Sperm morphology differences associated with pig fertility

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Abstract

Artificial insemination is routine in commercial pig breeding, and as such, the use of high-quality semen samples is imperative. Here, we have developed a novel, semi-automated, software-based approach to assess pig sperm nucleus morphology in greater detail than was previously possible. This analysis identified subtle morphological differences between samples assessed by the industry as normal and those assessed as abnormal. 50 normal and 50 abnormal samples that were initially categorised using manual assessment to industry standards, were investigated using this new method, with at least 200 fixed stained sperm heads analysed in each case. Differences in sperm nuclear morphology were observed between normal and abnormal samples; specifically, normal samples were associated with higher mean nuclear area, a consequence of a greater head width and a lower variability between sperm heads. This novel, unbiased and fast analysis method demonstrates a significant difference in sperm head morphology between normal and abnormal pig sperm and has the potential to be further developed to be used as a tool for sperm morphology assessment both in the pig breeding industry and potentially in human assisted reproductive technologies.

Keywords: Artificial insemination, morphology, nucleus, pig, sperm

1. Introduction

Male fertility is a consequence of both the number and quality of sperm [1]. In humans, many issues with male infertility are addressed using one of a range of assisted reproductive technology procedures, such as *in vitro* fertilisation and intracytoplasmic sperm injection. In agriculturally significant species such as pigs, cattle, and sheep, where a key goal is to maximise the production of meat at a low cost, male fertility is also a challenge [2]. To this end, improving reproductive traits is paramount. In such species, the critical aim is often to identify sub-fertile animals quickly and cheaply so that they can be removed from breeding schemes; boars from a nucleus herd with fertility problems have the potential to reduce litter sizes throughout the breeding population [3].

Artificial insemination (AI) is the oldest [4] and most routinely used technique in commercial animal breeding, especially in livestock species [4–10]. Over the past three decades, the use of AI has greatly benefited the pig breeding industry, particularly in Europe where over 80% of sows are bred through AI [4]. In North America, the technique is also widely implemented, especially in large farming units [9]. The principal objective of AI in the pig breeding industry is to permit the dissemination of genetics from high genetic merit boars to as many sows as possible. Without AI, more boars would be needed and hence animals of lower genetic merit would be required in breeding programmes. Moreover, the technique enables the opportunity to introduce superior genetic traits into sows whilst reducing the incidence of disease transmission, an advantage that does not exist with natural mating [11]. AI is achieved by depositing spermatozoa into the female genital tract using artificial devices and processes. The standardised method of insemination is the intra-cervical insemination technique, with the semen dose deposited in the posterior region of cervical canal [4].

In humans, semen analysis is widely used to evaluate male fertility [12] and may also be used for the determination of reproductive toxicity in therapeutic and environmental agents [13,14]. Various

physical characteristics of semen are assessed and whilst parameters such as volume, appearance, pH, and viscosity are considered important [15], several studies have shown that good sperm morphology is critical when determining semen quality and hence quantifying male fertility [16–21]. Generally, the cut-off values of what is considered “normal” vary, and are dependent on the fertility clinic. However, the following benchmarks were published in the World Health Organisation’s 5th edition of "normal semen analysis": morphology (≥4% normal forms), total motility (≥40%), vitality (≥58% live), sperm concentration (≥15,000,000 per mL) and volume (≥1.5 mL) [12,22]. To date, a number of studies have been performed to analyse semen composition [23,24] and to establish the relationship between sperm quality and fertility in men [25–30]. One such study used several comparative semen analyses of fertile and infertile men, to determine the most appropriate measurements that could be used in the determination of fertility potential in men [31]. Here, it was established that whilst threshold values for sperm motility, concentration and morphology could be used in the classification of males into fertile, indeterminate fertility, or sub-fertile categories, these measures cannot be used independently for the diagnosis of male infertility [31].

In livestock species, a key contribution to successful fertilisation following AI is also the use of high-quality semen during insemination. As such, routine assessment of semen quality is a standard process in the animal breeding industry [32]. An issue, however, is that the mammalian ejaculate does not contain a homogenous population of spermatozoa [33]; in general analysis of boar semen, semen quality is considered “normal” if the frequency of abnormal sperm heads does not exceed 10%. Normality 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 [34]. Some morphological abnormalities are regarded as significant due to their ability to hinder fertilisation and cause infertility [34]; some of these include decapitated spermatozoa, acrosomal plicae (knobbed defect), nuclear vacuoles (diadem defect), short tails (tail stump), coiled tails (dag defect) and corkscrewed mid-pieces [34].

93

94 As well as studying the morphology of whole spermatozoa, a limited number of studies have focused
95 on analysis of sperm nuclear morphometry in agricultural animals [35,36]. A number of
96 measurements of sperm nuclei have been compared in cattle, sheep, goats, and pigs using
97 computer-assisted sperm morphometry analysis-F (CASMA-F) [35,36]. Whilst mean numerical values
98 for area, perimeter, length, and width were identified for each of the four species studied, the key
99 findings of this study were that drying and fixation only has a minimal effect on sperm nuclear
100 morphometry and that variations between morphometric parameters do exist between the sperm
101 nuclei of each species [35]. A second study [36] used a combination of the CASMA-F method and
102 multivariate cluster analysis to identify subpopulations of spermatozoa within the same four species.
103 Based on these nuclear morphometrics, three subpopulations, namely, large, small-elongated and
104 small-round were identified. Whilst it has previously been shown that sperm shape differed between
105 high and low fertility bulls [37], such observations are limited in pigs [38]. Here, we demonstrate the
106 use of a semi-automated software-based approach to assess sperm nucleus morphometrics in pig
107 semen samples deemed as normal and abnormal.

108

109 ## 2. Material and Methods

110 ### *2.1 Semen collection*

111 Fresh ejaculated sperm samples from boars were collected using the 'gloved hand method' [47], by
112 trained staff at JSR Genetics Ltd. Samples were stored in Duragen extender, supplemented with no
113 less than: 500 IU per ml streptomycin; 500 IU per ml penicillin; 150 mg per ml lincomycin; and 300
114 mg per ml spectinomycin, diluted to 2.3 billion sperm per dose. Samples were stored at 17°C and
115 were prepared within two days following collection. No ethical approval was required for this study
116 as all semen doses used were collected as part of JSR Genetics Ltd.'s standard commercial
117 procedures. Semen samples identified as abnormal would have been discarded, those identified as
118 normal would have been used commercially.

119

120 ***2.2 Sample preparation***

121 Prior to preparation of samples for this study, semen samples were identified as either normal or
122 abnormal using computer assisted sperm analysis (CASA) followed by manual assessment.
123 Specifically, samples that had a normal morphology score of above 70% (obtained from CASA) and a
124 motility score of above 4 (motility was graded from 1 to 5, 1 being dead and 5 being excellent) were
125 graded as normal and those falling below these criteria were graded as abnormal.

126

127 50 normal and 50 abnormal samples were used in this study. 2mL of each semen sample was
128 centrifuged at 300g for 5 minutes at 17°C. The supernatant was discarded, the pellet was re-
129 suspended in 1.5mL of fixative solution (100% methanol and 100% acetic acid, added dropwise at a
130 3:1 ratio) and centrifuged at 300g for 5 minutes at 17°C. The supernatant was discarded, and the
131 pellet was re-suspended in 1.5mL of fixative solution. 10μL of each sample was then dropped onto
132 the centre of the surface of a labelled (sample ID, date), steam-warmed slide, immediately followed
133 by 10μL of fixative solution. Subsequently, slides were air-dried for two minutes before one drop of
134 fluorescent DAPI (4',6-diamidino-2-phenylindole) was added to the centre of the slide. Prepared
135 slides were air-dried in the dark, for at least 20 minutes prior to microscopy.

136

137 ***2.3 Image acquisition***

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

141

142 ***2.4 Data analysis***

143 Images were analysed using the ImageJ plugin 'Nuclear Morphology Analysis' (NMA) (see [43]),
144 freely available under the GNU General Public License version 3 from

145 https://bitbucket.org/bmskinner/nuclear_morphology/wiki/Home). The analysis software enables
146 automated recognition of round or asymmetric nuclei within an image of interest, and subsequent
147 morphological analysis of these nuclei. Initially developed for the analysis of mouse sperm [43], we
148 adapted the feature recognition to analyse pig sperm. The software generates a range of measures;
149 in this study, sperm heads were measured for: Area; Perimeter; Bounding Height and Bounding
150 Width, the dimensions of the rectangle perfectly enclosing the nucleus when vertically oriented;
151 Circularity, a measure between 0 and 1 indicating how circular the heads are, with 1 indicating a
152 perfect circle; Minimum Width across the centre of mass; Aspect, the ratio produced from height
153 divided by width; and Variability, a per-nucleus measure calculated as the square root of the sum-of-
154 squares difference at each index between the nuclear profile and the dataset median profile,
155 normalised to the length of the median profile.
156
157 Pig sperm are (mostly) symmetrical about the anterior-posterior axis. Without a distinctive hook, as
158 in mouse sperm, to act as a reference point, the tail attachment point was chosen to anchor the
159 angle profiles generated and orient nuclei. The tail attachment point is characterised by a 'dimple' in
160 the nucleus (Figure 1A, point 1). For consistent alignment of the nuclei, we placed the tail
161 attachment region directly below the centre of mass of the nucleus. The software output prior to
162 statistical analyses is summarised in Figure 1. This indicates how we generate an angle profile for a
163 given sample and how a consensus sperm head shape for the population is produced. These
164 refolded sperm heads are then used both to trace any identified abnormalities on a particular
165 segment of the refolded nucleus back to the angle profile, and to calculate a range of sperm head
166 parameters for analysis. Analysis was carried out using software version 1.13.5, and initial
167 correlation analysis was used to identify redundant variables. Non-redundant variables were then
168 further analysed; for initial comparisons between normal and abnormal animals, means of each
169 variable were compared by ANOVA in R version 3.4.4 [48], with the normality of residual distribution
170 assessed using scatter and quantile-quantile plots.

171

172 For the cluster analysis, samples were randomly allocated into two groups; a training group of 30
173 normal and 30 abnormal animals and a test group of 20 normal and 20 abnormal animals. Sperm
174 heads from the training group were used to determine if there were sperm of detectably different
175 types. This was done by cluster analysis, with Ward linkage, squared Euclidean distance and
176 standardised variables in Mintab v17. The representation of sperm heads from normal and abnormal
177 animals within in each identified cluster was then compared to that expected by chance. The
178 training group was then used to investigate if identified clusters could be used to predict fertility
179 status. This was done by using the mean cluster value from the training set to define the starting
180 partitions for a K-means clustering.

181

182 A ROC analysis was then used to test the diagnostic capacity of these clusters in predicting whether
183 a given semen sample would have a normal or abnormal semen profile based on the proportion of
184 sperm that fall into the clusters 1 and 3. ROC curves were plotted in R version 3.6.0 via the pROC
185 package [49] using the predictions derived from the cluster output.

186

187 **3. Results**

188 Analysis of sperm nuclear morphology from 50 normal and 50 abnormal animals yielded measures
189 from 11,534 and 11,326 nuclei, respectively. Correlation analysis of measured sperm head
190 characters indicated that, as expected, many of the measures were highly correlated (Table S1), and
191 further analysis was undertaken using measures of Area, Circularity, Variability, Bounding Height and
192 Bounding Width. Given the orientation of the sperm heads, Bounding Height and Width are
193 subsequently referred to as Maximum Height and Width.

194

195 Comparisons between normal and abnormal animals indicated that sperm heads in these groups
196 differ, but that there is a large amount of variation between individuals (Figure 2, and Figures S1-4).

197 Analysis of mean trait values from each animal indicates that sperm heads from fertile animals have
198 a higher overall area (Area, $F_{1,98} = 34.55$, $p < 0.001$), are wider (Width, $F_{1,98} = 11.58$, $p = 0.001$), longer
199 (Height, $F_{1,98} = 21.68$, $p < 0.001$) and are less variable in shape (Variability, $F_{1,98} = 24.98$, $p < 0.001$)
200 than those from abnormal animals. At this level of analysis, no difference between the sperm heads
201 of normal and abnormal animals in Circularity was detected (Circularity, $F_{1,98} = 1.80$, $p = 0.18$).
202
203 We hypothesised that individual sperm samples would contain different nucleus types, and that this
204 may allow the identification of abnormal animals. For example, a certain sperm head type might
205 only be found in abnormal animals or might be overrepresented within such animals. Such
206 differences might represent specific features within these abnormal animals, which could also be
207 used to investigate why their fertility was impaired, *i.e.,* this could be informative about the biology,
208 or the mechanism. Additionally, the presence of differences of this type might allow the fertility of
209 an animal to be predicted, thereby allowing the automated assessment of fertility. We therefore,
210 randomly allocated samples into two groups: a training group of 30 normal and 30 abnormal animals
211 in which we investigated the presence, or not, of different nuclei types; and a test group of 20
212 normal and 20 abnormal animals in which we investigated the ability of this type of analysis to
213 predict fertility status.
214
215 In the training group, cluster analysis (Ward linkage, squared Euclidean distance and standardised
216 variables) of 6,924 sperm heads from normal animals and 6,684 sperm heads from abnormal
217 animals supported the existence of three clusters (Figure 3). The identified clusters showed different
218 membership for the normal and abnormal sperm heads, with sperm heads from normal animals
219 overrepresented in the largest cluster and underrepresented in the smallest cluster.
220
221 Comparison of these clusters indicates that the cluster in which sperm heads from normal animals
222 are overrepresented is characterised by sperm heads with a low variability and a high area (Figure

223 4). In contrast, the cluster in which abnormal animals are overrepresented is characterised by sperm
224 heads with a lower area and a high variability (Figure 4).
225
226 The three clusters identified in the training group were also recovered in the test group (Figure S5),
227 with analysis of sperm head morphology showing the same differences between clusters (data not
228 shown). As in the training group, sperm heads from normal animals were overrepresented in cluster
229 1 and underrepresented in cluster 3 (Figure S5). This supports the idea that the frequency of certain
230 sperm head morphology types can be used to predict fertility status. Several approaches were
231 investigated to test this. Firstly, we used the mean trait values for the three clusters identified in the
232 training group to define the initial partitions for a K-means clustering of the sperm heads from the
233 test group of 20 normal and 20 abnormal animals. The proportion of nuclei from each animal that
234 was allocated to each cluster was then determined and compared to the proportions determined
235 from the training set (Figure 5). This indicates that this method does accurately predict fertility in
236 some animals, but that no scheme would correctly identify status for all animals. That is, if the aim
237 was to exclude any animal where abnormality was suggested, then 8 of the 20 normal animals and 6
238 of the 20 abnormal animals would be retained (Table S2). Conversely, when the aim was to retain
239 samples that were identified as normal through cluster membership, 13 of the 20 normal samples
240 and 6 of the 20 abnormal samples would be retained (Table S2). Similarly, attempts to predict
241 fertility using other approaches – for example defining thresholds based on rates of variability within
242 samples – also resulted in the inclusion of abnormal animals or the exclusion of normal ones. To
243 formally assess the diagnostic capacity of cluster membership, a receiver operating characteristic
244 (ROC) analysis was also performed using proportions in Table S1 as the range of cut-off values for
245 the predictor variables. This identified that using the proportion of sperm heads that do not fall into
246 cluster 3 results in the largest area under the curve (0.723, with 95% confidence interval of 0.537 –
247 0.868; Figure S6).
248

4. Discussion

Over the last decade, AI has become commonplace in the pig breeding industry, and as such it is an economical imperative to identify boars with prime fertility. To this end, various computer technologies have been developed, or adapted, to allow quantitative analysis of boar sperm characteristics. Examples of these are the various CASA systems that have been developed and improved over almost four decades that are now widely used in semen handling centres and spermatology laboratories. Currently, the Sperm Class Analyser (SCA) is considered to be the gold standard in automated sperm morphology analysis systems [39]; SCA has been shown to provide accurate measurements of head, mid-piece and tail morphometry in several mammalian species including humans [40], horses [41] and goats [42]. The absence of standardised morphometric parameters and corresponding values which could be used to identify both normal and abnormal sperm, however, is a limitation which is common to most species [39].

We previously developed Nuclear Morphology Analysis software for rapid and accurate assessment of nuclear morphology in mouse lines [43]. In contrast to other morphometric approaches, such as elliptic Fourier analysis [37], or Procrustes-based geometrics [44], our analysis can be run rapidly on many thousands of nuclei, using automatic detection of landmarks and semi-landmarks within the nucleus, and the results map cleanly back to the biological structure of the nucleus. Here, we have extended the capability of the software to recognise pig sperm for the first time, and have used this to compare sperm head morphometrics between semen samples previously identified as normal or abnormal by the industry. Results from these analyses support the hypothesis that subtle morphometric differences can be observed between sperm heads from normal and abnormal samples. The ability of the software to distinguish subtle morphological differences in pig sperm demonstrates the utility of this approach for other spatulate nuclei, as commonly found in other mammalian species, including humans [45].

275

276 Sperm head morphology in pigs is usually assessed as being either normal or abnormal based on
277 CASA data and manual assessment of morphology undertaken by the industry. These analyses have
278 identified differences between the sperm heads of these groups, with sperm heads from normal
279 samples having a higher overall area – a consequence of greater width and height – and being less
280 variable in shape than those from abnormal samples (Figures 2 and S1-4).

281

282 Our analysis also identifies three clusters of morphology types (Figure 3). These clusters group sperm
283 heads that have a low variability and a high area (cluster 1), those that are long and narrow (cluster
284 2), and those that have a low area and a high variability (cluster 3) (Figure 4). This mirrors the
285 clusters identified by previous work on sperm head morphology in pigs that used measures of area,
286 height (or length) and width [35,36,46]. Here however, our analysis also indicates that the variability
287 differs between these groups (Figure 4). We hypothesised that a certain type of sperm head might
288 only be present, or be overrepresented, in semen from abnormal samples. Whilst it is expected that
289 such differences in sperm head morphology would have an impact on the spermatozoa's fertilising
290 potential [46], there is limited research on which precise morphological parameters can in fact
291 impact fertility. Comparison of cluster membership indicated that sperm heads from normal samples
292 are overrepresented in cluster 1 and underrepresented in cluster 3, *i.e.*, abnormal samples have a
293 high incidence of sperm heads that have a low area and a high variability (Figure 4).

294

295 Given that automated approaches to identify abnormal semen samples would be of value in pig
296 production, we sought to determine if the clusters we identified could be used as a predictive tool
297 for semen analysis. Whilst grouping based on the previously identified clusters was able to
298 successfully distinguish some samples as normal or abnormal, it was found that cluster membership
299 alone could not accurately predict fertility in all 40 samples in the test group. To further test the
300 diagnostic capacity of these clusters, ROC analysis (Figure S6) was used to identify how suitable

cluster membership would be as a predictor variable in identifying whether a given semen sample has a normal or abnormal profile. This analysis revealed that if the proportion of sperm heads that do not fall into cluster 3 was used a predictor variable, there was a 72.3% chance of correctly distinguishing a normal semen sample from an abnormal sample, with an AUC of between 0.7 and 0.8 generally being considered to be acceptable.

5. Conclusions

Here we show that morphometric analysis of pig sperm using NMA software distinguishes morphologically distinct populations of nuclei. This analysis identifies differences in sperm nucleus morphology between animals deemed to be commercially acceptable and unacceptable using manual assessment to industry standards. These findings suggest that the use of NMA software provides a high-throughput and more accurate method to identify pig sperm with a higher fertilising potential. This approach may therefore have immediate utility for the pig breeding industry, and longer-term implications for human assisted reproductive technologies.

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Authors' contributions

Conceptualisation, BMS and KH; Methodology, BMS, KH, SH; Software and Validation, BMS; Investigation, AM; Data Curation and Formal Analysis, AM, SH; Visualisation, SH, BMS; Supervision and Project Administration, KH, SH; Writing - Original Draft, AM, KH; Writing - Review and Editing, BMS, KH, AM, SH; Resources, KH, GW. All authors gave final approval for publication.

Competing interests

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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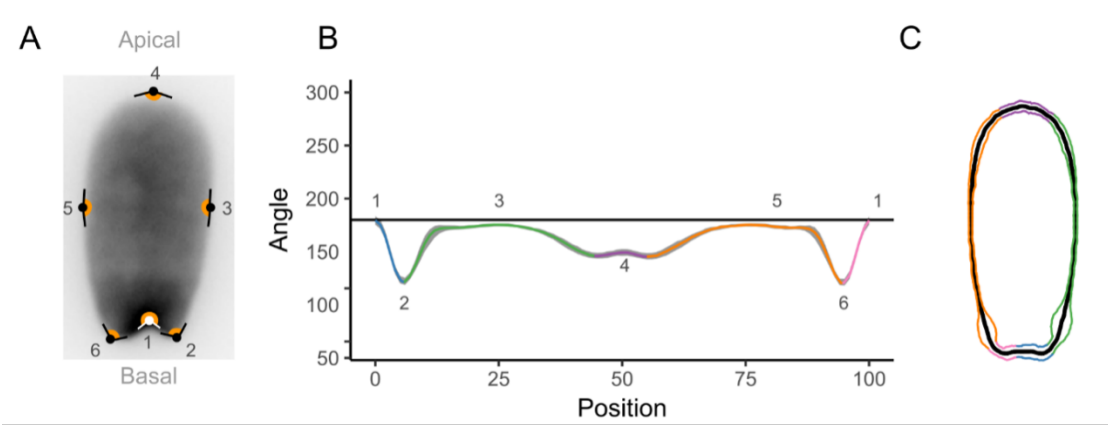


Figure 1: A representative summary of analysis workflow. (A) DAPI stained nucleus from a normal boar sample, captured using an Olympus IX83 fluorescence microscope with pre-installed CellSens software at 1,000x magnification. The software measures the interior angles along the perimeter of the nucleus as represented by point 1-6. (B) Schematic showing how these angles generate a profile. The figure shows the median and interquartile rages for one normal sperm sample and has been segmented at local maxima and minima. (C) The consensus normal pig sperm head shape, showing positions of profile segments in the nucleus.

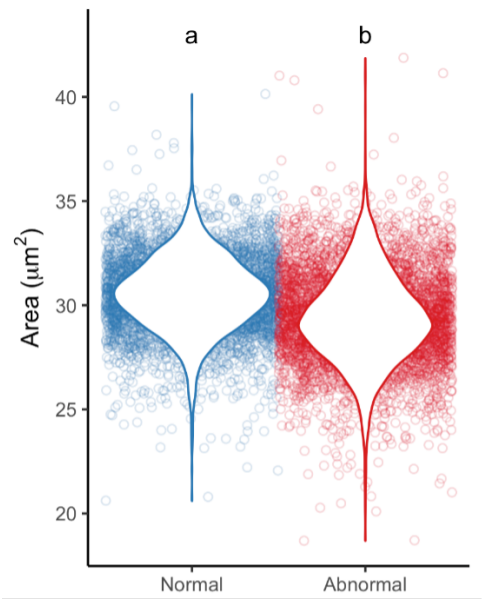


Figure 2: Comparison of sperm head area between 50 normal (blue) and 50 abnormal (red) samples. (A) Area of 11,534 sperm heads from normal samples (blue) and 11,326 sperm heads from abnormal samples (red).

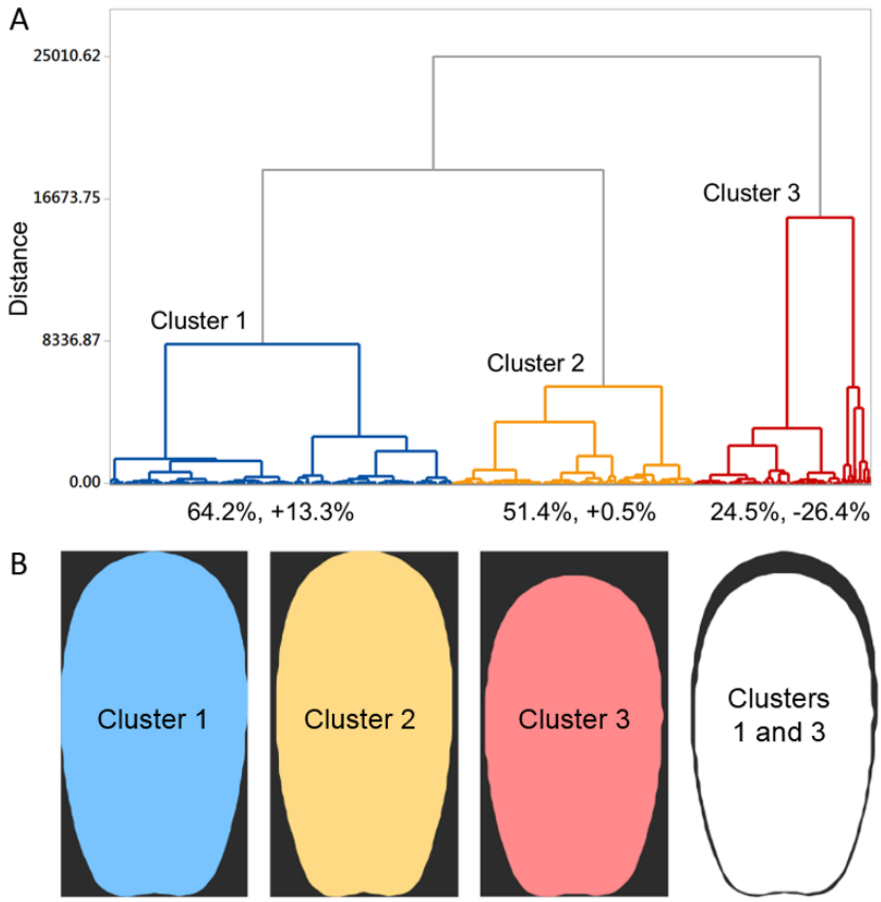


Figure 3: Analysis of the training group identifies distinct morphological clusters. (A) Cluster analysis of sperm from 30 normal and 30 abnormal individuals using measures of Area, Circularity, Variability, Maximum Height and Maximum Width by Ward linkage using squared Euclidean distance and standardised variables. This identifies three distinct clusters. Shown below each cluster is the percentage of sperm heads within that cluster that are from fertile animals and the difference in representation from that expected by chance (*i.e,.* a positive value indicates that fertile nuclei are overrepresented in a cluster and *vice versa* for a negative value). (B) The consensus shapes of the three clusters demonstrate the separation of nuclei on size and shape. Cluster 1 contains most of the normal sperm; cluster 2 has equal representation of normal and abnormal sperm; cluster 3 predominantly contains abnormal sperm. The increasing compaction of the nuclei is readily apparent overlaying consensus nuclei from clusters 1 and 3.

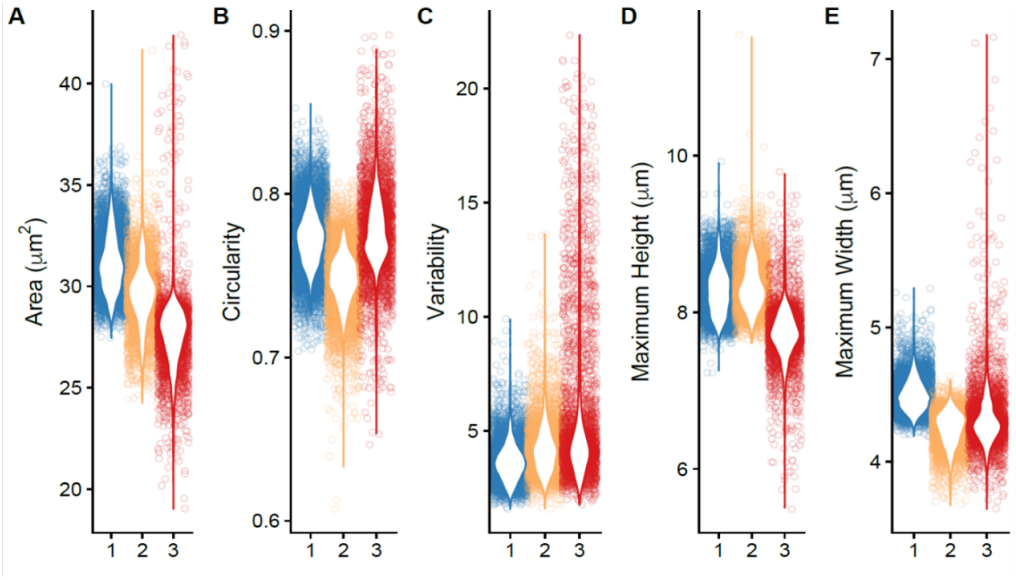


Figure 4: Sperm heads from the three clusters detected are morphologically distinct. Comparison of (A) Area, with 1>2>3 ($p < 0.001$ by pairwise *post hoc* test), (B) Circularity, with 3>1>2, (C) Variability, with 3>2>1, (D) Maximum Height, with 2>1>3, and (E) Maximum width, with 1>3>2, for the three clusters.

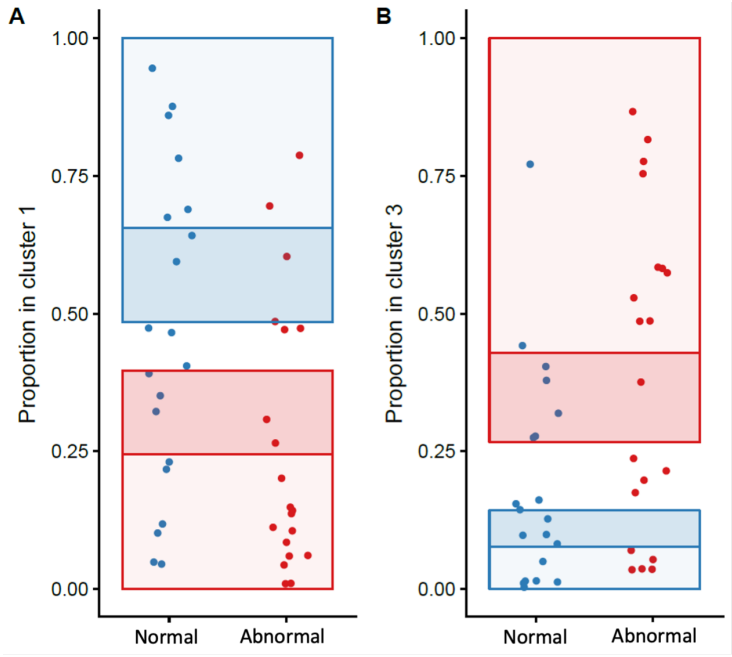


Figure 5: Representation of how cluster membership can be used to predict the status of a semen profile. The proportion of sperm heads from each sample in the test group that fall into (A) cluster 1 and (B) cluster 3 are illustrated. In both (A) and (B), the 95% confidence intervals from the training set are represented by the dark shaded rectangles and the areas between these confidence intervals and either 0 or 1 are represented by the light shaded rectangles. That is, blue points found in the regions falling onto the red rectangles represent normal samples that have been incorrectly classified as abnormal based on cluster membership and *vice versa* for red points falling into the regions defined by blue rectangles.

509 **Supplementary data**

510 ***Supplementary tables***

511 **Table S1.** Correlation matrix of measured parameters from the total dataset of 50 normal and 50

512 abnormal samples. Shown are the Pearson correlation values, all are significant at $p < 0.001$ except

513 for the correlation between Circularity and Variability where $p = 0.10$. Shaded boxes represent

514 Pearson correlation values > 0.8, and this was used to identify redundant variables.

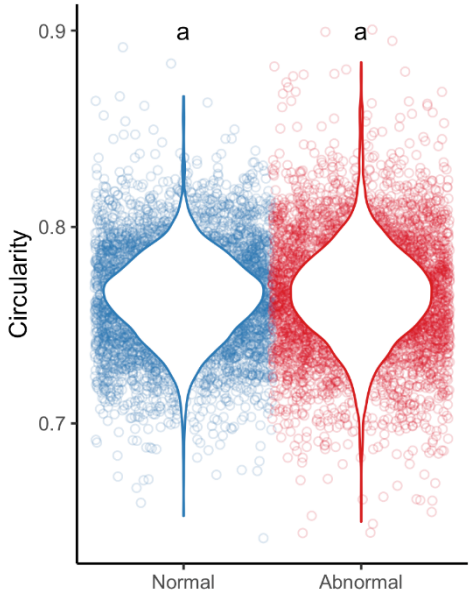
	Area	Perimeter	Maximum feret	Circularity	Minimum diameter	Aspect	Variability	Maximum Height
Perimeter	0.93							
Maximum feret	0.78	0.93						
Circularity	-0.12	-0.47	-0.64					
Minimum diameter	0.64	0.35	0.09	0.58				
Aspect	0.16	0.47	0.70	-0.88	-0.62			
Variability	-0.29	-0.26	-0.16	-0.01	-0.05	-0.15		
Maximum Height	0.78	0.92	1.00	-0.64	0.07	0.71	-0.17	
Maximum Width	0.61	0.34	0.09	0.54	0.96	-0.64	0.08	0.07

515

516 **Table S2:** Cluster membership as a predictive tool. Shown are the proportions of sperm heads falling
517 into clusters 1 and 3 for each of the test individuals. Predictions are made based on comparison to the
518 relevant 95% confidence interval derived from the training set, e.g., to be considered normal based
519 on the proportion of sperm heads in cluster 1 an individual would need to have a proportion higher
520 than the lower confidence limit. Prediction (exclusive) indicates a scenario where the aim is to exclude
521 the maximum number of samples and hence a sample is excluded if either prediction indicates
522 'abnormal'. Prediction (inclusive) indicates a scenario where the aim is to include the maximum
523 number of samples and hence a sample is included if either prediction indicates 'normal'.
524

Sample	Type	Proportion in cluster 1	Prediction from cluster 1	Proportion in cluster 3	Prediction from cluster 3	Prediction (exclusive)	Prediction (inclusive)
N1	N	0.11	AB	0.27	AB		
N2	N	0.22	AB	0.44	AB		
N4	N	0.94	N	0.04	N	N	N
N5	N	0.60	N	0.32	AB		N
N7	N	0.48		0.38	AB		
N13	N	0.11	AB	0.77	AB		
N17	N	0.04	AB	0.02	N		N
N18	N	0.47		0.16			
N21	N	0.87	N	0.01	N	N	N
N22	N	0.88	N	0.02	N	N	N
N24	N	0.64	N	0.02	N	N	N
N29	N	0.32	AB	0.02	N		N
N34	N	0.04	AB	0.41	AB		
N36	N	0.68	N	0.10	N	N	N
N41	N	0.40		0.14	N	N	N
N42	N	0.78	N	0.09	N	N	N
N44	N	0.67	N	0.17		N	N
N46	N	0.35	AB	0.27	AB		
N48	N	0.23	AB	0.13	N		N
N49	N	0.39	AB	0.10	N		N
A4	AB	0.21	AB	0.48	AB		
A6	AB	0.15	AB	0.81	AB		
A8	AB	0.00	AB	0.38	AB		
A9	AB	0.08	AB	0.48	AB		
A14	AB	0.11	AB	0.77	AB		
A15	AB	0.61	N	0.22		N	N
A16	AB	0.06	AB	0.87	AB		
A19	AB	0.27	AB	0.58	AB		
A20	AB	0.13	AB	0.76	AB		
A22	AB	0.15	AB	0.58	AB		
A29	AB	0.78	N	0.04	N	N	N
A33	AB	0.04	AB	0.53	AB		
A34	AB	0.00	AB	0.57	AB		
A35	AB	0.06	AB	0.19			
A36	AB	0.70	N	0.03	N	N	N
A38	AB	0.47		0.04	N	N	N
A39	AB	0.47		0.05	N	N	N
A40	AB	0.48		0.07	N	N	N
A43	AB	0.31	AB	0.18			
A49	AB	0.11	AB	0.23			

526 ***Supplementary figures***



527

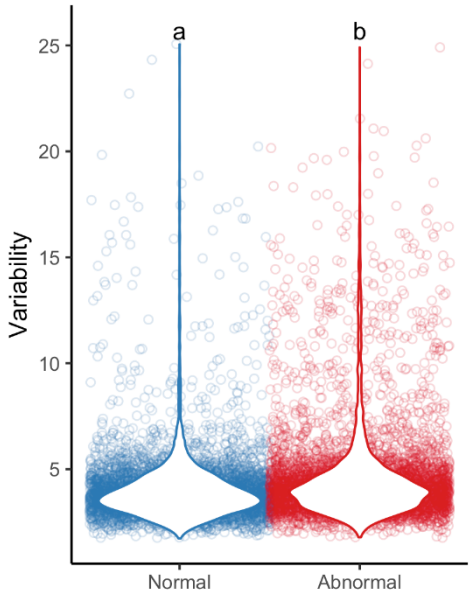
528 **Figure S1**: Comparison of sperm head Circularity between 50 normal (blue) and 50 abnormal (red)

529 samples. Representation of the difference in Circularity between 11,534 normal nuclei (blue) and

530 11,326 abnormal nuclei (red). Means with the same letter are not significantly different at the 95%

531 confidence interval.

532



533

534 **Figure S2:** Comparison of sperm head Variability between 50 normal (blue) and 50 abnormal (red)

535 samples. Representation of the difference in Variability between 11,534 normal nuclei (blue) and

536 11,326 abnormal nuclei (red). Means with the same letter are not significantly different at the 95%

537 confidence interval.

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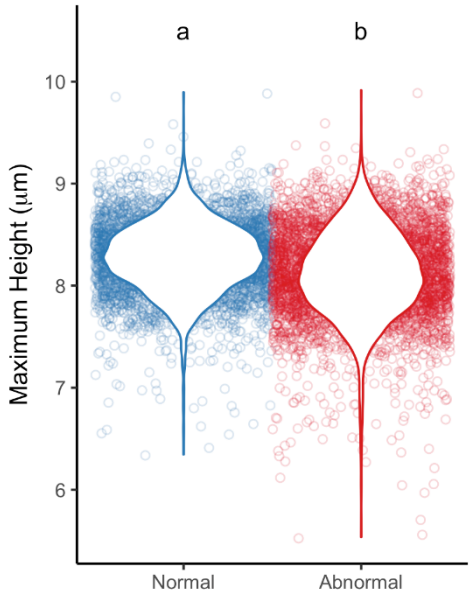

Figure S3: Comparison of sperm head Maximum Height (µm) between 50 normal (blue) and 50 abnormal (red) samples. Representation of the difference in Maximum Height (µm) between 11,534 normal nuclei (blue) and 11,326 abnormal nuclei (red). Means with the same letter are not significantly different at the 95% confidence interval.

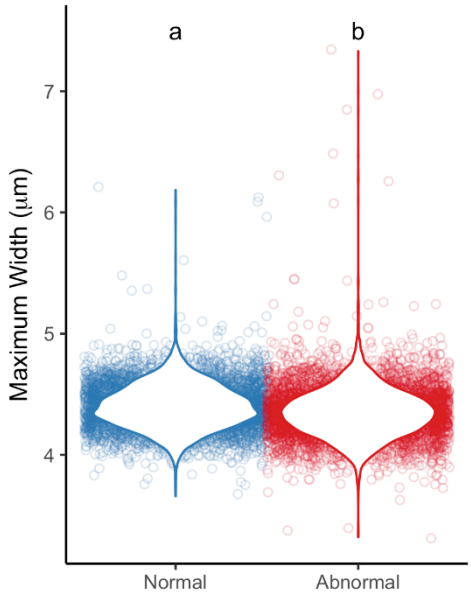

Figure S4: Comparison of sperm head Maximum Width (µm) between 50 normal (blue) and 50 abnormal (red) samples. Representation of the difference in Maximum Width (µm) between 11,534 normal nuclei (blue) and 11,326 abnormal nuclei (red). Means with the same letter are not significantly different at the 95% confidence interval.

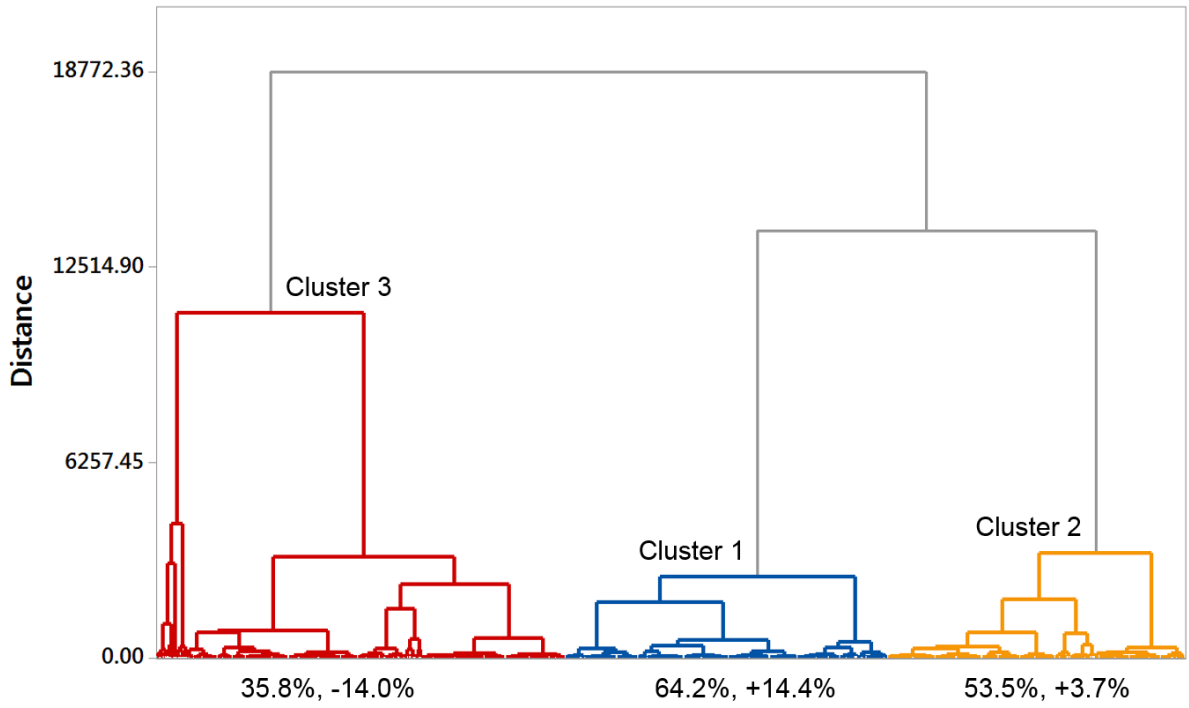

Figure S5: Analysis of the test group recovers the same clusters seen in the training group. Cluster analysis of sperm from 4,610 sperm heads from 20 normal samples and 4,642 sperm heads from 20 abnormal samples in the test group using measures of Area, Height, Width, Circularity and Variability by Ward linkage, squared Euclidean distance and standardised variables identifies the same three clusters that were seen in the training group. Below each cluster is the percentage of nuclei within that cluster that are from normal samples and the difference in representation from that expected by chance of (here, positive values show that normal nuclei are overrepresented in a cluster and vice versa for negative values).

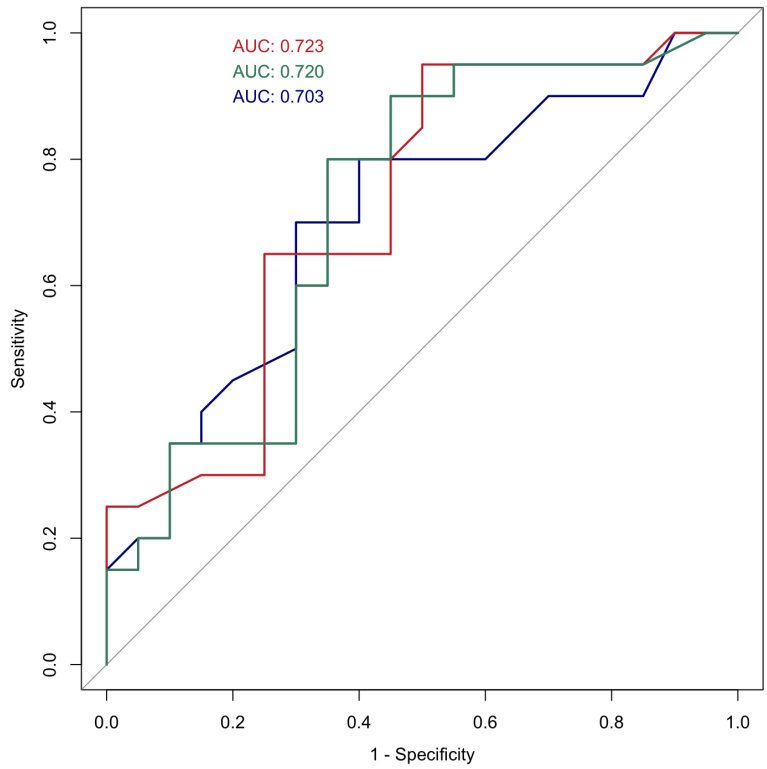

Figure S6: Empirical receiver operating curves based on the predictive functions of proportion in cluster 1 (blue), proportion not in cluster 3 (red) and the sum of proportion in cluster 1 and proportion not in cluster 3 (green). The figure also shows the respective values for the area under the curve (AUC) for each of the three predictor variables.