Contributed paper

CHARACTERISATION OF SPERMATOZOAL TRANSCRIPTOMES IN SHEEP AND THE INFLUENCE OF BREED AND SEMEN QUALITY

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SUMMARY

Reproductive success, particularly after AI, is dependent on a number of contributing factors on both the ewe and ram sides. While there has been considerable emphasis on characterising ewe side contributions to reproductive success, relatively little emphasis has been placed on defining ram side contributors. In this context, the quality of semen used in AI is a crucial factor. Research details that spermatozoa contain around 14,000 mRNA transcripts (Selvaraju *et al.* 2017), which are transferred to the ova on fertilisation, conceivably influencing early embryonic development and successful conception. Therefore, this study aims to characterise the ovine spermatozoal transcriptome and investigate whether spermatozoal transcriptomes differ between breeds and between semen samples with high or low quality. Semen was collected (n=45) across three breeds; Merino, Dohne and Poll Dorset, and each ejaculate was subjected to computer assisted semen analysis (CASA) for assessment of quality parameters. RNA Sequencing and differential gene expression analysis identified 754 differentially expressed genes that were identified to play crucial roles in a variety of physiological functions, including fertilisation, embryonic development, and offspring production.

INTRODUCTION

Artificial insemination (AI) is increasingly used in sheep breeding as it shortens the lambing period and allows for a single ejaculate to be used to inseminate a large number of ewes. While there are a number of contributing factors to conception success, a number are linked with seminal origin (Saacke 2008). Thus, it is crucial to characterise mechanisms underlying seminal factors which contribute to conception success. While it is generally accepted that semen quality influences conception outcomes, the magnitude of this influence has been difficult to characterize, primarily because visual assessment, frequently used to determine semen quality, can be highly subjective. However, computer assisted semen analysis (CASA), which enables repeatable assessment of semen quality parameters, now affords an objective alternative for determination of semen quality. Spermatozoa are known to contain a range of transcripts that can potentially influence fertilisation (Vijayalakshmy *et al*. 2018), and even offspring phenotype (Rando 2012). This study aims to characterise the transcriptome of three common sheep breeds in Australia, and determine whether spermatozoal transcriptome varies between breeds and between ejaculates of varying quality. These investigations could lead to the development of molecular markers and in vitro measures that could assist in predicting successful reproduction when specific ejaculates are used in AI programs.

MATERIALS AND METHODS

Animals and assessment. Semen was collected from 3 sheep breeds; Merino (n=16), Dohne Merino (n=16), and Poll Dorset (n=13). Rams were closely matched for age (~18 months old), location and management conditions. Immediately following collection, each ejaculate was split into 2 aliquots; 250µL was diluted 1:10 (Nutrixcell, IMV Technologies) maintained at 37^{O}C for 4 hours then assessed utilising a CASA, with remaining semen snap frozen and stored in liquid

nitrogen until RNA isolation. Each ejaculate was ranked (Gillian *et al.*, 2008) to identify ejaculates with high and low quality for RNA Sequencing analysis. Following RNA isolation (Kasimanickam and Kasimanickam 2019), the RNA integrity number was assessed and samples higher than 8 were kept for sequencing; Merino (n=12), Dohne (n=12), Poll Dorset (n=12).

RNA sequencing. Novogene (Singapore) utilised the NEBNext Ultra RNA Library Prep Kit for Illumina was used to fragment the RNA and synthesize the complementary DNA (cDNA) library. The sequencing of cDNA libraries was performed, obtaining 100 bp paired-end reads. Quality of reads was assessed, poor quality bases (Phred score Q<30), adaptors, and overrepresented sequences filtered out. Outliers and samples with low mapping rates to the ovine genome were also excluded.

Differential expression analysis. Quality control was performed and genes with low expression were removed. Differential gene expression analysis was performed within the R software environment to identify all genes that were either up or down regulated with a log fold change >1. A false discovery rate threshold of < 0.05 was applied to control type I error. Four contrasts were performed utilising Merino (n = 9), Dohne (n=10), and Poll Dorset (n = 12); three were between breeds (Dohne vs. Merino, Dohne vs. Poll Dorset, and Merino vs. Poll Dorset); and the fourth compared ejaculates of relatively high and low qualities fitting the breeds, to account for possible breed differences. The makeup per breed for the ejaculates ranked as being relatively low include Merino (n= 4), (Dohne (n= 5), and Poll Dorset (n = 6). Similarly, the ejaculates ranked as being relatively high include Merino (n= 5), (Dohne (n= 5), and Poll Dorset (n = 6).

RESULTS AND DISCUSSION

Spermatozoal cells contain a range of RNA transcripts that are transferred to the ovum during fertilisation. However, the physiological role of spermatozoal RNA, particularly in relation to fertility and embryonic development, remain largely unknown. Therefore, the key objectives of this study were to characterise the ovine spermatozoal transcriptome, and determine whether transcriptomic profiles varied between breeds, and between semen ejaculates of varying quality.

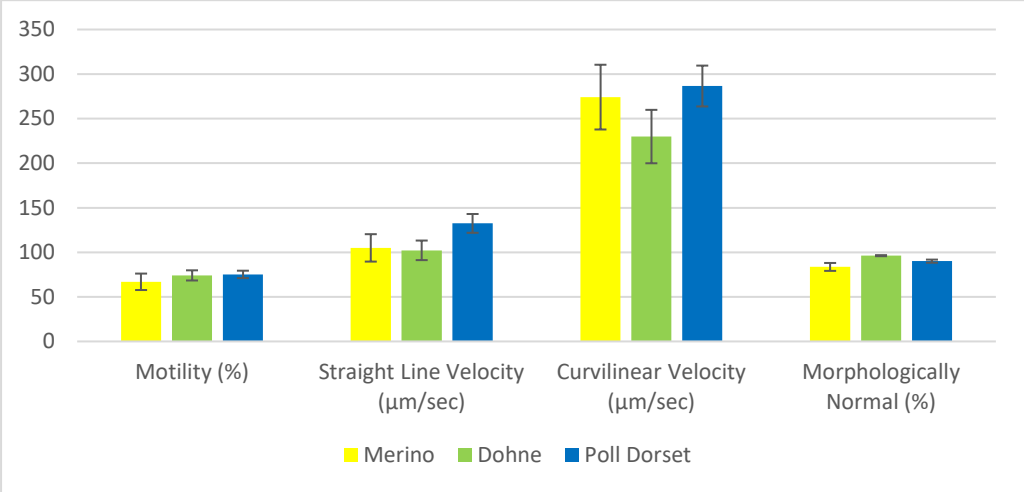

Figure 1. Semen quality parameters (Mean ± SE) for Dohne, Merino, and Poll Dorset

Differentially expressed genes. A total of 1,187,335,440 mapped reads across the three breeds sampled were mapped against the latest publicly available reference genome (Oar v.4.) at an average rate of 88%. According to the number of differentially expressed gene (DEGs) between contrasts, the transcriptomic profile of Merino and Dohne rams appear similar, in comparison to the

transcriptomic profile of the Merino compared to the Poll Dorset. Respectively; 570, 72, 73, and 39 DEGs were found between the breed comparisons Merino vs. Poll Dorset, Dohne vs. Merino, Dohne vs. Poll Dorset, and ejaculate quality contrasts (descriptive statistics for quality parameters shown in Figure 1). Figure 2 displays the DEGs found for each comparison group.

Of the 39 DEGs found when contrasting ejaculates that were determined to be relatively high and low quality, 10 were found in literature to be associated with reproduction. Most noteworthy DEGs associated with reproduction included *PRM3*, *SPEM2,* and *OXCT2*. *Protamine 3* (*PRM3*) is significantly enriched for spermatogenesis, gonad development and hormone synthesis in sheep following next generation sequencing of sheep testis (Yang *et al*. 2018). Stafuzza *et al*. (2019) found *SPEM family member 2* (*SPEM2*) to be associated with embryonic development and number of piglets born alive following a GWAS. Georgiadis *et al*. (2015) used *3-oxoacid CoA-transferase* 2 (*OXCT2*) as a post-fertilisation and early embryonic marker using quantitative polymerase chain reaction (qPCR) when investigating high quality RNA in human semen.

Eleven of the 39 DEGs found in ejaculate quality contrast are associated with growth and production traits. Key genes linked with growth and production are *BRI3BP*, *LYRM4*, *KLK10*, and *MFSD9*. Following GWAS conducted in cattle, *BRI3 binding protein* (*BRI3BP*) has been shown to be associated with carcass traits (Lee *et al*. 2012), and *LYR motif containing 4* (*LYRM4*) is significantly associated with rib eye area (Wang *et al*. 2020). Kern *et al*. (2016) found *kallirein related peptidase 10* (*KLK10*) to be up regulated in a study looking feed intake and efficiency in cattle, suggesting that it could play a similar role for growth and development in sheep. Likewise, Perland *et al*. (2018) validated *major facilitator superfamily domain containing 9* (*MFSD9*) as a central solute carrier which is expressed in the food regulatory areas of the brain, resulting in increased feed intake, and increased growth.

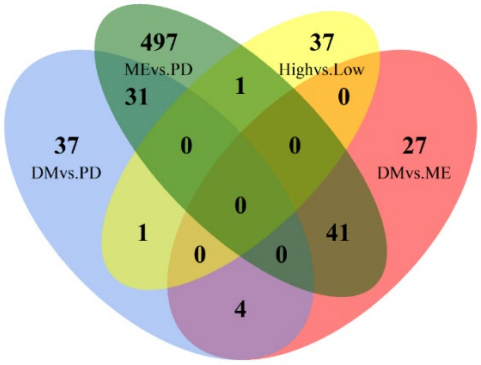

Figure 2. Venn diagram of DEGs for each breed contrast and semen quality contrast; DM: Dohne, PD: Poll Dorset, ME: Merino

Notable genes found when comparing three breeds sampled were subjected to a literature review. The *5'-aminolevulinate synthase 1* (*ALAS1*) is a gene of interest identified in the Merino vs. Dohne contrast, it is known to regulate circadian networks in cattle, which could influence the regulation of reproduction in seasonal breeding species like sheep (Wang *et al*. 2015). Likewise, Edwards *et al*. (2013) undertook a GWAS in cattle and found *capping protein regulator and myosin 1 linked* (*CARMIL1*) to be significantly associated with fertility.

In the Merino vs Poll Dorset contrast, *solute carrier family 35 member A5* (*SLC35A5*) and *integral membrane protein 2C* (*ITM2C*) were identified as key DEGs. In cattle, a GWAS found *SLC35A5* to be associated with fertility (Parker Gaddis *et al*. 2016). Similarly, expression of *ITM2C*

is significantly enriched in the epididymis and vas deferens in both humans and mice during sexual maturation (Rengaraj *et al*. 2007).

Key DEGs identified in the Dohne vs. Poll Dorset contrast included; *DNA polymerase lappa* (*POLK*), which is developmentally regulated in testis of human and mice, and is hypothesised to play a crucial role in spermatogenesis (Ogi e*t al*. 2001); and *mannosidase alpha class 1A Member 1* (*MAN1A1*), which is associated with 6 month weight in sheep in a GWAS (Gholizadeh *et al*. 2015).

CONCLUSION

The current study provides important insights into spermatozoal transcriptomes in sheep, and suggests that future investigations may target specific genes found to be differentially expressed in our study. Validation of our results in an independent population is also warranted. Furthermore, we have observed some of the differentially expressed genes are expressed stably within breeds, while others are expressed variably within breeds. This also deserves further scrutiny.

ACKNOWLEDGEMENTS

This work was financially supported by the Graham Centre (Charles Sturt University) and Apiam Animal Health. We also kindly thank the producers who assisted with this research.

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