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Original Research Article

High-throughput nuclear morphometric analysis for pig sperm quality assessment

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ABSTRACT

Artificial insemination (AI) is commonplace in commercial pig breeding, and as such, ensuring sperm sample quality is of utmost importance to avoid reduced farrowing rates and litter sizes. Here, we have used high-throughput nuclear morphometric analysis to compare pig sperm samples categorised as meeting the AI standard (AIS) or not meeting the AI standard (N-AIS) (50 vs. 44 samples from unique boars, respectively). We show that pig sperm nuclei are asymmetric, that samples contain phenotypic shape abnormalities that show continuous variation and that samples classed as N-AIS have more abnormally shaped sperm than AIS samples. This variation is not detected in conventional analysis. The specific phenotypes identified suggest the disruption of key aspects of spermiogenesis and indicate future avenues for improving pig sperm quality. Additionally, we find that the distribution and severity of abnormal sperm phenotypes were significantly associated with the day of semen collection, indicating that environmental factors may influence sperm morphology. High-throughput nuclear morphometric analysis therefore reveals significant differences in sperm head morphology between AIS and N-AIS pig sperm samples and has the potential to be further developed as a tool for sperm assessment both in the pig breeding industry and in other species.

1. Introduction

In humans, infertility is defined as the failure to achieve a pregnancy after one year of regular unprotected sexual intercourse, and this will affect around one in six people in their lifetime. Between 20 and 30% of these cases result solely from male factor infertility, with up to 50% of all instances of infertility being at least partly the result of male factor infertility [1]. The analysis of semen parameters is therefore an important part of infertility investigations, with semen volume, pH, and sperm parameters such as concentration, progressive motility and morphology all being routinely observed [2]. Many of these factors are determined during spermatogenesis, the complex process by which undifferentiated diploid spermatogonia become highly specialised haploid spermatozoa, with the process involving complicated cellular, proliferative, and developmental phases [3]. Which single sperm parameter is the most important for fertility is a source of conflict in the literature; some studies, for example, suggest that sperm progressive motility is positively associated with pregnancy rates [4,5], whilst others do not [6]. Similarly, research has indicated that sperm morphology may be correlated with positive pregnancy outcomes [7–9], but others have suggested that this plays only a limited role [10].

In agriculturally important species such as pigs, cattle, and sheep, semen analysis followed by artificial insemination (AI) is commonplace in breeding programmes [11], with 90% of sows in the world's primary pork producing countries resulting from AI [12]. As such, an industry-led working code of practice, the AI standard for pigs has been

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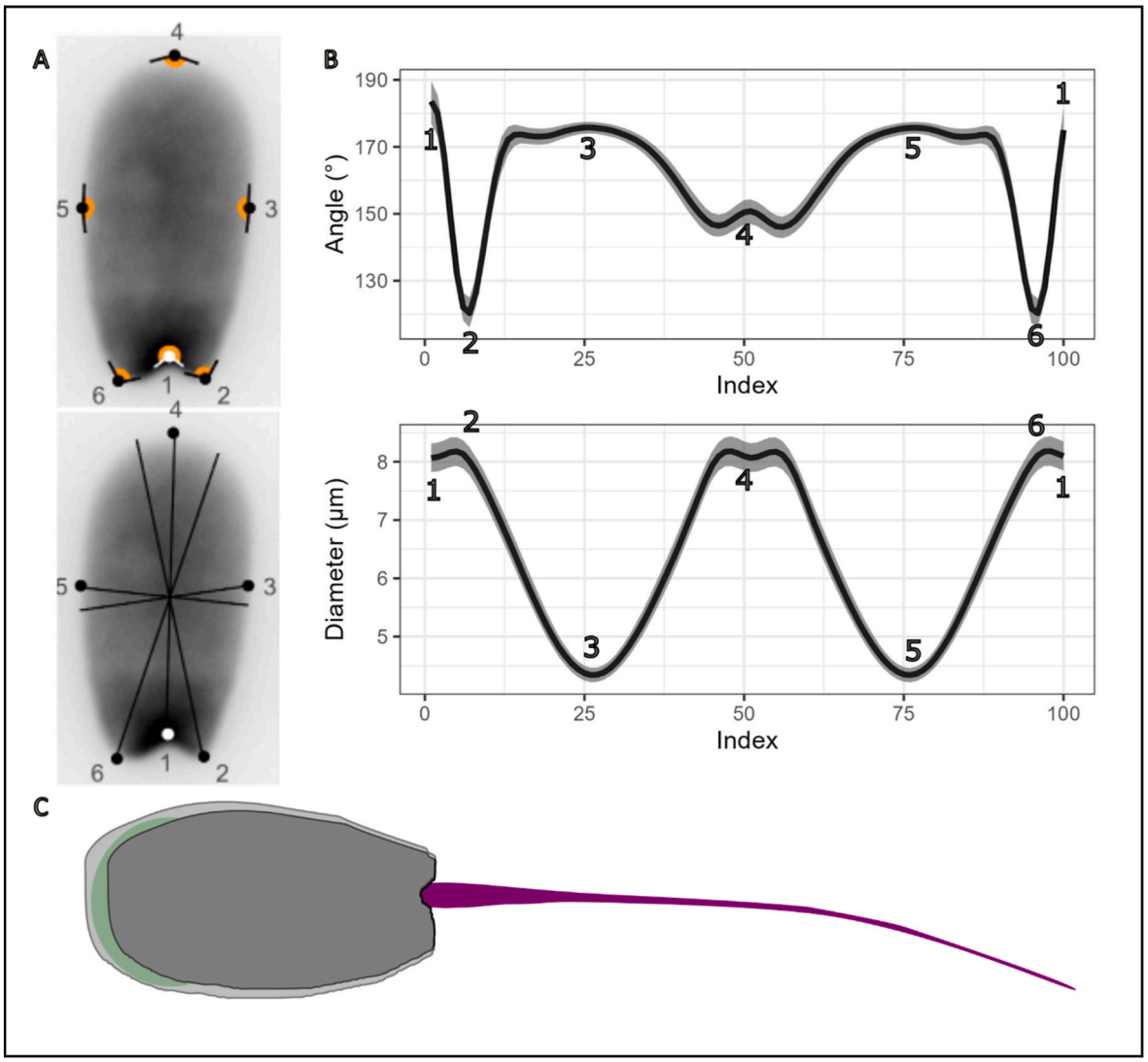


Fig. 1. – Example morphometric profiles of pig spermatozoa. A) angle profiles measure the internal angle around the perimeter of the nucleus. Diameter profiles measure diameter across the centre of mass. The numbered points are indicated in the profiles, demonstrating key landmarks can be identified. B) Profiles show median and interquartile range of the angles and diameters of cells. C) schematic of a pig spermatozoon; the sperm nucleus (dark grey) fills most of the sperm head, with the remainder of the head comprising the acrosome (green) and the residual cytoplasm (lighter grey). The tail (purple) is not shown to scale but is included to illustrate the attachment region. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

developed to assure standards of AI are adhered to by pork producers in the UK [13]. Generally, the goals of agricultural animal breeding are to maximise the production of meat at a low cost, and to efficiently disseminate the genetics of particularly valuable animals. As such, the use of high-quality semen doses is of paramount importance; for example in pigs the use of lower-quality doses is associated with lower farrowing rates and smaller litter sizes [14,15]. The fast identification of animals that consistently produce samples that do not meet the AI standard (N-AIS) is therefore important to ensure their removal from breeding programmes. It has been shown for example that boars with fertility problems in a nucleus herd have the potential to reduce litter sizes throughout the entire breeding population [16], in part due to one boar ejaculate commonly being used to serve between 15 and 25 sows [17] in routine AI. Semen parameters are therefore of key importance both in human fertility assessment, informing subsequent treatment, and in agricultural practice.

As is the case for all mammals, an ejaculate from a boar, bull or ram does not contain a homogenous population of sperm [18] making analysis, and the subsequent identification of sub-fertile individuals, challenging. In the general analysis of boar sperm morphology for example, a sample is deemed to be “fertile” if the frequency of abnormal sperm heads does not exceed 10%. Fertility can also be assumed if the frequency of abnormalities in acrosomes, mid-pieces, tails, or proximal cytoplasmic droplets is less than either 5% each, or 15% when combined [19]. In humans, the percentage of normal forms (in both fertile and sub-fertile men) is usually far less than 30% [20], and has been documented to be consistently (and staggeringly) low, with only 3-5% normal forms being identified in assisted reproductive technology (ART) cases [9,21,22].

There also remains much to learn about mammalian sperm biology. Only a limited number of studies to date have focused on the analysis of sperm nuclear morphometry in agricultural animals [23,24], and whilst it has previously been shown that sperm shape differs between high and low fertility bulls [25], such observations are limited in pigs [26,27], although variation in sperm head morphology has been identified by some [28,29]. Differences in morphometric variables that may be related to pig fertility are however yet to be elucidate [30]. Whilst the goals of the livestock industry are in stark contrast to human fertility treatment, furthering our understanding of the biology of sperm in general has potential benefits for both fields. Here, we have used

Nuclear Morphology Analysis (NMA) [31–35], morphometrics software for microscopy images, to measure and compare the shapes of sperm nuclei from 21,002 pig sperm cells. This is the largest reported number of individual sperm nuclei that have been analysed at this level of detail in any agricultural animal. Here, we sought to understand how sperm shape relates to samples being classified as AIS or N-AIS, and to determine if certain sperm features can be correlated with specific biological processes. If these differences can subsequently be linked to fertility, such analysis has the potential to provide an alternative to the livestock industry standard practices and additionally may provide transferrable insights into human sperm morphology.

2. Materials and methods

2.1. Semen collection

Fresh ejaculated sperm samples from 94 boars were collected using the gloved hand method [36], by trained staff at JSR Genetics Ltd. Samples were stored in Duragen extender, supplemented with no less than: 500 IU per ml streptomycin; 500 IU per ml penicillin; 150 mg per ml lincomycin; and 300 mg per ml spectinomycin, diluted to 2.3 billion sperm per dose. Samples were stored at 17 °C and were prepared within two days of collection. No specific ethical approval was required for this study as all semen doses used were collected as part of JSR Genetics Ltd.'s standard commercial procedures. Semen samples identified as N-AIS by the industry were discarded, and those that were identified as AIS by the industry were used commercially.

2.2. Sample preparation

Prior to preparation of samples for this study, semen samples were identified as either AIS or N-AIS using computer assisted sperm analysis (CASA) followed by manual assessment at JSR Genetics Ltd. Specifically, samples that had a morphology score of above 70% (obtained from CASA) and a motility score of above 4 (1 to 5, 1 being dead and 5 being excellent progressive forward movement) were graded as AIS and those falling below these criteria were graded as N-AIS.

50 AIS and 44 N-AIS samples were used in this study. 2 ml of each semen sample was centrifuged at 300 g for 5 min at 17 °C. The supernatant was discarded, the pellet was resuspended in 1.5 ml of fixative solution (100% methanol and 100% acetic acid, added dropwise at a 3:1 ratio) and centrifuged at 300 g for 5 min at 17 °C. The supernatant was discarded, and the pellet was resuspended in 1.5 ml of fixative solution. 10 μl of each sample was then dropped onto the centre of the surface of a labelled (sample ID, date), steam-warmed slide, immediately followed by 10 μl of fixative solution. Subsequently, slides were air-dried for 2 min before one drop of fluorescent DAPI (4′,6-diamidino-2-phenylindole) was added to the centre of the slide. Prepared slides were air-dried in the dark, for at least 20 min prior to microscopy.

2.3. Image acquisition

An Olympus IX83 inverted fluorescence microscope equipped with a XM10 monochrome CCD camera and CellSens Dimension version 1.9 (expandable imaging software for Life Science microscopy) was used for image capturing. A minimum of 200 nuclei per sample were imaged at 1000x magnification.

2.4. Data analysis

Images were analysed using Nuclear Morphology Analysis (NMA) v2.2.1 software [32,35], available here), that enables automated recognition of round or asymmetric nuclei within an image of interest, and the subsequent morphological analysis of these nuclei. We adapted the landmark recognition to detect and analyse pig sperm nuclei. The software generates 'profiles' describing the interior angles around the perimeter of the nucleus, and the diameter and radii across the centre of mass (Fig. 1). Pig sperm are (mostly) symmetrical about the anterior-posterior axis, so the tail attachment region, characterised by a 'dimple' in the nucleus, was chosen to anchor the profiles and orient the nuclei (Fig. 1A, reference point 1). For consistent alignment of the nuclei, the tail attachment region (as can be seen in Fig. 1 A point 1 and 1C) was placed directly below the centre of mass of the nucleus.

Data was exported from NMA and further processed in R (4.4.0) [37] using a custom analysis pipeline. Initially, k-means clustering on diameter profiles was used to identify distinct groups of shapes, with dimensionality reduction using uniform manifold approximation and projection (UMAP) [38,39] employed to visualise the shape profiles. Continuous phenotypes were then detected by selective focus on outlier cells, detected using the angle, radius and diameter profiles (Fig. 1B).

Cells were classed as normal or abnormal based on their morphometric profile data. Each profile comprised 100 indexes (Fig. 1A and B); at each index, potentially abnormal cells were detected as those cells with values outside 1.5x the interquartile range. Cells were then scored on how many indexes met the criteria for abnormality; consistent abnormal phenotypes have higher values, while more normal phenotypes have lower values. For each profile, the cells with a score higher than the mean number of abnormal indexes were determined to be morphological outliers. This approach enabled the detection of continuous morphological variation across cell populations. Cells were assigned to one of three categories: 'normal' (not identified as an outlier), 'intermediate abnormality' (outlier across a single profile type) or 'extreme abnormality' (outlier across multiple profile types).

Associations of breed, sample and date of collection with the severity of phenotype were tested using a Scheirer-Ray-Hare test (a non-parametric equivalent to a two-factor ANOVA). A significant association prompted the use of ordinal logistic regression to explore the association further. The samples were filtered to only known breeds and collection dates for which all samples had data (9321 cells). Ordinal logistic regression was implemented with the *polr* function in MASS [40] using a negative log-log link to account for higher proportions of normal phenotypes, modelling the phenotype cluster as the interaction of collection date and sample. The proportional odds assumption of the model was tested with the Brant-Wald test, and an intercept-only model was created to test the null hypothesis that the predictor variables added no information.

The predictive ability of morphometric data to classify samples as either AIS or N-AIS was tested using logistic regression via generalised linear models. Two models were created; the first using standard industry measurements (width, perimeter, area, ellipticity, elongation, roughness, regularity and height [19,41]), and the second using the morphometric profiles (angle, radius and diameter), modelling a sperm class as dependent on the morphometric parameters. The samples were split into training (70%) and test (30%) groups, and the probability of being in the AIS group was calculated for each sperm in a sample, and the mean probability was taken as the overall sample value.

Figures were generated using ggplot2 [42] UMAPs were generated using the UMAP package [38,39], Factoextra was used for hierarchical clustering and Caret was used for GLM validation. All scripts that were used in this analysis are available here.

3. Results

3.1. Pig sperm samples contain continuous phenotypic abnormalities

Analysis of sperm nuclear morphology from 50 AIS and 44 N-AIS ejaculates yielded measures from 11,416 to 9586 nuclei, respectively. Comparisons between these groups indicated that sperm heads differ, but that there is a large amount of overlap and variation between individuals (Fig. S1–4).

To identify different nuclear shape phenotypes in the samples, all samples were combined into a single dataset to enable a full unbiased

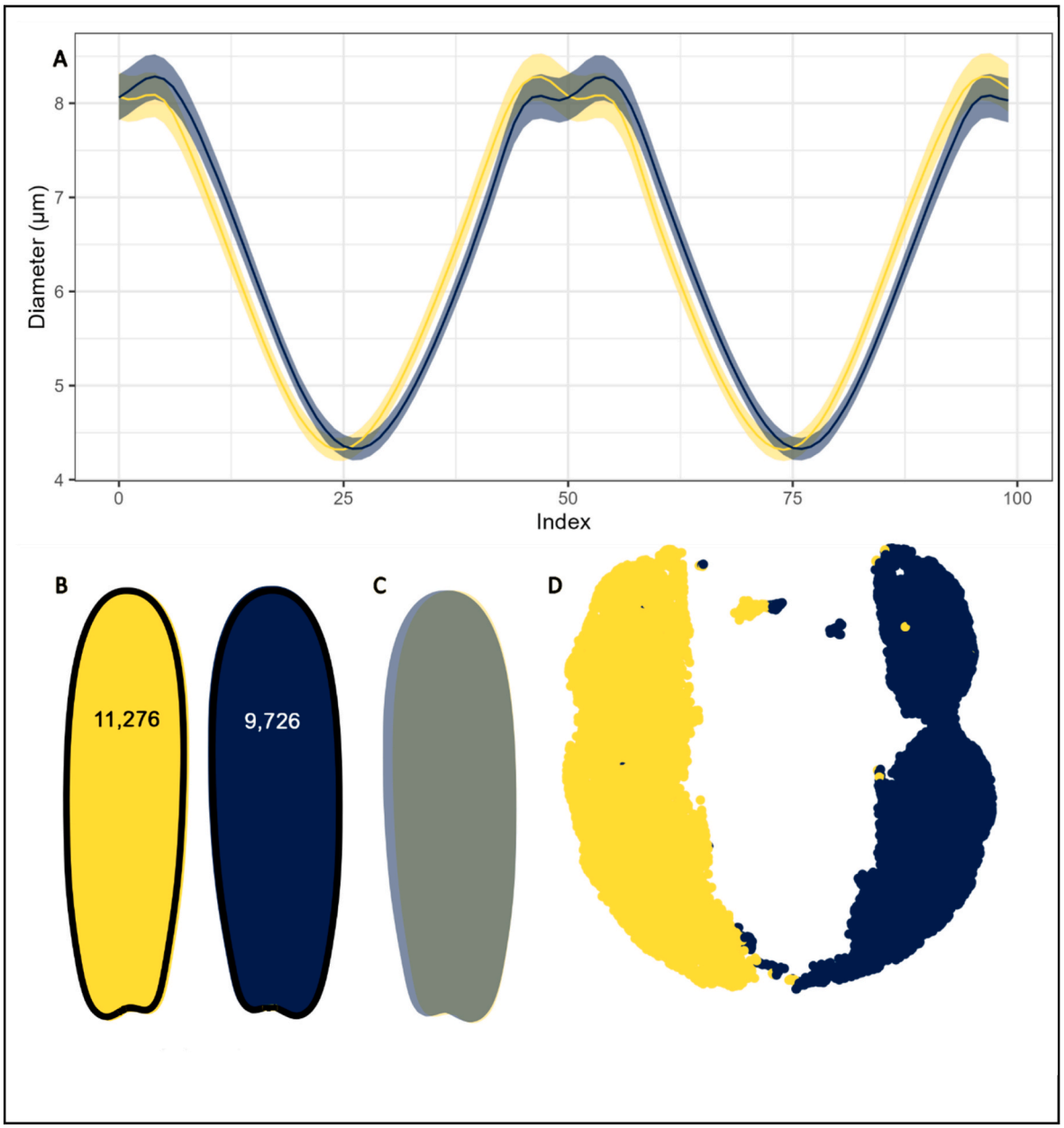


Fig. 2. – Cell shapes fall into two equally sized groups with mirroring around the vertical axis; A) diameter profiles for each cluster show the symmetrical clusters B) Consensus nucleus shapes show the main difference is at the tail attachment region with the more prominent side to the left or right respectively. C) the left cell in B mirrored across the vertical axis and overlaid on the right cell shows the two cell populations are mirrors of each other. D) Dimensionality reduction of diameter profiles by UMAP shows almost all cells fall cleanly into these two groups.

comparison of shapes. The first global observation indicated that sperm fell clearly into two equal sizes groups with distinct shape profiles (Fig. 2A). These two groups of sperm cells correspond to the differences in the shape of the tail attachment region, which is compressed on either the left or the right side (Fig. 2B). The profiles of the nuclei with compression on the right side were reversed to determine if the shapes were mirrored and superimposable. The two groups were deemed to be mirror images of each other around the tail attachment region (Fig. 2C), with almost all cells in the dataset falling neatly into these two major groups (Fig. 2D). This, and the general inflexibility of sperm chromatin, suggests the differences are unlikely to be due to compression from the angle of the tail at the time of fixation, but that the nucleus appears truly asymmetric, and the difference we observe is caused by the sperm head falling on its dorsal or ventral surface during slide preparation.

Since the shape around the tail attachment region was the primary factor that separated nuclei of different shapes, and UMAP showed continuous variation, standard clustering (*e.g.* Ref. [43]) to identify discrete shape groups was not feasible. Instead, outlier nuclei were aggregated with similar shape profiles to identify continuous phenotypes, iteratively grouping nuclei with the greatest differences to normal profiles. This amalgamation of outlier shapes yielded a normal phenotype group (containing 85.2% of nuclei), an intermediate phenotype group (12.7%) and an extreme phenotype group (2.1%). The progression from intermediate to extreme involved condensation of the basal region and bulbing of the anterior region (Fig. 3) with two main pathways; one being the compression with failure to elongate resulting a short pyriform phenotype (Fig. 3A; 7.75%) and the other being abaxial tail attachment and basal compression resulting in an abaxial pyriform phenotype (Fig. 3C; 6.25%). The intermediate phenotypes constituted the largest proportion of abnormal sperm that were observed. This group

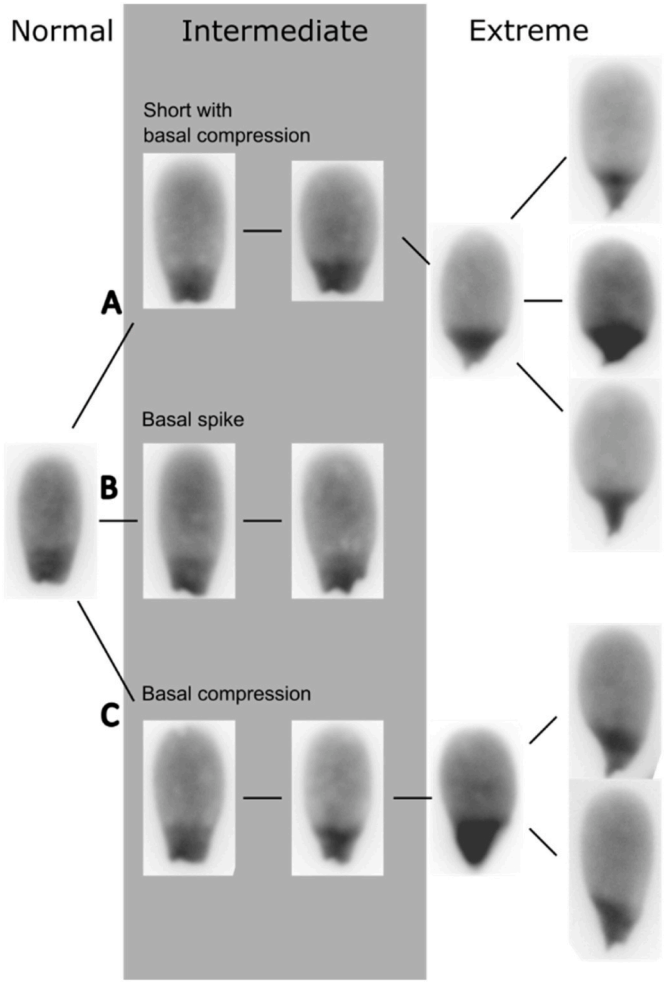


Fig. 3. – Example DAPI stained images of progressively abnormal sperm phenotypes. Three main classes of abnormality can be seen: A) short (compression anterior-posterior, leading to reduced bounding height) progressing to compression (tapering) around the base of the nucleus and a pyriform phenotype; B) extrusion of nuclear DNA forming a spike near the base of the nucleus with some tapering; C) abaxial compression (tapering) around the base of the nucleus, leading to a pyriform phenotype.

showed substantial morphological heterogeneity, from minor tapering and the formation of spikes at the basal region, to relatively well-formed pyriform cells (Fig. 1B; 1%).

3.2. Both AIS and N-AIS samples contain abnormal sperm phenotypes

Initial analysis indicated that *c.*15% of the sperm were abnormally shaped, however, as samples were pooled, this approach did not determine if these abnormalities were enriched in the N-AIS samples or if their prevalence was related to other sample characteristics. We therefore looked at three characteristics: whether the sample was classified as AIS or N-AIS, the breed of the boar (where known, n = 11,448 sperm heads) and the date of sample collection (as a proxy for environmental or technical factors). Intermediate and extreme sperm phenotypes were not evenly distributed across all samples (Fig. 4), and while the abnormal phenotypes were more prevalent (1.96:1 N-AIS:AIS more extreme, 1.44:1 N-AIS:AIS more intermediate) in the N-AIS samples, they were also found in AIS samples.

Given that the data suggested that there was variation between samples and between sample collection dates (Fig. S3 and S4), whether the distribution of phenotypes was associated with the date of sample collection or individual samples was tested. Significant associations were found for both variables and their interaction (Scheirer-Ray-Hare test, $p < 0.001$ for each). To explore these interactions in more detail, analysis focussed on samples for which breed information was known, with dates selected to ensure representation of all breeds. Ordinal logistic regression did not reveal a global association between phenotype severity and individual samples of known breed ($p > 0.05$), except for the White Duroc breed ($p = 0.01$), in which severe phenotypes were more common (Table S1). The odds of a sperm cell having an extreme phenotype relative to an intermediate or normal phenotype were 2.1 times higher in this breed.

The date of collection showed some global associations phenotype severity; for example, for samples collected 6th July, the odds of a sperm cell having an extreme phenotype relative to an intermediate or normal phenotype 3.3 times higher than on other dates ($p < 0.001$). Most other collection dates did not show global effects.

Interaction effects were detected between the date of collection and individual samples (see Table S1 for a full breakdown), indicating that, in this study, the environment had a larger impact on the quality of the ejaculate than the breed of the boar. This was confirmed by testing for differences in the shape profiles between breeds. Repeated Wilcoxon rank-sum tests, with multiple testing corrected with the Holm method showed no significant difference in the angle, radius or diameter profiles between breeds suggested that there is subtle nuclear morphological variation between breeds, with differences dominated by shared genetic or environmental factors.

3.3. Classification of normal/abnormal cell types does not align with industry assigned AIS/N-AIS classifications

We next considered if the extra morphometric information measured allowed for more robust classification of samples as AIS/N-AIS than previously possible. The samples were originally classified based on motility and shape, with 70% morphologically normal forms and a motility score of above 4 leading to a classification of AIS. Although motility information was not available for these samples, the predictive power of conventional morphometrics was compared. For this, a GLM was created to predict the probability of a cell originating from an AIS or N-AIS sample based on the width, perimeter, area, ellipticity, elongation, roughness, regularity and height, measurements that are commonly used in industry. Each sample was scored as the mean of its individual cell predictions (Fig. S2A). This score was then compared to a GLM that used the shape profiles for prediction (Fig. S2B). This comparison indicated that greater discrimination between the AIS and N-AIS samples was possible. The ability to classify samples directly by the proportion of normal, intermediate or extreme phenotypes is, in principle, a useful improvement on the simpler morphometric measures. AIS samples with a high rate of shape abnormalities indicate the presence of substantial morphological variation that warrants further investigation.

4. Discussion

Studies of sperm shape usually focus on simple morphometric measurements such as area and circularity [19,30,44–46]. These measurements can however miss subtle phenotypes, especially when large numbers of sperm are being screened. Here, 21,002 sperm nuclei were analysed at a high resolution, allowing an in-depth analysis of sperm nucleus shape. Previous sperm morphology studies report the analysis of between 3000 and 5550 sperm heads [45,47]. To the best of our knowledge, this is therefore the largest reported number of individual sperm nuclei that have been analysed at this level of detail in any agricultural species. The use of this large sample size has allowed us to detect and characterise subtle phenotypes that have previously been overlooked.

Previous studies measuring bright-field images have identified differences in sperm head size between pig breeds, with Saravia et al. concluding that Duroc sperm heads were 0.2 μM longer and 0.1 μM

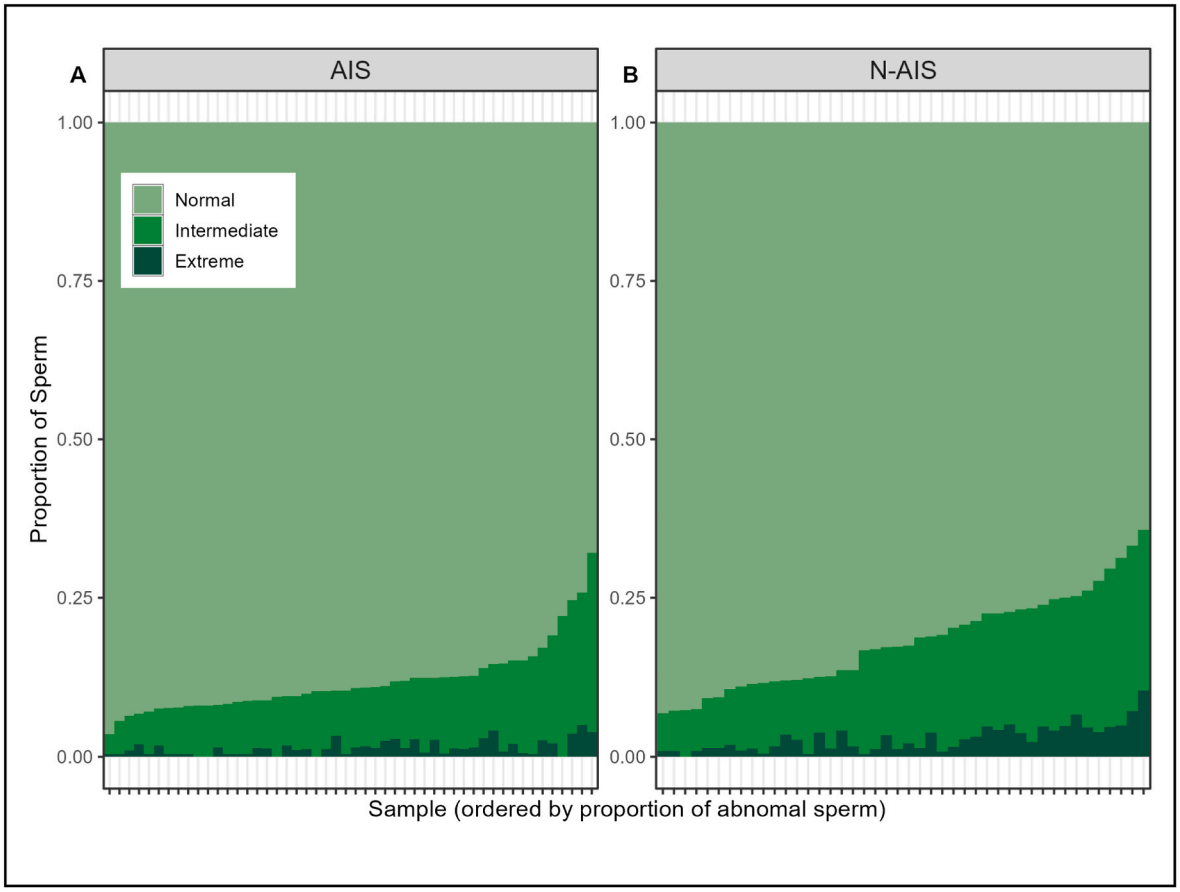


Fig. 4. – Proportion of sperm in normal, intermediate or extreme groups for A) AIS samples (50 samples) and B) N-AIS samples (44 samples). Intermediate and extreme phenotypes are more frequent in N-AIS samples but are also common in a subset of the AIS samples.

wider than other tested breeds [19], and Kawarasaki and colleagues finding Duroc sperm heads to be 0.1 μM longer and 0.03 μM wider [47]. Given that such differences were not observed here (Fig. S1), the size difference seen in other studies may be due to the structures outside of the nucleus, or due to sample variation. Of note however, is that the Duroc pig samples analysed in this study were from crossbred white Duroc animals. This crossbreeding may have resulted in the smaller sperm observed due to a lack of genetic diversity or a potential founder effect (Fig. S1).

As shown in Fig. 2, asymmetry in pig sperm nuclei was identified, with one side of the tail attachment being consistently larger than the other. Given that pig sperm nuclei have a flattened shape, we conject that this asymmetry reflects how the sperm cells fell on the slide during preparation; either dorsally or ventrally. Evidence from scanning electron microscopy also suggests that the asymmetry is present in the full sperm head, not just the nucleus [47].

The other shape phenotypes that were discovered in this study were continuous, with progressive abnormalities involving compression around the base of the nucleus (Fig. 3). Some of these shape phenotypes are similar to those reported before, including the division of intermediate and extreme phenotypes [45,47,48]. Basal nucleus compression causing a bulb-like shape has been observed in the sperm of other species such as humans [48], and has been studied extensively in cattle [49–53]. Notably, *Kif3a* knockouts in mice have also been shown to generate similar sperm phenotypes [54], although much of the characterisation of sperm phenotypes to date has been either manual or using CASA systems measuring simple morphometric values, and therefore lack the power required to detect subtle variations [23,24,30,45,47,55,56].

Some of the samples with the greatest proportion of abnormal sperm were collected on the same day, suggesting that environmental factors, either pre- or post-sample collection, may be as, if not more, important than genetic factors, as borne out by previous studies [57–59]. Purely technical factors in sample collection seem implausible to explain the range of phenotypes we observed. Whilst breeding seasons are unlikely to be the cause given that pigs are nonseasonal, it has been shown that stress and physical trauma can affect spermiogenesis, with stress-induced infertility being previously reported in mice, boars and humans [58,60,61]. Although chromatin integrity is important for fertility [62], morphological differences in sperm head shape are not directly attributable to chromatin integrity in stud boar sperm [19] or in humans [63,64], with the exception of rare, markedly round sperm heads observed in a small number of the pig samples. This suggests that chromatin integrity is unlikely to be an important factor in the structure of the predominant morphological phenotypes observed, as few sperm show known oxidative damage-induced phenotypes.

The sperm were organised into three groups that illustrate the progressive abnormalities (Fig. 3). These are (1) extrusion of a basal spike; (2) basal compression, developing asymmetrically; and (3) basal compression with failure of the remainder of the nucleus to elongate. Potential mechanisms that can generate these phenotypes have been described in mice, and information from other mammals can be used as an analogue for boar spermatogenesis [65,66].

Nuclear elongation during spermiogenesis is a complex, highly coordinated process shaped by five core structural elements: ectoplasmic specialisation of the Sertoli cell, which provides external compressive forces [67,68]; the acroplaxome, a cytoskeletal plate composed of f-actin and keratin, that supports elongation [67,69,70]; Sertoli cell-spermatid linkers (afadin/nectin complexes) which transmit these forces [71]; the manchette, a network of microtubules that directs basal nuclear shaping

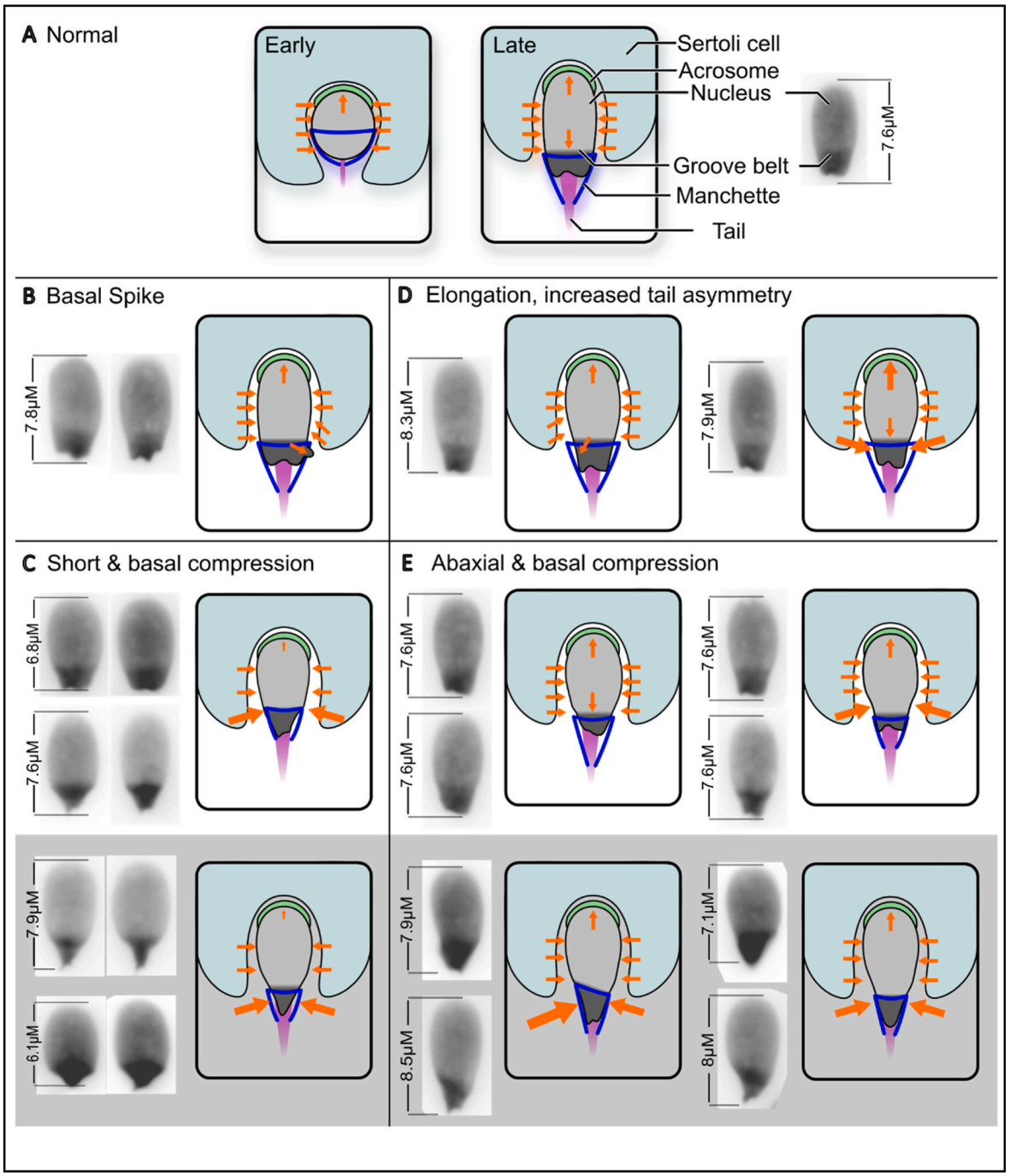


Fig. 5. – Summary of the forces involved in shaping the sperm nucleus and the abnormal phenotypes we observe. (A) the normal forces of elongation of a pig sperm exerted on and generated within the cell by the Sertoli cell ectoplasmic specialisation. The Basal spike (B) section shows the forces happening to the cells with an anterior bulge around the tail attachment region that is possibly either from the force of a broken tail or malformation of the manchette/ectoplasmic specialisation hoops slipping due to lack of acroplaxome attachment proteins below the manchette forcing out the bulge. The short with basal compression (C) phenotype has concentrated pressure around the base and a lack of intranuclear pressure which could come either from a lack of molecular motors or misalignment of ectoplasmic specialisation hoops that results in a lot of basal pressure causing constriction but not the elongation seen in other phenotypes. The elongation with increased tail asymmetry (D) is potentially due to manchette misattachment causing the asymmetry that is not severe enough to cause a full abaxial structure or abaxial misalignment of the hoops from the ectoplasmic specialisation without enough constricting force to cause full abaxial development. The elongation of this sperm phenotype is likely due to increased intranuclear pressure from the compression of the manchette and loops that are potentially due to increased molecular motor activity such as dyneins or kinesins or potentially moderated by the same acroplaxome attachment complex that allows for more force transfer due to more attachment. The Abaxial (E) phenotype with basal compression is likely due to a combination of manchette misalignment exacerbated by the basal compression due to the previously mentioned processes.

and tail attachment; and the groove belt which links the manchette to the nucleus where the acroplaxome ends [54,72,73]. These components are interconnected, and while not the only mediators of sperm shape, they are important for understanding how perturbations to the process of elongation in spermiogenesis can cause sperm defects. At a molecular level, many proteins are known to be involved in spermiogenesis, with various morphological abnormalities shown to be linked to the loss of function of specific genes. For example, SUN3 loss of function causes a complete failure of manchette formation [74], whilst SUN5 loss of function results in acephalic spermatozoa [75]. In Fig. 5 we indicate the forces that may be operating on the developing sperm to generate the phenotypes that we observe here.

We see over compression of the manchette and a dysfunction of nuclear elongation; potentially the absence or reduction in levels of one of these connector proteins or molecular motors would result in over-compression of the manchette and improper elongation [54,76]. One potential mechanism for this could involve the over-tightening of the hoops of the Sertoli cell ectoplasmic specialisation via uneven pressure exerted on the nucleus, either because the hoops lack constricting forces or because they lose their solid connection to the acroplaxome and prevent its function as a modulator of elongation.

Although we cannot be certain that these are the mechanisms at play in these boar samples, our analysis indicates that these structural changes are localised to specific regions of the sperm. This suggests that further study of the proteins involved in these regions will be important to understand variation in boar sperm development. If these components are affected by environmental factors, this may present opportunities for targeted interventions to improve sperm quality and ejaculate consistency, with potential benefits to the pig breeding industry.

This work, and Barquero et al. [77] both show a consistent distribution of morphological phenotypes, although Barquero et al. only used samples comparable to AIS samples in our study. Given the previously reported link between sperm head size and motility with simple morphological parameters such as height and width [78], and an association between the number of stillbirths in Piétrain boars and poor sperm motility [77], we see clear support for the well-established idea that boar semen quality is significantly affected by the environment [57,59,79]. Further investigation of this relationship is needed, particularly focussing on candidate proteins that may explain the observed phenotypes. A deeper understanding of sperm morphology may also further explain the relationship between morphology, motility and farrowing outcomes.

Evidence suggests that pig AI may be suboptimal [80–83], with this potentially due to the methods used to classify sperm at AIS or N-AIS. This interpretation is supported in this study, where a large overlap of morphology and the subtle intermediate phenotypes of abnormal sperm between AIS and N-AIS samples is seen. It would therefore be an important next step to test the fertility of samples classified as such using this new approach.

5. Conclusion

In this study we demonstrate that morphometric analysis of pig sperm using NMA software effectively distinguishes morphologically distinct populations of nuclei. Our analysis reveals differences in sperm nucleus morphology between animals classified as commercially acceptable (AIS) and unacceptable (N-AIS) based on assessment to industry standards. Use of NMA has offered additional insights into pig sperm morphology. Importantly, the distribution of sperm phenotypes between different collection dates and between breeds suggests that environmental factors in sperm collection and transport can greatly influence sperm morphology. Further study is needed to understand how these phenotypes develop, and if there are functional consequences of these phenotypes on boar fertility. This method may also be useful in the categorisation of sperm head phenotypic distributions in human samples, and has the potential to provide insights into the complex and subtle morphological differences associated with environmental factors, fertility and disease [84].

CRediT authorship contribution statement

A.A. Mandawala: Writing – original draft, Methodology, Investigation. **J.C. Perry:** Writing – review & editing, Visualization, Investigation, Formal analysis. **B.M. Skinner:** Writing – review & editing, Supervision, Software. **G.A. Walling:** Writing – review & editing, Resources. **S.C. Harvey:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **K.E. Harvey:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Availability of data and materials

Data are available at: https://github.com/JosephCliffordPerry/Pig-scripts.

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Conflict of interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Grant Walling reports a relationship with Topigs Norsvin that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2026.117874.

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