UNDERSTANDING THE IMPACT OF CLIMATE ON THE BOAR AND PROGENY THROUGH SPERM NON-CODING RNA

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Final Report prepared for the Australasian Pork Research Institute Limited (APRIL)

By

A/Prof Jeremy Cottrell[1]
A/Prof Mark Green[1]
Dr Darryl D'Souza[2]
Prof Anthony Hannan[1]

[1]The University of Melbourne
[2]Sunpork Group
Contact: jcottrell@unimelb.edu.au

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Executive Summary

This project aimed to investigate the effect of seasonal heat stress on boar sperm characteristics and non-coding RNA (sncRNA) profiles to provide insight into how these may impact fertility and production traits. As expected, sperm quality was significantly reduced in ejaculates collected over summer when compared with winter, with reduced motility and increased deformities evident. Due to boar availability, health, managerial interventions and sample quality the intended number of samples could not be collected and complete the full analytical pipeline. However, the final data set of 11 paired samples between summer and winter showed two sncRNAs that were significantly down-regulated in the summer, as well as numerous microRNAs that demonstrated substantial up- and down-regulation in the summer, including several microRNAs known to regulate spermatogenesis. With this low sample, statistical comparisons were not possible for fertility and production traits and a larger data set will be required to meet the project goals.

An immediate recommendation is the introduction of on-farm CASA quality control cut-offs for progressive motility and morphological criteria to remove the most heat-stressed ejaculates from being used in commercial breeding programs, negating detrimental effects on fertility and production traits. The second recommendation is to undertake a larger study to identify seasonal changes in sncRNA profiles that can be associated with favorable reproductive and progeny traits, as well as to determine the least heat stress susceptible genetic lines.

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1. Introduction

Background
Semen quality follows a seasonal pattern, with poorer quality in the summer months (Liu et al., 2022). While sperm are considered transcriptionally and translationally inactive, their transcriptome is known to be influenced by the transfer of small non-coding RNA (sncRNA) molecules secreted by the epididymis during the final maturation stage prior to ejaculation. Thus, sperm contain a rich population of coding and non-coding RNA molecules (Jodar et al. 2013), with functions that have been related to spermatogenesis (Ostermeier et al. 2002; Ablondi et al. 2021), sperm chromatin reorganization (Martins and Krawetz 2005; Hamatani 2012), fertility potential (Jodar et al. 2015), early embryo development (Sendler et al. 2013) and trans-generational epigenetic inheritance (Rando 2016) in many species. Hence, the study of the sperm transcriptome in the pig is crucial for understanding how sncRNA can be influenced by temporal climatic heat events (as well as other factors such as diet and physical activity) and ultimately drive progeny phenotype.

The aim of this project was to improve understanding of sncRNAs during seasonal heat events and how this drives improvement in progeny phenotype and output measures. While it is well known that summer results in declining sperm quality in multiple species, the mechanisms responsible, i.e. sncRNAs, the genes of interest and how these then result in changes in progeny, are yet to be elucidated. In the pig to date, previous proof of concept studies report that differences in sperm sncRNAs exist between summer and winter, but it remains to be seen whether this results in differences in herd performance.

The specific project aims were:

1. An improved understanding of the basis for paternal non-genomic contribution to progeny phenotype and industry performance measures, as well as how these are affected by season.
2. Quantifying the effect of heat-stress on boar sperm parameters and investigating management practices that could negate any detrimental effects to maximise the genetic heritable gains to manifest in progeny phenotype.
3. Development of a tool to aid the selection of genetic lines and increased rates of gain, due to the ability to test sperm before artificial insemination for fitness by sncRNA transcripts profiles for appropriate lines/stock for summer production.

2. Methodology

2.1 Animals and study design

The experiment was conducted at the PIC Genetic Nucleus facility at Grong Grong (NSW) following animal ethics approval (AEC Application 45B, CHM Alliance Animal Ethics Committee). PIC genetics are nationally significant, comprising 43% of the national sow herd. The PIC genetics comprises of five major lines and the objective for this experiment was to collect paired ejaculates from six individual boars in 5 genetic lines under Summer and “Winter”. The boars were selected based upon their prior reproductive performance (total number of services, average day 35 conception rate, farrowing rate, average number of piglets born alive, and average birth weight) within the Grong Grong Genetic Nucleus herd, with desired inclusion criteria of a minimum of 10 single sire matings. As spermatogenesis in boars requires three months for completion the experiment collected samples in late February (heat-stress sample), which is at the end of the warmest period of summer, and the control sample was collected in June. The collection at this time reflected *de novo* spermatogenesis through Autumn, to minimize any impact of cold stress. The environmental conditions at the time of semen collection were recorded from Narrandera Airport (20 km away) and the Temperature Humidity Index (THI) calculated, using the Equation $THI = 0.8*T + RH*(T-14.4) + 46.4$. In addition, boar phenotypic information (age, ejaculations frequency and health) was collected. Parameters of boar sperm quality, such as motility, concentration and count, were quantified by Computer Aided Sperm Analysis (CASA; Hamilton Thorne Software for boars, Beverly, MA, USA).

2.2 Sample collection, quality assessment and statistical analysis

Semen samples were collected manually from boars by farm staff and processed following the standard farm protocol. Specifically, total ejaculate volume was recorded by weight and quality assessed by CASA with a minimum total motility threshold of 75% for commercial use. Parameters from the CASA analysis (e.g. sperm count, concentration, motility and morphology) were recorded. These parameters were analysed to account for any seasonal differences between the summer and winter ejaculates. A total of 18 boars had paired collections under both summer and winter conditions, but there was a total of 41 boars collected from for the total experiment ("unpaired" collections), these data sets were analysed using the Wilcoxon and Mann-Whitney tests, respectively with GraphPad Prism, Version 10 (San Diego, CA, USA). These parameters were also analysed to account for any seasonal differences between genetic lines, where possible, using a one-way ANOVA performed using SAS (SAS Institute, Cary, NC, USA). All data are presented as mean ± standard deviation, with $P < 0.05$ denoted as statistically significant.

Sperm for sncRNA analysis were collected using a "swim up" protocol modified from Navarro-Serna et al. (2021). Briefly, the ejaculate was left to liquify for 10 minutes in the dark at room temperature. One millilitre of liquified sample was removed from the upper surface of the sample and placed under 0.6 mL of warm (38.5˚C) Gx-IVF medium (Vitrolife; Gothenburg, Sweden) in a 15 mL Conical tube (Falcon; Thermo Fisher Scientific, Scoresby, Aus). The tube was then placed at a 45˚ angle in a tube holder in an incubator set at 38.5˚C for 40 mins. For each boar, triplicate swim-up assays were undertaken. After 40 mins, the top 0.6 mL of each swim-up was transferred and combined into a 2.0 mL RNAse free conical tube (Thermo Fisher Scientific) and centrifuged at 300 *g* for 10 mins at room temperature. The fluid was aspirated, and the pellet snap frozen in dry ice before being stored at -80 ˚C until analysis.

2.3 RNA Extraction

Total RNA was extracted from boar sperm samples using the miRNeasy Micro Kit (Cat# 217084, Qiagen, Netherlands). Frozen sperm samples were thawed on ice and RNA was isolated in 1000 µL Qiazol supplemented with 100 µL of Bond-Breaker TCEP solution (Cat# 77720, Thermo Fisher Scientific). Samples were homogenised via shearing by repeated aspiration through a 26 G needle (BD Biosciences, Macquarie Park, NSW). The homogenate was then incubated within the Qiazol solution for 1 hour at 4°C, and subsequently for 5 mins at room temperature. Subsequently, 200 µL of chloroform was added to the tube, shaken vigorously and incubated at room temperature for 3 mins. Following a 15-minute centrifugation at 12,000 *g* at 4°C, the upper aqueous phase of each sample was transferred to a new sterile 1.5 mL collection tube, to which was added 525 µL of ice-cold isopropanol supplemented with 1µL of (5 mg/mL) glycogen (Cat# AM9510, Ambion, Invitrogen). Samples were incubated overnight at -20°C. The following day, samples were transferred into RNeasy MinElute spin column in 2 mL collection tubes and centrifuged at 8000 *g* for 15 seconds at room temperature. Following this 700 µL of RWT buffer was added to the column and centrifuged at 8000 *g* for 15 seconds. The column was further washed with 500 µL of RPE and centrifuged at 8000 *g* for 15 seconds, followed by a final wash with 500 µL of 80% ethanol and centrifugation at 8000 *g* for 2 mins. Flow through was discarded at each stage. The RNeasy MinElute spin column was placed into a new, sterile 2 mL collection tube and centrifuged at 14000 *g* for 5 minutes to dry the membrane of the column. Finally, RNA was eluted with 14 µL of RNase-free water and centrifuged for 1 min at 14000 *g* at 4°C.

2.4 RNA sequencing

Eluted RNA was submitted to the Australian Genome Research Facility (AGRF; Melbourne Victoria) for RNA quality control (RIN) and quantification assessment (ng/µL) determined using an Agilent Tape Station, prior to small RNA library preparation, Next Generation Sequencing and mapping to the reference genome. Only boars with both a paired summer and winter sample with a total RNA of > 62 ng were selected for the NEXTFLEX small RNA v4 library preparation. Samples of 150 bp paired end runs were sequenced at a depth of 50 million reads on the Illumina NovaSeq X Plus platform. The primary bioinformatics analysis involved demultiplexing and quality control. Data were then processed through a small RNA-seq expression analysis

workflow, which included trimming, alignment, and quantification. The extraction and sequencing steps were similar to those previously described (Short et al., 2016; 2017). The primary sequence data were generated using the Illumina DRAGEN BCL Convert 07.021.645.4.0.3 pipeline. The sequence files are generated in a standard FASTQ format. The sequence reads from all samples were analysed according to AGRF quality control measures. The per base sequence quality had >62% bases above Q30 across all samples. The reads were also screened for the presence of any Illumina adapter/overrepresented sequences and cross-species contamination. The cleaned sequence reads were then aligned against the *Sus scrofa* genome (Sscrofa 11.1). The STAR aligner (v2.5.3a) was used to map reads to the genomic sequences using parameters that are optimised for small RNA-sequences reads.

EdgeR (version 4.2.1) was used to perform the differential expression analysis using R 4.2.1. EdgeR is a package used to detect and quantify differential expression of digital gene expression data, that is, counts of reads mapped for each gene. The default TMM normalisation method of edgeR was used to normalise the counts between samples for false discovery rates. A generalised linear model was then used to quantify the differential expression of all sncRNA (miRNA and other sncRNAs) between the summer and winter groups based on an adjusted P-value ($P<0.05$) or the Fold Change (up or down-regulated by two-fold, equivalent to a 1 $\log_2$FC). A second sub-analysis was also undertaken, in which only miRNAs were analysed to determine those that were differentially expressed based on an adjusted P-value ($P<0.05$) or the Fold Change (up or down-regulated by 1.5-fold, equivalent to a 0.58 $\log_2$FC).

3. Outcomes

3.1 Weather and Thermal Humidity Index (THI)

Ejaculate sample collections were undertaken on 19th/20th February (summer) and 24th/25th June (winter) 2024. The maximum daily temperature in Jan/Feb was 33°C and May-Jun 19°C, but noting that spermatogenesis can take 3 months to complete and the sncRNA would represent changes over that time and just not the collection day. The daily temperature at 3 pm and THI for the months of February and June are presented in Figure 1. Notably, in February the maximum temperatures (Figure 1A) typically exceeded 30°C and overwhelmingly were in the "moderate" range of heat stress for the month. Note that the 3pm readings were not the maximum for the day, but allowed calculation of the THI.

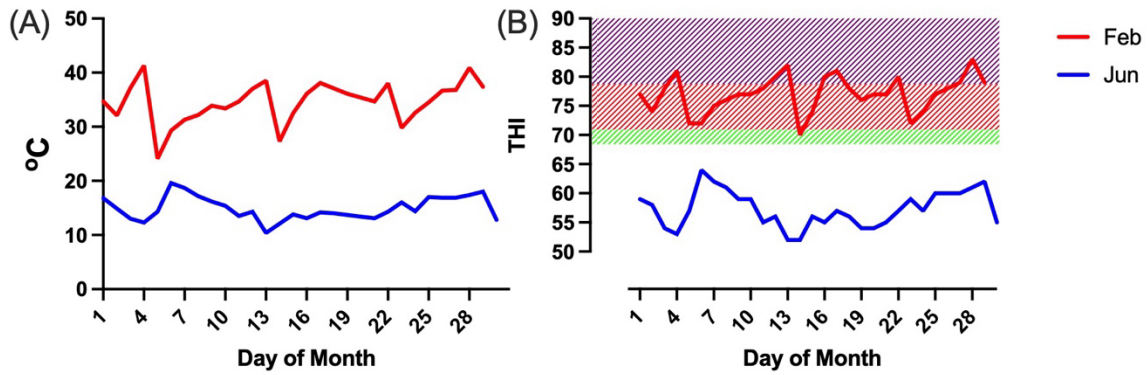

Figure 1(A) Maximum daily temperatures (°°C) and (**B)** Thermal Humidity Index (THI) at 3 pm for the months of February (Summer) and June (Winter) 2024 at Narrandera, NSW. For **(B)** hatching represents mild, moderate and severe heat stress conditions.

3.2 Sample numbers and analyses

The objective for the experiment was to collect 60 paired samples from 30 boars from five genetic lines. Ultimately, samples were collected from 23 out of 30 planned boars in summer (mean age 394.3 ± 39.6 days)

and 18 boars in the winter (mean age 519.3 ± 39.6 days), due to boar availability, health, managerial interventions and sample quality. Whilst in total 41 samples were collected across seven genetic lines, only genetic lines B and C had > four boars per line to allow possible comparisons between lines.

Sperm characteristics were analysed for the 41 unpaired and 18 paired samples. RNA was extracted from all 41 available samples. Based on boars having only one sample (n=5) or a total RNA yield of > 62 ng in both samples from an individual (n=9), then only 26 samples (13 paired samples) were selected for small RNA analyses. Following initial principal component analysis to investigate the effect of season on sncRNA profiles, data from two boars (4 samples) were removed based on these being obvious outliers. These two boars had the highest 'slow motility' sperm percentages > 17%, whereas the rest of the cohort averaged ~7%. Notably, these high proportion of slow sperm were evident in both winter and summer ejaculates for these two boars, thus likely to confound analyses when trying to identify differences in miRNA profiles between seasons.

Supplementary Table 1 (Appendix 1) summarises individual samples used for the various data analyses of sperm characteristics, reproductive performance, as well as sncRNA profiles.

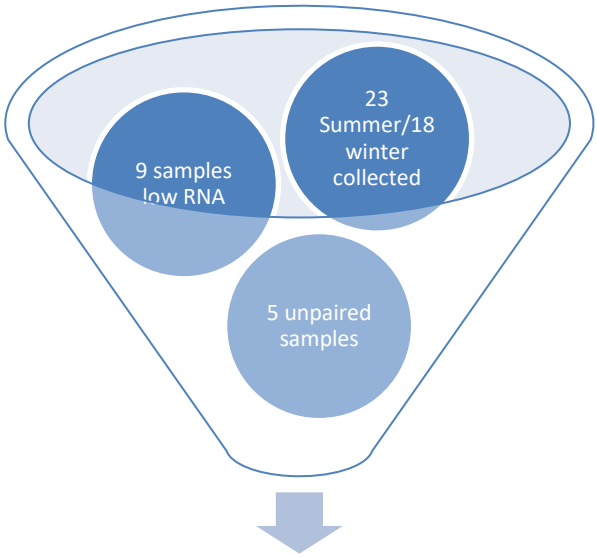

Figure 2: The experiment aimed to collect 30 paired ejaculates from individual boars in Summer and Winter. While 41 samples were analysed for sperm characteristics, ultimately 22 samples (11 paired) were collected for the sncRNA final analysis, through a combination of on farm factors, low RNA in samples and statistical outliers identified during analysis.

3.3 Reproductive performance

Evaluation of reproductive performance data revealed a wide range in the number of matings per boar (0 to 63 services), average day 35 pregnancy rate (64 to 100%), as well as average number of live piglets per litter and average birth weight (Table 1). Because matings and reproductive outcome data were from across the year (not in defined winter and summer season) and due to low sample size, as well as number of boars per genetic line, the data set was not sufficiently large enough to perform statistical analyses.

Table 1: Reproductive performance characteristics of 23 boars included in the study.

Boar ID	Genetic Line - deidentify	Age at summer (d)	Age at winter (d)	Total Services	Av. d35 Conception Rate (%)	Farrowed	Av. piglets born alive	Av. Birth Weight (kg)
1	A	389	514	2	100	1	7	1.9
2*	A	367	492	0	0	0	0	0
3*	B	480	605	24	75	17	12	1.3
4	B	442	567	30	77	3	12	1.2
5	B	412	537	6	83	5	13	1.2
6	B	400	525	8	88	7	13.7	1.3
7	B	360	485	4	100	4	12.5	1.2
8	C	472	597	63	94	48	12	1.3
9*	C	427	552	47	79	23	12	1.2
10	C	393	518	22	95	18	11	1.5
11	C	379	504	1	100	1	12	1.4
12	C	308	433	0	0	0	0	0
13	D	427	552	20	95	16	12.1	1.3
14	D	412	537	47	81	36	12	1.3
15	D	395	520	22	95	20	11	1.2
16*	D	383	508	14	93	12	13	1.1
17	E	382	507	7	100	7	9	1.4
18	E	329	454	0	0	0	0	0
19	F	390	515	12	75	6	7	1.2
20	F	388	513	11	90	6	10	0.9
21*	F	353	478	0	0	0	0	0
22	G	393	518	9	67	4	6	1.7
23	G	388	513	11	64	3	9	1.1

* Denotes only summer ejaculate collected.

3.4 Sperm characteristics

Analysis was undertaken of all samples (unpaired, n=41 samples from 23 boars) and those for which a paired summer and winter sample was collected (paired, n=36 samples from 18 boars). Effects of season on sperm morphology were evident, with the proximal and distal droplets increased, resulting in a lower normal fraction in the summer (P=0.004 and <0.0001 for unpaired and paired respectively, Table 2). Furthermore, the paired analysis also showed an increased concentration and fraction of sperm with bent tails (P=0.046). A notable decrease in the percentage of progressive motility was identified in the summer for the paired samples (P=0.010). Likewise, there was a trend for a lower total sperm count (P=0.089) and percentage of motile sperm in the summer (P=0.093), while trends for a higher static count (P=0.093) and Distal Midpiece Reflex (DMR, P=0.086) were also observed in paired samples.

Collectively, these results highlight functional negative differences in sperm quality when collected in summer compared with winter. Specifically, there is a lower sperm count, impaired motility (reduced progressive and motile fractions) and impaired morphology (bent tails, presence of droplets and reduced normal sperm fraction) at ejaculation.

Table 2: Boar sperm characteristics quantified by computer aided sperm analysis (CASA) of ejaculates collected in the winter and summer.

		Unpaired (N = 41)			Paired (N = 36)		
Characteristic		**Summer**	**Winter**	**P**	**Summer**	**Winter**	**P**
Total count	Count	445 ± 126	494 ± 147	0.29	428 ± 116	494 ± 147	*0.089*
	M/mL	460 ± 179	485 ± 239	0.99	440 ± 148	485 ± 239	0.31
Motility							
Static	M/mL	51.5 ± 49.8	33.1 ± 31.3	0.34	46.4 ± 43.5	33.1 ± 31.3	0.23
	%	12.9 ± 13.3	8.10 ± 8.30	0.17	12.3 ± 12.7	8.10 ± 8.30	*0.093*
Progressive	M/mL	344 ± 179	385 ± 217	0.67	330 ± 154	385 ± 217	0.17
	%	72.8 ± 16.0	78.2 ± 11.1	0.27	72.9 ± 15.3	78.2 ± 11.1	**0.010**
Motile	M/mL	408 ± 187	452 ± 240	0.73	394 ± 162	452 ± 240	0.16
	%	87.1 ± 13.3	91.9 ± 8.30	0.17	87.7 ± 12.7	91.9 ± 8.30	*0.093*
Slow	M/mL	33.2 ± 30.9	36.2 ± 26.0	0.35	33.7 ± 32.6	36.2 ± 26.0	0.58
	%	7.60 ± 6.50	7.80 ± 5.50	0.62	7.80 ± 6.70	7.80 ± 5.50	0.48
Morphology							
Bent Tail	M/mL	15.2 ± 12.1	10.7 ± 8.31	0.20	15.9 ± 13.1	10.7 ± 8.31	**0.046**
	%	3.50 ± 2.80	2.40 ± 1.70	0.28	3.80 ± 3.10	2.40 ± 1.70	**0.034**
Coiled Tail	M/mL	3.44 ± 4.82	1.76 ± 1.50	0.49	3.91 ± 5.42	1.76 ± 1.50	0.25
	%	0.90 ± 1.30	0.40 ± 0.30	0.43	1.00 ± 1.40	0.40 ± 0.30	0.22
Distal midpiece	M/mL	7.43 ± 13.0	8.02 ± 15.7	0.51	8.64 ± 14.4	8.02 ± 15.7	0.45
reflex (DMR)	%	1.90 ± 4.00	1.80 ± 4.00	0.43	2.30 ± 4.50	1.80 ± 4.00	*0.086*
Distal Droplet	M/mL	10.5 ±9.85	6.68 ± 6.94	0.13	10.1 ± 9.14	6.68 ± 6.94	*0.062*
	%	2.30 ± 2.00	1.30 ± 1.30	**0.018**	2.50 ± 2.20	1.30 ± 1.30	**0.012**
Proximal Droplet	M/mL	15.7 ± 12.6	7.36 ± 5.60	**0.019**	15.8 ± 13.07	7.36 ± 5.60	**0.0004**
	%	3.60 ± 3.10	1.70 ± 1.50	**0.008**	3.80 ± 3.40	1.70 ± 1.50	**0.0014**
Normal Fraction	%	91.2 ± 5.10	95.1 ± 3.01	**0.004**	90.5 ± 5.60	95.1 ± 3.00	**<0.0001**

Data analysed for the entire dataset (unpaired, n=41 samples from 23 boars), or limiting to paired samples collected in summer and winter for the same boar (paired, n=36 samples from 18 boars).

3.5 sncRNA analyses

Analyses were undertaken using the data from the 26 paired samples from 13 boars. Following the initial screening of normalised data, the paired samples from two boars were found to be noticeable outliers in the PCA plot and failed to cluster as a pair in the heat map. Evaluation of their sperm characteristics revealed that irrespective of season these four samples had the highest percentage of slow motile sperm (>17.0%), hence were removed from subsequent analyses, due to this parameter likely being associated with changes in sncRNA and thus confounding analyses with respect to season. The remaining 22 paired samples from 11 boars were then analysed.

Figure 3 shows the PCA plot based on the distance between samples corresponding to the leading log fold change between each pair of RNA Seq samples using the 440 sncRNAs found to be differentially expressed for the 11 boars. No clear clustering of samples by season was identified when 43% of the variation was accounted for between the two dimensions. Equally, for the secondary sub-analysis a PCA plot using only the 315 miRNA was produced (**Figure 4**). Again, no clear clustering of samples by season was evident, despite 76% of the variation being captured in the two dimensions. **Figure 5** shows the overall heatmap of the 440 sncRNAs found to be differentially expressed between summer and winter. However, only two sncRNAs were significantly different (P<0.05) between seasons, miR9843 and LOC110259929 (Figure 6). When only miRNAs were analysed, using different threshold cut-offs, then 315 miRNAs were found to be differentially expressed (Figure 7), with none found to differ significantly (P<0.05) between seasons. Table 3 shows the highest and lowest differentially expressed miRNAs based on fold-changes.

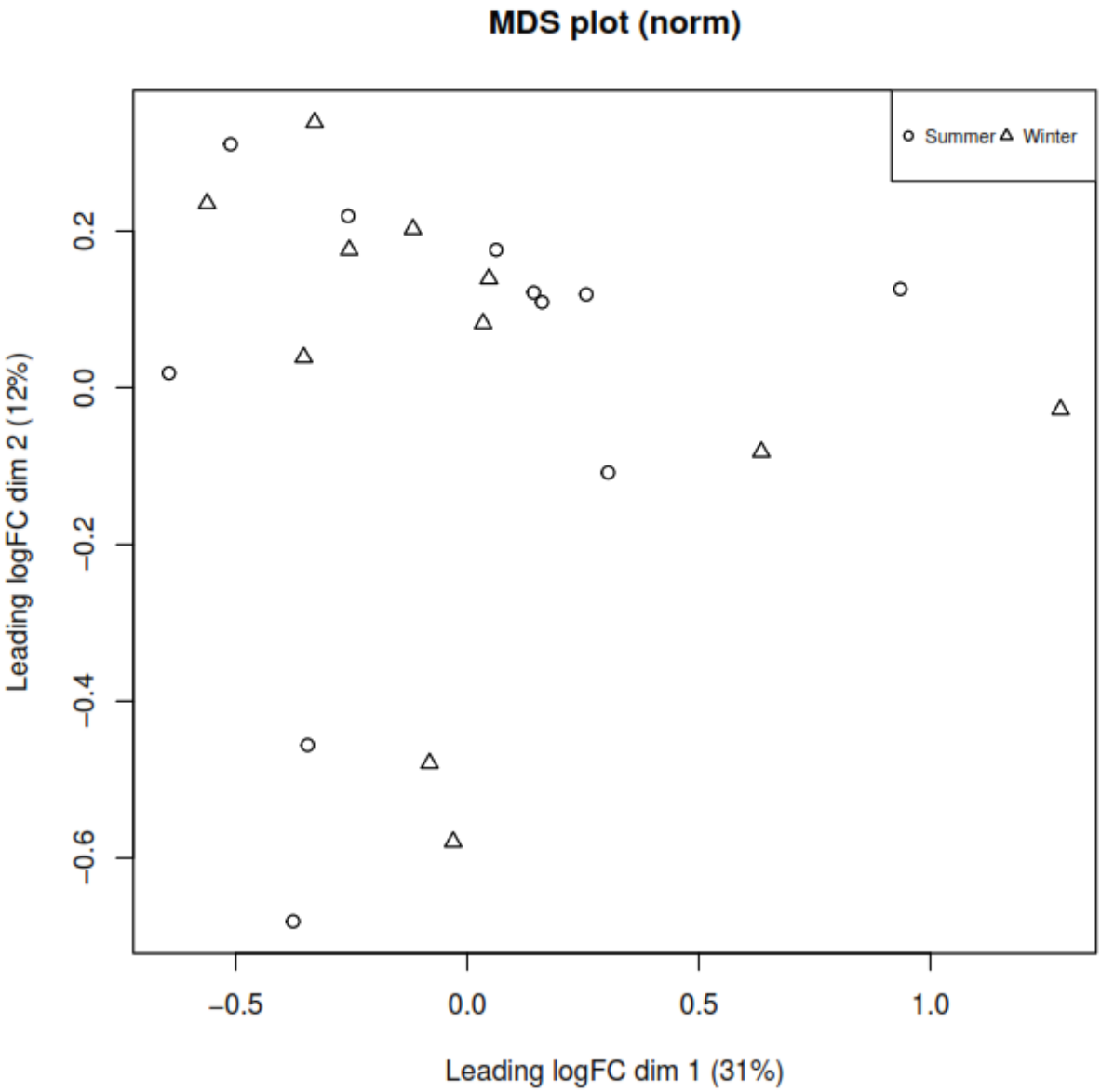

Figure 3: PCA plot depicting the clustering of samples performed for the 440 sncRNAs that were differentially expressed**.** The distance between the samples corresponds to the leading log fold change between each pair of RNAseq samples (n=22 samples, n=11 pairs) in winter (green font) and summer (blue font). The text = Boar ID. No clear clustering based on season is evident.

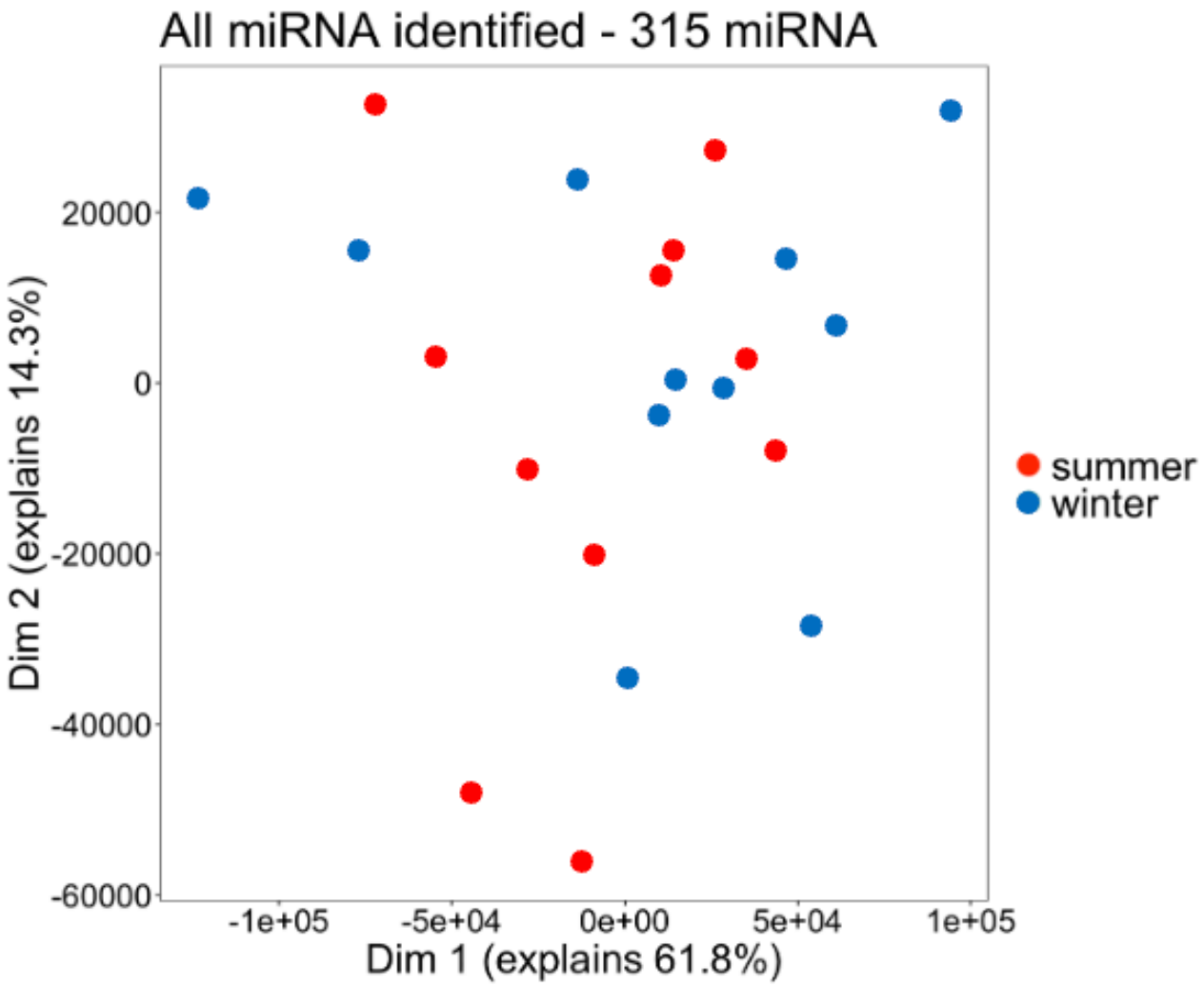

Figure 4: PCA plot depicting clustering of miRNAs for 22 sperm samples. The distance between the samples corresponds to the leading log fold change between each pair of RNAseq samples (n=22 samples, n=11 pairs) in winter (blue font) and summer (red font). The text = Boar ID. No clear clustering based on season is evident.

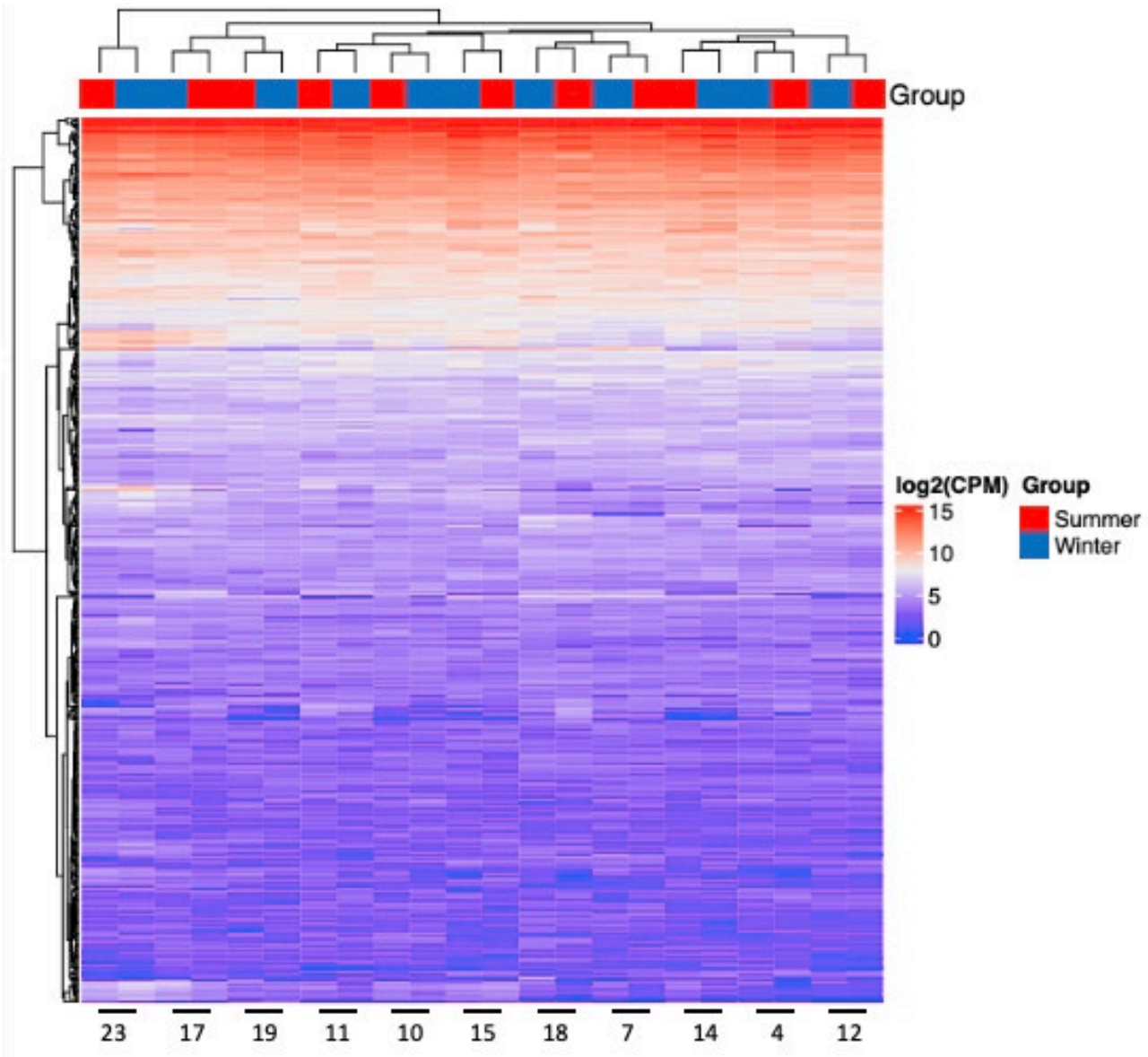

Figure 5: Overall heatmap for the 440 differentially expressed sncRNAs in the 22 sperm samples. The heatmap values are log_2 counts of expression in summer versus winter samples. The colour scale (blue to red) represents the log_2 counts (low to high, negative to positive). S= Summer, W= Winter and the de-identify boar ID.

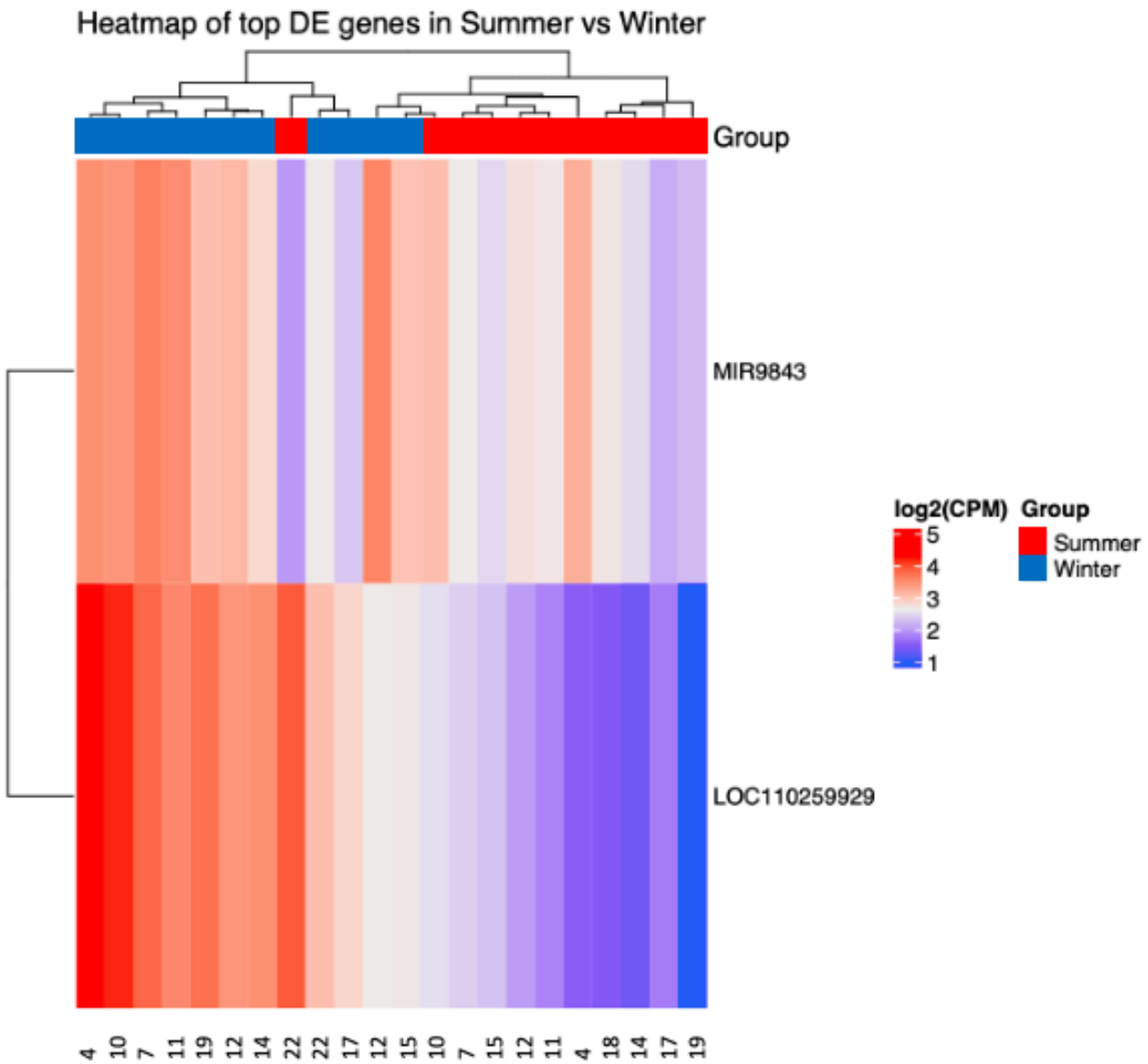

Figure 6: A heat map comparison for the two significantly differentially expressed (DE) sncRNAs. These sncRNAs are mIR9843 and LOC110259929. They have an adjusted P value of <0.05. S= Summer, W= Winter and the de-identify boar ID.

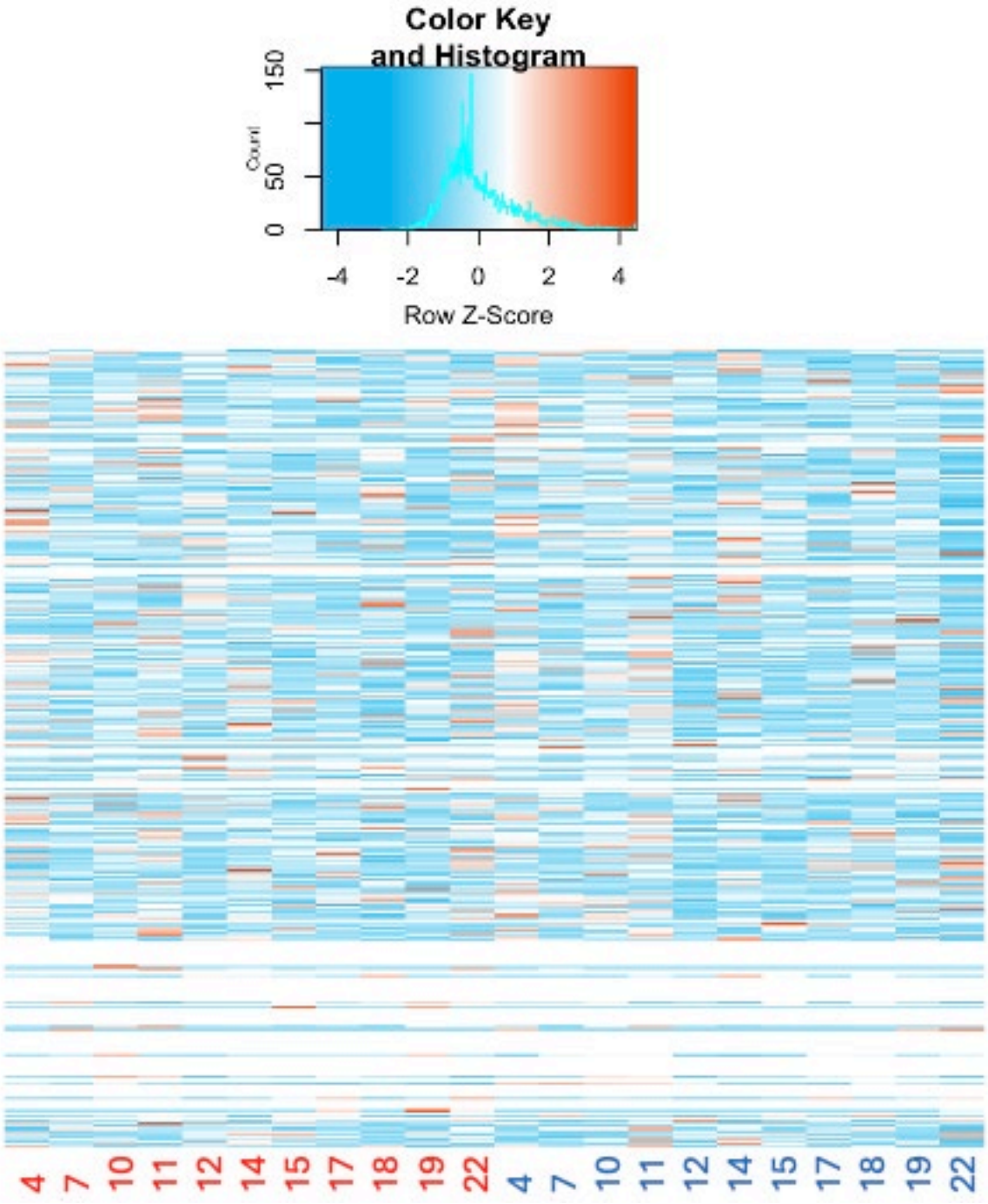

Figure 7: Heatmap for the 315 differentially expressed miRNAs in the 22 sperm samples. The heatmap values are $\log_2$ counts of expression in summer versus winter samples. The colour scale (blue to red) represents the $\log_2$ counts (low to high, negative to positive). Red = Summer, Blue = Winter and the number = de-identify boar ID.

Table 3: The miRNAs with the highest and lowest differential expression based on fold changes (log_2FC) cut-offs of 0.5 and -0.5 for sperm samples (n=22) collected from boars in the Summer and Winter.

Up-regulated miRNA		Down-regulated miRNA	
miRNA	**Log_2FC value**	**miRNA**	**Log_2FC value**
miR9858	3.13	miR214	-1.68
miR9796	2.73	miR9-3	-1.20
miR181B-1	1.47	miR371	-1.07
miR451	1.45	miR365-2	-0.95
miR196A-1	1.42	miR24-1	-0.80
miR1271	1.10	miR708	-0.77
miR615	1.07	miR219B	-0.62
miR208B	0.88	miR9843	-0.61
miR9812	0.76	miR135-1	-0.61
miR9795	0.76	miR133B	-0.60
miR339-2	0.76	miR218-1	-0.56
miR181B-2	0.76	miR9823	-0.56
miR224	0.68	miR124A-1	-0.56
miR7138	0.65	miR24-2	-0.54
miR19B-1	0.63	miR30A	-0.54
miR9810	0.56	miR150-1	-0.52
miR452	0.52		
miR218B	0.51		

4. Application of Research

4.1 Effects of season on sperm characteristics

Sperm quality was significantly reduced in ejaculates collected over summer when compared with winter. This was characterized by reduced motility and increased deformities, such as bent tails and retained droplets, in agreement with earlier research (Stone, 1982; Huang et al., 2000; Zasiadczyk et al., 2015), and is likely a major factor behind why matings need to be increased in summer months to achieve the same number of piglets born (Liu et al., 2022). Currently, there are not firm guidelines on the required percentage of total motility as a quality control threshold, therefore, an immediate and easy-to-implement finding of this study is to introduce CASA quality control cut-offs for progressive motility and morphological criteria when assessing ejaculates on farm. This can be adopted by the Grong Grong Nucleus herd and other producers to have direct commercial benefits to negate the major negative impacts of heat stress on reproductive performance and progeny production traits. Future studies can then be conducted to determine the cost benefits on production systems derived from excluding ejaculates severely comprised by heat stress.

4.2 Association of sncRNA with season

For a variety of on-farm and technical reasons the current study was unable to obtain the planned 60 samples from 30 boars or to quantify sncRNA variations between genetic lines. However, based on the analysis of the 11 paired samples a preliminary interrogation of the impact of season on sncRNA profiles was possible. The analysis revealed indistinct clustering of both 440 differentially expressed sncRNAs and the sub-analysis of 315 differentially expressed miRNA, which indicates that the observed changes in sncRNA profiles are likely due to many factors, with season not being solely responsible for the sncRNA profiles. Whilst it should be acknowledged that the lack of distinct clustering may also be attributed to limited sample numbers, the current study is the largest undertaken to date in the pig. This study did identify two novel miRNAs (miR9843 and an uncharacterised sncRNA) that were significantly down-regulated in the summer. In addition, analysis of only the miRNA population determined numerous miRNAs to be substantially up- or down-regulated, many of which have not been described in pig spermatozoa before. From those shown to be differentially expressed, miR214 is of particular interest, which was the most down-regulated miR. This miRNA is known to be involved in regulating sperm maturation (Belleannée et al., 2013) and is thus potentially a prime candidate facilitating the negative effects of heat stress on sperm quality.

Collectively, findings from the current study provide a promising insight into identifying sncRNAs that may be responsible in mediating heat stress effects. Future larger studies are thus warranted to determine seasonal changes in sncRNA profiles. The inclusion of more boars and inclusion of progeny traits would allow the association between specific sperm characteristics (e.g. bent tails) to be investigated, and understanding how these profiles alter progeny traits, as well as the identification of specific sncRNAs that may be invention targets. Application of this knowledge would thus be commercially useful for producers to maximise environmental and genetic interactions resulting in financial gains despite having a limited genetic pool and an inability to introduce heat stress resistant alleles into the population.

5. Conclusions

This study identified that boar sperm motility and morphology parameters are negatively impacted by summer heat stress. An immediate and easy to implement commercially beneficial finding of this study is to introduce CASA quality control cut-offs for progressive motility and morphological criteria when assessing ejaculates on farm. This would remove the most severely heat-stress affected ejaculates from commercial breeding programs and thus likely negate the most pronounced negative impacts on reproductive performance and progeny production traits. Analysis of sncRNA profiles in the limited number of available samples identified two sncRNAs that were significantly down-regulated in the summer, as well as numerous miRNAs that demonstrated substantial up- and down-regulation in the summer, including several miRs known to regulate spermatogenesis. Future studies are warranted with larger numbers as well as reproductive and progeny performance data to robustly determine seasonal sncRNA profile changes and understand how these mediate commercially desirable improvements in reproductive and progeny traits.

6. Limitations/Risks

Introduction of CASA quality control cut-offs for progressive motility and morphological criteria is quick to implement with no perceivable risks. One limitation of the current study was that in the summer of 2024 boars only experienced mild summer heat-stress, relative to previous years, thus this could affect the ability to easily determine differential expression of sncRNAs between seasons. Future sncRNA studies are limited by the availability of sufficient boars, along with their associated high-quality season specific reproductive and progeny performance data (including daily feed intake, average daily gain and FCR), as well as the optimisation of RNA sample isolation and processing techniques.

7. Recommendations

As a result of the outcomes in this study the following recommendations are advocated:

1. The introduction of CASA quality control cut-offs for progressive motility and morphological criteria when assessing ejaculates on farm to remove the most heat stressed ejaculates from being used in commercial breeding programs.
2. Investment in a larger study to identify seasonal changes in sncRNA profiles that can be associated with favorable reproductive and progeny traits, as well as to determine the least heat stress susceptible genetic lines.

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Appendix 1 - Notes

Confidential Information

Information regarding the genetic lines of boars used in this project have been deidentified, the true genetic lines remain confidential.

Deficient Report

If APRIL reasonably forms the view that the Final Report does not adequately set out matters referred to, it must notify the Researcher of the extent to which it believes the Final Report is deficient.

Appendices

Appendix 1:

Supplementary Table 1: Detailed summary of individual samples used for the various data analyses of sperm characteristics, reproductive performance, as well as sncRNA profiles.

Boar ID	Genetic Line	Season	Used in sperm analyses	RNA extracted	Sample sequenced	Used in paired sncRNA analyses	Exclusion reason
1	A	Summer	Yes	Yes	Yes	No	> 17% slow sperm motility, consistent in both seasons
1	A	Winter	Yes	Yes	Yes	No	> 17% slow sperm motility, consistent in both seasons
2	A	Summer	Yes	Yes	No	No	Not a paired sample
3	B	Summer	Yes	Yes	No	No	Not a paired sample
4	B	Summer	Yes	Yes	Yes	Yes	
4	B	Winter	Yes	Yes	Yes	Yes	
5	B	Summer	Yes	Yes	No	No	< 62 ng total RNA
5	B	Winter	Yes	Yes	No	No	< 62 ng total RNA
6	B	Summer	Yes	Yes	Yes	No	> 17% slow sperm motility, consistent in both seasons
6	B	Winter	Yes	Yes	Yes	No	> 17% slow sperm motility, consistent in both seasons
7	B	Summer	Yes	Yes	Yes	Yes	
7	B	Winter	Yes	Yes	Yes	Yes	
8	C	Summer	Yes	Yes	No	No	< 62 ng total RNA
8	C	Winter	Yes	Yes	No	No	< 62 ng total RNA
9	C	Summer	Yes	Yes	No	No	Not a paired sample
10	C	Summer	Yes	Yes	Yes	Yes	
10	C	Winter	Yes	Yes	Yes	Yes	
11	C	Summer	Yes	Yes	Yes	Yes	
11	C	Winter	Yes	Yes	Yes	Yes	
12	C	Summer	Yes	Yes	Yes	Yes	
12	C	Winter	Yes	Yes	Yes	Yes	
13	D	Summer	Yes	Yes	No	No	< 62 ng total RNA
13	D	Winter	Yes	Yes	No	No	< 62 ng total RNA
14	D	Summer	Yes	Yes	Yes	Yes	
14	D	Winter	Yes	Yes	Yes	Yes	
15	D	Summer	Yes	Yes	Yes	Yes	
15	D	Winter	Yes	Yes	Yes	Yes	
16	D	Summer	Yes	Yes	No	No	Not a paired sample
17	E	Summer	Yes	Yes	Yes	Yes	
17	E	Winter	Yes	Yes	Yes	Yes	
18	E	Summer	Yes	Yes	Yes	Yes	
18	E	Winter	Yes	Yes	Yes	Yes	
19	F	Summer	Yes	Yes	Yes	Yes	
19	F	Winter	Yes	Yes	Yes	Yes	
20	F	Summer	Yes	Yes	No	No	< 62 ng total RNA
20	F	Winter	Yes	Yes	No	No	< 62 ng total RNA
21	F	Summer	Yes	Yes	No	No	Not a paired sample
22	G	Summer	Yes	Yes	Yes	Yes	
22	G	Winter	Yes	Yes	Yes	Yes	
23	G	Summer	Yes	Yes	No	No	< 62 ng total RNA
23	G	Winter	Yes	Yes	No	No	< 62 ng total RNA