Frequencies of Morphological Sperm Abnormalities in Australian Bulls and Rams

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Table of contents

1. Abstract

Levels of abnormal sperm within an ejaculate can influence the reproductive productivity in livestock. This study aimed to establish sperm abnormality frequencies as per the Australian Cattle Veterinarians eight-category system amongst Australian bulls and rams. It was hypothesised rams would present lower levels of sperm abnormalities than bulls based on the higher normal sperm sheep industry standard. Semen samples were sourced from bulls (n = 300) and rams (n = 149) of various breeds located throughout NSW and SA and were fixed in 5% formalin in buffered saline and stored in a 4°C before assessment. Differential inference contrast optics at x400 magnification were used to count 100 cells per sample, which were categorised as per the Australian Cattle Veterinarians eight-category system. Both rams and bulls followed similar trends in frequencies across all eight sperm morphology categories with loose heads/abnormal tails being the most prevalent (11.54% and 10.23% respectively). Percentage mean of normal sperm was significantly higher in bulls (74.03%) when compared to rams (68.05%), rejecting the hypothesis ($P < 0.01$). The findings in this paper allows a preliminary understanding of what can be expected to appear in the average Australian ram ejaculate. Further investigation into abnormality frequencies as characterised by the eight-category system within a larger ram population would gain understanding of normal levels of sperm abnormalities within a ram ejaculate.

Key words

Bulls, frequencies, rams, sperm abnormality, sperm evaluation, sperm morphology

2. *Introduction*

The prevalence of abnormal sperm within an ejaculate can influence the sample quality and fertility of the male. In comparison to cattle, morphological sperm assessments in rams are seldom performed alongside breeding soundness evaluations. Knowing average frequencies of sperm abnormalities provides clinicians with insight into the level of normal and abnormal sperm likely to be seen in a population of bulls or rams.

Acceptable thresholds of outlined sperm abnormalities in livestock determines whether individuals pass or fail breeding soundness exams based on sperm morphology. Thresholds for normal levels of abnormal sperm in bulls is well established amongst literature. Fordyce et al. (2006) outlines proximal cytoplasmic droplets, pyriform heads and knobbed acrosomes abnormalities have an acceptable threshold of 20%. Midpiece abnormalities, loose heads/abnormal tails, vacuoles and teratoids and swollen acrosomes have an acceptable threshold of 30% within bull ejaculates (Fordyce et al., 2006). The threshold determined in rams is more simply, no more than 15% of abnormal sperm present in an ejaculate (S.P. de Graaf, personal communication). In rams, morphological standards are not set to the same standard creating an ambiguous threshold of acceptable levels of abnormal sperm and their links to fertility. Little is known about the representation of various types of sperm abnormalities within the ejaculate of ram semen, with current data characterising ejaculate makeups of small sample sizes ($n \leq 20$; Almadaly et al., 2016; Azubuike et al., 2017; Basioura et al., 2022). Furthermore, industry standards of normal levels of abnormal sperm and their relationship to ram fertility are yet to be determined. Frequencies of abnormalities will vary depending on how sperm are categorised.

Detailed classification systems, such as the eight-category system used with Australian bulls (Fordyce et al., 2006; Koziol & Armstrong, 2018), provide reproductive biologists with insight

into which specific abnormality is impacting the percentage of normal sperm. As certain abnormalities have been linked to external stressors and sire reproductive performance, this knowledge can inform the identification of sub-fertile males (Barth, 1986; Söderquist et al., 1991). When assessing ram semen, sperm are simply classified as normal or abnormal (Evans & Maxwell, 1987). Without characterising subcategorization of the abnormal category, type and subsequent frequency of abnormalities remains unknown. Without detailed categorisation of abnormalities, it hinders insight into the root causes of these abnormalities as well as their potential relationship with negative fertility.

By having detailed categorisation of abnormalities, identifying the presence of particular abnormalities can pinpoint influences on the fertility of individual animals. Of all possible abnormalities, head morphometry has demonstrated the closest link to livestock fertility in current literature (Barth et al., 1992). This has been stated as early as 1927 by Williams and Savage (Saacke, 2004). In rams, sperm subpopulations based on head dimensions have been correlated to fertility rate (Bravo et al., 2014; Maroto-Morales et al., 2015; Santolaria et al., 2015). Furthermore, sperm with detached heads or tailless sperm are considered immobile because they can't migrate through the female tract. Bulls with over 70% of detached heads or tailless sperm are considered to have decreased fertility (Perry, 2021). Knowing this link between abnormalities and fertility should drive future research to further understanding causes of sperm abnormalities. This can provide more insight into why individuals not meeting breeding soundness thresholds set by the cattle or sheep industries.

This study aimed to establish frequencies of sperm abnormalities in ram and bull ejaculates from properties across New South Wales (NSW) and South Australia (SA), Australia, using the eight-category system. By characterising ram ejaculates in populations over 20 individuals this study addresses the gap in the literature to provide a preliminary understanding of the expected percentage of normal and abnormal sperm within Australian ram ejaculates and compare this

to bull ejaculates. Industry thresholds for accepted abnormalities within ram ejaculates is 15% (S.P. de Graaf, personal communication), while in cattle this is doubled at 30% (Perry, 2021). Based on these thresholds, it is hypothesised there will be lower frequencies of sperm abnormalities present amongst rams when compared to bulls.

3. Materials and methods

3.1. Morphological assessment of sperm

Samples from rams (n = 149) and bulls (n = 300) were sourced from 16 Australian properties across the states of New South Wales and South Australia. They were fixed in 5% formalin in buffered saline and stored in a 4°C walk in freezer prior to analysis. Samples were vortexed to ensure homogeneity prior to assessment, then 9µl of each sample was pipetted onto a glass slide and covered with a 22x22mm coverslip. As an observational study, samples were observed under an Olympus BX53 microscope with Differential Inference Contrast (DIC) optics at 400x magnification to count 100 sperm per sample. Overlapping or partially focused sperm were excluded from counts to avoid incorrect categorisation.

Sperm were categorised as per the ACV eight-category system into normal sperm, proximal cytoplasmic droplets, midpiece abnormalities, loose heads and abnormal tails, pyriform heads, knobbed acrosomes, vacuoles and teratoids and swollen acrosomes (each category detailed further in Perry (2021)). This category system was chosen because it is used in the ACV standardised BullCheck Scheme in assessing bull fertility for breeding (BullCheck Scheme, 2024). Frequencies for individual sires and categories were recorded in Microsoft Excel (version 2407).

3.2. Methods of accuracy/reliability

To ensure observer accuracy prior to data collection training samples were used to ensure consistency with expert morphologists. Prior to commencing assessment, the observer was required to reliably record results within ± 10% of the expert morphologists' results. Accuracy of counting was ensured by using an automatic counter which alerted the user when 100 sperm were recorded. Sessions were broken up with multiple breaks to reduce fatigue and ensure reliability of the observer. In cases of sperm with multiple abnormalities, abnormalities closer to the head took precedence, due to the close link between head abnormalities and fertility (Sprecher & Coe, 1996).

3.3. Minimising bias

Observer bias was managed in the categorisation of sperm using descriptions of normality/abnormality provided in Perry (2021), and comparison of visual aids as per Seymour et al. (2023).

3.4. Data analysis

To ensure confidentiality, data identifiers were removed including owner, property name and sire ID/name and replaced with generic numeric identifiers. In Microsoft Excel (version 2407) descriptive statistics of each morphological category were calculated, as well as figures obtained using Pivot Charts and Pivot Tables. Standard one tailed t-tests were conducted to make interspecies comparisons of normal levels of sperm in ejaculates.

4. Results

4.1. Mean percentages of sperm abnormality categories were similar for bulls and rams

The mean percentage of each category in both rams and bulls was compared (Figure 1). There was a similar trend in average across all eight categories in both rams and bull. The highest mean value recorded was loose heads/abnormal tails for both bulls and rams (10.23% and 11.54% respectively). Midpiece abnormalities (7.65% in bulls, 10.83% in rams) and proximal cytoplasmic droplets (5.69% in bulls, 7.95% in rams) were found next most prevalent in ejaculates. Amongst midpiece and loose heads/abnormal tail abnormalities, bulls displayed more extreme outliers in comparison to rams. The lowest recorded mean value of any abnormality was swollen acrosomes in both rams and bulls (0% and 0.03% respectively).

4.2. Percentage of mean normal sperm was significantly higher in bulls in comparison to rams

Variations were present in mean percentage of normal sperm (PNS) found within the ejaculates of each species (Figure 2A). The mean of normal sperm was significantly higher ($P < 0.01$) in bull ejaculates (74.03%) in comparison to ram ejaculates (68.05%). Interestingly, the standard deviation was higher in rams (SD = 25.83) than bulls (SD = 18.98). PNS in the bull population had a minimum of 5% and maximum of 99% (Figure 2B). Similarly, rams had a minimum PNS of 10% and maximum of 100% (Figure 2C).

4.3. Bulls passed the cattle industry threshold of >70% percentage normal sperm significantly more than rams

Finally, the proportion of individuals that passed cattle (>70%) and sheep (>85%) industry normality standards was quantified for this population (Figure 3). The number of bulls that passed the cattle industry threshold was significantly higher than rams ($P < 0.001$). When the

threshold was >85%, only one third of ejaculates from each species pass the sheep industry standard threshold.

5. Discussion

This study aimed to establish frequencies of sperm abnormalities in Australian ram and bull ejaculates from 16 properties across NSW and SA, Australia. Abnormalities were categorised as per the complex eight-category system used in bulls. Frequencies of abnormalities in all categories followed a similar trend when comparing bulls and rams. Industry thresholds outline normal sperm levels need to be higher than <70% for cattle and <85% for sheep ejaculates, and hence hypothesised lower levels of sperm abnormalities would be present within rams than bulls. The findings from this study reject this hypothesis.

Frequencies of sperm abnormalities were comparably similar of bulls and rams across all eight categories. Mean percentage of abnormalities followed similar trend in both rams and bulls (Figure 1). Loose heads/abnormal tails were the most prevalent (10.23% in bulls, 11.54% in rams), while swollen acrosomes were non-existent to rare (0.03% in bulls, 0% in rams) in ejaculates of both species. Loose heads and abnormal tails found in this study were much higher than previous literature. In bulls this abnormality is found in frequencies <6% (Azubuike et al., 2017; Callaghan et al., 2016; Menon et al., 2011a; Söderquist et al., 1991). More limited in rams, the small number of findings available showed these frequencies do not exceed 5% within an ejaculate which is contrast to the 10.23% observed in the population of rams in this study (Azubuike et al., 2017; Savage, 1984; Yániz et al., 2012). More similarly to our finding, 15.25% of abnormal tails were observed within a ram ejaculate, however it was identified as having varicocele (Basioura et al., 2022).

Swollen acrosomes were non-existent in our ram population and rare amongst bulls (0.03%, Figure 1). These were consistent with ram findings of no distorted or twisted acrosomes the

study of López Armengol et al. (2018). They were similarly identified in bulls at 0.1% (Callaghan et al., 2016), as well as present within 6% of bulls in the work of Andersson et al. (1989), however the frequency of this abnormality is not mentioned. Acrosomal swelling is considered normal after sperm undergo the acrosome reaction, suggesting swollen acrosomes present after premature offset of the acrosome reaction (Perry, 2021). The low prevalence of swollen acrosomes found in both species could be relevant to the levels of knobbed acrosomes abnormality within an ejaculate (Perry, 2010), of which were also found in low prevalence in this study. Considering the location of properties being in NSW and SA, animals are at a higher risk of experiencing heat stress during warmer months. This may explain the high frequency of abnormal tails and proximal cytoplasmic droplet abnormalities (Barth & Bowman, 1994; Perry, 2021) shown in this study, but would need to be confirmed with future studies.

The mean percentage of normal sperm (PNS) present within an ejaculate was varied between rams and bulls (Figure 2). Significantly higher levels of normal sperm were present in bulls (74.03%) than rams (68.05%), meaning more abnormalities were present in ram (Figure 2A), rejecting the hypothesis. PNS values of other bull studies ranged from 76% - 92.4%, only slightly higher (< 2%) than what was observed in this population (Al-Saedi & Abdulkareem, 2022; Callaghan et al., 2016; Freneau et al., 2010; Menon et al., 2011a; Menon et al., 2011b; Walters et al., 2004). PNS values of rams were notably higher amongst other studies, ranging from 82.25% - 96.58% (Basioura et al., 2022; Hassan et al., 1970; Yániz et al., 2012). The difference in PNS values between literature could be attributed to the different definitions of normality considered from each paper. Within this study normal bull and ram sperm were characterised as those with an intact acrosome, a smooth, rounded paddle shaped head and a long tail that lacks twists, breaks, coils or bends. Furthermore, abaxial tails, bent midpieces (bent to at least 90° without folding or breaking), segmental aplasia, distal cytoplasmic droplets slightly pyriform heads and narrow heads were also considered normal due to their lack of

influence on fertility, as per Perry (2021). Koziol and Armstrong (2018) also characterises distal cytoplasmic droplets as normal sperm in bull assessments. Given the differences in PNS values of this study and literature in both species, more notably sheep, the characterisation of normality and abnormality should be stated in future sperm morphology studies.

Interestingly, rams showed more variability in the standard deviation of mean PNS than bulls (SD = 25.83 in rams, SD = 18.98 in bulls). This finding indicates bulls may have a higher susceptibility to internal or external stressors in comparison to rams. Stressors could include environmental, health-related or handling practises, including semen collection (Fernandez-Novo, 2020; Palmer, 2005). Current literature shows there are various reasons for abnormalities presenting within the ejaculate. Heat stress has been linked to being the cause of multiple abnormalities including loose/detached heads, abnormal tails, proximal cytoplasmic droplets (Basioura et al., 2022; López Armengol et al., 2018; Perry, 2021). Variation could also stem from the number of properties ram ejaculates were sourced from when compared to bull ejaculates (11 and 5 respectively), despite the sample size of rams being almost halved. Two ram studies noted a PNS value of 5%, where in both cases rams were affected by varicocele (Ott et al., 1982; Savage, 1984), which more similarly relates to the minimum PNS found in rams was 10% (Figure 2C). It would be interesting to explore whether this was due to disease like varicocele or another factor in future. The higher variability found in ram ejaculates could be attributed to lower susceptibility to stressors or number of properties ejaculates were sources from in comparison to bulls.

Using different industry threshold for acceptable levels of normal sperm within an ejaculate altered the number of individuals that passed these thresholds. Significantly more bulls than rams passed the cattle industry standard of >70% PNS in an ejaculate (Figure 3). Interestingly, when increased to the sheep industry standard of >85%, only one third of either species passed the threshold. Industry thresholds of PNS are used as an indicator for fertility success in

breeding soundness exams (Bravo et al., 2014; Söderquist et al., 1991). The bull industry threshold of >70% PNS (Koziol & Armstrong, 2017) is more feasible in practice because there will be more sires included in the breeding program. Using the sheep industry standard of >85%, may not be appropriate for this population due to the low pass rate of ~30% of bulls or rams.

Whilst the ram population size (n = 149) in this study is the largest of any previous literature found (n ≤ 20), it is recognised the sample size may not provide an accurate representation of the total NSW, SA or Australian ram population. The findings of this study do provide a preliminary insight into the makeup of the average ejaculate of NSW and SA rams and the frequencies of normal and abnormal sperm in samples. and how this can be applied to fertility data already done in large scale studies performed in bulls. There is literature available comparing the effectiveness of sperm evaluation methodologies in cattle. This is not the case currently in rams and is suggested to be performed in future to see whether using these cattle-based protocols is truly appropriate for use in sheep studies. Building upon the population size in future research would be an interesting comparison to these findings and may gain further insight into the makeup of normal ram ejaculates. Lastly, a limitation of manual sperm assessments relies on the characterisation of abnormalities by the observer, where in this study was only a single morphologist, increasing the potential for bias in this study. Bias of the morphologist was minimised through cross referencing training samples with expert morphologist data, as well as closely following written and visual references (Perry, 2021; Seymour et al., 2023).

This study has significance in being the first study to focus on the frequency of sperm abnormalities in the ram in a population over 20 individuals, to provide a preliminary insight into the makeup of a ram ejaculate based on a detailed sperm categorisation system. It was hypothesised rams would have lower levels of sperm abnormalities in an ejaculate in

comparison to bulls. Bulls had a significantly higher PNS than rams, rejecting the hypothesis. Bulls and rams follow similar patterns of normality and abnormality frequencies, with loose heads/abnormal tails, midpiece and proximal cytoplasmic droplets being most prevalent among both species. Similarly, swollen acrosomes were the least prevalent in both species. Levels of abnormalities amongst larger populations of rams and bulls should be explored to further the findings of this study in the future. The information presented will be useful for breeders and clinicians by allowing them to categorise sperm abnormalities more accurately and understand what could be expected to appear in an average ejaculate.

6. *Figures and tables*

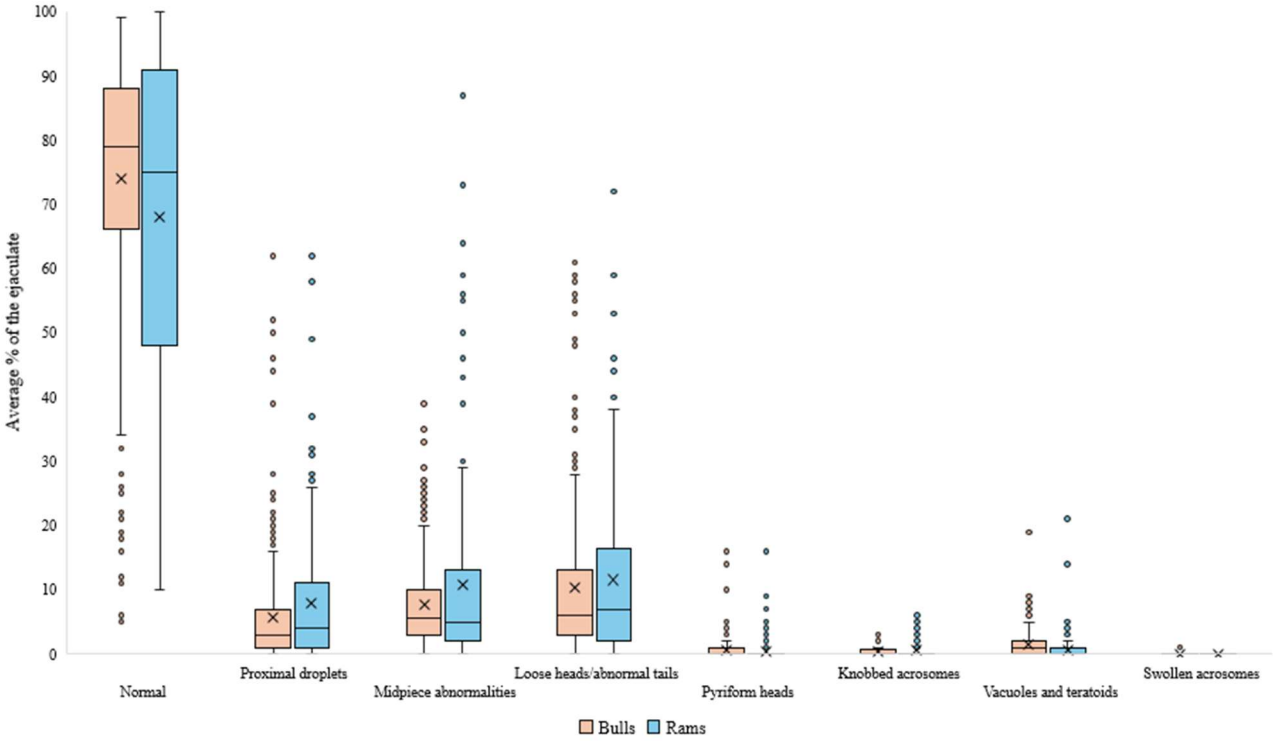

Figure 1: Sperm morphology characteristics of bull (n = 300) and ram (n = 149) ejaculates classified using an eight-category system (1. Normal sperm, 2. Midpiece abnormalities, 3. Proximal cytoplasmic droplets, 4. Loose heads/abnormal tails, 5. Pyriform heads, 6. Knobbed acrosomes, 7. Vacuoles and teratoids, 8. Swollen acrosomes).

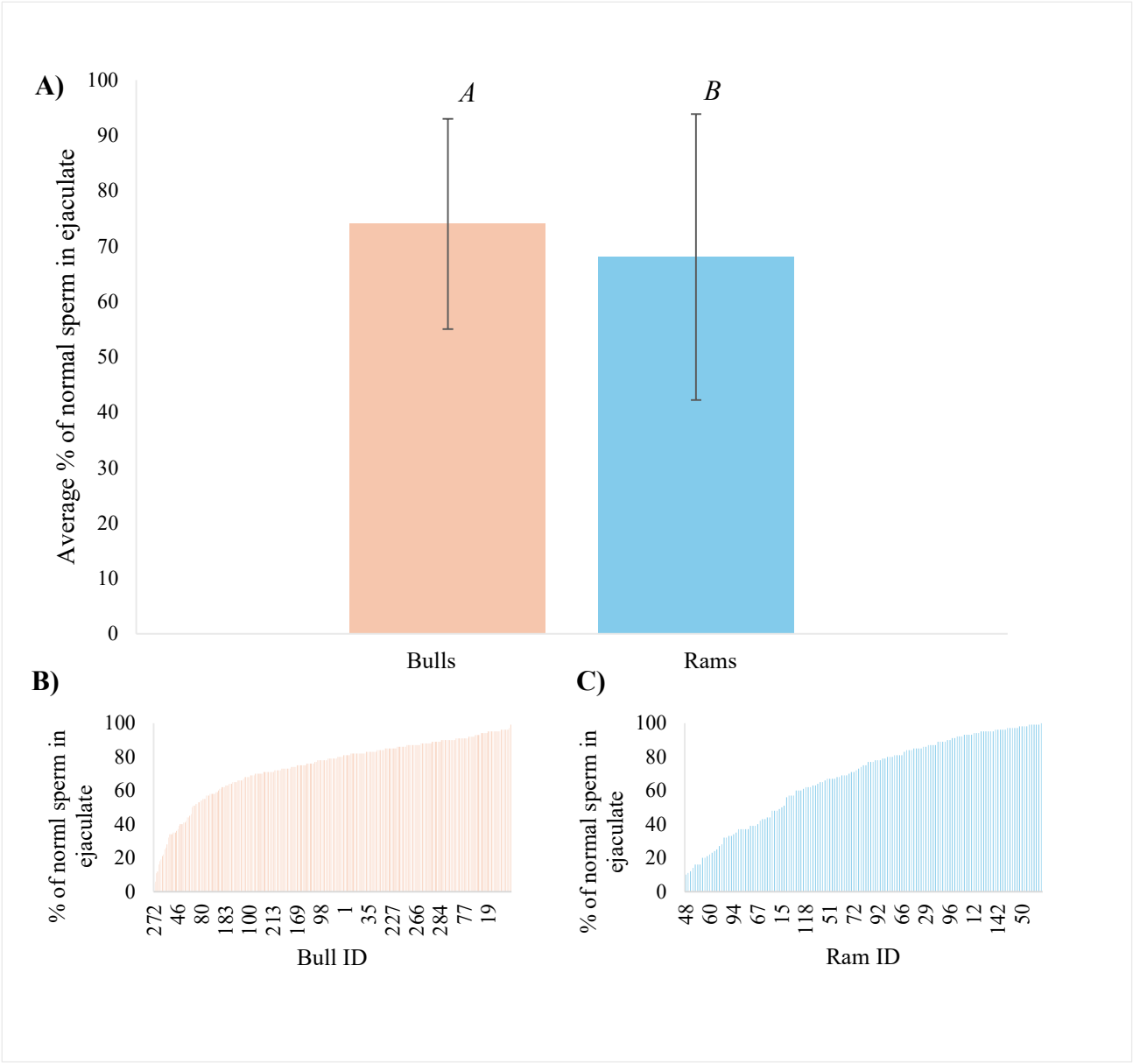

Figure 2: ***A)*** *Variation in percentage of normal sperm (PNS) in bull (n = 300, SD = 25.83) and ram (n = 149, SD = 18.98) ejaculates. Values without common superscripts differ significantly (P < 0.001).* ***B)*** *Range of normal sperm amongst bull ejaculates (n = 300, min = 99%, max = 5%).* ***C)*** *Range of normal sperm amongst ram ejaculates (n = 149, min = 100%, max = 10%).*

Table 1: Proportion of ejaculates in bulls (n = 300) and rams (n = 149) that pass respective cattle and sheep industry thresholds of normal sperm within an ejaculate to satisfy soundness breeding evaluations. Values without common superscripts differ significantly (P < 0.001).

	Cattle industry standard (>70% PNS)	**Sheep industry standard (>85% PNS)**
Bulls	69.3% (208/300)[A]	32% (96/300)
Rams	53.7% (80/149)[B]	33.6% (50/149)

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