelines of the Australian Code of Practice for the Use of Animals for Scientific Purposes and the work approved by both the University of Adelaide Animal Ethics Committee (Approval number S-2017-068) and Primary Industries and Regions South Australia (PIRSA)(Approval number 14/17). The work was conducted across two sites: 1) CSIRO Armidale; New England Highway, Armidale NSW 2350 (Armidale) and 2) Centreplus Merino Stud; The Bogan Highway, Tullamore NSW 2874

(Tullamore). The work was conducted on 216 Merino ewes at Armidale and 293 Merino ewes at Tullamore.

Armidale

216 Merino ewe lambs were randomly selected from a syndicate natural mating commencing in 2018, and lasting for 35 days, DNA testing was used to determine birth type and subsequent sex of the co twin in these ewes. All ewes were weighed at weaning and again prior to ram entry. They were mated in their maiden year, at 1.5 years of age, using a syndicate natural mating system at 2.3% rams per 100 ewes (n = 15 rams, with all rams 1.5 years old). Mating commenced in early Autumn 2020 (southern hemisphere), with rams remaining with the ewes for 35 days. All animals were provided feed and water *ad libitum* and reared under the same conditions. All ewes and lambs were managed under standard husbandry practice, based on the farm management at the CSIRO Armidale site. At approximately day 60 of gestation all ewes were scanned for pregnancy. Those pregnant or not pregnant were recorded and separated accordingly for the remainder of pregnancy. DNA testing was used to determine the number of lambs born to each ewe.

Tullamore

293 Merino ewe lambs were randomly selected from a single sire mating in 2018, DNA testing was used to determine birth type and subsequent sex of the co twin in these ewes. All ewes were weighed prior to ram entry. Ewes were mated in their maiden year (1.5 years old) in a single sire mate on natural pasture. Mating commenced in late Summer 2020 (southern hemisphere), with single sires remaining with ewes for 42 days. They were provided feed and water *ad libitum* and reared under the same conditions. All ewes and lambs were managed under standard husbandry practice, based on the farm management at the Centre plus Merino Stud. At approximately day 60 of gestation all ewes were scanned for pregnancy. Those

pregnant or not pregnant were recorded and separated accordingly for the remainder of pregnancy. DNA testing was used to determine the number of lambs born to each ewe.

Blood sampling and AMH measurement

To determine pre-pubertal (AMH) concentrations for all Merino ewe lambs, a 9 ml blood sample was collected from each animal at two ages. The timing of sample collection differed between sites, and was timed to coincide with routine husbandry practices. At Armidale, samples were collected from 216 Merino ewe lambs at 44 days (lamb marking) and 292 days of age. At Tullamore samples were collected from 293 ewes at 82 days (lamb weaning) and 324 days of age. All samples were collected at the same time of day, and within the same period of time at each collection point. All samples were collected via jugular venepuncture into 9 ml lithium heparin vacuum tubes (BD Biosciences, Provet, Adelaide, Australia) and stored on ice. Samples were centrifuged at 1500rpm for 15 minutes, and stored at -20°C until assayed for AMH. Plasma AMH concentration was measured in 50 µl plasma samples in duplicate for each of the time points sampled using an Ovine AMH ELISA kit (AL—155 Ansh Labs, Webster, TX; MDC:0.025ng/ml) (5.62%, 0.83% intra and inter-assay coefficient of variation, respectively for Tullamore, and 8.63%, 0.72% intra and inter-assay coefficient of variation, respectively for Armidale) according to manufacturer's instructions. Variation determined over 12 assay plates for Tullamore samples and 15 plates for Armidale samples, including re-analysed samples (80 samples per plate). MDC calculated by the interpolation mean plus two standard deviations of 24 replicates of calibrator A (0 ng/ml) and low calibrator (0.37 ng/ml). Upper limit of standard curve at absorbance of 3.12 (20 ng/ml) for Tullamore and 3.09 (20 ng/ml) Armidale measured at 450 nm.

Statistics

As explained above, the ewes used in this study were housed and managed at two different locations, and therefore under different conditions. As a result, analysis of all results has been completed and separated according to location. Site 1: Armidale and Site 2: Tullamore. Differences in AMH between ewes that failed to conceive, had one or had two lambs were determined using an ANOVA, unbalanced design and Fisher's LSD post hoc test for between group comparisons of litter size. Data is presented as mean ± SEM. An ANOVA, unbalanced design was used to determine the relationship between AMH and birth type, sex of co twin, and sire. An ANOVA, unbalanced design was used to determine the relationship between weight, birth type, sex of cotwin and number of fetuses at scanning and number of lambs born. Pearson's Correlations were used to determine the relationship between plasma AMH concentration, weaning and mating weights. As per McGrice *et al*. (2020) quartiles were calculated for AMH concentrations. AMH concentration at 44-, 82-, 292- and 324- days of age were divided into low (lowest quartile), medium (middle two quartiles) and high (upper quartile) categories according to plasma AMH concentrations at each of these stages (Table 1). Lambs with AMH concentrations below the detectable range were included in the low-AMH group for analyses. One- way ANOVA unbalanced design was used to assess differences in litter size and pregnancy rates between quartile groups. All analysis was completed using SPSS Version 26 (IBM Corp.)

Results

Relationship between AMH concentration and fertility

Site 1: Armidale

Mean AMH concentrations were 42.4 ± 1.5 ng/ml at 44- days of age and 3.6 ± 0.64 ng/ml at 292- days of age, with ewe lambs weighing 23.4 ± 0.30 kg and 35.2 ± 0.30 kg at 44- and 292-

days of age, respectively. AMH concentration at 44- days of age was similar between Merino ewes that scanned and birthed 0, 1 or 2 lambs (Table 2).

When grouped according to AMH concentration quartile ranges, ewes that were categorised as having high AMH (>3.66 ng/ml) at 292- days of age had fewer fetuses at pregnancy scanning compared with those in the low or medium AMH groups (Table 3; P<0.05). Per 100 ewes scanned, those with high AMH scanned 27 fewer lambs than those in the medium group. Per 100 ewes, those in the medium AMH group at 292- days of age gave birth to 24 more lambs than the low group and 30 more lambs than the high group. There was no difference in the number of lambs born between the low and high AMH groups at 292- days of age (Table 3).

Site 2: Tullamore

Average AMH concentration for Tullamore Merino ewes at 82- days of age was 5.1 ± 0.22 ng/ml and at 324- days of age was 4.0 ± 0.11 ng/ml. AMH concentration did not differ between ewes at Tullamore that did or did not became pregnant (Table 4). AMH concentration was not different between ewes that had 0, 1 or 2 fetuses at scanning or birthed 0, 1 or 2 lambs (Table 4).

When grouped according to AMH quartile ranges, ewes at Tullamore with a high AMH at 82- days of age tended to have fewer fetuses at pregnancy scanning (Table 5; P=0.051) compared with those with medium but not low AMH, although there was no difference in the number of lambs born between these groups. Therefore, per 100 ewes, those with high AMH scanned 19 fewer fetuses. At Armidale, ewes with high AMH at 292 days of age also had a lower number of pregnant ewes (Table 5; P=0.154) and 6 to 10 fewer ewes pregnant per 100 ewes. No relationship was found between AMH group and fertility parameters at 324- days of age.

Factors affecting AMH concentration

Birth type and sex of the co-twin

There was no effect of birth or rear type on AMH concentrations at 44- or 292-days of age in the ewes at Armidale (Table 6). Ewes that were born a singleton tended to have lower AMH concentrations at 82-days of age compared with those that were born a multiple (P=0.087) at Tullamore. Similarly, those that were raised a single or those that were born with a male co-twin had lower concentrations of AMH at 82-days age compared with twin ewe lambs born with a female co-twin (Table 6). At Armidale, ewes which reared a single scanned and birthed more lambs compared with those which reared a twin (Table 7; P<0.05). No differences in number of fetuses at scanning or number of lambs born were found between birthing parameters at Tullamore.

Weaning and premating weight

Pre-mating weight did not differ between those ewes that failed to conceive or birthed a single lamb at Armidale or Tullamore (Table 8; P>0.05). Pre-mating weight was significantly (P< 0.001) greater for those ewes that birthed twins compared with those that did not birth, or birthed a single lamb at Armidale and Tullamore (Table 8). Correlation analysis showed that weaning weight decreased as AMH concentration at 44 days increased (Figure 1). AMH concentration at both 82-days and 324-days was lower (P<0.05) in ewes with a high pre- mating weight at Tullamore (Figure 2).

Sire effect

Of the 15 rams utilised at Armidale, 2 rams produced ewe lambs with significantly (P<0.001) higher AMH concentrations at 292-days of age (9.6 ± 6.38 and 22.5 ± 9.07 ng/ml). In the remaining 13 rams ewes lambs, the mean AMH ranged from 1.6 ± 0.33 ng/ml to 3.6 ± 0.64 ng/ml. At 44-days of age, the progeny from one ram had higher AMH concentrations, with another ram producing ewe lambs with low AMH (20.5 ± 3.09 ng/ml), in the progeny from the remaining sires average AMH ranged between 28.3 ± 2.88 and 56.7 ± 9.27 mg/ml). There was also a significant (P=0.005) difference in number of fetuses at scanning and number of live born (P<0.005) at Armidale between sires. Two rams produced significantly fewer (P< 0.001) fetuses (0.6 ± 0.17 and 0.7± 0.18) and lambs (0.6 ± 0.16 and 0.8 ± 0.2) all other sires ranged 0.9 ± 0.21 to 1.6 ± 0.15 fetuses scanned and live lambs born. Whilst not significant at 324-days of age, there was a strong sire effect in the Tullamore ewe lambs in AMH concentrations at 82-days of age. 4 rams produced ewe lambs with consistently lower AMH concentrations (3.0 ± 0.47, 3.1 ± 0.49, 3.8 ± 0.62 and 2.9 ± 0.97 ng/ml). At Tullamore, there was no difference between rams in the number of fetuses present at pregnancy rate or the number of lambs born.

Discussion

To the best of my knowledge, this is the first study to demonstrate that there is an optimum range of AMH concentration in ewe lambs to predict fertility and fecundity following first mating in commercial Australian Merino flocks. In both flocks, and at both ages, AMH was similar for ewes regardless of pregnancy status or the number of lambs born. However, when ewes were divided into groups based on their AMH concentrations, Armidale ewes with medium AMH concentrations at 292-days of age produced the most lambs following their first mating. Similarly, Tullamore ewes with high AMH at 82-days of age had fewer fetuses at pregnancy scanning. Together these data suggest that the relationship between AMH concentrations at the time points measured and ewe fertility and fecundity may not be as simple as indicated in previous studies (Lahoz *et al.* 2012; Torres-Rovira *et al.* 2016) and that optimal concentrations of AMH, rather than just high or low, may need to be identified.

AMH concentrations were significantly higher at 44-days of age in the ewe lambs at Armidale compared with any other sampling point. Across all Merinos, there is a peak in ovarian activity at 6-weeks of age (chapter three), and this phenomenon is also evident in Sarda ewe lambs (Torres-Rovira *et al.* 2016). It is common for AMH concentration to peak in 6-week old Merino ewe lambs (chapter three), and the measurement of AMH in 44-day old ewe lambs at Armidale was designed to determine if peak AMH concentrations correlated with ewe fertility at first mating. AMH concentrations by 82-days in the Merino ewe lambs and Tullamore was considerably lower, and was similar to concentrations observed at both 292- and 324-days of age at Armidale and Tullamore, respectively. This is unsurprising given previous literature has demonstrated a decline in the number growing follicles by 8-weeks of age (Jorio *et al.* 1991) with numbers remaining low up until 16-weeks of age (Kennedy 1974). Similarities in AMH concentrations at 292- and 324-days of age in the current study would support previous suggestions that from 8-weeks of age the antral follicle pool is established. Thus, sampling from ewe lambs pre-pubertally, close to 2-months of age, in the current study could potentially relate to differences in fertility in adulthood. For the purpose of flock management, the aim was to determine a relationship between AMH prior to, or at, weaning to allow for selection of ewe lambs early in life and coincident with standard, routine husbandry practices (i.e. lamb marking and/or weaning). Results presented in Chapter three suggest that timing of AMH sampling may need to be scheduled close to the time of the intervention to be informative. Therefore, taking samples from the ewe close to 12-months of age would allow for adequate time to determine the management of the flock prior to breeding, i.e. selling ewes with a potentially reduced fertility and preparing the remainder for mating.

There is an extensive body of literature from the past decade investigating the correlation between AMH and markers of reproductive potential in cattle and sheep, with conflicting results. Evidence to support the use of AMH to select more productive donors for inclusion in assisted reproductive technology programs is strong in cattle. Cattle with higher AMH possessed more antral follicles, and produced more recoverable oocytes, and a greater number of transferable embryos when enrolled in superovulation protocols (Monniaux *et al.* 2010; Rico *et al.* 2012; Souza *et al.* 2015; Ghanem *et al.* 2016) or laparoscopic ovum pick (Batista *et al.* 2016) (LOPU) protocols. Similarly in sheep, ewes with higher AMH concentrations were response to the respective hormonal protocols used in an FSH ovarian reserve test (EFFORT) (Torres-Rovira *et al.* 2014) (Torres-Rovira *et al.* 2016), multiple ovulation and embryo transfer (MOET) (Lahoz *et al.* 2015), hormonal stimulation for inducing ovulation for a natural mating at 10-months of age (Lahoz *et al.* 2012), LOPU (Lahoz *et al.* 2014) or eCG prior to a natural mating (Lahoz *et al.* 2016); producing more CLs, more punctured follicles or resulting in a greater fertility at first or second service. The similarities in the relationship between circulating AMH and response to exogenous hormones in both species, demonstrate that AMH is a strongly, positively correlated with the ovarian response to hormonal stimulation.

In contrast, the current study suggests simply using high or low AMH as a marker of fertility in a natural mating system is insufficient. It appears categorising animals based on a range of AMH is much more useful, as reported in our previous work within an assisted reproductive technology. In contrast to previous studies in sheep (Lahoz *et al.* 2012; Lahoz *et al.* 2016; Torres-Rovira *et al.* 2016), AMH concentration was not correlated to pregnancy outcomes or the number of fetuses produced and lambs born at either of the two locations tested. Similarly, using Katahdin ewes aged from 7-months to 7-years of age, Acharya et al. (2016) reported no correlation between AMH and estimated breeding values for number of lambs born, and no relationship between AMH and number live born even when broken down into quartiles. However, when ewes were divided into groups based on AMH concentrations in the current study, there was evidence from both sites that AMH has the potential to predict the outcomes following first mating. At Armidale (Site 1), ewes with an AMH between 0.4 and 3.66 ng/ml, at 292-days of age (approximately 10 months) produced the most lambs. This is a promising finding for the industry, as it allows for cut off points to be established allowing quick selection of ewes for breeding. Whilst no relationship was found at 324-days of age (approximately 11 months), ewe lambs with an AMH ranging from 0.21 to 0.64 ng/ml from samples collected at 82-days of age (approximately 3 months) had more fetuses at pregnancy scanning than those with an AMH greater than 0.64 ng/ml at Tullamore (Site 2). To date, three other studies have found a relationship between animals categorised based on AMH and reproductive outcomes. Lahoz *et al.,* (2016) allocated ewes lambs to either a low or a high AMH group, with Torres-Rovira *et al.*, (2014 and 2016) allocating ewe lambs to groups based on having either a low, medium or high antral follicle count. In these studies, animals allocated to the high treatment had higher pregnancy rates and fertility at first mating. Our work shows an optimal range for AMH concentration, rather than a greater concentration of AMH resulted in a better outcome. In women with polycystic ovarian syndrome, and enrolled in an ART program, high AMH concentrations resulted in a reduced response to ovulation induction (Mumford *et al.* 2016) and lower live birth rates (Tal *et al.* 2020; Guo *et al.* 2021). Our current work supports this, indicating that increased concentrations of AMH may not necessarily reflect a healthy, growing follicle pool.

It is possible that metabolism plays a key role in the development of the ovarian reserve. The relationship between weight and ovarian reserve is fairly well established, with feed restriction associated with a smaller ovarian reserve and negative impacts on the primordial follicle pool (Aiken *et al.* 2013). This same group also reported transgenerational impacts of feed restriction, whereby AMH concentrations were lower in the granddaughters of rats, even though the granddaughters had not had any feed restriction imposed on them directly (Aiken *et al.* 2015). Maternal undernutrition in cows results in female offspring with a reduced antral follicle count and reduced concentrations of AMH as well as increased FSH concentrations (Mossa *et al.* 2013). Feed restricted ewes produced offspring with fewer ovarian follicles (Rae *et al.* 2001). Similarly, overnutrition in sheep restricts placental growth and follicular development (Da Silva *et al.* 2003). A review by Monniaux (2018), suggests that a diet change, whether it be undernutrition, overnutrition or high fat diets alone, only alters the ovarian reserve if accompanied by a change in metabolism. An extensive review by Kenyon (2014) reported a curvilinear relationship between ewe body condition and performance. Furthermore, in cattle, a small but positive relationship between antral follicle count and birth weight has been identified (Cushman *et al.* 2009). Together these studies suggest that the metabolism and condition of the animal play a key role in productivity. In the current study, a negative relationship between AMH and weight has been identified. At Armidale, AMH concentrations at 44-days of age were higher in ewe lambs that had a lower weaning weight; however, this relationship was no longer evident in the ewes once they reached 292-days of age. Further, for this location there was no relationship between AMH and pre-mating weight. Conversely, AMH concentrations at both 82- and 324-days of age in ewes at Tullamore were lower in ewes that had a higher premating weight. There is evidence in humans that obese women (Body mass index >30) have lower AMH concentration compared with normal weight women (body mass index<25) (Su *et al.* 2008), therefore in sheep, an estimate of body condition at time of mating may be informative to determine whether an excessive body condition was the underlying cause of this inverse relationship. Ultimately, there may be differences in the metabolism of ewe lambs pre-pubertally and this has the potential to alter the ovarian reserve, but not fertility, in the animal.

In the current study, the relationship between weight and AMH concentration was interesting, and may somehow be related to the birth type (single or twin). One study in sheep has identified that twins have a greater percentage of fat mass by two years of age (Hancock *et al.* 2012). Up until weaning, twin lambs remained smaller than singles but grew much more rapidly thereafter (Hancock *et al.* 2012) suggesting a major change in the metabolism of these animals. Although not significant, in the current study singleton ewes had a lower AMH in comparison with twins. These animals likely had a different metabolism from birth and this may reflect reduced AMH concentrations. Similarly, ewes with a greater bodyweight at weaning/ mating may also have different metabolism resulting in the decreased AMH. This is further supported given our results in chapter one, where Suffolk ewe lambs AMH concentration differed to Merino ewe lambs, resulting in some minor differences in the ovarian follicle pool. Together this may suggest a relationship between metabolism and ovarian reserve development in the Merino, but more research is required to quantify this.

The results from the current study suggest that further investigation is required into the effects of uncontrollable factors; birth type, rear type and sex of the co-twin on AMH concentration. There was no difference in AMH concentration between ewes born as a single or a twin at Armidale or Tullamore and this is surprising given literature suggests that animals born a twin have impaired reproduction as a result of decreased liveweight and competition *in utero* (Cushman *et al.* 2012). In support of this, ewes that were raised as a single at Armidale produced significantly more lambs compared with those that were raised as a twin. Ewes born with a female co-twin on average had a 0.5 ng/ml increase in AMH compared with those born with a male co-twin. Whilst JIVET outcomes were unaffected by birth type, the number of blastocysts produced from both embryos cleaved and cumulus-oocyte complexes collected, was higher for female lambs born with a female co-twin (Kelly *et al.* 2017), similar to the current study. In addition, the number of follicles recovered in singles versus twins was lower in this study, although not significantly, and there were no differences in the number of blastocysts produced (Kelly *et al.* 2017). When comparing AMH between co-twins of different sexes, it is evident that a male co-twin may reduce ovarian reserve. This has also been reported in humans, whereby women born with a male co-twin have a reduced lifetime reproductive success compared to those with a female co-twin (Lummaa *et al.* 2007). Similarly, female Soay sheep born with a male co-twin were lighter than those with a female co-twin which had long term impacts on lifetime reproductive success (Korsten *et al.* 2009).

Conclusion

Categorising ewes based on a quartile range of AMH at 292-days of age suggests that those with an AMH concentration of 3.66 ng/ml or higher had 27 fewer fetuses at pregnancy scanning compared with those with an AMH concentration lower than this. Furthermore, ewes with an AMH concentration ranging between 0.40 and 3.66 ng/ml at this age birthed 24 and 30 more lambs compared with those in the low or high group, respectively. Together these data suggest that selection of ewes with an AMH concentration between 0.40 and 3.66 ng/ml may be used to increase the number of lambs born in a commercial system. Alternatively, selecting ewe lambs at 82-days with an AMH concentration less than 0.64 ng/ml may increase the number of lambs born, as those with an AMH concentration greater than this tended to have fewer fetuses at pregnancy scanning, and up to 10 fewer ewes pregnant per 100 ewes. Understanding the impact of a variety of external factors in the current study requires further investigation. The negative correlation between AMH concentration at 44-days of age, and with pre-mating weight at both 82- and 324-days of age is interesting and unexpected, and further investigation is warranted to determine the cause of this. Additionally, the effect of birth type and rear type differed between sites was interesting as to the slight effect of the sex of the co-twin. Finally, the large sire effect on AMH was a major finding in the current study and further investigation is required.

Based on our findings in our previous chapter, AMH may also reflect the responsiveness of the ovary to gonadotrophins, which under natural conditions may not vary sufficiently to express differences in fertility. Therefore, the major findings from this chapter support the use of categorising AMH concentration at key ages, 88-days (weaning) and 292-days of age, to select ewes that will increase the number of lambs born within a commercial flock. Overall, this study has been effective in determining options for the timing of AMH sampling that may be used to optimise the number of lambs born within a farming system, and also reiterated the effect of weight on the ovarian reserve as well as the impact of external factors.

List of tables

Table 1. AMH concentration ranges of ewes classified as having low, medium or high values at 72 and 292 days of age at Armidale and 84 and 324 days of age at Tullamore.

	AMH concentration (ng/ml)		
	Low	Medium	High
Armidale *n*	*72*	*108*	*53*
44 days	<9.71	9.71-61.41	>61.41
292 days	<0.40	0.40- 3.66	>3.66
Tullamore *n*	*73*	*147*	*73*
82 days	<0.21	0.21-0.64	>0.64
324 days	<1.21	1.21-5.06	>5.06

Table 2: AMH concentration (mean ± SEM) at 44 and 292 days of age for ewes according to pregnancy status, number of fetuses at scanning and number of lambs born at Armidale

	n	AMH 44 days	*n*	AMH 292 days
Pregnant				
No	*49*	43.86 ± 3.16	*51*	2.52 ± 0.25
Yes	*166*	41.90 ± 1.76	*165*	4.28 ± 0.9
Number of fetuses at scanning				
0	*49*	43.86 ± 3.16	*51*	2.52 ± 0.25
1	*84*	40.65 ± 2.38	*83*	4.63 ± 1.3
2	*82*	43.19 ± 2.61	*82*	3.92 ± 1.24
Number lambs born				
0	*49*	43.86 ± 3.16	*51*	2.52 ± 0.25
1	*84*	40.95 ± 2.37	*83*	4.72 ± 1.3
2	*82*	42.88 ± 2.62	*82*	3.84 ± 1.24

Table 3: Average number of pregnant ewes, fetuses at scanning and number of lambs born for ewes grouped according to high, medium or low AMH at 44 or 292 days of age at Armidale. [ab] *Within column, superscripts indicate significant differences (P< 0.05)*

	Low	n	Medium	n	High	n
AMH at 44 days of age	*<9.71*	*72*	*9.71-61.41*	*108*	*>61.41*	*53*
Pregnant	0.79 ± 0.05		0.76 ± 0.04		0.77 ± 0.06	
Number of fetuses at scanning	1.13 ± 0.09		1.14 ± 0.07		1.15 ± 0.11	
Number lambs born	1.14 ± 0.09		1.13 ± 0.07		1.15 ± 0.11	
AMH at 292 days of age	*<0.40*	*71*	*0.40 – 3.66*	*108*	*>3.66*	*54*
Pregnant	0.77 ± 0.05		0.81 ± 0.04		0.70 ± 0.06	
Number of fetuses at scanning	1.04 ± 0.08^{a}		1.27 ± 0.07^{b}		1.00 ± 0.11^{a}	
Number lambs born	1.04 ± 0.08^{a}		1.28 ± 0.07^{b}		0.98 ± 0.1^{a}	

Table 4: AMH concentration (mean ± SEM) at 82 and 324 of age for ewes according to pregnancy status, number of fetuses at scanning and number of lambs born at Tullamore

	n	AMH 82 days	*n*	AMH 324 days
Pregnant				
No	*42*	5.45 ± 0.6	*24*	4.27 ± 0.35
Yes	*251*	5.02 ± 0.24	*246*	4.01 ± 0.11
Number of fetuses at scanning				
0	*41*	5.58 ± 0.6	*41*	4.26 ± 0.36
1	*133*	5.31 ± 0.34	*129*	3.97 ± 0.16
2	*118*	4.69 ± 0.34	*117*	4.05 ± 0.17
Number Lambs Born				
0	*80*	4.82 ± 0.37	*80*	3.93 ± 0.21
1	*148*	5.39 ± 0.34	*144*	4.12 ± 0.16
2	*64*	4.75 ± 0.46	*63*	4.01 ± 0.23

Table 5: Average number of pregnant ewes, number of fetuses at scanning and number of lambs born for ewes grouped according to high, medium or low AMH at 82 and 324 days of age at Tullamore ^{ab} Within column, superscripts indicate significant differences ($P< 0.05$)

	Low	*n*	Medium	*n*	High	*n*
AMH at 82 days of age	*<0.21*	*73*	*0.21-0.64*	*147*	*>0.64*	*73*
Pregnant	0.85 ± 0.04		0.89 ± 0.03		0.79 ± 0.05	
Number of fetuses at scanning	1.29 ± 0.08^{a}		1.33 ± 0.06^{a}		1.10 ± 0.08^{b}	
Number lambs born	0.95 ± 0.09		0.98 ± 0.06		1.04 ± 0.16	
AMH at 324 days of age	*<1.21*	*78*	*1.21-5.06*	*143*	*>5.06*	*72*
Pregnant	0.88 ± 0.04		0.85 ± 0.03		0.83 ± 0.04	
Number of fetuses at scanning	1.31 ± 0.08		1.25 ± 0.06		1.24 ± 0.09	
Number lambs born	1.13 ± 0.15		0.91 ± 0.06		0.99 ± 0.08	

Table 6: Effects of birthing parameters on circulating AMH concentration at 44 and 292 days of age at Armidale 82 and 324 days of age at Tullamore ^within column, indicates trend (P<0.1)

		Armidale			Tullamore	
	n	44 days	292 days	*n*	82 days	324 days
Birth Type						
Single	*93*	41.68 ± 2.33	3.67 ± 1.14	*68*	4.38 ± 0.45	4.08 ± 0.2
Multiple	*122*	42.85 ± 2.05	3.51 ± 0.71	*225*	5.30 ± 0.26^	3.94 ± 0.13
Rear Type						
Single	*126*	41.39 ± 2.03	3.37 ± 0.88	*113*	4.77 ± 0.35	3.95 ± 0.17
Multiple	*89*	43.70 ± 2.34	3.90 ± 0.94	*180*	5.28 ± 0.29	3.99 ± 0.15
Sex of Co Twin						
Female				*95*	5.53 ± 0.43	3.93 ± 0.17
Male				*93*	5.03 ± 0.37	4.02 ± 0.23

Table 7: Effects of birthing parameters on fertility outcomes at Armidale and Tullamore * within column, indicates significant difference (P< 0.05)

		Armidale					Tullamore
	n	Number of fetuses at scanning	Number of live born	*n*	Number of fetuses at scanning	*n*	Number of live born
Birth Type							
Single	*93*	1.61 ± 0.24	1.61 ± 0.24	*133*	1.78 ± 0.39	*148*	1.78 ± 0.36
Multiple	*122*	1.51 ± 0.55	1.51 ± 0.52	*118*	1.84 ± 0.43	*65*	1.88 ± 0.64
Rear Type							
Single	*126*	1.47 ± 0.23	1.48 ± 0.23	*133*	1.62 ± 0.42	*148*	1.60 ± 0.40
Multiple	*89*	1.34 ± 0.50*	1.32 ± 0.50*	*118*	1.64 ± 0.44	*65*	1.68 ± 0.58
Sex of Co-Twin							
Female				88	1.56 ± 0.53	*96*	1.53 ± 0.51
Male				78	1.49 ± 0.57	*44*	1.41 ± 0.75

Table 8: Effects of weight on litter size born at Armidale and Tullamore ^{ab} within column, indicates significant difference ($P < 0.05$)

n	Litter size	Armidale pre-mating weight	*n*	Armidale weaning weight	*n*	Tullamore pre-mating weight
49	0	48.53 ± 0.87^{a}	*49*	23.68 0.66^{a}	*79*	51.15 ± 0.60^{a}
84	1	47.10 ± 0.42^{a}	*83*	22.29 0.42^{a}	*148*	52.38 ± 0.50^{a}
82	2	52.28± 0.45^{b}	*82*	24.48 0.30^{b}	*64*	55.30 ± 0.99^{b}

List of figures

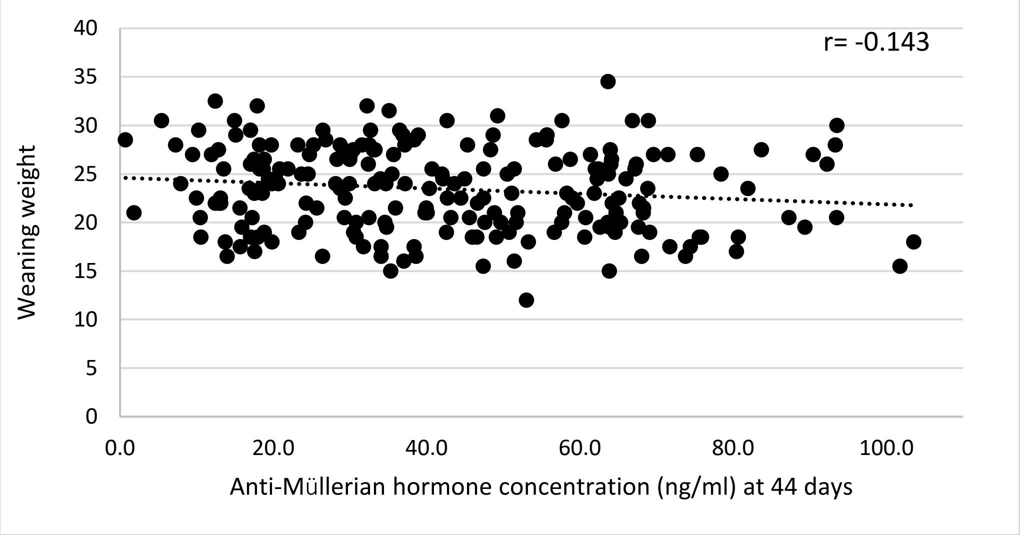

Figure 1: Correlation between weaning weight and AMH at 44 days old in Armidale ewe lambs

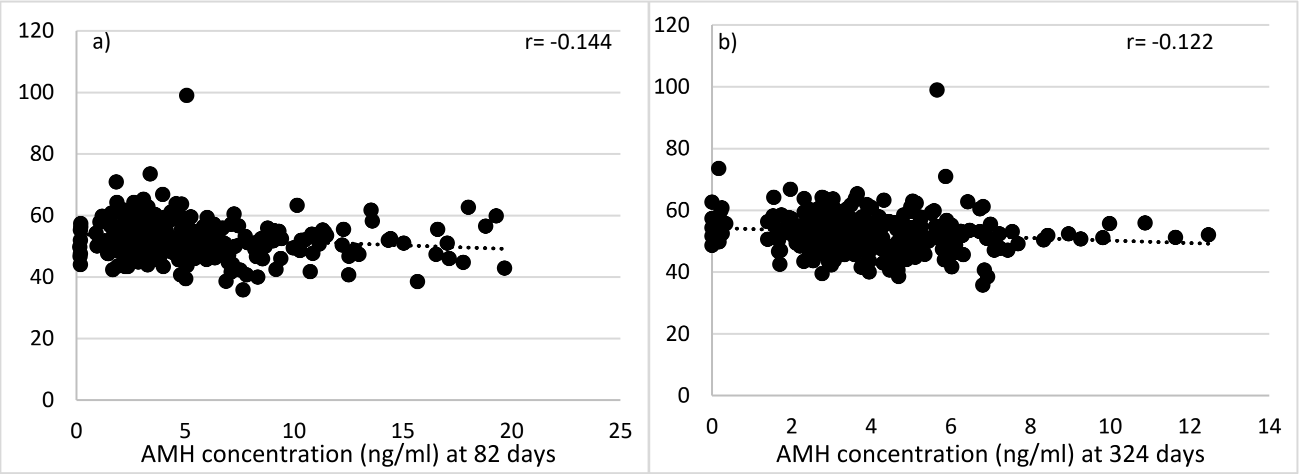

Figure 2: Correlation between pre- mating weight and AMH concentration at a) 82 days and b) 324 days of age in Tullamore ewe lambs

Chapter seven: Short communication: circulating anti-Müllerian hormone (AMH) from 5-month-old Australian Merino ewe lambs predicts birthing rates at their first service accepted with major revisions in ‘Reproduction in Domestic Animals’.

Short Communication: circulating anti-Müllerian hormone (AMH) from 5-month-old Australian Merino ewe lambs predicts birthing rates at their first service

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Statement of Authorship

Title of Paper	Short Communication: circulating anti-Müllerian hormone (AMH) from 5-month-old Australian Merino ewe lambs predicts birthing rates at their first service	
Publication Status	☐ Published ☐ Submitted for Publication	■ Accepted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Reproduction in Domestic Animals	

Principal Author

Name of Principal Author (Candidate)	Jamee Daly		
Contribution to the Paper	Design of the research within the manuscript, performed the experiments and data analysis within the manuscript. Interpreted the results. Wrote the initial manuscript and completed all drafting edits as well as approval of the final manuscript.		
Overall percentage (%)	80		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	18/05/2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	William van Wettere		
Contribution to the Paper	(10%). Conception and design of the project. Interpretation of results. Edited and revised the manuscript for approval of final version		
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Name of Co-Author	Jennifer Kelly		
Contribution to the Paper	(5%). Drafting of manuscript and interpretation of results		
Signature		Date	18/05/2022

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Contribution to the Paper	(5%). Drafting of manuscript and interpretation of results		
Signature		Date	18/05/2022

Abstract

Anti-Müllerian hormone (AMH) is a marker of antral follicle numbers and responsiveness to ovarian stimulation in cattle and sheep. In Sarda and Rasa Aragonesa ewe lambs, plasma AMH at 1.5 - 3.6 months of age has been shown to positively correlate with fertility at first mating. The aim of this study was to determine if a similar relationship between pre-pubertal AMH and fertility exists in Australian Merinos when sampled at a time that coincides with husbandry procedures. Plasma AMH from 86 Merino ewe lambs (5-months of age) was measured. At 18 months, the ewes were housed with Merino rams for 35 days. Pregnancy status was determined by scanning (day 60) and subsequent lambing recorded. Pre-pubertal AMH concentrations were compared for lambing and non-lambing ewes by Independent t-test (SPSS, IBM Statistics 26). AMH concentrations at 5 months were higher ($P<0.05$) for singleton (2.74 ± 0.23 ng/ml) (n=62) and twin-bearing (3.34 ± 0.81 ng/ml) ewes (n=10) compared with non-lambing (1.26 ± 0.48 ng/ml) ewes (n=14). A cut-off AMH concentration of 1.29 ng/ml at 5 months of age was determined to predict Merino ewes likely to become pregnant at first mating, with a sensitivity of 86.1% and specificity of 64.3%. This data demonstrates that circulating AMH concentrations are higher at 5 months of age in Merino ewes that produce a lamb following their first mating at 18 months of age, when compared with those that fail to establish a pregnancy.

Key words: Anti-Müllerian hormone, ewe lamb, fertility marker, Australian Merino, pregnancy, first service

Introduction

There is a need to select Merino ewes with greater fertility and fecundity to improve the reproductive performance of the breed. Merino and Merino cross ewes make up 76% of the

Australian flock (Australia 2018); however, for every 100 Merino ewes mated 84 lambs are reared to 12 weeks of age, compared with 97 lambs for non-Merino ewes (Australia 2018). In females, anti- Müllerian hormone (AMH) is expressed exclusively by the granulosa cells of growing antral follicles (Rico *et al.* 2009). As such, AMH accurately reflects the size of the ovarian follicle reserve, and is currently the most reliable method of assessing both ovarian reserve and the ovarian response to stimulation in women (Fleming *et al.* 2015), cattle (Monniaux *et al.* 2010), goats (Monniaux *et al.* 2011) and sheep (Lahoz *et al.* 2014).

In Sarda and Rasa Aragonesa ewe lambs aged 1.5 and 3.6 months respectively, plasma AMH was indicative of lambing success at their first mating (Lahoz *et al.* 2012; Torres-Rovira *et al.* 2016). However, it remains to be established whether AMH can be used as a predictor of pregnancy rates and mating outcomes in Australian Merinos. The objective of this study was to determine whether a pre-pubertal plasma AMH sample taken at 5 months of age could predict fertility at first mating (18 months of age) in Australian Merino ewes.

Methods

All procedures were conducted at the Turretfield Research Centre, Rosedale, South Australia (34°38'S, 138° 44'E), and approved by the Animal Ethics Committees of University of Adelaide and Primary Industries and Regions South Australia (PIRSA). Merino ewe lambs (n=86) born within the same month were used.

Blood sampling and AMH measurement

A blood sample was collected at weaning (5-months of age) from all lambs by jugular venepuncture into 9 ml lithium heparin vacuum tubes (Vacuette; Southern Cross Science). All samples were collected at the same time of day and stored on ice until processing. Samples were centrifuged at 1500 gfor 15 minutes and plasma was recovered and stored at -

20°C. Plasma AMH concentrations were measured in duplicate using two AMH (Ovine) ELISA (AL-155, Ansh Labs, Webster, Tx), according to the manufacturer's instructions. Intra-assay variation was 8.22% and 6.42% respectively.

Ewe Management

All 86 eighteen-month-old ewes were housed in a paddock with 6 entire two-year-old Merino rams over two consecutive oestrous cycles (approximately 35 days) in Summer (mid-December - January). Rams were fitted with sire-sine harnesses and a marking crayon, and crayon marks were checked daily to determine mating date. Ewes were scanned for pregnancy at approximately day 60 post-mating using a Sonosite M-Turbo Vet ultrasound fitted with an abdominal convex transducer (C60X: 2—5 MHz). During lambing in Autumn (late May - June), paddock observations were made at 12 hourly intervals and new lambs were tagged.

Statistics

Differences in mean AMH concentrations between ewes with different litter sizes (0,1,2) were determined using an Independent-Samples Kruskal-Wallis test, as data was not normally distributed. A Receiver Operating Curve (ROC) analysis was performed to determine a cut-off plasma AMH value capable of predicting fertility at first mating. Sensitivity was defined as the ratio of ewes correctly predicted as pregnant above determined AMH value and all the pregnant ewes. Specificity was the ratio of ewes correctly predicted as non-pregnant below determined AMH value and all non- pregnant ewes. Data are presented as mean ± SEM. All data analysis was completed using SPSS, IBM Statistics 26.

Results

Plasma AMH concentrations were significantly higher ($P<0.05$) at 5 months of age in singleton and twin-bearing ewes compared with non-lambing ewes, but did not differ between singleton and twin-bearing ewes (Figure 1).

The ROC curve (Figure 2) represents the trade-off between specificity and sensitivity of AMH concentration at 5 months of age to predict the occurrence of live birth at first mating at 18 months of age. The predictive performance of plasma AMH from lambs at 5 months of age, assessed by area under the ROC curve, was 0.821 ± 0.55, where a result above 0.8 is considered good (Lahoz *et al.* 2012). The cut-off plasma AMH concentration for lambs at 5 months of age that could be used to predict the ability of a ewe to become pregnant at first mating was found to be 1.29 ng/ml. At this value sensitivity was 86.1% and specificity was 64.3%.

Discussion

In this study, circulating AMH at five months of age was higher in Australian Merino ewes that became pregnant and lambed at 18 months of age compared with non-pregnant ewes. This is in agreement with previous reports that Sarda and Rasa Aragonesa ewe lambs with higher AMH at 50 days of age (Torres-Rovira *et al.* 2016) and at 3.6 months of age (Lahoz *et al.* 2012), respectively, were more fertile at first mating. First mating occurred at 10 months of age in Rasa Aragonesa ewes (Lahoz *et al.* 2012), and at 16.5 months of age in Sarda ewes (Torres-Rovira *et al.* 2016), demonstrating a similar long-term relationship between pre-pubertal AMH and fertility at first mating to that observed in the Australian Merino at 18 months, in the current study. However, this is the first study to demonstrate a correlation between a phenotypic marker of ovarian function in the most utilised breed of sheep in Australia and fertility, and the first to suggest a breed specific cut-off value for selection.

Additionally, the timing of sampling aligns within Australian sheep husbandry practices as it allows for selection of ewes at 5 months of age and 12 months of management prior to current standard industry practice of first mating maiden ewes at 18 months of age. Similarly, within cohorts of 11- to 15-month-old Holstein heifers, Jimenez-Krasse *et al* (2015) reported that individuals within the lower quartile range for AMH had a shorter productive herd life and were less likely to become pregnant over successive matings.

Developing breed specific cut-off ranges to identify high, or low, fertility Australian Merino ewes is warranted given AMH concentrations and antral follicle count are higher in beef breeds compared with dairy breeds of cattle, and vary among genetic groups and breeds within dairy cattle (Mossa *et al.* 2017). Based on our data, a plasma AMH concentration at five-months of age of >1.29 ng/ml is predictive of Australian Merino ewes most likely to become pregnant at first mating. This value is higher than the determined cut-off value for predicting positive super-ovulatory response in Sarda ewe lambs (0.097 ng/ml) (Lahoz *et al.* 2012) and Santa Ines meat ewes (0.168 ng/ml) (Pinto *et al.* 2018). These differences may be due to breed (Merino) and age (5-month sample collection and 18-month mating).

This is the first study to demonstrate that circulating AMH concentrations in 5-month-old Australian Merino ewe lambs are higher in ewes which birth a lamb following first mating and those with an AMH concentration greater than 1.29 ng/ml at 5 months of age have an 86% chance of being pregnant following this first mating. The Australian sheep industry is the world’s largest exporter of sheepmeat, despite only accounting for 7% of the global sheep population, and 76% of Australian sheep is Merino. Therefore, developing breed specific cut—off values and identifying a means with which to select the most reproductively viable Merino ewes prior to puberty is of great interest to the Australian sheep industry.

Conflict of Interest

The authors declare that there are no conflicts of interest

Reference List

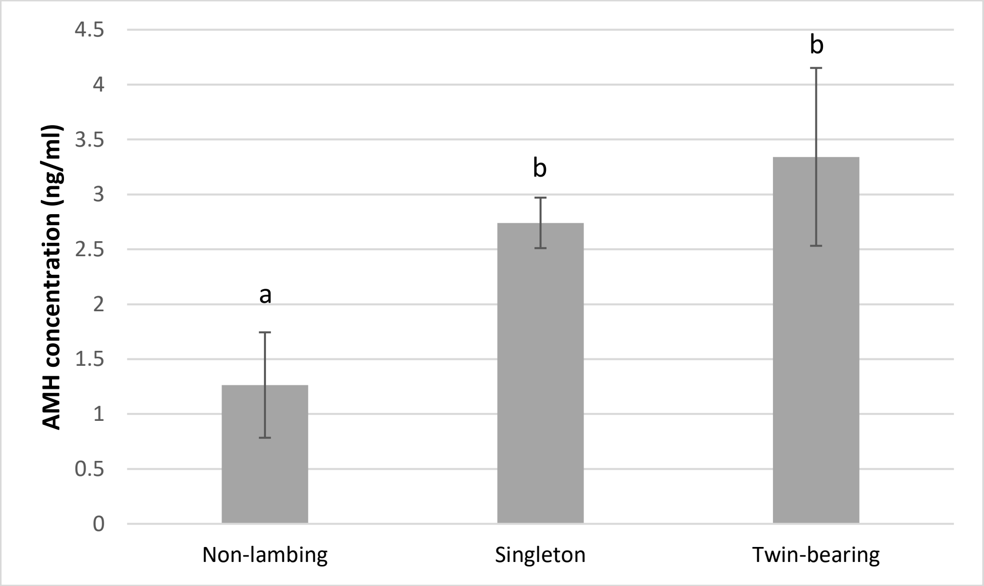

Figure 1: AMH (ng/ml) at 5-months of age for Merino ewes that were subsequently not-pregnant (n= 14), singleton-bearing (n= 62) or twin-bearing (n=10) following first natural mating at 18-months of age. Data are presented as Mean + SEM. Different superscripts represent significant difference ($P<0.05$).

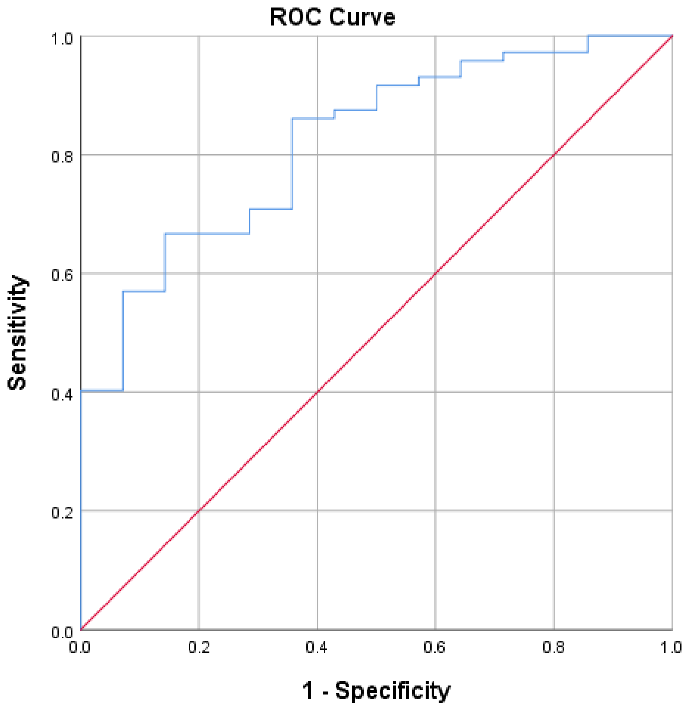

Figure 2: Receiver operating characteristic (ROC) curve for predicting live birth following first service of Merino ewe lambs through their AMH determined at 5 months of age

Chapter eight: General discussion

Introduction

Improving ewe fertility and fecundity, both within natural mating systems or when included in assisted reproductive technologies (ARTs), would markedly improve the productivity and sustainability of both the Australian and global sheep industries. The development of a reliable, quantifiable phenotypic marker of ewe fertility, would be a valuable selection tool to identify more fertile replacement ewe lambs and ewes as well to identify suitable donors for inclusion in ARTs. Based on the available literature, anti-Müllerian hormone (AMH), a glycoprotein of the transforming growth factor (TGF-β) family, has shown the greatest potential. AMH concentration has been reliably correlated to antral follicle count in humans (Fleming *et al.* 2015), mice (Durlinger *et al.* 1999), cattle (Ireland *et al.* 2008)and sheep (Torres-Rovira *et al.* 2014). However, before AMH can be utilised within the Australian sheep industry, and more broadly worldwide, as a phenotypic marker of fertility multiple gaps in the literature need to be addressed. To begin, natural fluctuations in AMH in the prepubertal and mature ewe are yet to be extensively investigated. This was therefore an area of work addressed in this thesis, to provide us with the best foundation to investigate the value of using AMH as a marker of fertility within a natural mating system, or within an ART.

Based on an extensive review of the literature, it is clear that while AMH concentrations differ significantly between breeds of cattle (Ribeiro *et al.* 2014; Batista *et al.* 2016), breed differences in sheep are yet to be determined. One of the objectives of chapter three was to determine whether AMH concentrations differ in pre-pubertal and mature Merino and Suffolk ewes. The second objective was to determine the optimal time to measure AMH in pre-pubertal and mature ewes to achieve an accurate prediction of reproductive potential. Sarda ewe lambs exhibit one of two distinct profiles in the pre-pubertal period (Torres-Rovira *et al.* 2016), with these differences in AMH reflected in differences in pregnancy rates, suggesting that pre-pubertal AMH concentrations may indicate reproductive potential. Although AMH is highly repeatable within and across oestrous cycles in cattle (Rico *et al.* 2009; Ireland *et al.* 2011; El-Sheikh Ali *et al.* 2013; Monniaux *et al.* 2013), studies reporting repeatability within ewes are variable and conflicting (Lahoz *et al.* 2014). A better understanding of AMH changes in the mature ewe is required given there is only one study that has mapped AMH concentration in ewes over 9 days of an induced oestrous cycle (Waheeb 2017). By doing so, it will allow informed decisions to be made regarding the need to synchronise ewes prior to measuring AMH, or whether AMH concentrations on a specific day of the oestrous cycle are more reflective of ovarian follicle development and predictive of fertility outcomes. Therefore, the focus of chapter four was to identify how AMH concentrations fluctuate during the ovine oestrous cycle and how repeatable it is within individual ewes.

Multiple studies have successfully determined the ability of AMH to predict the response of individuals to exogenous hormone stimulation (Rico *et al.* 2009; Monniaux *et al.* 2010; Lahoz *et al.* 2014; Souza *et al.* 2015; Ghanem *et al.* 2016; Lahoz *et al.* 2016; Torres-Rovira *et al.* 2016); however, few look specifically at fertility outcomes within the ART. There are limited studies in cattle (Ribeiro *et al.* 2014; Jimenez-Krassel *et al.* 2015) and sheep (Lahoz *et al.* 2012; Lahoz *et al.* 2016) suggesting AMH may indicate fertility outcomes in a natural protocol thereby warranting the need for additional research in this area. As a result, the focus of chapters five and six were to optimise the use of AMH within an ART (chapter five) and within a natural mating system (chapter six) to predict fertility outcomes in the Australian sheep industry.

Relationship between AMH and the antral follicle pool

This discussion assumes that changes in AMH concentration reflect changes in the antral follicle pool, with previous studies (Torres-Rovira *et al.* 2016) demonstrating a strong, positive correlation between AMH and growth and development of the antral follicle pool.

Furthermore, the relationship between the number of 1 - 3 mm antral follicles and AMH is evident across multiple chapters of this thesis. Pre-pubertal Suffolk and Merino ewes exhibit positive relationships between follicles of this size and AMH across different ages in chapter three. Additionally, the data presented in chapter five demonstrates a strong, positive correlation between AMH concentration and the number of cumulus-oocyte complexes (COCs) recovered following FSH stimulation of 7-week old Merino ewe lambs. Our criteria for aspiration are all follicles > 2mm in size for sheep ovaries. Therefore, numerous follicles close to 2mm in size enables the greatest recovery of oocytes, as the surface area of the ovary is more efficiently covered by follicles containing oocytes at an appropriate stage of development. In agreement with our findings, peak AMH concentrations were found in primordial, primary and secondary follicles, and small antral follicles (around 2mm in size), with concentrations decreasing significantly once follicles grow beyond 3 mm (Campbell *et al.* 2012).

Pre-pubertal differences in AMH concentration in Merino and Suffolk ewes

Pre-pubertal breed differences and patterns of AMH

In chapter three, similar patterns of AMH, but significant differences in pre-pubertal AMH concentrations between Merino and Suffolk ewe lambs were determined. However, this did not translate to differences in pre-pubertal ovarian development, aside from an increase in primary and secondary follicles in the Suffolk. Based on this, it is suggested that for pre-pubertal AMH concentration to be used as a phenotypic marker of fertility, breed specific cut-off values need to be developed; however, timing of sampling may be similar for different breeds. The data presented in chapter three demonstrates a peak in AMH concentration at 6-weeks of age in both the Suffolk and Merino. Additionally, in chapter five 7-week old ewe lambs also exhibited a peak in AMH concentration, which, when combined with previous work in Sarda ewe lambs (Torres-Rovira *et al.* 2016), suggests that a proliferation in ovarian activity occurs in multiple breeds at this age. From 6-weeks of age there was no difference in the pattern of AMH between either cohort or breed, with all ewe lambs exhibiting a similar profile of gradually declining AMH from 6- to 18-weeks of age. In both the Merino and Suffolk AMH concentrations at 2-weeks of age were similar to those observed from 10-weeks of age onwards. In support of this, in Chapter five, AMH concentration in Merino ewe lambs at 7-days of age was similar to that at 12- and 14-months of age. Therefore, from chapters three and five, it is clear that there is a significant proliferation in ovarian activity at approximately 6-weeks of age in the Merino and the Suffolk and relatively little fluctuation in AMH and, therefore, size of the antral follicle pool outside of this peak.

Pre-pubertal AMH profiles within breed

Previous literature in sheep (Torres-Rovira *et al.* 2016) demonstrates two distinct profiles of pre-pubertal AMH concentration, which are associated with differences in antral follicle count (AFC) and fertility outcomes. The other focus of Chapter three was therefore, to identify if differences in the pattern of AMH exist within breed and to determine whether pre-pubertal AMH predicts AFC and fertility in the Merino or the Suffolk. Within each breed circulating AMH displayed one of two distinct profiles, with the Merino exhibiting a profile that has not yet been identified in any breed. Similar to previous work in Sarda ewe lambs (Torres-Rovira *et al.* 2016), one cohort of Suffolk ewes showed a rapid increase in AMH from 2- to 6-weeks of age, whilst the other cohort showed no discernible peak (Chapter three; Figure 2). Similarly, in one cohort of Merino ewe lambs, AMH was low at 2-weeks of age and then increased steadily to peak at 6-weeks of age. Based on the findings from (Torres-Rovira *et al.* 2016) it is likely that there is a major difference in FSH concentrations between these cohorts, suggesting differences in the function of the pituitary gland between cohorts. It is possible, that like Sarda ewe lambs, there is a sharp peak in FSH production shortly after birth, and this programs but does not control the development of the follicle pool over the next 18 weeks (Torres-Rovira *et al.* 2016). As the maturation of the negative feedback loop mediated by ovarian hormones on the hypothalamo-pituitary complex is attained *in utero* (Rhind *et al.* 2001), it is hypothesised that the low FSH is a reflection of the high number of follicles present in the cohort of Sarda ewes lambs with the high AFC (Torres-Rovira *et al.* 2016). With this in mind, the correlation between MIVET ewes AMH at 14 months of age and their dam at 5 months found in chapter five of this thesis warrants further investigation. It becomes clear the development in utero is clearly affecting post-natal AMH concentration in Suffolk ewe lambs. However in the other cohort of Merino ewe lambs, AMH was high at 2-weeks of age and then declined to less than 5 ng/ml by 4-weeks of age followed by a sharp peak at 6-weeks of age. Therefore, Merino ewe lambs could be divided into two distinct cohorts based on have either low (< 10 ng/ml) or high (> 15 ng / ml) AMH at 2-weeks of age, indicative of divergent patterns of ovarian follicle development at this time (Chapter three, Figure 1). In regard to ovarian follicle development and fertility, the Suffolk ewes with no discernible change in AMH had a significantly smaller primordial follicle pool. Similarly, the Merino ewe lambs that exhibited a sharp decline in AMH at 4-weeks of age had a numerically, but not significantly, smaller primordial follicle pool, but produced almost significantly (p= 0.054) fewer oocytes with the ability to develop into blastocysts *in vitro* (Chapter three, Table 4). From this chapter it can be suggested that AMH concentrations at 4-weeks of age in the Merino may predict fertility outcomes within an ART. AMH at 4-weeks of age was the only significantly different concentration in the Merino, whereas in the Suffolk significant differences were seen from 2- through to 10-weeks of age. This suggests breed specific values and ranges are of significant importance when validating AMH as a fertility marker within the Australian sheep industry.

Interestingly, AMH concentration in the mature Merino and Suffolk did not differ significantly, although Suffolk ewes were numerically higher both prior to and following oestrous synchronisation using a progesterone pessary. Anecdotally, the percentage of Suffolk ewes that were pregnant in the first replication of this trial were significantly greater in comparison to the Merino. Therefore, it is possible that breed differences in fertility exist, and AMH concentration alone is not sufficient to capture these differences. Alternatively, a larger data set may be required to tease apart significant fertility differences between breed and AMH.

Repeatability of AMH within oestrous cycle

From this point, the remainder of this discussion is focussed on the Merino. Considering that 76% of the Australian sheep flock is Merino, improvements to reproduction in this breed are likely to have the most profound benefit for the productivity of the national flock. The advantage of determining the repeatability of AMH across a natural oestrous cycle in a Merino ewe is two-fold. First; it informs us as to whether ewes need to be synchronised prior to sampling for AMH and second; determines whether there is a particular day of the oestrus cycle that will prove the most informative. To date, only one study has assessed AMH concentration over 9 days of the oestrous cycle in Barki ewes with ovulation being induced using exogenous hormones. Therefore, the focus of chapter four was to determine how AMH changes and the repeatability of AMH within an oestrous cycle. The use of progesterone to manipulate the oestrous cycle in the ewe, and other species, is common practice and relatively effective. The main aim of this treatment is to synchronise the oestrous cycle of all animals within the treatment; however, timing of ovulation can differ from 12 hours up to 48 hours post pessary removal. Therefore, a secondary objective in chapter four was to determine the relationship between AMH concentration and progesterone throughout the oestrous cycle as well as within an intravaginal progesterone pessary treatment protocol. By doing so, it allows us to determine whether AMH can be used to predict the response to this treatment which would reduce the variation, through targeting treatments and reducing losses to the production system.

Repeatability of AMH across a 14-day natural oestrous cycle

In chapter four I found that in the Merino ewe AMH concentrations are repeatable throughout the oestrous cycle, with or without the treatment of a pessary. This is unsurprising given the available literature and species similarities to cattle, as well as the data collected in chapters three and five. There was little variation in AMH in individual ewe lambs between 6- and 18-weeks (chapter three; figures 1 and 2) and little variation in concentration between 7-days, 12- or 14-months of age in JIVET/MIVET ewes in chapter five, (chapter five; table 2 and figure 1). It appears that AMH concentration is simply a reflection of the antral follicle pool, as there were no correlations within the natural oestrous cycle and follicle dynamics or parameters of ovulation.

Use of AMH to predict outcomes of oestrous synchronisation

It must be noted that I only had access to a small number of animals within this study (10 per treatment) and this was a major limitation. Even so, the small dataset indicated that AMH concentration prior to the insertion of the pessary was strongly negatively correlated to the timing of the onset of oestrus, and subsequently the day of ovulation, supporting the evidence presented in chapter five that AMH concentration may be useful as a predictive tool within an ART. The benefit of assessing AMH concentration at the beginning of a progesterone pessary treatment for oestrous synchronisation is being able to predict the day of ovulation the secondary treatment; whether that be artificial insemination or a recipient animal for embryo transfer, timing can be optimised to align more closely with ovulation. This will reduce/limit the variation in the outcomes.

Assessment of AMH as a marker of fertility within an assisted reproductive technology beneficial to the Australian sheep industry

The *in vitro* work conducted in chapter five was designed to determine the link between oocyte and embryo quality and AMH concentration at different ages. This study highlighted the importance of timing of the AMH sample prior to either JIVET or MIVET. AMH concentration at 7-days of age was positively correlated to the number and quality of oocytes collected when ewe lambs were stimulated with FSH at 7-weeks of age. Importantly, this chapter determined that 7-day old ewe lambs with an AMH concentration ranging between 1.03 ng/ml and 4.78 ng/ml produced the most A grade oocytes and the highest percentage of embryos. This thesis suggests for the first time, that there may be an optimal range of AMH based on the age of the animal, and that animals with the highest AMH may not be the most fertile or fecund. This work was the first to suggest that AMH concentration could not be used pre-pubertally to assess fertility in the Merino undergoing an *in vitro* fertilisation and embryo transfer program in adulthood. Furthermore, this work suggested that AMH concentrations had little ability to predict the outcomes from animals stimulated with FSH at 14-months of age, regardless of the timing of sampling. The current data provides further evidence that the ovarian response to FSH stimulation is higher in juvenile compared with mature ewes, with FSH stimulation in mature ewes causing a very poor ovarian response. The poorer response of mature animals together with the lack of relationships between AMH and parameters of *in vitro* development further suggest that AMH concentration may simply reflect the antral follicle pool. Additionally, in a natural setting, AMH may only be useful in predicting fertility when significant variation within the flock exists, an optimal range of AMH is identified, or an extremely large sample size is used. Once again, AMH plays critical roles in modulating FSH and recruitment of follicles from the primordial pool, and that of the selected follicles for ovulation. This could explain why AMH is predictive of the response and outcome from animals that respond to exogenous hormone treatment with FSH.

Assessment of AMH as a marker of fertility within a natural mating system

The final assessment of the potential of AMH to predict fertility came from the natural mating of three separate cohorts of Merino ewes, a small pilot study in South Australia, and two larger scale farm sites in NSW. The small-scale study in South Australia demonstrated that AMH concentrations taken at 5-months of age predicted pregnancy rates and the number of live-born lambs per ewe, and demonstrated that 86 out of 100 ewes will become pregnant if all ewes with an AMH concentration greater than 1.29 ng/ml are selected (Daly et al., In press, chapter seven). From chapter six, across the two large- scale sites, the animals were tested at different ages; 44- and 292-days of age at one site and 82- and 324-days at the other. Overall, I have tested the correlation between fertility outcomes and AMH concentrations at approximately 1.5, 2.5, 5, 9.5 and 10.5 months of age for ewes mated at 18-months of age. Unless broken down into categories, AMH concentration was unable to predict fertility at any time other than 5-months of age. Sampling occurred at the times listed above mainly based around time of marking or weaning within each production system, and given the peak at 6-weeks of age, and plateau after 8-weeks of age it was thought that sampling near these times may have proved useful. Furthermore, the decision to sample animals closer to the time of mating was based on the moderate relationship between AMH concentrations in 12-month old ewes and reproductive parameters within the MIVET protocol. Animals classified as having mid-range AMH (0.40-3.66 ng/ml) at 9.5-months of age had more fetuses at pregnancy scanning and gave birth to more lambs, whilst animals with high AMH (>0.64ng/ml) at 2.5-months of age produced fewer lambs. These results come from animals that were unstimulated and these values of AMH are naturally occurring. As in women, it is likely that an increased natural circulating concentration of AMH may be detrimental. It should be noted, that the two trial sites in NSW undergo rigorous selection of their ewes for reproductive outcomes in comparison with limited selection of the ewes from the site in South Australia. This means that there was greater variation between ewes in the South Australian flock and therefore, differences in fertility were increased thereby optimising the use of AMH as a predictor. Therefore, chapter six has shown that selecting animals based on a range of AMH at a certain age will lead to increases in the reproductive potential of a flock. Given ewe lambs at 82-days of age (2.5 months) with an AMH ≥ 0.64ng/ml scanned the lowest number of fetuses, selecting animals with AMH below this at weaning (approximately 2.5 months) may 1) improve the number of lambs born within a flock and 2) allow for significant management decisions to be made to optimise the structure of the flock.

External factors affecting AMH concentration in a naturally mated commercial system

In chapter six, the relationship between external factors known to affect fertility and AMH concentration were investigated. First, an inverse relationship was found between AMH concentration and either weaning or mating weight depending on the site location (Armidale or Tullamore) and age of the ewe, suggesting growth rate plays a role in ovarian development. Puberty attainment and bodyweight have profound positive effects on fertility (Baptiste 2002; Rosales Nieto *et al.* 2015; Karthik *et al.* 2021), and in the current study ewes that carried and birthed twins were on average 3 kg heavier at mating at both large-scale trial sites (chapter six). Therefore, it is possible that ovarian development and fertility are not directly related but may be affected by growth rate. In chapter six, I determined there was a tendency that ewes that were born a singleton have lower AMH concentration in comparison with those that were born a twin (p=0.087) and that AMH concentration was lower in twin ewe lambs that were born with a male, compared with a female co-twin. It is evident from the literature that singletons perform better than multiples in terms of production and reproduction (Cushman *et al.* 2012) and for ewes born to a male co-twin to have impaired reproduction (Korsten *et al.* 2009). Further research comparing the follicle pool, response to stimulation and AMH concentration over multiple ages is required to better understand the effect of twins, and the sex of the co-twin on the development of the ovarian follicle pool. Finally, chapter six determined a significant sire effect across each of the two large-scale trial sites and AMH concentration in the offspring. Whilst no maternal relationship was found to AMH concentration in chapter three, ewes that had a higher AMH concentration at 5-months of age birthed offspring that produced more blastocysts at 14-months of age when used in a MIVET procedure. Together, these results also indicate maternal and paternal heritage on the ovarian follicle pool and fertility. The outcomes from chapter six reiterate the importance of accounting for sire, birth type and sex of the co-twin when predicting fertility and indicate an effect on ovarian follicle pool as well.

Areas of future work

Based on the data presented in the chapters contained in this thesis there appears to be three key areas of future work. First, determining how differences in pre- and post- natal growth of ewes affect their ovarian reserve, follicle development and AMH. Second, determining the relationship between AMH and both LH and FSH, and how these relate to differences in follicle function and fertility. Third, large scale trials utilising the sampling windows we have determined within this thesis to select ewes for use within ARTs or natural matings. This will determine the true commercial capability of AMH as a phenotypic marker of fertility in sheep, and in particular the Australian sheep industry.

Growth rate and ovarian reserve

Within chapters three and six of this thesis, it is evident that growth rate and AMH concentration are related. From chapter three it appears that the Suffolk may exhibit a clear pattern of ovarian follicle growth prior to puberty, with increases in the number of primary and secondary follicles neatly aligned to AMH concentrations at similar time points. In contrast, neither primordial or antral follicle growth in the Merino was clearly related to AMH concentration. Periods of anoestrus differ among sheep breeds (Murphy and Freking 2020) and therefore may contribute to differences in development of the follicle pool. However, these differences in AMH and follicle growth prior to puberty between breeds in this chapter may be an indication of differences in the growth rate. Especially as we consider the inverse relationship between AMH concentration and weaning and pre-mating weights at different ages across different flocks, in chapter six. Together, these observations suggest a relationship between growth rate and AMH concentration.

Suffolk ewe lambs in the current study gain, on average, an extra 150 grams per day compared with the Merino ewe lambs, which may have affected the development of the ovarian follicle pool. It is possible that ewes with a more rapid weight gain through to puberty may be directing their energy into growth instead of the development of the ovarian follicle pool. Similarly, a significant but weak inverse relationship (r=-0.143) between weaning weight and AMH concentration at 44-days of age and between premating weight and AMH at both 82- (r=-0.144) and 324- (r=-0.122) days of age suggests patterns of growth rate at key ages affect ovarian development.

It is possible that a lower AMH concentration and greater bodyweight is not necessarily detrimental, rather an external factor affecting the development of the follicle pool, especially as we consider limited direct correlations between AMH and fertility in this thesis. One study assessed the effect of the *FecB* (Booroola) mutation on bodyweight and growth rate, this mutation increased the average number of lambs born from 1.06 to 2.44 if one copy is carried, and 2.55 if two copies are carried in a ewe (Gootwine *et al.* 2006). Ewes with the FecB mutation also had slower post-weaning growth rates as well as lighter mature bodyweights

(Gootwine *et al.* 2006) indicating an inverse relationship between growth rate and fertility. Additionally, a review discussing the perspective of utilising ewe lamb matings reiterates the importance of metabolism on the attainment of puberty and reproduction (Rosales Nieto *et al.* 2018). Importantly, Leptin, a hormone released by adipose tissues, provides information to the hypothalamus whether metabolic status is adequate for the initiation of reproduction (Scaramuzzi *et al.* 2011) .In this thesis, pre-pubertal AMH was negatively correlated to weaning weight at two sites, growth rate may not have yet been sufficient to produce sufficient leptin to signal the onset reproduction, reducing the growth of the follicle pool at this time. In the case where this correlation was still seen at 324 days of age, as reviewed in (Rosales Nieto *et al.* 2018)the link between adipose tissue and reproduction has been clearly identified it is possible that differences in the deposition of adipose tissue within the first year of life affects the maturation of the follicle pool, without significantly impacting reproductive potential when mated at 18 months of age. Similarly, in cattle, heifers with a high pre-pubertal average daily gain attained puberty earlier; however, this did not result in any differences in conception or calving fate following first mating (Moriel *et al.* 2017). Furthermore, JIVET lambs in chapter five with high AMH produced the greatest number of oocytes and blastocysts. However, blastocyst development as a percentage of oocytes recovered did not differ from MIVET ewes, even though the response was significantly lower compared with JIVET lambs. This demonstrates that high AMH concentrations may not align directly to fertility outcomes. Further research in this area is needed to quantify the effect of pre-pubertal growth rate on ovarian development in ewes, as well as the long- term effects of growth rate, AMH concentration and fertility.

Generally speaking, Suffolk ewes, as with other breeds purposely bred for meat production, have a greater fertility rate. A comparison of South African Merinos bred for different purposes determined a fertility rate of 92% in Mutton Merino, where meat production is the focus, compared with 81.4% in pure wool Merino and 86.2% for dual-purpose Dohne Merino (Fourie and Cloete 1993). Therefore, as growth rate and fertility differ between breeds, it is worth addressing whether the production of AMH within and by the ovary, and the expression of AMH by ovarian follicles differs between breeds. As mentioned in chapter three, breed differences exist in the expression of FSH between Chinese Hu and Chinese Merino ewes (Li *et al.* 2019). Therefore, it is possible that the expression of AMH differs between breeds, as does the size of follicles producing AMH.

FSH, gonadotropins, AMH and follicular dynamics

I found, and have published work determining a positive relationship between AMH concentration at 5-months of age and fertility within a largely variable population of sheep (Daly et al. In Press). However, outside this age, a tightly defined range of AMH is required within a commercial natural mating production system (chapter six). Importantly, AMH concentration appears most useful as a marker of fertility when used as part of ARTs involving stimulation of ovarian follicle growth or manipulation of the oestrous cycle. AMH concentrations were predictive of outcomes within a JIVET protocol, and AMH concentrations prior to progesterone pessary insertion were correlated with the onset of oestrous and the day of ovulation. This shows that the follicles producing AMH may interact differently depending on the treatment intervention as a result of the concentrations of AMH within the animal.

Pre-pubertal AMH concentrations are similar to that of post-pubertal AMH concentrations but the response to exogenous FSH stimulation is vastly different (chapter five). Additionally, there was no association between AMH concentration pre-pubertally and in adult life in Rasa Aragonesa ewes (Lahoz *et al.* 2014), even though in the current study AMH concentrations are similar at 7-days and 12- or 14-months in chapter five. Therefore, a better understanding of the relationships between AMH, FSH and differences pre and post pubertally are needed to optimise the use of AMH as a fertility marker. Pre-pubertally, during the first 9 weeks of life AMH and FSH concentrations were negatively correlated in Sarda ewes (Torres-Rovira *et al.* 2016). As puberty approaches, the patterns of FSH and AMH are reversed, FSH concentrations continue to increase while AMH concentrations decrease and stabilise (Torres-Rovira *et al.* 2014; El-Sheikh Ali *et al.* 2017). Therefore, the relationship between FSH and AMH in ewes in both the pre and post pubertal period requires further investigation.

Currently we know that the response of granulosa cells from cows with a low antral follicle count to FSH is much poorer than those from cows with a high antral follicle count, suggesting differences in the responsiveness of the follicles to FSH stimulation (Scheetz *et al.* 2012). Treatment with FSH decreases circulating AMH concentration in women (Eldar-Geva *et al.* 2005) and decreases AMH mRNA expression in rats (Baarends *et al.* 1995), while other studies show no effect of FSH administration on AMH in humans (Voutilainen and Miller 1987; Pellatt *et al.* 2007). Additionally, different regimes of FSH affect the developmental capacity of embryos derived using ovum pick-up in sheep (Berlinguer *et al.* 2004). This suggests FSH play an important role in developmental competence and therefore the need to look at the relationship to AMH when considering the relationship between oocyte quality and AMH found in chapter five. Scheetz, (2012) suggests that the type and / or size of the follicle may impact the capacity of granulosa cells to produce AMH in response to FSH. Furthermore, the oestrous cycle is governed by changes in follicle sensitivity to FSH and LH, as the switch in sensitivity from gonadotrophin responsive to gonadotrophin dependant is a requirement for the selection of the dominant follicle for ovulation (Driancourt 2001). AMH concentration plays a regulatory role in the selection of dominant follicles (Umer *et al.* 2019) specifically, AMH modifying the growth of pre-antral and small antral follicles by decreasing their responsiveness to FSH, whilst also dictating the recruitment of large antral follicles into the pre-ovulatory stage (Durlinger *et al.* 2002b). This is important because AMH expression was greatest in small pre-antral follicles, where the response to FSH treatment was also the highest (Campbell *et al.* 2012). Therefore, it is worth investigating further the relationship between follicle size, AMH and gonadotropins, as well as breed differences in these interactions.

Optimising the timing of AMH sampling, and range of AMH depending on the age and use of the ewes

Within this thesis, a variety of sampling times were compared across production systems; however, the optimal timing still needs to be determined. First, additional work to optimise the range of AMH, within a natural mating system, that will increase the number of pregnant, and successfully lambing, ewes, is a priority. In chapter six, ewe lambs with AMH greater than 0.64 ng/ml at 8- days of age produced fewer fetuses, therefore currently I recommend selecting ewe lambs with an AMH below this concentration. However future work to optimise the range of AMH at 82-days of age that will include the most fertile, productive ewes would be beneficial. Optimising the range as early as 82-days of age, or at weaning, will allow producers additional time to coordinate management of the animals. Second, the promising results between AMH concentration at 7-days of age and response within a JIVET protocol suggest that selecting animals based on the range of AMH determined in chapter five and assessing overall production rates would be of huge benefit. Optimising the response of animals within a JIVET protocol, and reducing variation has the potential to increase the uptake of JIVET within the Australian production system.

The data contained in this thesis demonstrated little relationship between AMH concentration and parameters of the ovarian follicle pool or oocyte quality over the first 18-weeks of life. It would be useful to continue the blood sampling of animals into adulthood with regular transrectal follicle scanning. This would allow more accurate correlations between pre- and post-pubertal AMH concentrations and ovarian and follicle development.

Finally, the results in chapters three and six suggest two other time points may be beneficial for predicting fertility. In chapter three, there is potential to use a single blood sample at 4-weeks of age as a differentiation point for Merino ewes, given the vast difference in AMH at this time and the correlation to *in vitro* blastocyst development. A blood sample at this time, and then subsequently mating the ewes as ewe lambs (7 to 9 months old) or hoggets (~18 months old) would be of interest, given timing at 1.5, 2.5, 9.5 and 10.5 months of age showed no direct correlations. Similarly, the sample at 5-months of age and the correlation to fertility within a largely variable population also requires further explanation. It would be beneficial to take blood samples for another population of animals at 5-months of age at each of the large-scale sites to determine whether AMH concentration at 5-months of age has the same predicative ability within a less reproductively variable population. In addition to this, taking each of the key times highlighted from this thesis; 7-days, 4-weeks, 82-days (~12 weeks/ weaning), 5- months and 9.5-months of age and assessing the lifetime performance of a Merino flock, across multiple lambing periods, would provide valuable insight into the optimal timing of sampling, as well as the reliability of these times for repeated fertility outcomes.

Strengths and limitations of this thesis

The major strength of the studies in this thesis is the broad assessment of procedures that are easily applicable within an industry setting. Further, the lack of research in the area of AMH in sheep meant a broad, overview structure in the experimental work was required to begin to assess the validity of using AMH concentration as a marker of fertility within the Australian sheep industry. To date this thesis is the first to study AMH concentration throughout 14 days of the oestrous cycle, and its relationship to progesterone pessaries and circulating concentrations of progesterone. Additionally, this body of work is the first to assess the relationship between AMH concentration and fertility in ewes in a completely natural system, and is the first to investigate the effects of external factors (birth type, sire or bodyweight) on

AMH. The methods in this experiment are straightforward and robust, which enabled accurate assessment of AMH concentration within the studies described and in relation to the variables listed above. Importantly, the methodology allowed for sampling across multiple ages, within multiple production systems to encompass the true potential of AMH as a marker of fertility in the Australian industry. Conversely, the major limitations from this study also came from the desire to create a dataset that was industry applicable. Much of the data in this thesis could have benefitted from the analysis of additional hormones, and more frequent sampling for AMH concentration. Additionally, across chapters three and four the inclusion of more animals would have been ideal to better understand the relationships between AMH and characteristics of the ovarian follicle pool, oestrous cycle and other parameters of reproduction.

Conclusion

In conclusion, the content of this thesis has successfully determined how AMH concentrations change in pre-pubertal ewe lambs and within a natural oestrous cycle. As a result, this thesis plays a fundamental role in increasing our understanding of AMH within sheep. This understanding has helped develop areas of future work, and been useful in identifying the optimal timing of sampling to determine whether AMH can be used as a phenotypic marker of fertility within an ART or natural mating program in the Australian sheep industry. This thesis is the first to describe the pattern of circulating AMH over the first 18-weeks of life in Suffolk and Merino ewes in Australia and compare the relationship between AMH and parameters of the pre-pubertal ovary. Furthermore, I have determined, for the first time, that differences in AMH concentration exist between the pre-pubertal Suffolk and Merino ewe, suggesting breed specific cut-off values would be necessary within the Australian sheep industry. An important finding was a unique profile for the Merino ewe lambs seen at 4-weeks of age, which may be a point of difference for fertility outcomes and therefore a target for sampling times. Additionally, this is the first study to describe the pattern of circulating AMH across 14 days of a natural oestrous cycle, and to confirm the repeatability of AMH across the cycle. For both JIVET and a natural mating system, the results from this thesis indicate that AMH may only have the potential to predict fertility of ewes if utilising a range of AMH concentration. However, AMH may also have the potential to identify the timing of oestrus/ day of ovulation from progesterone pessary treatment.

Therefore, the recommendation from the conclusion of this thesis would be to direct future research into the optimisation of cut-off values and ranges of AMH, perhaps a standardised range for each breed, within a natural mating system and a JIVET protocol. Otherwise the selection of Merino ewes at 82-days of age with an AMH concentration < 0.64 ng/ml into a natural mating protocol at 18-months of age could be used to improve the reproductive efficiency of Australian Merino sheep flocks. Similarly selecting ewe lambs at 7-days of age with an AMH concentration between 1.03 ng/ml and 4.78 ng/ml into a JIVET protocol at 7-weeks of age will reduce the variation in response, and therefore improve outcomes.

Reference list

Acharya, M., Burke, J.M., Smyth, E., Ngere, L., and Rorie, R.W. (2016) 1724 Serum anti-mullerian hormone as an indicator of fertility in Katahdin ewes. *Journal of Animal Science* **94**(suppl5), 840-840

Adams, G.P., Matteri, R.L., and Ginther, O.J. (1992) Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. *Journal of reproduction & fertility* **96**(2), 627-640

Afolayan, R.A., Fogarty, N.M., Gilmour, A.R., Ingham, V.M., Gaunt, G.M., and Cummins, L.J. (2008) Reproductive performance and genetic parameters in first cross ewes from different maternal genotypes. *Journal of Animal Science* **86**(4), 804-814

Aiken, C.E., Tarry-Adkins, J.L., and Ozanne, S.E. (2013) Suboptimal nutrition in utero causes DNA damage and accelerated aging of the female reproductive tract. *Faseb j* **27**(10), 3959-65

Aiken, C.E., Tarry-Adkins, J.L., and Ozanne, S.E. (2015) Transgenerational Developmental Programming of Ovarian Reserve. *Scientific reports* **5**, 16175-16175

Amiridis, G.S., and Cseh, S. (2012) Assisted reproductive technologies in the reproductive management of small ruminants. *Animal Reproduction Science* **130**(3-4), 152-161

Armstrong, D.T. (2001) Effects of maternal age on oocyte developmental competence. *Theriogenology* **55**(6), 1303-1322

Armstrong, D.T., Kotaras, P.J., and Earl, C.R. (1997) Advances in production of embryos in vitro from juvenile and prepubertal oocytes from the calf and lamb. *Reproduction Fertility and Development* **9**(3), 333-339

Aungier, S.P.M., Roche, J.F., Diskin, M.G., and Crowe, M.A. (2014) Risk factors that affect reproductive target achievement in fertile dairy cows. *Journal of Dairy Science* **97**(6), 3472-3487

Meat and Livestock Australia (2018) Industry Projection 2018 Australian Sheep. Sydney, Australia: Meat and Livestock Australia (MLA).

Meat and Livestock Australia (2021) Global Snapshot Sheepmeat. Sydney, Australia: Meat and Livestock Australia (MLA).

Baarends, W.M., Uilenbroek, J.T., Kramer, P., Hoogerbrugge, J.W., van Leeuwen, E.C., Themmen, A.P., and Grootegoed, J.A. (1995) Anti-Müllerian hormone and anti-Müllerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. *Endocrinology* **136**(11), 4951-62

Baby, T.E., and Bartlewski, P.M. (2011) Circulating concentrations of ovarian steroids and follicle-stimulating hormone (FSH) in ewes with 3 or 4 waves of antral follicle emergence per estrous cycle. *Reproductive biology* **11**(1), 19-36

Baird, D.T., Campbell, B.K., Mann, G.E., and McNeilly, A.S. (1991) Inhibin and estradiol in the control f FSH-secretion in the sheep *Journal of Reproduction and Fertility*, 125-138

Baird, D.T., and McNeilly, A.S. (1981) Gonadotrophic control of follicular development and function during the oestrous cycle of the ewe. *J Reprod Fertil Suppl* **30**, 119-33

Baldrighi, J.M., Sá Filho, M.F., Batista, E.O.S., Lopes, R., Visintin, J.A., Baruselli, P.S., and Assumpção, M. (2014) Anti-Mullerian Hormone Concentration and Antral Ovarian Follicle Population in Murrah Heifers Compared to Holstein and Gyr Kept Under the Same Management. *Reproduction in domestic animals* **49**(6), 1015-1020

Bancsi, L., Broekmans, F.J.M., Eijkemans, M.J.C., de Jong, F.H., Habbema, J.D.F., and te Velde, E.R. (2002) Predictors of poor ovarian response in in vitro fertilization: a prospective study comparing basal markers of ovarian reserve. *Fertility and Sterility* **77**(2), 328-336

Baptiste, Q.S. (2002) The effects of diet and weight on induction of puberty and fertility response in yearling beef heifers. ProQuest Dissertations Publishing, Bartlewski, P.M., Baby, T.E., and Giffin, J.L. (2011) Reproductive cycles in sheep. *Animal Reproduction Science* **124**(3-4), 259-268

Batista, E.O.S., Guerreiro, B.M., Freitas, B.G., Silva, J.C.B., Vieira, L.M., Ferreira, R.M., Rezende, R.G., Basso, A.C., Lopes, R., Renno, F.P., Souza, A.H., and Baruselli, P.S. (2016) Plasma anti-Mtillerian hormone as a predictive endocrine marker to select Bos taurus (Holstein) and Bos indicus (Nelore) calves for in vitro embryo production. *Domestic Animal Endocrinology* **54**, 1-9

Batista, E.O.S., Macedo, G.G., Sala, R.V., Ortolan, M., Sa, M.F., Del Valle, T.A., Jesus, E.F., Lopes, R., Renno, F.P., and Baruselli, P.S. (2014) Plasma Antimullerian Hormone as a Predictor of Ovarian Antral Follicular Population in Bos indicus (Nelore) and Bos taurus (Holstein) Heifers. *Reproduction in Domestic Animals* **49**(3), 448-452

Berlinguer, F., Leoni, G., Bogliolo, L., Pintus, P.P., Rosati, I., Ledda, S., and Naitana, S. (2004) FSH different regimes affect the developmental capacity and cryotolerance of embryos derived from oocytes collected by ovum pick-up in donor sheep. *Theriogenology* **61**(7), 1477-1486

Boni, R., Cocchia, N., Silvestre, F., Tortora, G., Lorizio, R., and Tosti, E. (2008) Juvenile and Adult Immature and In Vitro Matured Ovine Oocytes Evaluated in Relation to Membrane Electrical Properties, Calcium Stores, IP3 Sensitivity and Apoptosis Occurrence in Cumulus Cells. *Molecular Reproduction and Development* **75**(12), 1752-1760

Borys, B., and Osikowski, M. (1999) Productive and economic efficiency of meat and dairy utilization of ewes coming from crossing Polish Merino and prolific breeds. In '6th International Symposium on the Milking of Small Ruminants', Sep 26-Oct 01 1998, Athens, Greece, pp. 424-426. (WAGENINGEN)

Broadbent, P.J., Stewart, M., and Dolman, D.F. (1991) Recipient management and embryo transfer. *Theriogenology* **35**(1), 125-139

Burns, D.S., Jimenez-Krassel, F., Ireland, J.L.H., Knight, P.G., and Ireland, J.J. (2005) Numbers of antral follicles during follicular waves in cattle: Evidence for high variation among animals, very high repeatability in individuals, and an inverse association with serum follicle-stimulating hormone concentrations. *Biology of Reproduction* **73**(1), 54-62

Campbell, B.K., Clinton, M., and Webb, R. (2012) The Role of Anti-Mullerian Hormone (AMH) During Follicle Development in a Monovulatory Species (Sheep). *Endocrinology* **153**(9), 4533-4543

Cardoso, C.J.T., de Oliveira, J.S., Kischel, H., da Silva, W.A.L., Arruda, E.D.D., Souza-Caceres, M.B., de Oliveira, F.A.M., Nogueira, E., Nogueira, G.D., and Melo-Sterza, F.D. (2018) Anti-Mullerian hormone (AMH) as a predictor of antral follicle population in heifers. *Animal Reproduction* **15**(1), 12-16

Chang, H.-M., Cheng, J.-C., Klausen, C., and Leung, P.C.K. (2013) BMP15 suppresses progesterone production by down-regulating StAR via ALK3 in human granulosa cells. *Molecular endocrinology (Baltimore, Md.)* **27**(12), 2093-2104

Ciftci, H.B. (2014) Regulation of Follicular Growth and Development in Sheep. *Iranian Journal of Applied Animal Science* **4**(1), 21-31

Clarke, I.J. (1995) The preovulatory LH surge - A case of a neuroendocrine switch. *Trends in Endocrinology and Metabolism* **6**(7), 241-247

Clarke, I.J., and Cummins, J.T. (1984) Direct pituitary effects of estrogen and progesterone on gonadotropin secretion in the ovariectomized ewe. *Neuroendocrinology* **39**(3), 267-74

Clemente, M., de la Fuente, J., Fair, T., Al Naib, A., Gutierrez-Adan, A., Roche, J.F., Rizos, D., and Lonergan, P. (2009) Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? *Reproduction* **138**(3), 507-517

Cox, J.F., and Alfaro, V. (2007) In Vitro Fertilization and Development of OPU Derived Goat and Sheep Oocytes. *Reproduction in domestic animals* **42**(1), 83-87

Cruz, R.S., Cushman, R.A., and Vinoles, C. (2018) Antral follicular count is a tool that may allow the selection of more precocious Bradford heifers at weaning. *Theriogenology* **119**, 35-42

Cushman, R.A., Allan, M.F., Kuehn, L.A., Snelling, W.M., Cupp, A.S., and Freetly, H.C. (2009) Evaluation of antral follicle count and ovarian morphology in crossbred beef cows: investigation of influence of stage of the estrous cycle, age, and birth weight. *J Anim Sci* **87**(6), 1971-80

Cushman, R.A., Miles, J.R., Rempel, L.A., Lents, C.A., McNeel, A.K., Eborn, D.R., Echternkamp, S.E., Leymaster, K.A., and Vallet, J.L. (2012) Litter Size Influences Lamb Weight and Ovarian Reserve, but Not Reproductive Tract Development or Expression of Genes Involved in Early Follicular Development. *Biology of reproduction* **87**(Suppl_1), 342-342

Da Silva, P., Aitken, R.P., Rhind, S.M., Racey, P.A., and Wallace, J.M. (2003) Effect of maternal overnutrition during pregnancy on pituitary gonadotrophin gene expression and gonadal morphology in female and male foetal sheep at day 103 of gestation. *Placenta* **24**(2-3), 248-257

Daly, J., Smith, H., McGrice, H.A., Kind, K.L., and van Wettere, W.H.E.J. (2020) Towards improving the outcomes of assisted reproductive technologies of cattle and sheep, with particular focus on recipient management. *Animals* **10**(2)

de Brun, V., Meikle, A., Fernandez-Foren, A., Forcada, F., Palacin, I., Menchaca, A., Sosa, C., and Abecia, J.A. (2016) Failure to establish and maintain a pregnancy in undernourished recipient ewes is associated with a poor endocrine milieu in the early luteal phase. *Animal Reproduction Science* **173**, 80-86

De Loos, F., Van Vliet, C., van Maurik, P.v., and Kruip, T.A. (1989) Morphology of immature bovine oocytes. *Gamete research* **24**(2), 197-204

de Moraes, F.L.Z., Morotti, F., Costa, C.B., Lunardelli, P.A., and Seneda, M.M. (2019) Relationships between antral follicle count, body condition, and pregnancy rates after timed-AI in Bos indicus cattle. *Theriogenology* **136**, 10-14 de Souza-Fabjan, J.M.G., Panneau, B., Duffard, N., Locatelli, Y., De Figueiredo, J.R., Freitas, V., and Mermillod, P. (2014) In vitro production of small ruminant embryos: Late improvements and further research. *Theriogenology* **81**(9), 1149-1162

Dewailly, D., Andersen, C.Y., Balen, A., Broekmans, F., Dilaver, N., Fanchin, R., Griesinger, G., Kelsey, T.W., La Marca, A., Lambalk, C., Mason, H., Nelson, S.M., Visser, J.A., Wallace, W.H., and Anderson, R.A. (2014) The physiology and clinical utility of anti-Mllerian hormone in women. *Human Reproduction Update* **20**(3), 370-385

Dewailly, D., and Laven, J. (2019) AMH as the primary marker for fertility. *European Journal of Endocrinology* **181**(6), D45-D51

di Clemente, N. (1994) Cloning, expression, and alternative splicing of the receptor for anti-Mullerian hormone. *Molecular endocrinology (Baltimore, Md.)* **8**(8), 1006-1020

Díaz, P.U., Rey, F., Gareis, N.C., Notaro, U.S., Matiller, V., Belotti, E.M., Stassi, A.F., Salvetti, N.R., and Ortega, H.H. (2018) Altered Expression of Anti-Müllerian Hormone during the Early Stage of Bovine Persistent Ovarian Follicles. *Journal of comparative pathology* **158**, 22-31

Donadeu, F.X., Schauer, S.N., and Sontakke, S.D. (2012) Involvement of miRNAs in ovarian follicular and luteal development. *Journal of Endocrinology* **215**(3), 323-334

Dong, J.W., Albertini, D.F., Nishimori, K., Kumar, T.R., Lu, N.F., and Matzuk, M.M. (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383**(6600), 531-535

dos Santos, G.M.G., Silva-Santos, K.C., Barreiros, T.R.R., Morotti, F., Sanches, B.V., de Moraes, F.L.Z., Blaschi, W., and Seneda, M.M. (2016) High numbers of antral follicles are positively associated with in vitro embryo production but not the conception rate for FTAI in Nelore cattle. *Animal Reproduction Science* **165**, 17-21

Driancourt, M.A. (2001) Regulation of ovarian follicular dynamics in farm animals. Implications for manipulation of reproduction. *Theriogenology* **55**(6), 1211-1239

Driancourt, M.A., Gibson, W.R., and Cahill, L.P. (1985) Follicular dynamics throughout the estrous-cycle in sheep- A review. *Reproduction Nutrition Development* **25**(1A), 1-15

Durlinger, A.L.L., Gruijters, M.J.G., Kramer, P., Karels, B., Ingraham, H.A., Nachtigal, M.W., Uilenbroek, J.T.J., Grootegoed, J.A., and Themmen, A.P.N. (2002a) Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology* **143**(3), 1076-1084

Durlinger, A.L.L., Kramer, P., Karels, B., de Jong, F.H., Uilenbroek, J.T.J., Grootegoed, J.A., and Themmen, A.P.N. (1999) Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. *Endocrinology* **140**(12), 5789-5796

Durlinger, A.L.L., Visser, J.A., and Themmen, A.P.N. (2002b) Regulation of ovarian function: the role of anti-Mullerian hormone. *Reproduction* **124**(5), 601-609

Earl, C.R., Armstrong, D.T., and Irvine, B. (1995) Juvenile *in vitro* fertilisation-embryo transfer (JIVET): The *in vitro* production of viable embryos from oocytes obtained from gonadotrophin-stimulated juvenile calves and lambs. *Proceedings of the Australian Association of Animal Breeding and Genetics* **11**, 94-96

Edwards, S.J., and Juengel, J.L. (2017) Limits on hogget lambing: the fertility of the young ewe. *New Zealand Journal of Agricultural Research* **60**(1), 1-22

Edwards, S.J., Smaill, B., O'Connell, A.R., Johnstone, P.D., Stevens, D.R., Quirke, L.D., Farquhar, P.A., and Juengel, J.L. (2016) Reduced ovulation rate, failure to be mated and fertilization failure/embryo loss are the underlying causes of poor reproductive performance in juvenile ewes. *Animal Reproduction Science* **167**, 125-132

El-Sheikh Ali, H., Kitahara, G., Nibe, K., Yamaguchi, R., Horii, Y., Zaabel, S., and Osawa, T. (2013) Plasma anti-Mullerian hormone as a biomarker for bovine granulosa-theca cell tumors: comparison with immunoreactive inhibin and ovarian steroid concentrations. *Theriogenology* **80**(8), 940-9

El-Sheikh Ali, H., Kitahara, G., Takahashi, T., Mido, S., Sadawy, M., Kobayashi, I., Hemmi, K., and Osawa, T. (2017) Plasma anti-Müllerian hormone profile in heifers from birth through puberty and relationship with puberty onset†. *Biology of Reproduction* **97**(1), 153-161

Eldar-Geva, T., Ben-Chetrit, A., Spitz, I.M., Rabinowitz, R., Markowitz, E., Mimoni, T., Gal, M., Zylber-Haran, E., and Margalioth, E.J. (2005) Dynamic assays of inhibin B, anti-Mullerian hormone and estradiol following FSH stimulation and ovarian ultrasonography as predictors of IVF outcome. *Human reproduction (Oxford)* **20**(11), 3178-3183

Elvin, J.A., Yan, C., and Matzuk, M.M. (2000) Growth differentiation factor-9 stimulates progesterone synthesis in granulosa cells via a prostaglandin E2/EP2 receptor pathway. *Proceedings of the National Academy of Sciences* **97**(18), 10288-10293

Eppig, J.J. (2001) Oocyte control of ovarian follicular development and function in mammals. *Reproduction* **122**(6), 829-838

Erickson, B.H. (1966) Development and senescence of the postnatal bovine ovary. *J Anim Sci* **25**(3), 800-5

Erickson, G.F., and Danforth, D.R. (1995) Ovarian Control of Follicle Development. *American Journal of Obstetrics and Gynecology* **172**(2), 736-747

Estienne, A., Pierre, A., di Clemente, N., Picard, J.-Y., Jarrier, P., Mansanet, C., Monniaux, D., and Fabre, S. (2015) Anti-Müllerian Hormone Regulation by the Bone Morphogenetic Proteins in the Sheep Ovary: Deciphering a Direct Regulatory Pathway. *Endocrinology (Philadelphia)* **156**(1), 301-313

Evans, A.C.O., Mossa, F., Walsh, S.W., Scheetz, D., Jimenez-Krassel, F., Ireland, J.L.H., Smith, G.W., and Ireland, J.J. (2012) Effects of Maternal Environment During Gestation on Ovarian Folliculogenesis and Consequences for Fertility in Bovine Offspring. *Reproduction in Domestic Animals* **47**, 31-37

Evans, G., and Robinson, T.J. (1980) Reproductive potential and endocrinological responses of sheep kept under controlled lighting. I. Comparative reproductive performance of four breed types of ewe. *Animal Reproduction Science* **3**(1), 23-37

Fleming, R., Seifer, D.B., Frattarelli, J.L., and Ruman, J. (2015) Assessing ovarian response: antral follicle count versus anti-Mullerian hormone. *Reproductive Biomedicine Online* **31**(4), 486-496

Fogarty, N.M., and Mulholland, J.G. (2014) Seasonal reproductive performance of crossbred ewes in intensive lamb-production systems. *Animal Production Science* **54**(6), 791-801

Fortune, J.E. (2003) The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. *Animal reproduction science* **78**(3), 135-163

Fouladi-Nashta, A., Gutierrez, C., Gong, J., Garnsworthy, P., and Webb, R. (2007) Impact of Dietary Fatty Acids on Oocyte Quality and Development in Lactating Dairy Cows. *Biology of reproduction* **77**, 9-17

Fourie, A.J., and Cloete, S.W.P. (1993) Reproductive-performance of commercial Merino, Dohne Merino and SA Mutton Merino flocks in the Southern Cape. *South African Journal of Animal Science-Suid-Afrikaanse Tydskrif Vir Veekunde* **23**(3-4), 104-110

Geenty, K.G., Brien, F.D., Hinch, G.N., Dobos, R.C., Refshauge, G., McCaskill, M., Ball, A.J., Behrendt, R., Gore, K.P., Savage, D.B., Harden, S., Hocking-Edwards, J.E., Hart, K., and van der Werf, J.H.J. (2014) Reproductive performance in the Sheep CRC Information Nucleus using artificial insemination across different sheep-production environments in southern Australia. *Animal Production Science* **54**(6), 715-726

Geisert, R.D., Fox, T.C., Morgan, G.L., Wells, M.E., Wettemann, R.P., and Zavy, M.T. (1991) Survival of bovine embryos transferred to progesterone treated asynchronous recipients. *Journal of Reproduction and Fertility* **92**(2), 475-482

Ghanem, N., Jin, J.I., Kim, S.S., Choi, B.H., Lee, K.L., Ha, A.N., Song, S.H., and Kong, I.K. (2016) The Anti-Mullerian Hormone Profile is Linked with the In Vitro Embryo Production

Capacity and Embryo Viability after Transfer but Cannot Predict Pregnancy Outcome. *Reproduction in Domestic Animals* **51**(2), 301-310

Gilchrist, R.B., Lane, M., and Thompson, J.G. (2008) Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Human reproduction update* **14**(2), 159-177

Gleicher, N., Weghofer, A., and Barad, D.H. (2010) Anti-Mullerian hormone (AMH) defines, independent of age, low versus good live-birth chances in women with severely diminished ovarian reserve. *Fertility and Sterility* **94**(7), 2824-2827

Gobikrushanth, M., Dutra, P.A., Bruinje, T.C., Colazo, M.G., Butler, S.T., and Ambrose, D.J. (2017) Repeatability of antral follicle counts and anti-Mullerian hormone and their associations determined at an unknown stage of follicular growth and an expected day of follicular wave emergence in dairy cows. *Theriogenology* **92**, 90-94

Gonzalez-Bulnes, A., Santiago-Moreno, J., Cocero, M.J., and Lopez-Sebastian, A. (2000) Effects of FSH commercial preparation and follicular status on follicular growth and superovulatory response in Spanish Merino ewes. *Theriogenology* **54**(7), 1055-1064

Gootwine, E., Rozov, A., Bor, A., and Reicher, S. (2006) Carrying the FecB (Booroola) mutation is associated with lower birth weight and slower post-weaning growth rate for lambs, as well as a lighter mature bodyweight for ewes. *Reproduction, Fertility and Development* **18**(4), 433-437

Gou, K.M., Guan, H., Bai, H.H., Cui, X.H., Wu, Z.F., Yan, F.X., and An, X.R. (2009) Field evaluation of juvenile in vitro embryo transfer (JIVET) in sheep. *Animal Reproduction Science* **112**(3-4), 316-324

Granleese, T., Clark, S.A., Swan, A.A., and van der Werf, J.H.J. (2015) Increased genetic gains in sheep, beef and dairy breeding programs from using female reproductive technologies combined with optimal contribution selection and genomic breeding values. *Genetics Selection Evolution* **47**, 13

Granleese, T., Clark, S.A., Swan, A.A., and van der Werf, J.H.J. (2017) Increased genetic gains in multi-trait sheep indices using female reproductive technologies combined with optimal contribution selection and genomic breeding values. *Animal Production Science* **57**(10), 1984-1992

Guerreiro, B.M., Batista, E.O.S., Vieira, L.M., Sa, M.F., Rodrigues, C.A., Netto, A.C., Silveira, C.R.A., Bayeux, B.M., Dias, E.A.R., Monteiro, F.M., Accorsi, M., Lopes, R., and Baruselli, P.S. (2014) Plasma anti-mullerian hormone: an endocrine marker for in vitro embryo production from Bos taurus and Bos indicus donors. *Domestic Animal Endocrinology* **49**, 96-104

Gunn, R.G., Sim, D.A., and Hunter, E.A. (1995) Effects of nutrtion in-utero and in early life on the subsequent lifetime reproductive performance of scottish blackface ewes in 2 management systems. *Animal Science* **60**, 223-230

Guo, Y., Liu, S., Hu, S., Li, F., and Jin, L. (2021) High Serum Anti-Müllerian Hormone Concentrations Are Associated With Poor Pregnancy Outcome in Fresh IVF/ICSI Cycle but Not Cumulative Live Birth Rate in PCOS Patients. *Front Endocrinol (Lausanne)* **12**, 673284

Hancock, S.N., Oliver, M.H., McLean, C., Jaquiery, A.L., and Bloomfield, F.H. (2012) Size at birth and adult fat mass in twin sheep are determined in early gestation. *J Physiol* **590**(5), 1273-85

Hawker, H., and Kennedy, J.P. (1978) Puberty and subsequent oestrous activity in young Merino ewes. *Australian journal of experimental agriculture* **18**(92), 347

Hinch, G.N., and Brien, F. (2014) Lamb survival in Australian flocks: a review. *Animal Production Science* **54**(6), 656-666

Hirayama, H., Naito, A., Fukuda, S., Fujii, T., Asada, M., Inaba, Y., Takedomi, T., Kawamata, M., Moriyasu, S., and Kageyama, S. (2017) Long-term changes in plasma anti-Mullerian hormone concentration and the relationship with superovulatory response in Japanese Black cattle. *Journal of Reproduction and Development* **63**(1), 95-100

Horton, B. (1996) A method of using a genetic algorithm to examine the optimum structure of the Australian sheep breeding industry: Open-nucleus breeding systems, MOET and AI. *Australian Journal of Experimental Agriculture* **36**(3), 249-258

Hunter, M.G., Robinson, R.S., Mann, G.E., and Webb, R. (2004) Endocrine and paracrine control of follicular development and ovulation rate in farm species. *Animal Reproduction Science* **82-3**, 461-477

Ilha, G.F., Rovani, M.T., Gasperin, B.G., Ferreira, R., de Macedo, M.P., Neto, O.A., Duggavathi, R., Bordignon, V., and Goncalves, P.B.D. (2016) Regulation of Anti-Mullerian Hormone and Its Receptor Expression around Follicle Deviation in Cattle. *Reproduction in Domestic Animals* **51**(2), 188-194

Iliodromiti, S., Kelsey, T.W., Wu, O., Anderson, R.A., and Nelson, S.M. (2014) The predictive accuracy of anti-Mullerian hormone for live birth after assisted conception: a systematic review and meta-analysis of the literature. *Human Reproduction Update* **20**(4), 560-570

Meat and Livestock Australia and Australian Wool Innovation. (2021) MLA and AWI Wool and Sheepmeat Survery. Sydney, Australia: Meat and Livestock Australia (MLA).

Ireland, J.J., Smith, G.W., Scheetz, D., Jimenez-Krassel, F., Folger, J.K., Ireland, J.L.H., Mossa, F., Lonergan, P., and Evans, A.C.O. (2011) Does size matter in females? An overview of the impact of the high variation in the ovarian reserve on ovarian function and fertility, utility of anti-Mullerian hormone as a diagnostic marker for fertility and causes of variation in the ovarian reserve in cattle. *Reproduction Fertility and Development* **23**(1), 1-14

Ireland, J.L.H., Scheetz, D., Jimenez-Krassel, F., Themmen, A.P.N., Ward, F., Lonergan, P., Smith, G.W., Perez, G.I., Evans, A.C.O., and Ireland, J.J. (2008) Antral Follicle Count Reliably Predicts Number of Morphologically Healthy Oocytes and Follicles in Ovaries of Young Adult Cattle. *Biology of Reproduction* **79**(6), 1219-1225

Jackson, L.M., Mytinger, A., Roberts, E.K., Lee, T.M., Foster, D.L., Padmanabhan, V., and Jansen, H.T. (2013) Developmental Programming: Postnatal Steroids Complete Prenatal

Steroid Actions to Differentially Organize the GnRH Surge Mechanism and Reproductive Behavior in Female Sheep. *Endocrinology* **154**(4), 1612-1623

Jamil, Z., Fatima, S.S., Cheema, Z., Baig, S., and Choudhary, R.A. (2016) Assessment of ovarian reserve: Anti-Mullerian hormone versus follicle stimulating hormone. *Journal of Research in Medical Sciences* **21**(11), 5

Jimenez-Krassel, F., Scheetz, D.M., Neuder, L.M., Ireland, J.L.H., Pursley, J.R., Smith, G.W., Tempelman, R.J., Ferris, T., Roudebush, W.E., Mossa, F., Lonergan, P., Evans, A.C.O., and Ireland, J.J. (2015) Concentration of anti-Mullerian hormone in dairy heifers is positively associated with productive herd life. *Journal of Dairy Science* **98**(5), 3036-3045

Jorio, A., Mariana, J.C., and Lahlou-Kassi, A. (1991) Development of the population of ovarian follicles during the prepubertal period in D'man and Timahdite sheep. *Animal Reproduction Science* **26**(3), 239-250

Juengel, J.L., Cushman, R.A., Dupont, J., Fabre, S., Lea, R.G., Martin, G.B., Mossa, F., Pitman, J.L., Price, C.A., and Smith, P. (2021) The ovarian follicle of ruminants: the path from conceptus to adult. *Reproduction fertility and development* **33**(10), 621-642

Kane, N., Jones, M., Brosens, J.J., Saunders, P.T.K., Kelly, R.W., and Critchley, H.O.D. (2008) Transforming growth factor-beta1 attenuates expression of both the progesterone receptor and Dickkopf in differentiated human endometrial stromal cells. *Molecular endocrinology (Baltimore, Md.)* **22**(3), 716-728

Karsch, F.J., Cummins, J.T., Thomas, G.B., and Clarke, I.J. (1987) Steroid feedback inhibition of pulsatile secretion of gonadotropin-releasing-hormone in the ewe. *Biology of Reproduction* **36**(5), 1207-1218

Karsch, F.J., and Evans, N.P. (1996) Feedback actions of estradiol on GnRH secretion during the follicular phase of the estrous cycle. *Acta Neurobiologiae Experimentalis* **56**(3), 715-725

Karthik, D., Suresh, J., Reddy, Y.R., Sharma, G.R.K., Ramana, J.V., Gangaraju, G., Pradeep Kumar Reddy, Y., Yasaswini, D., Adegbeye, M.J., and Reddy, P.R.K. (2021) Farming systems in sheep rearing: Impact on growth and reproductive performance, nutrient digestibility, disease incidence and heat stress indices. *PloS one* **16**(1), e0244922-e0244922

Kato, N., Iwase, A., Sugita, A., Goto, M., Nakahara, T., Nakamura, T., Kondo, M., Osuka, S., Mori, M., Saito, A., and Kikkawa, F. (2015) Anti-Mullerian hormone as a possible predictor of fecundability in subfertile women over 38 years: a retrospective cohort study. *Gynecological Endocrinology* **31**(1), 22-25

Kaya, A., Gunes, E., and Memili, E. (2018) Application of reproductive biotechnologies for sustainable production of livestock in Turkey. *Turkish Journal of Veterinary & Animal Sciences* **42**(3), 143-151

Kelly, J.M., Kleemann, D.O., McGrice, H., Len, J.A., Kind, K.L., van Wettere, W., and Walker, S.K. (2017) Sex of co-twin affects the in vitro developmental competence of oocytes derived from 6-to 8-week-old lambs. *Reproduction Fertility and Development* **29**(7), 1379-1383

Kelly, J.M., Kleemann, D.O., and Walker, S.K. (2005) Enhanced efficiency in the production of offspring from 4-to 8-week-old lambs. *Theriogenology* **63**(7), 1876-1890

Kelly, M.J., Swan, A.A., and Atkins, K.D. (2007) Optimal use of on-farm fibre diameter measurement and its impact on reproduction in commercial Merino flocks. *Australian Journal of Experimental Agriculture* **47**(5), 525-534

Kennedy, J.P., Worthington, C.A., and Cole, E.R. (1974) The post-natal development of the ovary and uterus of the Merino lamb. *Journal of Reproduction and Fertility* **36**, 275-282

Kezele, P., and Skinner, M.K. (2003) Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: Endocrine model of follicle assembly. *Endocrinology* **144**(8), 3329-3337

Kezele, P.R., Ague, J.M., Nilsson, E., and Skinner, M.K. (2005) Alterations in the Ovarian Transcriptome During Primordial Follicle Assembly and Development1. *Biology of Reproduction* **72**(1), 241-255

Khader, A., Lloyd, S.M., McConnachie, A., Fleming, R., Grisendi, V., La Marca, A., and Nelson, S.M. (2013) External validation of anti-Mullerian hormone based prediction of live birth in assisted conception. *Journal of Ovarian Research* **6**, 7

Kleemann, D.O., and Walker, S.K. (2005) Fertility in South Australian commercial Merino flocks: sources of reproductive wastage. *Theriogenology* **63**(8), 2075-2088

Korsten, P., Clutton-Brock, T., Pilkington, J.G., Pemberton, J.M., and Kruuk, L.E.B. (2009) Sexual conflict in twins: male co-twins reduce fitness of female Soay sheep. *Biology letters (2005)* **5**(5), 663-666

Kotsampasi, B., Chadio, S., Papadomichelakis, G., Deligeorgis, S., Kalogiannis, D., Menegatos, I., and Zervas, G. (2009) Effects of Maternal Undernutrition on the Hypothalamic-Pituitary-Gonadal Axis Function in Female Sheep Offspring. *Reproduction in Domestic Animals* **44**(4), 677-684

La Marca, A., Malmusi, S., Giulini, S., Tamaro, L.F., Orvieto, R., Levratti, P., and Volpe, A. (2004) Anti-Müllerian hormone plasma levels in spontaneous menstrual cycle and during treatment with FSH to induce ovulation. *Human reproduction (Oxford)* **19**(12), 2738-2741

Lahoz, B., Alabart, J.L., Cocero, M.J., Monniaux, D., Echegoyen, E., Sanchez, P., and Folch, J. (2014) Anti-Mullerian hormone concentration in sheep and its dependence of age and independence of BMP15 genotype: An endocrine predictor to select the best donors for embryo biotechnologies. *Theriogenology* **81**(2), 347-357

Lahoz, B., Alabart, J.L., Cocero, M.J., Monniaux, D., Fabre, S., and Folch, J. (2015) The use of plasma anti-Müllerian hormone in sheep as an endocrine marker of the ovarian response to follicle-stimulating hormone in multiple-ovulation and embryo transfer programs *Reproduction fertility and development* **27**(1), 264

Lahoz, B., Alabart, J.L., and Folch, J. (2016) Relationship between Anti-Mullerian hormone (AMH) and the ovulatory response measured in three-month old ewe lambs with their reproductive efficiency in adulthood. *Itea-Informacion Tecnica Economica Agraria* **112**(4), 392-404

Lahoz, B., Alabart, J.L., Monniaux, D., Mermillod, P., and Folch, J. (2012) Anti-Mullerian hormone plasma concentration in prepubertal ewe lambs as a predictor of their fertility at a young age. *Bmc Veterinary Research* **8**, 9

Lalit, Malik, Z.S., Dalal, D.S., Patil, C.S., and Dahiya, S.P. (2017) Genetic studies on growth, reproduction and wool production traits in Harnali sheep. *Indian Journal of Animal Research* **51**(5), 813-816

Land, R.B. (1970) Number of oocytes present at birth in the ovaries of pure and finnish landrace cross blackface and welsh sheep. *Journal of reproduction & fertility* **21**(3), 517-521

Ledda, S., Bogliolo, L., Calvia, P., Leoni, G., and Naitana, S. (1997) Meiotic progression and developmental competence of oocytes collected from juvenile and adult ewes. *Reproduction* **109**(1), 73-78

Li, L., Lu, S., Ma, Q., Wan, P., Liu, C., Yang, H., Yang, H., Yang, Y., Dai, R., and Shi, G. (2019) The comparison of reproductive hormone receptor expressions of the sheep ovary and hormone concentrations in two Chinese breeds. *Reprod Domest Anim* **54**(6), 892-901

Loi, P., Ptak, G., Dattena, M., Ledda, S., Naitana, S., and Cappai, P. (1998) Embryo transfer and related technologies in sheep reproduction. *Reproduction Nutrition Development* **38**(6), 615-628

Lonergan, P., and Forde, N. (2015) The Role of Progesterone in Maternal Recognition of Pregnancy in Domestic Ruminants. In 'Regulation of Implantation and Establishment of Pregnancy in Mammals: Tribute to 45 Year Anniversary of Roger V. Short's Maternal Recognition of Pregnancy. Vol. 216.' (Eds. RD Geisert and FW Bazer) pp. 87-104. (Springer International Publishing Ag: Cham)

Long, N.M., Nijland, M.J., Nathanielsz, P.W., and Ford, S.P. (2010) The effect of early to mid-gestational nutrient restriction on female offspring fertility and hypothalamic-pituitary-adrenal axis response to stress. *Journal of Animal Science* **88**(6), 2029-2037

Lukaszuk, K., Liss, J., Kunicki, M., Jakiel, G., Wasniewski, T., Woclawek-Potocka, I., and Pastuszek, E. (2014) Anti-Mullerian hormone (AMH) is a strong predictor of live birth in women undergoing assisted reproductive technology. *Reproductive Biology* **14**(3), 176-181

Lummaa, V., Pettay, J.E., and Russell, A.F. (2007) Male Twins Reduce Fitness of Female Co-Twins in Humans. *Proceedings of the National Academy of Sciences - PNAS* **104**(26), 10915-10920

Mahdi, D., and Khallili, K. (2008) Relationship between follicle growth and circulating gonadotrophin levels during postnatal development of sheep. *Animal Reproduction Science* **106**(1-2), 100-112

Martinez, M.F., McLeod, B., Tattersfield, G., Smaill, B., Quirke, L.D., and Juengel, J.L. (2015) Successful induction of oestrus, ovulation and pregnancy in adult ewes and ewe lambs out of the breeding season using a GnRH plus progesterone oestrus synchronisation protocol. *Animal Reproduction Science* **155**, 28-35

Martinez, M.F., Sanderson, N., Quirke, L.D., Lawrence, S.B., and Juengel, J.L. (2016) Association between antral follicle count and reproductive measures in New Zealand lactating dairy cows maintained in a pasture-based production system. *Theriogenology* **85**(3), 466-475

McGee, E., and Hsueh, A. (2000) Initial and cyclic recruitment of ovarian follicles. *Endocrine Reviews* **21**, 200-214

McGrice, H., Kelly, J.M., Kleemann, D.O., Kind, K.L., Hampton, A.J., Hannemann, P., Walker, S.K., and van Wettere, W.H.E.J. (2020) Plasma anti-Müllerian hormone concentration as a predictive endocrine marker for selection of donor lambs to improve success in juvenile in vitro embryo transfer programs. *Reproduction fertility and development* **32**(4), 383-391

McNatty, K.P., Fidler, A.E., Juengel, J.L., Quirke, L.D., Smith, P.R., Heath, D.A., Lundy, T., and O'Connell, A. (2000) Growth and paracrine factors regulating follicular formation and cellular function. *Molecular and Cellular Endocrinology* **163**(1-2), 11-20

McNatty, K.P., Smith, P., Hudson, N.L., Heath, D.A., Tisdall, D.J., O, W.S., and Brawtal, R. (1995) Development of the sheep ovary during fetal and early neonatal life and the effect of fecundity genes. *Journal of Reproduction and Fertility*, 123-135

Monniaux, D., Barbey, S., Rico, C., Fabre, S., Gallard, Y., and Larroque, H. (2010) Anti-Mullerian hormone: a predictive marker of embryo production in cattle? *Reproduction Fertility and Development* **22**(7), 1083-1091

Monniaux, D., Baril, G., Laine, A.L., Jarrier, P., Poulin, N., Cognie, J., and Fabre, S. (2011) Anti-Mullerian hormone as a predictive endocrine marker for embryo production in the goat. *Reproduction* **142**(6), 845-854

Monniaux, D., Drouilhet, L., Rico, C., Estienne, A., Jarrier, P., Touzé, J.-L., Sapa, J., Phocas, F., Dupont, J., Dalbiès-Tran, R., and Fabre, S. (2012) Regulation of anti-Müllerian hormone production in domestic animals. *Reproduction fertility and development* **25**(1), 1-16

Monniaux, D., Drouilhet, L., Rico, C., Estienne, A., Jarrier, P., Touze, J.L., Sapa, J., Phocas, F., Dupont, J., Dalbies-Tran, R., and Fabre, S. (2013) Regulation of anti-Mullerian hormone production in domestic animals. *Reproduction Fertility and Development* **25**(1), 1-16

Moriel, P., Lancaster, P., Lamb, G.C., Vendramini, J.M.B., and Arthington, J.D. (2017) Effects of post-weaning growth rate and puberty induction protocol on reproductive performance of Bos indicus -influenced beef heifers. *Journal of animal science* **95**(8), 3523-3531

Morton, K. (2008) Developmental Capabilities of Embryos Produced In Vitro from Prepubertal Lamb Oocytes. *Reproduction in Domestic Animals* **43**, 137-143

Morton, K.M., Catt, S.L., Maxwell, W.M.C., and Evans, G. (2005) An efficient method of ovarian stimulation and in vitro embryo production from prepubertal lambs. *Reproduction Fertility and Development* **17**(7), 701-706

Mossa, F., Carter, F., Walsh, S.W., Kenny, D.A., Smith, G.W., Ireland, J.L.H., Hildebrandt, T.B., Lonergan, P., Ireland, J.J., and Evans, A.C.O. (2013) Maternal Undernutrition in Cows Impairs Ovarian and Cardiovascular Systems in Their Offspring1. *Biology of Reproduction* **88**(4)

Mossa, F., Duffy, P., Naitana, S., Lonergan, P., and Evans, A.C.O. (2007) Association between numbers of ovarian follicles in the first follicle wave and superovulatory response in ewes. *Animal Reproduction Science* **100**(3-4), 391-396

Mossa, F., Jimenez-Krassel, F., Scheetz, D., Weber-Nielsen, M., Evans, A.C.O., and Ireland, J.J. (2017) Anti-Mullerian Hormone (AMH) and fertility management in agricultural species. *Reproduction* **154**(1), R1-R11

Mossa, F., Walsh, S.W., Butler, S.T., Berry, D.P., Carter, F., Lonergan, P., Smith, G.W., Ireland, J.J., and Evans, A.C.O. (2012) Low numbers of ovarian follicles >= 3 mm in diameter are associated with low fertility in dairy cows. *Journal of Dairy Science* **95**(5), 2355-2361

Mulders, A.G.M.G.J. (2004) Changes in anti-Mullerian hormone serum concentrations over time suggest delayed ovarian ageing in normogonadotrophic anovulatory infertility. *Human reproduction (Oxford)* **19**(9), 2036-2042

Mumford, S.L., Legro, R.S., Diamond, M.P., Coutifaris, C., Steiner, A.Z., Schlaff, W.D., Alvero, R., Christman, G.M., Casson, P.R., Huang, H., Santoro, N., Eisenberg, E., Zhang, H., and Cedars, M.I. (2016) Baseline AMH Level Associated With Ovulation Following Ovulation Induction in Women With Polycystic Ovary Syndrome. *The Journal of clinical endocrinology and metabolism* **101**(9), 3288-3296

Munsterberg, A., and Lovellbadge, R. (1991) Expression of the mouse anti-Mullerian hormone gene suggests a role in both male and female sexual-differentiation. *Development* **113**(2), 613-624

Murphy, T.W., and Freking, B.A. (2020) Comparison of performance of F1 Romanov crossbred ewes with wool and hair breeds during fall lambing and body weight and longevity through six production years. *Journal of Animal Science* **99**(1):skaa400

Nagdy, H., Mahmoud, K.G.M., Kandiel, M.M.M., Hemy, N.A., Ibrahim, S.S., Nawito, M.F., and Othman, O.E. (2018) PCR-RFLP of bone morphogenetic protein 15 (BMP15/FecX) gene as a candidate for prolificacy in sheep. *International Journal of Veterinary Science and Medicine* **6**, S68-S72

Nakhuda, G.S., Chu, M.C., Wang, J.G., Sauer, M.V., and Lobo, R.A. (2006) Elevated serum mullerian-inhibiting substance may be a marker for ovarian hyperstimulation syndrome in normal women undergoing in vitro fertilization. *Fertility and Sterility* **85**(5), 1541-1543

Nasrin Ferdousy, R., Kereilwe, O., and Kadokawa, H. (2020) Anti-Müllerian hormone is expressed and secreted by bovine oviductal and endometrial epithelial cells. *Animal science journal* **91**(1), e13456-n/a

Nelson, S.M., Fleming, R., Gaudoin, M., Choi, B., Santo-Domingo, K., and Yao, M. (2015) Antimullerian hormone levels and antral follicle count as prognostic indicators in a personalized prediction model of live birth. *Fertility and Sterility* **104**(2), 325-332

Nestor, C.C., Bedenbaugh, M.N., Hileman, S.M., Coolen, L.M., Lehman, M.N., and Goodman, R.L. (2018) Regulation of GnRH pulsatility in ewes. *Reproduction* **156**(3), R83-R99

Nilsson, E.E., Schindler, R., Savenkova, M.I., and Skinner, M.K. (2011) Inhibitory actions of Anti-Müllerian Hormone (AMH) on ovarian primordial follicle assembly. *PloS one* **6**(5), e20087-e20087

Obrien, J.K., Catt, S.L., Ireland, K.A., Maxwell, W.M.C., and Evans, G. (1997) In vitro and in vivo developmental capacity of oocytes from prepubertal and adult sheep. *Theriogenology* **47**(7), 1433-1443

Obrien, J.K., Dwarte, D., Ryan, J.P., Maxwell, W.M.C., and Evans, G. (1996) Developmental capacity, energy metabolism and ultrastructure of mature oocytes from prepubertal and adult sheep. *Reproduction Fertility and Development* **8**(7), 1029-1037

Paganoni, B.L., Ferguson, M.B., Ferrio, S., Jones, C., Kearney, G.A., Kenyon, P.R., Macleay, C., Vinoles, C., and Thompson, A.N. (2014) Early reproductive losses are a major factor contributing to the poor reproductive performance of Merino ewe lambs mated at 8-10 months of age. *Animal Production Science* **54**(6), 762-772

Pankhurst, M.W. (2017) A putative role for anti-Mullerian hormone (AMH) in optimising ovarian reserve expenditure. *Journal of Endocrinology* **233**(1), R1-R13

Paramio, M.T., and Izquierdo, D. (2014) Current Status of In Vitro Embryo Production in Sheep and Goats. *Reproduction in Domestic Animals* **49**, 37-48

Pellatt, L., Hanna, L., Brincat, M., Galea, R., Brain, H., Whitehead, S., and Mason, H. (2007) Granulosa cell production of anti-Müllerian hormone is increased in polycystic ovaries. *J Clin Endocrinol Metab* **92**(1), 240-5

Pellatt, L., Rice, S., Dilaver, N., Heshri, A., Galea, R., Brincat, M., Brown, K., Simpson, E.R., and Mason, H.D. (2011) Anti-Mullerian hormone reduces follicle sensitivity to follicle-stimulating hormone in human granulosa cells. *Fertility and Sterility* **96**(5), 1246-U216

Peterson, A.J., and Lee, R.S.F. (2003) Improving successful pregnancies after embryo transfer. *Theriogenology* **59**(2), 687-697

Pfeiffer, K.E., Jury, L.J., and Larson, J.E. (2014) Determination of anti-Mullerian hormone at estrus during a synchronized and a natural bovine estrous cycle. *Domestic Animal Endocrinology* **46**, 58-64

Pielecka-Fortuna, J., Chu, Z., and Moenter, S.M. (2008) Kisspeptin acts directly and indirectly to increase gonadotropin-releasing hormone neuron activity and its effects are modulated by estradiol. *Endocrinology* **149**(4), 1979-86

Pierre, A., Estienne, A., Racine, C., Picard, J.-Y., Fanchin, R., Lahoz, B., Alabart, J.L., Folch, J., Jarrier, P., Fabre, S., Monniaux, D., and di Clemente, N. (2016) The Bone Morphogenetic Protein 15 Up-Regulates the Anti-Müllerian Hormone Receptor Expression in Granulosa Cells. *The journal of clinical endocrinology and metabolism* **101**(6), 2602-2611

Pinto, P.H.N., Balaro, M.F.A., Souza-Fabjan, J.M.G., Ribeiro, L.D., Braganca, G.M., Leite, C.R., Arashiro, E.K.N., Silva, K.D., Da Fonseca, J.F., and Brandao, F.Z. (2018) Anti-Mullerian hormone and antral follicle count are more effective for selecting ewes with good potential for in vivo embryo production than the presence of FecG(E) mutation or eCG pre-selection tests. *Theriogenology* **113**, 146-152

Poole, D.H., Ocon-Grove, O.M., and Johnson, A.L. (2016) Anti-Mullerian hormone (AMH) receptor type II expression and AMH activity in bovine granulosa cells. *Theriogenology* **86**(5), 1353-1360

Ptak, G., Loi, P., Dattena, M., Tischner, M., and Cappai, P. (1999) Offspring from one-month-old lambs: Studies on the developmental capability of prepubertal oocytes. *Biology of Reproduction* **61**(6), 1568-1574

Ptak, G., Tischner, M., Bernabo, N., and Loi, P. (2003) Donor-dependent developmental competence of oocytes from lambs subjected to repeated hormonal stimulation. *Biology of Reproduction* **69**(1), 278-285

Rae, M.T., Kyle, C.E., Miller, D.W., Hammond, A.J., Brooks, A.N., and Rhind, S.M. (2002) The effects of undernutrition, in utero, on reproductive function in adult male and female sheep. *Animal Reproduction Science* **72**(1-2), 63-71

Rae, M.T., Palassio, S., Kyle, C.E., Brooks, A.N., Lea, R.G., Miller, D.W., and Rhind, S.M. (2001) Effect of maternal undernutrition during pregnancy on early ovarian development and subsequent follicular development in sheep fetuses. *Reproduction* **122**(6), 915-922

Rawlings, N.C., Evans, A.C., Honaramooz, A., and Bartlewski, P.M. (2003) Antral follicle growth and endocrine changes in prepubertal cattle, sheep and goats. *Anim Reprod Sci* **78**(3-4), 259-70

Reader, K.L., Cox, N.R., Stanton, J.A.L., and Juengel, J.L. (2015) Mitochondria and vesicles differ between adult and prepubertal sheep oocytes during IVM. *Reproduction Fertility and Development* **27**(3), 513-522

Redmond, J.S., Macedo, G.G., Velez, I.C., Caraty, A., Williams, G.L., and Amstalden, M. (2011) Kisspeptin activates the hypothalamic-adenohypophyseal-gonadal axis in prepubertal ewe lambs. *Reproduction* **141**(4), 541-548

Rey, R., Lukas-Croisier, C., Lasala, C., and Bedecarras, P. (2003) AMH/MIS: what we know already about the gene, the protein and its regulation. *Molecular and Cellular Endocrinology* **211**(1-2), 21-31

Rhind, S.M., Rae, M.T., and Brooks, A.N. (2001) Effects of nutrition and environmental factors on the fetal programming of the reproductive axis. *Reproduction* **122**(2), 205-214

Ribeiro, E.S., Bisinotto, R.S., Lima, F.S., Greco, L.F., Morrison, A., Kumar, A., Thatcher, W.W., and Santos, J.E.P. (2014) Plasma anti-Mullerian hormone in adult dairy cows and associations with fertility. *Journal of Dairy Science* **97**(11), 6888-6900

Rico, C., Drouilhet, L., Salvetti, P., Dalbies-Tran, R., Jarrier, P., Touze, J.L., Pillet, E., Ponsart, C., Fabre, S., and Monniaux, D. (2012) Determination of anti-Mullerian hormone concentrations in blood as a tool to select Holstein donor cows for embryo production: from the laboratory to the farm. *Reproduction Fertility and Development* **24**(7), 932-944

Rico, C., Fabre, S., Medigue, C., di Clemente, N., Clement, F., Bontoux, M., Touze, J.L., Dupont, M., Briant, E., Remy, B., Beckers, J.F., and Monniaux, D. (2009) Anti-Mullerian Hormone Is an Endocrine Marker of Ovarian Gonadotropin-Responsive Follicles and Can Help to Predict Superovulatory Responses in the Cow. *Biology of Reproduction* **80**(1), 50-59

Rico, C., Medigue, C., Fabre, S., Jarrier, P., Bontoux, M., Clement, F., and Monniaux, D. (2011) Regulation of Anti-Mullerian Hormone Production in the Cow: A Multiscale Study at Endocrine, Ovarian, Follicular, and Granulosa Cell Levels. *Biology of Reproduction* **84**(3), 560-571

Rosales Nieto, C.A., Ferguson, M.B., Thompson, H., Briegel, J.R., Macleay, C.A., Martin, G.B., and Thompson, A.N. (2015) Relationships among Puberty, Muscle and Fat, and Liveweight Gain during Mating in Young Female Sheep. *Reprod Domest Anim* **50**(4), 637-42

Rosales Nieto, C.A., Thompson, A.N., and Martin, G.B. (2018) A new perspective on managing the onset of puberty and early reproductive performance in ewe lambs: a review. *Animal production science* **58**(11), 1967

Rosasco, S.L., Beard, J.K., Hallford, D.M., and Summers, A.F. (2019) Evaluation of estrous synchronization protocols on ewe reproductive efficiency and profitability. *Animal reproduction science* **210**, 106191-106191

Rowe, J.B. (2010) The Australian sheep industry - undergoing transformation. *Animal Production Science* **50**(11-12), 991-997

Roy, S., Gandra, D., Seger, C., Biswas, A., Kushnir, V.A., Gleicher, N., Kumar, T.R., and Sen, A. (2018) Oocyte-Derived Factors (GDF9 and BMP15) and FSH Regulate AMH Expression Via Modulation of H3K27AC in Granulosa Cells. *Endocrinology (Philadelphia)* **159**(9), 3433

Ryan, K.D., Goodman, R.L., Karsch, F.J., Legan, S.J., and Foster, D.L. (1991) Patterns of Circulating Gonadotropins and Ovarian Steroids during the First Periovulatory Period in the Developing Sheep1. *Biology of Reproduction* **45**(3), 471-477

Safari, E., Fogarty, N.M., and Gilmour, A.R. (2005) A review of genetic parameter estimates for wool, growth, meat and reproduction traits in sheep. *Livestock Production Science* **92**(3), 271-289

Safari, E., Fogarty, N.M., Gilmour, A.R., Atkins, K.D., Mortimer, S.I., Swan, A.A., Brien, F.D., Greeff, J.C., and van der Werf, J.H.J. (2007) Genetic correlations among and between wool, growth and reproduction traits in Merino sheep. *Journal of Animal Breeding and Genetics* **124**(2), 65-72

Scaramuzzi, R.J., Adams, N.R., Tsonis, C.G., Baird, D.T., Campbell, B.K., Downing, J.A., Findlay, J.K., Henderson, K.M., Martin, G.B., McNatty, K.P., and McNeilly, A.S. (1993) A model for follicle selection and the determination of ovulation rate in the ewe. *Reproduction fertility and development* **5**(5), 459-478

Scaramuzzi, R.J., Baird, D.T., Campbell, B.K., Driancourt, M.-A., Dupont, J., Fortune, J.E., Gilchrist, R.B., Martin, G.B., McNatty, K.P., McNeilly, A.S., Monget, P., Monniaux, D., Viñoles, C., and Webb, R. (2011) Regulation of folliculogenesis and the determination of ovulation rate in ruminants. *Reproduction, Fertility and Development* **23**(3), 444-467

Scheetz, D., Folger, J.K., Smith, G.W., and Ireland, J.J. (2012) Granulosa cells are refractory to FSH action in individuals with a low antral follicle count. *Reproduction fertility and development* **24**(2), 327-336

Seccafien, J., Kelly, J., McGrice, H., Kleemann, D., Kind, K., and van wettere, W. (2019) 74 Follicular fluid anti-Müllerian hormone concentration predicts juvenile ovine in vitro embryo development. *Reproduction, Fertility and Development* **31**, 162

Silva-Santos, K.C., dos Santos, G.M.G., Siloto, L.S., dos Santos, J.T., de Oliveira, E.R., Machado, F.Z., Rosa, C.O., and Senedaa, M.M. (2014a) The correlation between the number of antral follicles and ovarian reserves (preantral follicles) in purebred Bos indicus and Bos taurus cows. *Animal Reproduction Science* **151**(3-4), 119-125

Silva-Santos, K.C., Santos, G.M.G., Koetz, C., Morotti, F., Siloto, L.S., Marcantonio, T.N., Urbano, M.R., Oliveira, R.L., Lima, D.C.M., and Seneda, M.M. (2014b) Antral Follicle Populations and Embryo Production - In Vitro and In Vivo - of Bos indicus-taurus Donors from Weaning to Yearling Ages. *Reproduction in Domestic Animals* **49**(2), 228-232

Silva-Santos, K.C., Santos, G.M.G., Siloto, L.S., Hertel, M.F., Andrade, E.R., Rubin, M.I.B., Sturion, L., Melo-Sterza, F.A., and Seneda, M.M. (2011) Estimate of the population of preantral follicles in the ovaries of Bos taurus indicus and Bos taurus taurus cattle. *Theriogenology* **76**(6), 1051-1057

Skinner, M.K. (2005) Regulation of primordial follicle assembly and development. *Human Reproduction Update* **11**(5), 461-471

Smith, C. (1986) Use of embryo transfer in genetic improvement of sheep. *Animal Science* **42**(1), 81-88

Smith, P., Wilhelm, D., and Rodgers, R.J. (2014) Development of mammalian ovary. *Journal of Endocrinology* **221**(3), R145-R161

Snowder, G.D., and Fogarty, N.M. (2009) Composite trait selection to improve reproduction and ewe productivity: a review. *Animal Production Science* **49**(1), 9-16

Songsasen, N., Woodruff, T.K., and Wildt, D.E. (2011) In vitro growth and steroidogenesis of dog follicles are influenced by the physical and hormonal microenvironment. *Reproduction (Cambridge, England)* **142**(1), 113-122

Souza, A.H., Carvalho, P.D., Rozner, A.E., Vieira, L.M., Hackbart, K.S., Bender, R.W., Dresch, A.R., Verstegen, J.P., Shaver, R.D., and Wiltbank, M.C. (2015) Relationship between circulating anti-Mullerian hormone (AMH) and superovulatory response of high-producing dairy cows. *Journal of Dairy Science* **98**(1), 169-178

Souza, C.J.H., Campbell, B.K., and Baird, D.T. (1997) Follicular dynamics and ovarian steroid secretion in sheep during the follicular and early luteal phases of the estrous cycle. *Biology of Reproduction* **56**(2), 483-488

Souza, C.J.H., Campbell, B.K., and Baird, D.T. (1998) Follicular waves and concentrations of steroids and inhibin A in ovarian venous blood during the luteal phase of the oestrous cycle in ewes with an ovarian autotransplant. *Journal of Endocrinology* **156**(3), 563-572

Stevenson, J.S., and Call, E.P. (1983) Influence of early estrus, ovulation, and insemination on fertility in postpartum holstein cows. *Theriogenology* **19**(3), 367-375

Stojsin-Carter, A., Mahboubi, K., Costa, N.N., Gillis, D.J., Carter, T.F., Neal, M.S., Miranda, M.S., Ohashi, O.M., Favetta, L.A., and King, W.A. (2016) Systemic and local anti-Mullerian hormone reflects differences in the reproduction potential of Zebu and European type cattle. *Anim Reprod Sci* **167**, 51-8

Su, H.I., Sammel, M.D., Freeman, E.W., Lin, H., DeBlasis, T., and Gracia, C.R. (2008) Body size affects measures of ovarian reserve in late reproductive age women. *Menopause (New York, N.Y.)* **15**(5), 857-861

Tal, R., Seifer, C.M., Khanimov, M., Seifer, D.B., and Tal, O. (2020) High serum Antimullerian hormone levels are associated with lower live birth rates in women with polycystic ovarian syndrome undergoing assisted reproductive technology. *Reproductive biology and endocrinology : RB&E* **18**(1), 20-20

Tassell, R., Chamley, W.A., and Kennedy, J.P. (1978) Gonadotrophin levels and ovarian development in the neonatal ewe lamb. *Aust J Biol Sci* **31**(3), 267-73

Thompson, J.G., Bell, A.C.S., McMillan, W.H., Peterson, A.J., and Tervit, H.R. (1995) Donor and recipient ewe factors affecting in vitro development and post-transfer survival of cultured sheep embryos. *Animal Reproduction Science* **40**(4), 269-279

Torres-Rovira, L., Gonzalez-Bulnes, A., Succu, S., Spezzigu, A., Manca, M.E., Leoni, G.G., Sanna, M., Pirino, S., Gallus, M., Naitana, S., and Berlinguer, F. (2014) Predictive value of antral follicle count and anti-Mullerian hormone for follicle and oocyte developmental competence during the early prepubertal period in a sheep model. *Reproduction Fertility and Development* **26**(8), 1094-1106

Torres-Rovira, L., Succu, S., Pasciu, V., Manca, M.E., Gonzalez-Bulnes, A., Leoni, G.G., Pennino, M.G., Spezzigu, A., Gallus, M., Dattena, M., Monniaux, D., Naitana, S., and Berlinguer, F. (2016) Postnatal pituitary and follicular activation: a revisited hypothesis in a sheep model. *Reproduction* **151**(3), 215-225

Turzillo, A.M., and Nett, T.M. (1999) Regulation of GnRH receptor gene expression in sheep and cattle. *Journal of Reproduction and Fertility*, 75-86

Umer, S., Zhao, S.J., Sammad, A., Sahlu, B.W., Pang, Y.W., and Zhu, H.B. (2019) AMH: Could It Be Used as A Biomarker for Fertility and Superovulation in Domestic Animals? *Genes* **10**(12), 14

van Asselt, K.M., Kok, H.S., Pearson, P.L., Dubas, J.S., Peeters, P.H., Te Velde, E.R., and van Noord, P.A. (2004) Heritability of menopausal age in mothers and daughters. *Fertility and sterility* **82**(5), 1348-1351

van Rooij, I.A.J., Broekmans, F.J.M., te Velde, E.R., Fauser, B., Bancsi, L., de Jong, F.H., and Themmen, A.P.N. (2002) Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. *Human Reproduction* **17**(12), 3065-3071

Veiga-Lopez, A., Gonzalez-Bulnes, A., Garcia-Garcia, R.M., Dominguez, V., and Cocero, M.J. (2005) The effects of previous ovarian status on ovulation rate and early embryo development in response to superovulatory FSH treatments in sheep. *Theriogenology* **63**(7), 1973-1983

Veiga-Lopez, A., Ye, W., and Padmanabhan, V. (2012) Developmental programming: prenatal testosterone excess disrupts anti-Mullerian hormone expression in preantral and antral follicles. *Fertility and Sterility* **97**(3), 748-756

Visser, J.A., and Themmen, A.P.N. (2005) Anti-Mullerian hormone and folliculogenesis. *Molecular and Cellular Endocrinology* **234**(1-2), 81-86

Visser, J.A., and Themmen, A.P.N. (2014) Role of anti-Mullerian hormone and bone morphogenetic proteins in the regulation of FSH sensitivity. *Molecular and Cellular Endocrinology* **382**(1), 460-465

Voutilainen, R., and Miller, W.L. (1987) Human müllerian inhibitory factor messenger ribonucleic acid is hormonally regulated in the fetal testis and in adult granulosa cells. *Mol Endocrinol* **1**(9), 604-8

Waheeb, R.S. (2017) Changes in Serum Levels of Anti-Mullerian Hormone and Ovarian Steroids in Barki Sheep during Follicular and Early Luteal Phase of Estrous Cycle. *Pakistan Veterinary Journal* **37**(4), 421-426

Walkom, S.F., Brien, F.D., Hebart, M.L., Fogarty, N.M., Hatcher, S., and Pitchford, W.S. (2016) Season and reproductive status rather than genetic factors influence change in ewe weight and fat over time. 4. Genetic relationships of ewe weight and fat score with fleece, reproduction and milk traits. *Animal Production Science* **56**(4), 708-715

Ward, F., Lonergan, P., Jimenez-Krassel, F., Ireland, J.J., and Evans, A.C.O. (2006) 240 Relationship between peak number of antral follicles and follicular waves, hormone concentrations, superovulatory response, and embryo quality in beef heifers. *Reproduction, Fertility and Development* **18**(2), 228

Webb, R., Campbell, B.K., Garverick, H.A., Gong, J.G., Gutierrez, C.G., and Armstrong, D.G. (1999) Molecular mechanisms regulating follicular recruitment and selection. *Journal of Reproduction and Fertility*, 33-48

Webb, R., Garnsworthy, P.C., Campbell, B.K., and Hunter, M.G. (2007) Intra-ovarian regulation of follicular development and oocyte competence in farm animals. *Theriogenology* **68**, S22-S29

Wildeus, S. (2000) Current concepts in synchronization of estrus: Sheep and goats. *Journal of animal science* **77**(E-Suppl), 1

Appendix One: Published literature review: Towards improving the outcomes of Reproduction; assisted and natural in sheep, with a focus on the Australian Merino and anti-Müllerian hormone in ‘Animals’

animals

MDPI

Review

Towards Improving the Outcomes of Assisted Reproductive Technologies of Cattle and Sheep, with Particular Focus on Recipient Management

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Received: 30 December 2019; Accepted: 11 February 2020; Published: 13 February 2020

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Simple Summary: The Australian livestock industry has grown significantly over the last decade. In order to meet national consumer demands, as well as the growing export market, more efficient means of producing lamb and beef products are required. Assisted reproductive technologies (ARTs) can be used to increase genetic gain and improve overall herd reproductive potential. This review focuses particularly on the management and selection of recipient animals utilised in ARTs. The condition and quality of the recipient animal is pivotal to the efficiency of reproductive technologies, as the inability of an embryo to establish and maintain pregnancy is the most significant cause of reproductive losses. A variety of external, uncontrollable factors affect the reproductive potential of an individual within any given reproductive season, limiting reproductive efficiency. Therefore, improved selection and management of recipient animals can help to increase the productivity of the Australian livestock industries.

Abstract: The Australian agricultural industry contributes AUD 47 billion to the Australian economy, and Australia is the world’s largest exporter of sheep meat and the third largest for beef. Within Australia, sheep meat consumption continues to rise, with beef consumption being amongst the highest in the world; therefore, efficient strategies to increase herd/flock size are integral to the success of these industries. Reproductive management is crucial to increasing the efficiency of Australian breeding programs. The use of assisted reproductive technologies (ARTs) has the potential to increase efficiency significantly. The implementation of multiple ovulation and embryo transfer (MOET) and juvenile in vitro fertilization and embryo transfer (JIVET) in combination with genomic selection and natural mating and AI is the most efficient way to increase genetic gain, and thus increase reproductive efficiency within the Australian livestock industries. However, ARTs are costly, and high variation, particularly between embryo transfer recipients in their ability to maintain pregnancy, is a significant constraint to the widespread commercial adoption of ARTs. The use of a phenotypic marker for the selection of recipients, as well as the better management of recipient animals, may be an efficient and cost-effective means to increase the productivity of the Australian livestock industry.

Keywords: recipient management; recipient animal; anti-Müllerian hormone; AMH; assisted reproductive technology; ART; sheep; cow

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1. Introduction

Commensurate with population growth, there has been a 58% increase in global meat consumption over the last 20 years, with 4% of this growth attributed to increases in per person consumption [1].

Review

Towards Improving the Outcomes of Reproduction; assisted and natural in sheep, with a focus on the Australian Merino and Anti-Mullerian Hormone

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Simple Summary: The reproductive efficiency of the Australian sheep flock needs to be improved in order to match the increasing global demand for sheep meat. Genetic selection alone is not fast or effective enough to make rapid improvements within the industry. A variety of external, uncontrollable factors affect the reproductive potential of an individual within any given reproductive season, limiting reproductive efficiency. This together with the fact a combination of natural matings and assisted reproductive technologies is the best practice method to improve overall flock numbers, a phenotypic marker of selection may be of use. A phenotypic marker, AMH specifically, will consider the impact of external pressures on each individual, and can be readily applied within a production system undergoing any reproductive management practice.

Abstract: The Australian sheep industry is the worlds largest exporter of sheepmeat, despite only making up 7% of the world's domestic sheep population. Together with a 58% increase in meat consumption, strategies need to be implemented to improve the efficiency of the Australian sheep flock. Conceptions rates for the Australian Merino are 82%, compared with 102% for crossbred ewes, despite making up 75% of the Australian sheep population. Together with the fact that 60% of the lambs weaned are Merino, better selection is needed for breeding ewes within in this flock. A combination of adjusting natural mating procedures, artificial insemination (AI), and other assisted reproductive technologies (ARTs) has been identified as the most effective way to increase the reproductive efficiency of the current population. Unfortunately, significant variation exists amongst all these factors and genetic selection alone will not overcome this. External factors post- conception significantly impacts the reproductive development of an individual, and this is not accounted for through genetic selection. A phenotypic marker of the ovarian environment will consider external stressors and allow for the selection of the most reproductively viable animals. Anti-Mullerian hormone (AMH) reflects the ovarian follicle pool and is potentially an exceptional marker of reproductive efficiency.

Keywords: reproduction; Australian Merino; anti-Müllerian hormone; AMH; assisted reproductive technology; ART; sheep; oestrous cycle

Appendix two: Conference Abstracts

The Joint Annual Scientific Meetings of the Endocrine Society of Australia and the Society for Reproductive Biology 2018

J. Seccafien, J. Kelly, H. McGrice and W.H.E.J. van Wettere, 'The effect of oestrous synchronisation and breed on anti-Mullerian hormone (AMH) concentrations in Merino and Suffolk ewes'

Anti-Möllerian hormone (AMH) is a transforming growth factor family glycoprotein expressed exclusively by the granulosa cells of growing antral follicles. AMH plays an inhibitory role during initial primordial follicle growth recruitment and regulates the continued growth of large pre-antral and small antral follicles. AMH is an endocrine marker of ovarian reserve in humans, a reliable marker of the response of cows to super-ovulatory stimulation protocols, and can be used to identify which ewes should be used as donors for laparoscopic ovum pick up. This study had two primary aims; one, to determine if there is an optimum time to measure AMH relative to synchronisation of the oestrous cycle, and, two, to determine whether AMH concentrations differ between breeds of sheep. Suffolk (n = 19) and Merino (n = 19) ewes were used, and oestrus cyclicity was synchronised using an intravaginal progesterone insert, which was removed after 12 days. Blood samples were collected into lithium heparin coated tubes on the day of the insertion of the intravaginal progesterone insert (day 0), and again on the day of its removal (day 12). Samples were centrifuged at 1500 rpm for 15 minutes and AMH concentrations measured in the plasma using an ovine AMH ELISA kit (ANSH labs, Texas, USA). Data were analysed using an ANOVA (unbalanced design; Genstat 15th Edition SP2). Mean AMH concentrations were similar on Day 0 and Day 12 (2.61 ± 0.25 and 2.64 ± 0.25 pg/ml, respectively; P = 0.315) and for Merino and Suffolk ewes (2.55 ± 0.25 and 2.69 ± 0.25 pg/ml; P = 0.315). The current data indicates no differences in AMH concentrations between non-synchronised and synchronised ewes, or between breeds, suggesting it may be a useful tool with which to select donor ewes, regardless of breed, prior to commencing oestrous synchronisation.

International Embryo Technology Society (IETS) 2019 45th Annual Conference

J.E. Seccafien, J.M. Kelly, H. McGrice, D.O. Kleemann, K.L. Kind and W.H.E.J. van Wettere, 'Follicular fluid anti-Mullerian hormone (AMH) concentration predicts in vitro ovine oocyte developmental competence'

Currently, the commercial viability of assisted reproductive embryo technologies within the Australian livestock industry is restricted by individual variability in response to treatment protocols as well as oocyte developmental competence. The majority of losses come from embryo wastage, resulting from poor developmental competence during *in vitro* embryo production. Follicular fluid is readily available when oocytes are collected for *in vitro* embryo production from juvenile or mature ewes making it an appropriate target for analysis of phenotypic markers of oocyte developmental competence. Plasma anti-Müllerian hormone (AMH) is correlated with pregnancy losses, oocyte recovery and blastocyst development in sheep and cattle and is an indicator for donors that respond best to gonadotrophin stimulation protocols in sheep, cattle and goats. The aim of the current work was to determine the relationship between follicular fluid AMH and *in vitro* embryo production outcomes in sheep. Briefly, pairs of ovaries from 38 abattoir-derived lambs were collected individually and transferred to the laboratory. Ovaries were aspirated for *in vitro* embryo production following previously described methods (Walker *et al.* 1996 Biol. Reprod. **55**, 703-8) and follicle counts completed. Aspirated oocytes from each of the 38 individual lamb's pair of ovaries were pooled (n= 4.11 ± 0.53 COCs matured/ lamb; Total COC matured= 156), and remained as such during maturation, fertilisation and culture. The remaining follicular fluid was centrifuged for 10 minutes at 3000 rpm to remove excess cells and frozen at -20 °C. AMH was measured in follicular fluid by a human AMH Gen II ELISA kit validated for ovine samples (Beckman Coulter USA, A79766). Correlations between follicular fluid AMH concentrations and oocyte maturation and blastocyst development were determined using simple linear regression. Animals were divided into groups based on AMH concentrations (LOW (0.5- 10.8 ng/ml), MED (10.81- 17.89 ng/ml) or HIGH (17.9- 19.25 ng/ml)), with an unbalanced ANOVA used to determine group effects on oocyte maturation and blastocyst development (Genstat 18th edition). Follicular fluid AMH was positively correlated ($P< 0.05$) with the number of follicles greater than 2 mm ($r^2=0.120$) and the proportion of cumulus oocyte complexes cleaved from recovered oocytes ($r^2=0.134$). The number of COC matured per lamb was greater for those with High and Medium versus Low AMH (5.6 ± 0.97 and 4.4 ± 0.72 versus 2.1 ± 0.97 COC / lamb). Animals with high AMH produced more blastocysts than those with medium or low AMH, when expressed as a proportion of COC recovered ($P<0.002$) or cleaved ($P<0.009$) oocytes. High AMH was also correlated with a greater number of expanded blastocysts produced from cleaved oocytes ($P<0.042$).

The current data supports previous evidence that AMH concentrations positively correlate to higher antral follicle counts. The correlation between AMH and components of oocyte developmental competence suggest intra-follicular AMH may indicate the best oocytes to use for an *in vitro* embryo production system.

19th International Congress on Animal Reproduction (Cancelled Satellite meeting July 2020)

J. Daly, J. Kelly, K. Kind and W. van Wettere, 'Circulating anti-Mullerian hormone from 5 month old Merino ewe lambs can predict birthing rates at their first service'

Anti-Müllerian hormone (AMH) is an accurate phenotypic marker of antral follicle numbers and responsiveness to stimulation protocols in cattle and sheep. However, the relationship between pre-pubertal AMH and fertility of mature breeding females is poorly understood. Fertility at first mating and AMH has been correlated in Rasa Aragonesa and Sarda ewe lambs. The relationship between pre-pubertal AMH and the fertility of Australian Merino ewes has not yet been investigated. This study determined whether pregnancy and birthing rates of Australian Merinos following their first mating was related to plasma AMH concentration at 5 months old.

At 5 months of age, a single blood sample was collected from 86 Merino ewe lambs managed under routine husbandry at a Research Centre in South Australia. At 18 months of age, ewes were housed with harnessed Merino rams for 35 days (24 hours / day). Ultrasound was used to determine pregnancy status, at an average of day 60 pregnancy. AMH concentrations in plasma were measured using an ovine specific AMH ELISA kit (Ansh laboratories, Texas, USA). Differences in AMH between groups of ewes that became pregnant and lambed and those that did not were determined using an ANOVA, unbalanced design (Genstat 19th Edition; VSC International). Data is presented as mean ± SEM.

Ewes scanned pregnant (n=70) had higher AMH at 5 months of age than those scanned not-pregnant (n=16) (2.74± 0.22 vs 1.64± 0.48 ng/ml; P<0.05). AMH was higher (P<0.05) for ewes which birthed (n=71) a lamb compared with those which did not (n=15) (2.83± 0.21 vs 1.26± 0.48 ng/ml). AMH concentrations at 5 months of age were higher (P<0.05) for singleton and twin-bearing ewes compared with non-lambing ewes (2.74± 0.23 and 3.34± 0.81 vs 1.26± 0.48 ng/ml, respectively), but similar for singleton and twin-bearing ewes.

To the best of our knowledge, this the first data in the Merino to demonstrate that circulating AMH concentrations at 5 months of age are higher for hoggets which produce a lamb following their first mating. This finding is consistent with previous evidence that AMH can predict fertility in Rasa Aragonesa and Sarda ewes. Ongoing studies by our group will determine the relationship between AMH at weaning and lifetime fertility and productivity of Merino ewes.

J. Daly, J. Kelly, K. Kind and W. van Wettere, 'Post-natal anti-Mullerian hormone profiles for Australian Merino and Suffolk ewe lambs'

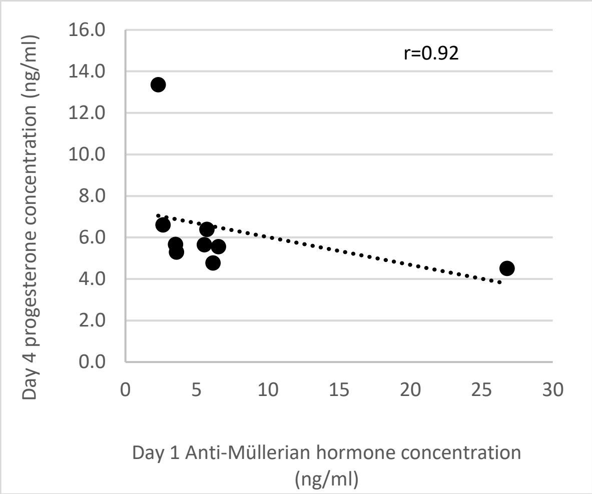
In sheep, anti-Müllerian hormone (AMH) can predict ovarian response to FSH stimulation, ovarian reserve, and fertility at first mating. Therefore, AMH may be a suitable phenotypic marker for fertility in sheep; however, AMH profiles have not be established for breeds utilised in Australia. This experiment determined how circulating AMH concentrations change between two and 18 weeks of age in Suffolk and Merino ewe lambs.

Blood samples were collected fortnightly from two to 18 weeks of age from 42 ewe lambs (n=20 Suffolk and n=22 Merino) and plasma AMH was determined using an ovine specific AMH ELISA kit (Ansh laboratories, Texas, USA). Differences between breeds were determined using an ANOVA, unbalanced design (Genstat 18th Edition, VSC international). Data are presented as Mean ± SEM.

Compared with Suffolk ewe lambs, AMH concentrations were higher (P < 0.01) in Merino ewe lambs at 2 weeks (13.1 ± 2.57 vs 2.8 ± 0.93 ng/ml), 6 weeks (26.1 ± 2.46 vs 11.1 ± 1.55 ng/ml), 10 weeks (10.8 ± 1.37 vs 5.6 ± 0.92 ng/ml) and 18 weeks (1.55 ± 0.21 vs 0.81 ± 0.08 ng/ml). For each breed, two distinct AMH profiles were evident. In 13 Suffolk lambs (PEAK), AMH peaked between 6 and 8 weeks of age (15.1 ± 1.37 ng/ml) and decreased thereafter, with no distinct peak observed in the remaining animals (FLAT). Average AMH differed between these cohorts of Suffolk lambs (FLAT; 1.5 ± 0.22 vs PEAK; 6.0 ± 0.39 ng/ml, P< 0.05). In contrast, an AMH peak was observed in all Merino lambs at 6 weeks of age. However, in 14 lambs this peak was preceded by a trough at 4 weeks of age (AMH = 3.8 ± 2.76 ng/ml), with no drop observed in the remaining animals (AMH = 19.4 ± 3.76 ng/ml) ($P < 0.05$).

There appears to be two distinct AMH profiles for both of the widely utilised breeds in Australia. Similar to the profiles reported for Sarda ewe lambs, Suffolk ewe lambs show either a single AMH peak at approximately 6 weeks of age, or a gradual increase in AMH. In contrast, and not previously described, AMH peaked in all Merino ewes lambs; however, in a proportion of animals this peak was preceded by a distinct trough. The current data demonstrate that post-natal AMH profiles differ between breeds; whether this is reflected in differences in ovarian development requires further investigation.

Appendix three: Additional correlation graphs corresponding to chapter three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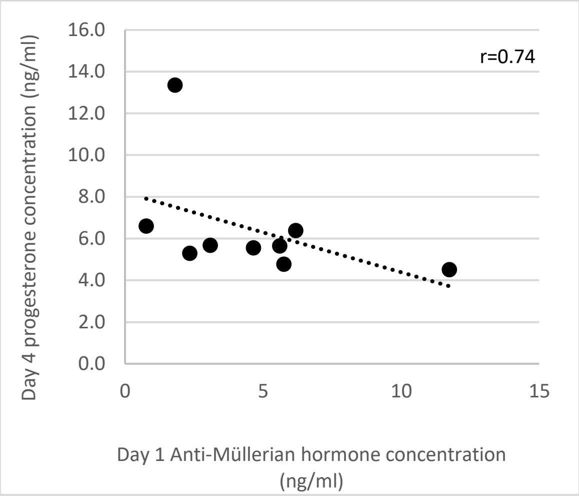
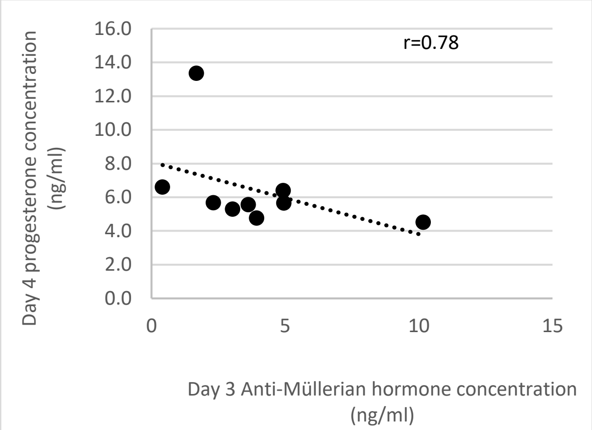
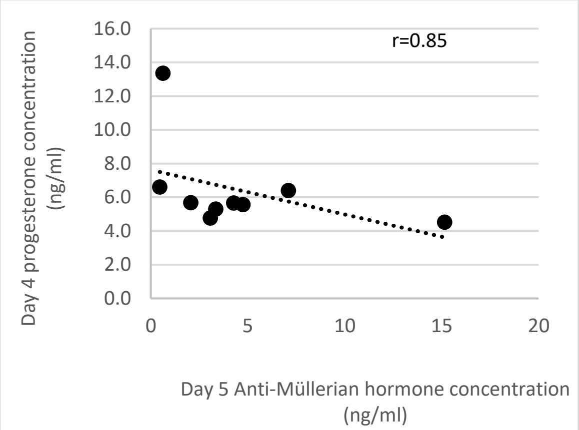
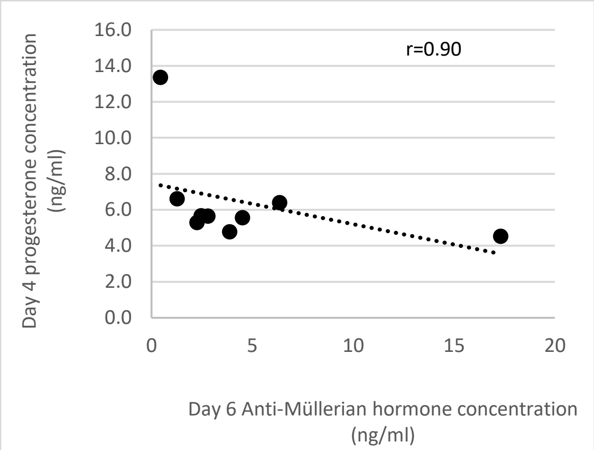
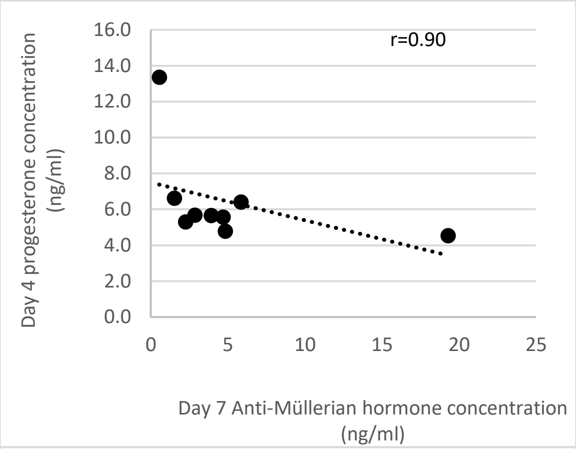
Day 4 progesterone concentration (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
25
30
Day 1 Anti-Müllerian hormone concentration (ng/ml)

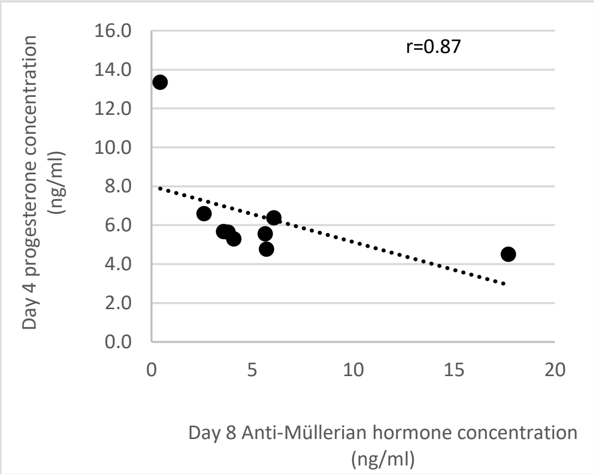

r=0.74
Day 4 progesterone concentration (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
Day 1 Anti-Müllerian hormone concentration (ng/ml)

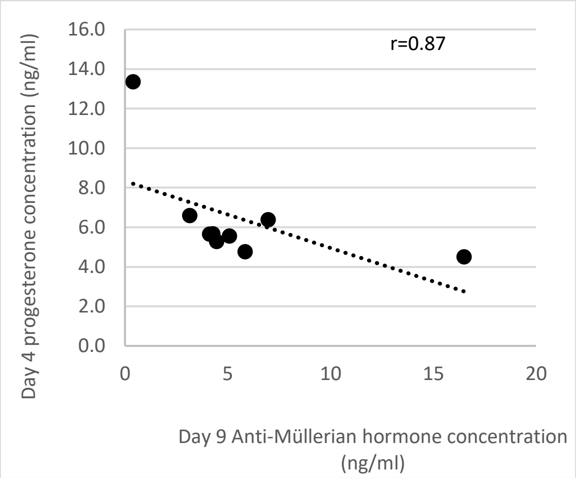

r=0.78
Day 4 progesterone concentration (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
Day 3 Anti-Müllerian hormone concentration (ng/ml)

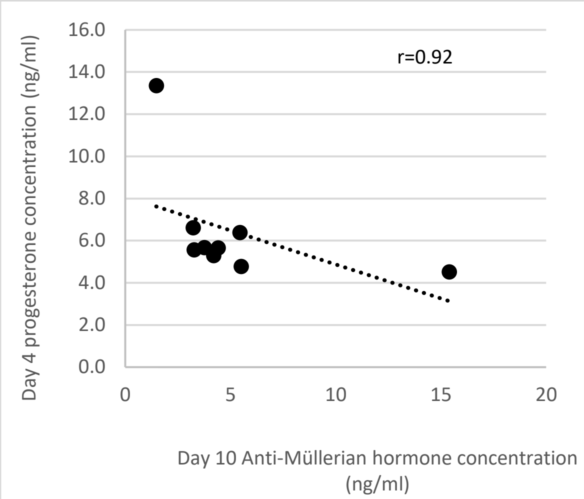

r=0.85
Day 4 progesterone concentration (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 5 Anti-Müllerian hormone concentration (ng/ml)

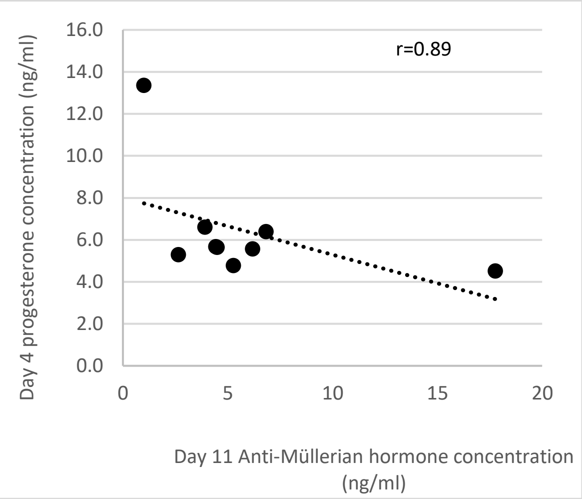

r=0.90
Day 4 progesterone concentration (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 6 Anti-Müllerian hormone concentration (ng/ml)

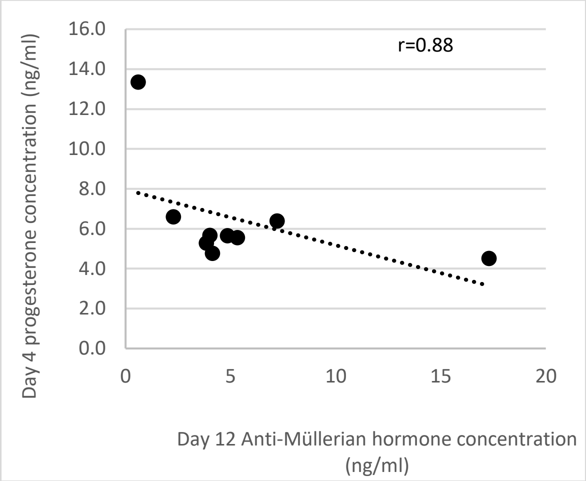

r=0.90
Day 4 progesterone concentration (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
25
Day 7 Anti-Müllerian hormone concentration (ng/ml)

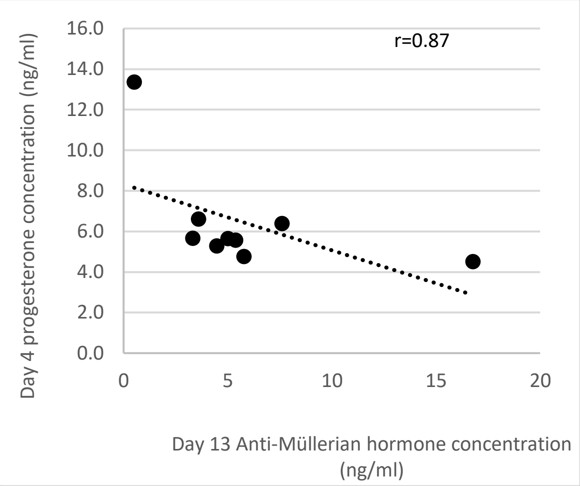

r=0.87
Day 4 progesterone concentration (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 8 Anti-Müllerian hormone concentration (ng/ml)

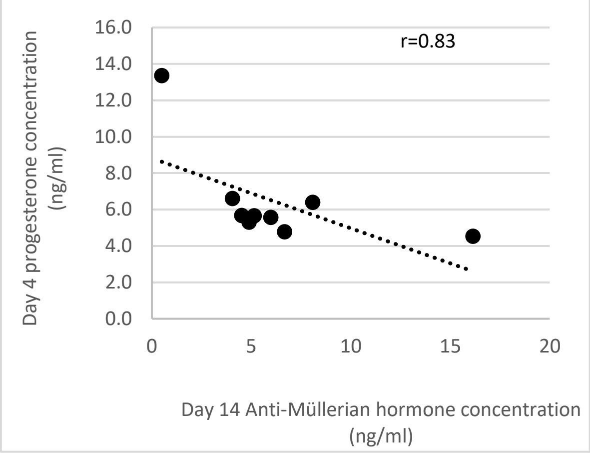

r=0.87
Day 4 progesterone concentration (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 9 Anti-Müllerian hormone concentration (ng/ml)

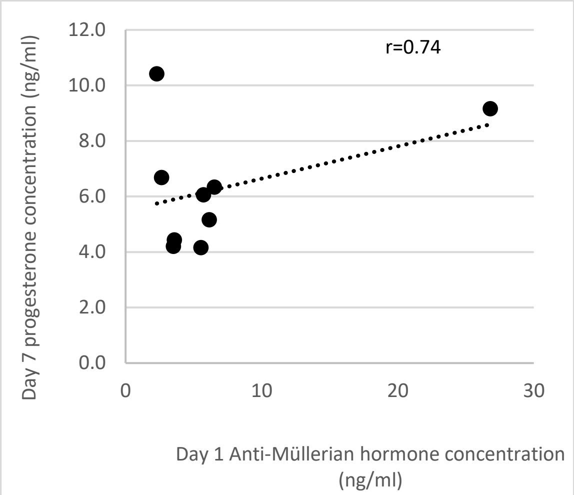

r=0.92
Day 4 progesterone concentration (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 10 Anti-Müllerian hormone concentration (ng/ml)

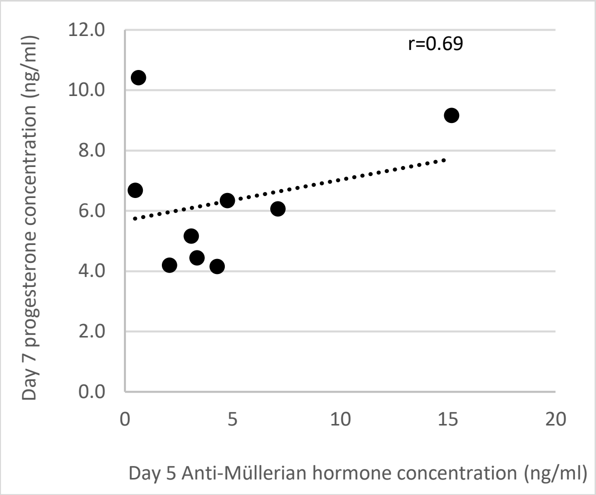

r=0.89
Day 4 progesterone concentration (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 11 Anti-Müllerian hormone concentration (ng/ml)

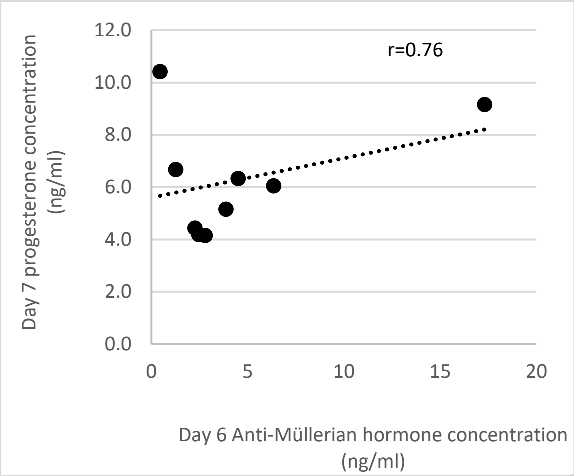

r=0.88
Day 4 progesterone concentration (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 12 Anti-Müllerian hormone concentration (ng/ml)

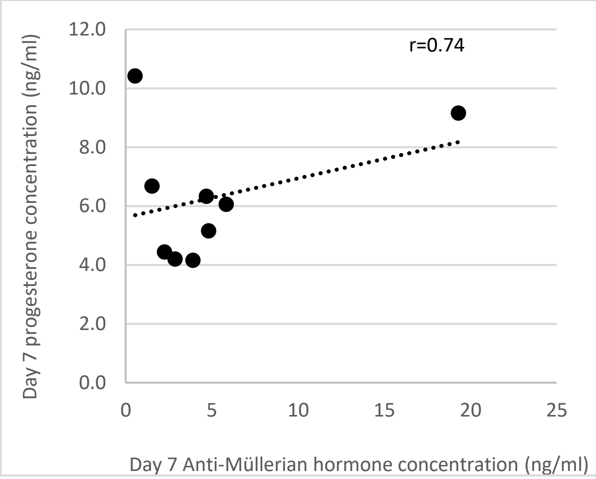

r=0.87
Day 4 progesterone concentration (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 13 Anti-Müllerian hormone concentration (ng/ml)

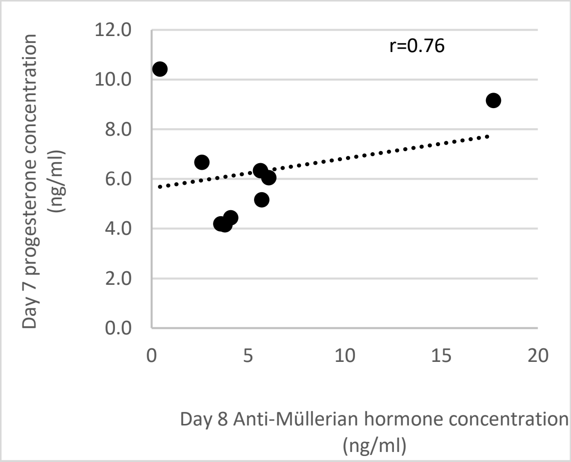

r=0.83
Day 4 progesterone concentration (ng/ml)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 14 Anti-Müllerian hormone concentration (ng/ml)

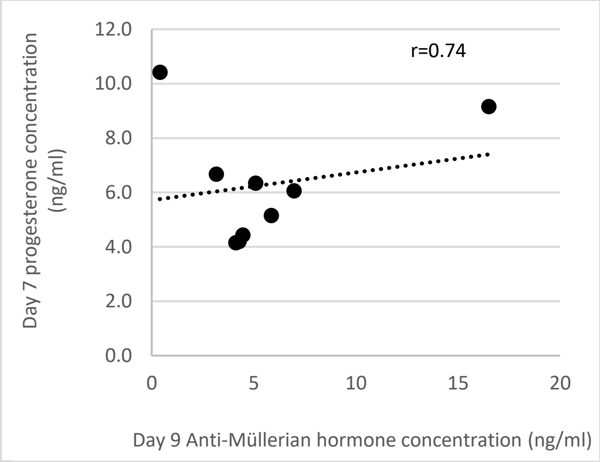

Day 7 progesterone concentration (ng/ml)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
10
20
30
r=0.74
Day 1 Anti-Müllerian hormone concentration (ng/ml)

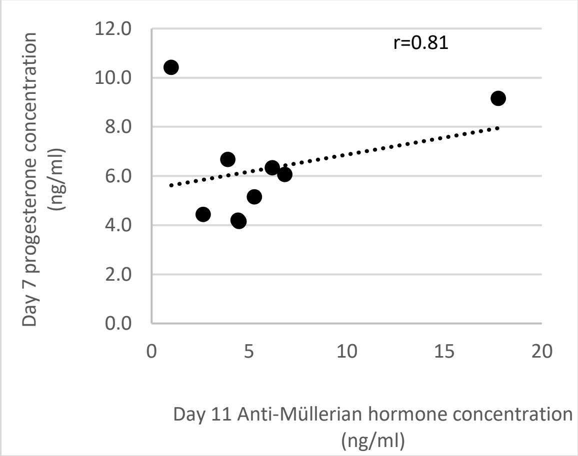

Day 7 progesterone concentration (ng/ml)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
r=0.69
Day 5 Anti-Müllerian hormone concentration (ng/ml)

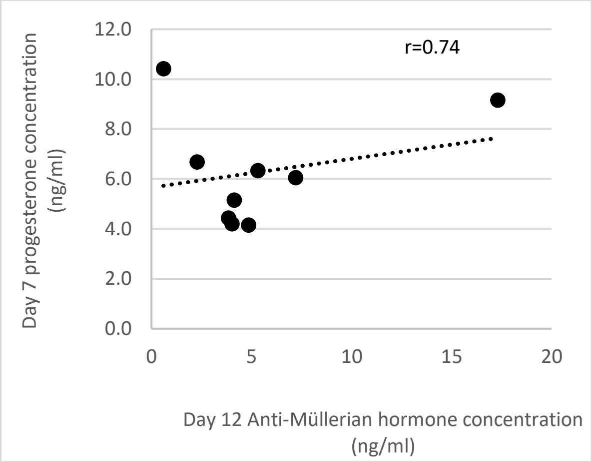

Day 7 progesterone concentration (ng/ml)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
r=0.76
Day 6 Anti-Müllerian hormone concentration (ng/ml)

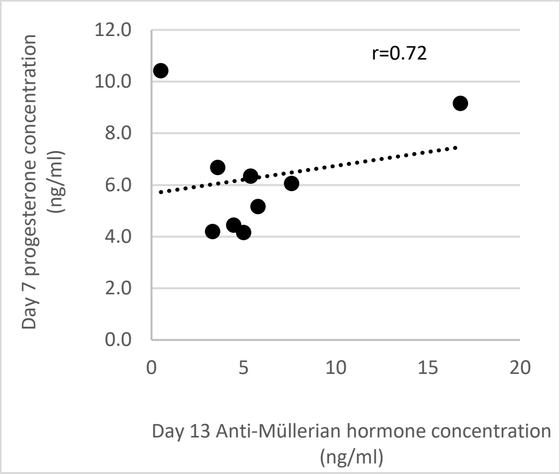

Day 7 progesterone concentration (ng/ml)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
25
r=0.74
Day 7 Anti-Müllerian hormone concentration (ng/ml)

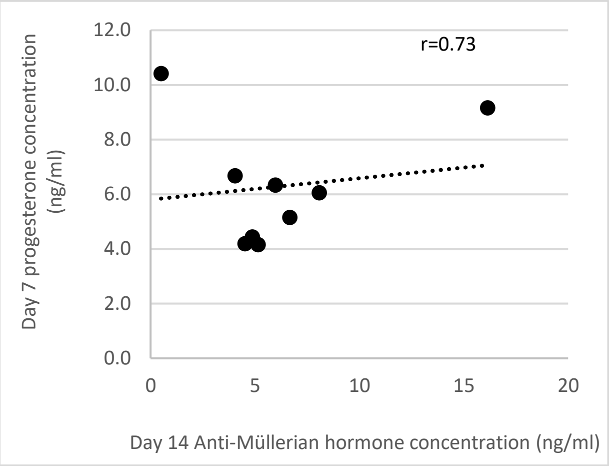

Day 7 progesterone concentration (ng/ml)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
r=0.76
Day 8 Anti-Müllerian hormone concentration (ng/ml)

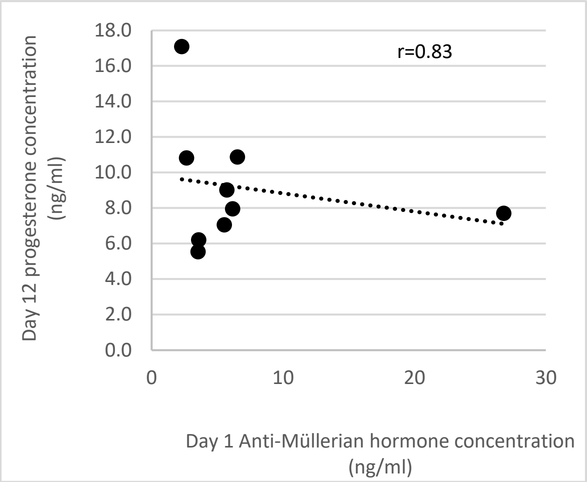

Day 7 progesterone concentration (ng/ml)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
r=0.74
Day 9 Anti-Müllerian hormone concentration (ng/ml)

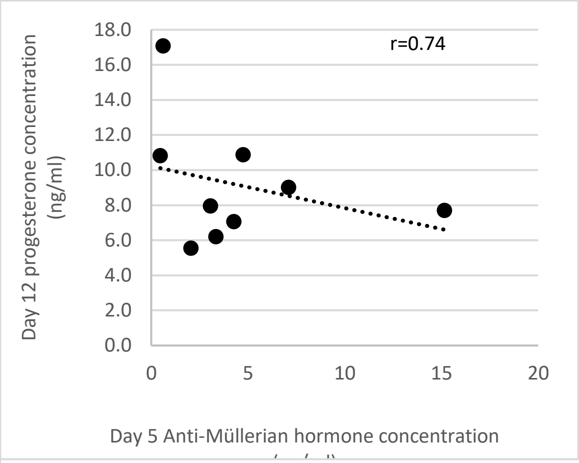

Day 7 progesterone concentration (ng/ml)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
r=0.81
Day 11 Anti-Müllerian hormone concentration (ng/ml)

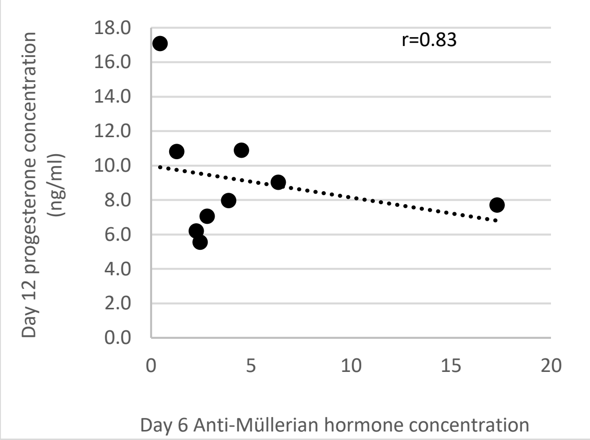

Day 7 progesterone concentration (ng/ml)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
r=0.74
Day 12 Anti-Müllerian hormone concentration (ng/ml)

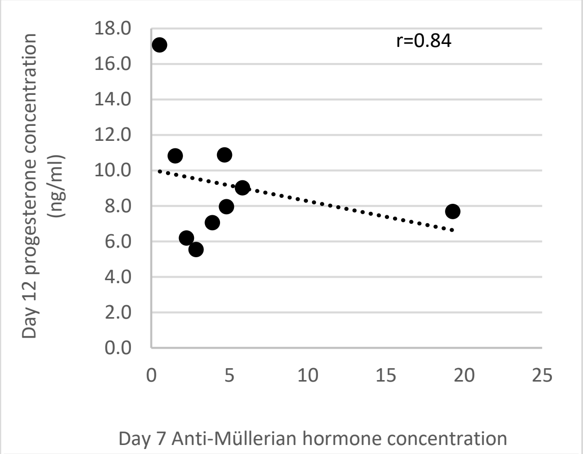

r=0.72
Day 7 progesterone concentration (ng/ml)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 13 Anti-Müllerian hormone concentration (ng/ml)

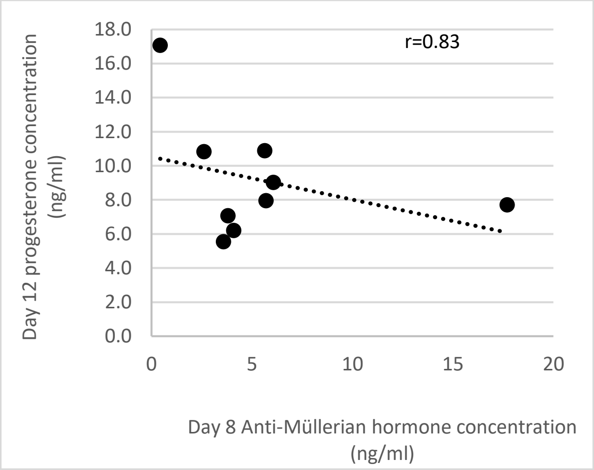

r=0.73
Day 7 progesterone concentration (ng/ml)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 14 Anti-Müllerian hormone concentration (ng/ml)

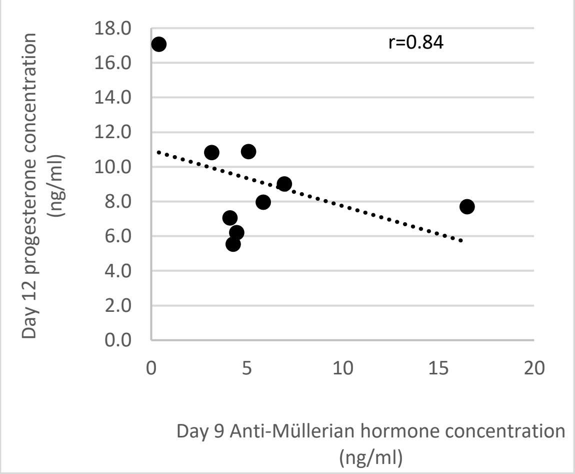

r=0.83
Day 12 progesterone concentration (ng/ml)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
10
20
30
Day 1 Anti-Müllerian hormone concentration (ng/ml)

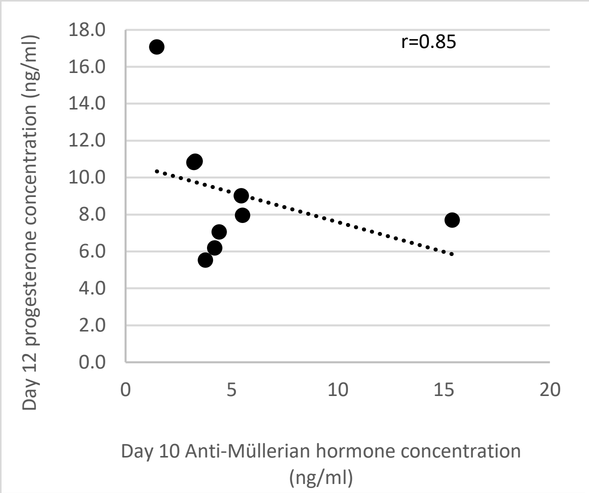

r=0.74
Day 12 progesterone concentration (ng/ml)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 5 Anti-Müllerian hormone concentration

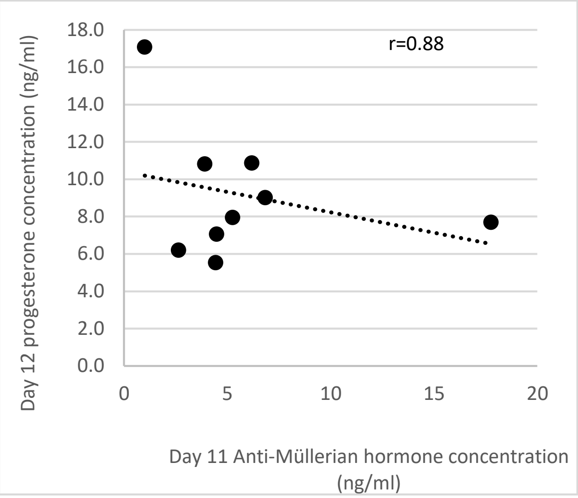

r=0.83
Day 12 progesterone concentration (ng/ml)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 6 Anti-Müllerian hormone concentration

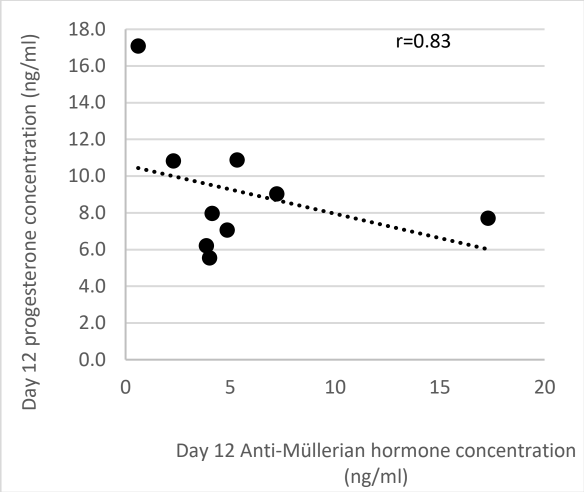

r=0.84
Day 12 progesterone concentration (ng/ml)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
25
Day 7 Anti-Müllerian hormone concentration

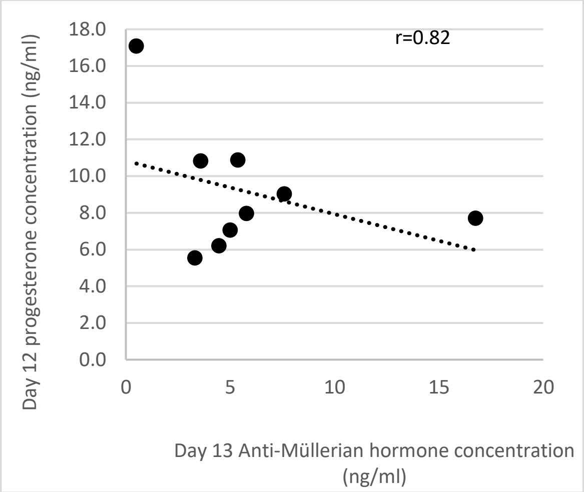

r=0.83
Day 12 progesterone concentration (ng/ml)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 8 Anti-Müllerian hormone concentration (ng/ml)

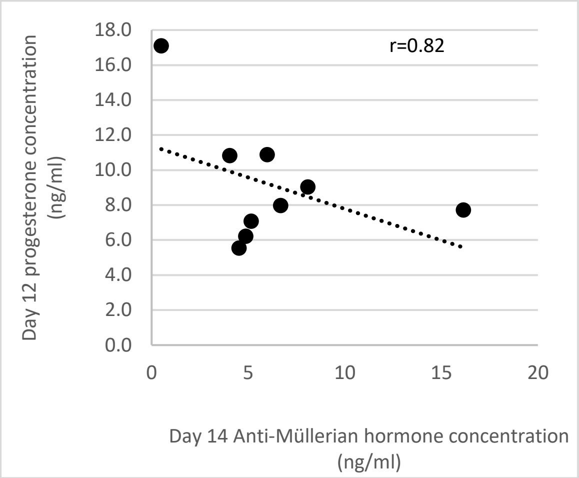

r=0.84
Day 12 progesterone concentration (ng/ml)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 9 Anti-Müllerian hormone concentration (ng/ml)


r=0.85
Day 12 progesterone concentration (ng/ml)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 10 Anti-Müllerian hormone concentration (ng/ml)


r=0.88
Day 12 progesterone concentration (ng/ml)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 11 Anti-Müllerian hormone concentration (ng/ml)


r=0.83
Day 12 progesterone concentration (ng/ml)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 12 Anti-Müllerian hormone concentration (ng/ml)


r=0.82
Day 12 progesterone concentration (ng/ml)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 13 Anti-Müllerian hormone concentration (ng/ml)


r=0.82
Day 12 progesterone concentration (ng/ml)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
5
10
15
20
Day 14 Anti-Müllerian hormone concentration (ng/ml)