

Tropical Summer Induces DNA Fragmentation in Boar Spermatozoa: Implications for Evaluating Seasonal Infertility

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Abstract

Summer infertility continues to undermine pig productivity, costing the pig industry millions in annual losses. The boar's inefficient capacity to sweat, non-pendulous scrotum, and the extensive use of European breeds in tropical conditions, can make the boar particularly vulnerable to the effects of heat stress, however, the link between summer heat stress and boar sperm DNA damage has not been demonstrated. Semen from five Large White boars was collected and evaluated during the early dry, late dry and peak wet seasons to determine

the effect of seasonal heat stress on the quality and DNA integrity of boar spermatozoa. DNA damage in spermatozoa during the peak wet was 16-fold greater than early dry and nearly 9-fold greater than the late dry season. Sperm concentration was 1.6-fold lower in the peak wet than early dry while no difference was found across several motility parameters as determined by computer-assisted sperm analysis. These results demonstrate that tropical summer (peak wet season) induces DNA damage and reduces concentration, without depressing motility in boar spermatozoa; suggesting that traditional methods to evaluate sperm motility may not detect inherently compromised spermatozoa. Boar management strategies (such as antioxidant supplementation) need to be developed to specifically mitigate this problem.

Introduction

Forty percent of global meat consumption is pork (National Pork Board, 2017), with at least 4 tropical countries (Brazil, Vietnam, The Philippines and Mexico) among the top 10 pork producers in the world (National Pork Board, 2011). With rising populations and increasing demand for animal protein, emerging tropical economies in Asia and elsewhere, are projected to significantly contribute to the global food crisis (Pingali, 2007; Alexandratos and Bruinsma, 2012). However, the production efficiency of pigs in tropical and sub-tropical regions is known to be affected by seasonal or summer infertility; a syndrome characterised by an overall reduction in the reproductive performance of the breeding herd. This poor performance can be caused by a number of factors including: ambient temperatures greater than the animal's thermal comfort zone (i.e. 18-20° C; Stone, 1982; Prunier *et al.*, 1997), humidity, photoperiod, and management practices (Love, 1981; Hennessy and Williamson, 1984; Auvigne *et al.*, 2010) including genetic background (Sonderman and Luebbe, 2008); causing significant reduction to profitability in the pig industry. For example, at least \$300 million are

lost annually in swine alone and billions across the US livestock industry due to heat stress (St-Pierre *et al.*, 2003).

Summer infertility is mainly characterized by 1) reduced expression of oestrus in gilts and sows; 2) increased rates of pregnancy failure (Paterson *et al.*, 1978; Hughes and van Wettere, 2010); and/or 3) decreased breeding efficiency in boars (Wettemann *et al.*, 1976; Boma and Bilkei, 2006; Auvigne *et al.*, 2010). Even in a temperate climate such as southern France over a 5 year period, mean fertility, based on ultrasound pregnancy diagnosis 28 days after insemination, was at its lowest in summer (81.2%; end of August) compared to its peak of 86.8% in winter (end of March; Auvigne *et al.*, 2010). In Australia, the adjusted farrowing rate dropped to 77.1% in summer/autumn compared to 91.9% in spring (O'Leary, 2010).

While in the tropical Philippines, farrowing rate, percent live born, litter size at weaning, and pigs weaned/sow/year were significantly less around the third quarter of the year after exposure to higher ambient temperatures. This was compounded by reduced voluntary feed intake and lower feed quality, with small to medium farms being the most severely affected (Vega and Agbisit Jr, 2009; Vega *et al.*, 2010).

The boar is particularly vulnerable to the effects of heat stress due to a number of notable characteristics. Pigs generally are known to be inefficient at sweating (Ingram, 1965; Mount, 1968). While apocrine sweat glands appear to be abundant in the skin of pigs, they are located deeper in the dermis and in subcutaneous tissue except for eccrine type in the snout and dorsal nasal regions (Sumena *et al.*, 2010). Moreover, the boar scrotum is not pendulous (Knox, 2003); and boar spermatozoa tend to be more susceptible to temperature shock (Einarsson *et al.*, 2008). Stone (1982) demonstrated that spermatogenesis in boars is impaired when ambient temperatures rise above 29° C. Thus, heat stress in boars has been shown to

result in lower semen volume (Cameron and Blackshaw, 1980), reduced sperm concentration (Egbunike and Dede, 1980), lower motility and higher rates of abnormal spermatozoa (Egbunike and Dede, 1980; Heitman *et al.*, 1984; Barranco *et al.*, 2013), interference in testosterone production (Stone and Seamark, 1984), extended ejaculation time (Egbunike and Dede, 1980), and reduced libido (Flowers, 1997).

Moreover, the relatively high, unsaturated fatty acids in the plasma membrane (Cerolini *et al.*, 2001) and low antioxidant activity of seminal plasma (Brzezińska-Ślebodzińska *et al.*, 1995), all contribute to boar sperm's high sensitivity to peroxidative damage. We have recently proposed that such mechanisms may make boar spermatozoa highly prone to DNA damage during periods of heat stress (Peña *et al.*, 2017). Recent studies in mice have conclusively demonstrated that heat stress induces sperm DNA damage, which causes abnormal and arrested embryo development, and ultimately embryo and foetal loss (Paul *et al.*, 2008). This suggests that heat stress-induced DNA damage in boar sperm may contribute significantly to seasonal pregnancy failure and reduced litter size in sows (Peña *et al.*, 2017). In pigs, Didion *et al.* (2009) have proposed that sperm with greater than 6% DNA fragmentation can cause both decreased farrowing rates and average number of piglets born. However, definitive evidence of the link between heat stress and DNA damage in boar spermatozoa is limited. While boar sperm collected in spring-summer appeared to have relatively higher percentage of DNA damaged spermatozoa, a significant increase was only evident in fractionated ejaculates (F1 and F2) from two out of five boars (Zasiadczyk *et al.*, 2015). By contrast, Petrocelli *et al.* (2015) reported that neither season, photoperiod nor genetic line affected sperm DNA fragmentation. Both studies however, were conducted in temperate climates where ambient temperatures may not be sufficient to induce significant DNA damage compared to pigs raised in the tropics. Thus, the aim of this study was to

determine the effect of seasonal heat stress on the quality and DNA integrity of spermatozoa obtained from boars housed in the dry tropics of Townsville, North Queensland, Australia; a climate more similar to that experienced by pig producers in developing tropical Asian countries.

Materials and Methods

Boars and Location

Power analysis (PASS 14 Power Analysis and Sample Size Software 2016, NCSS LLC, Kaysville, Utah, USA) was undertaken to determine the minimum number of animals needed to show a significant change in sperm DNA damage due to heat stress according to the principles of the Three Rs (EU Directive 2010/63/EU; Kilkenny *et al.*, 2010;). A sample size of n=3 boars was sufficient under the following estimated conditions: mean difference in DNA fragmentation index between control boars and heat stressed boars = 24%; standard deviation = ± 10 ; assuming equal variance (Fernandes *et al.*, 2008; Evenson *et al.*, 2009). As a contingency against failed sample collections, and to align with other boar studies (Dubé *et al.*, 2004; Boe-Hansen *et al.*, 2005; Alkmin *et al.*, 2013; Zasiadczyk *et al.*, 2015), n=5 Large White boars were purchased at 11-12 months of age from a commercial piggery and reared in an open, gable roof-type facility within individual 3 x 3 metre pens at the College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Queensland, Australia (19°19'46.4"S, 146°45'40.3"E). Boars were exposed to prevailing winds and ambient temperatures throughout the day. Each boar was fed 1.8 - 2.3 kg/day of a commercial pelleted diet (Barastoc, Ridley AgriProducts, Victoria, Australia) to maintain a body score between 3 - 3.5. Water was provided *ad libitum* via an automatic pig nipple waterer. Experiments were approved by the James Cook University Animal Ethics Committee.

Temperature, Relative Humidity and Temperature-Humidity Index

Townsville is situated in the dry tropics; with a climate that has less rainfall than other comparable regions in the wet tropics (Bureau of Meteorology, 2011a). The dry season (late April to October) is typically cooler and dry, while the wet season (November to early April) tends to be hot and wet, with monsoon rains from late December to early April. Mean, minimum and maximum daily temperatures as well as mean, 6:00 am and 3:00 pm daily relative humidity (corresponding to the coolest and hottest times of the day, respectively; Bureau of Meteorology, 2011b) for Townsville were obtained from the Australian Bureau of Meteorology. Mean, minimum and maximum temperature-humidity indices (THI) were generated for each day using mean, minimum and maximum daily temperatures coupled with mean, 6 am and 3 pm daily relative humidity values, respectively. This was achieved using an online heat index calculator (National Weather Service, 2016a), validated and interpreted using a Temperature-Humidity Index chart (Thom, 1959; Hahn *et al.*, 2009). Mean values were calculated for all parameters spanning the 42-day period immediately before each seasonal semen collection time point. This period encompasses the ambient environmental conditions to which boars were exposed for one complete cycle of spermatogenesis in the boar (Franca and Cardoso, 1998; França *et al.*, 2005).

Seasonal Semen Collection and Processing

Boars were sexually mature (20 - 28 months old) at the time of the experiment and met minimum standards of sperm quality (70% motility, 65% morphologically normal spermatozoa and an ejaculate volume of at least 100 ml) in order to qualify for the study. To avoid measuring DNA damage associated with dead/degenerating spermatozoa stored for prolonged periods in the epididymis, semen was routinely collected from boars by the same

person 2-3 times every two weeks prior to experimental sampling. One ejaculate was analysed from each of the same $n=5$ boars at each sampling time point during the late dry (warm and humid; October 2014), peak wet (hot and wet; February 2015) and early dry (cool and dry; end of May 2015) seasons. Semen was collected using a dummy sow (Minitube, USA) and the gloved hand technique (Hancock and Hovell, 1959) into a plastic semen collection bag fitted inside a collection cup and covered with non-woven tissue filters (all Minitube, Victoria, Australia) to remove the gel fraction. The collection bag was then placed inside an insulated container containing 38° C water and immediately brought to the laboratory for processing. Raw semen from each boar was diluted 1:3 with 38° C pre-warmed Beltsville Thawing Solution (BTS; pH 7.2; Pursel and Johnson, 1975) containing 205 mM D-glucose, 20 mM sodium citrate tribasic dihydrate, 3 mM ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate, 10 mM potassium chloride, 15 mM sodium bicarbonate, 0.1% (v/v) gentamicin reagent solution (Life Technologies, Victoria, Australia) in nanopure deionized water. All reagents were sourced from Sigma-Aldrich (Sydney, New South Wales, Australia), unless otherwise stated. One aliquot was evaluated for sperm concentration using a Neubauer haemocytometer, using standard protocols (WHO, 2010), a second aliquot adjusted to 20×10^6 sperm/mL in BTS for evaluation of sperm motility characteristics using a computer-assisted sperm analyser (CASA; IVOS version 10, Hamilton Thorne Research, Beverly, MA, USA), and a third aliquot evaluated for DNA damage.

Determination of motility characteristics by CASA

About 3 μ l of 20×10^6 sperm/mL semen was loaded into each chamber of 38° C pre-warmed Leja Standard Count 4 Chamber Slides (Leja Products, Nieuw-Vennep, Netherlands) and loaded into the CASA machine. At least 200 spermatozoa across five random fields were examined per sample. Motility characteristics of spermatozoa were analysed as previously

described (Peña *et al.*, 2015). The CASA software was calibrated to the following settings: analysis set-up #7: BOAR; frames acquired, 40/sec; frame rate, 50 Hz; minimum contrast, 60%; minimum cell size, 2 pixels; minimum static contrast, 30%; straightness threshold, 71.4%; low VAP cut-off, 5.0 $\mu\text{m}/\text{sec}$; medium VAP cut-off, 22.0 $\mu\text{m}/\text{sec}$; low VSL cut-off, 11.0 $\mu\text{m}/\text{sec}$; head size (non-motile), 2 pixels; head intensity (non-motile), 70 pixels; static head size, 0.10 to 10.0 pixels; static head intensity, 0.10 to 0.95 pixels; static elongation, 0 to 60; count slow cells as motile, YES; magnification, 3.20; video source, camera; video frequency, 50; bright field, NO; illumination intensity, 2381; and temperature, 38° C. The following characteristics were evaluated: total motility, progressive motility of the whole sample, average-path velocity (VAP; $\mu\text{m}/\text{sec}$), straight-line velocity (VSL; $\mu\text{m}/\text{sec}$), curvilinear velocity (VCL; $\mu\text{m}/\text{sec}$), amplitude of lateral head displacement (ALH; μm), beat cross frequency (BCF; Hertz), straightness (STR; ratio of VSL/VAP), linearity (LIN; ratio of VSL/VCL), and elongation (ELO; ratio in % of head width to head length) as previously described (Mortimer, 2000).

Sperm DNA Integrity Assay

BTS-diluted semen samples were purified by Percoll gradient centrifugation to remove seminal plasma and possibly dead and damaged spermatozoa (Grant *et al.*, 1994). Two mL of 40% Percoll solution (GE Healthcare, Uppsala, Sweden) in BTS was layered on top of 2 mL of 80% Percoll solution in BTS in a 15 mL centrifuge tube. Six mL of 1:3 diluted semen in BTS solution was layered on top of the Percoll gradients and centrifuged at 700 x g for 25 min. The supernatant was removed and the remaining pellet was washed twice in 5 mL BTS by spinning tubes at 1000 x g for 5 min each. The final sperm pellet was adjusted to 5×10^6 sperm/mL in BTS.

Boar spermatozoa was stained using the Terminal deoxynucleotidyl transferase dUTP nick end labelling assay according to manufacturer's instructions (TUNEL; *In Situ* Cell Death Detection Kit, Fluorescein, Version 17, Nov 2012, Roche Diagnostics, Mannheim, Germany) with modifications. Briefly, boars were randomly divided and collected in two groups of 2-3 boars to facilitate timely processing. Six control samples (2 positive, 2 negative, and 2 unlabelled) were prepared in parallel using pooled semen from each batch of boars tested and were individually stained for each of the six controls. These were used to accurately gate different populations of spermatozoa in the flow cytometer before experimental samples were analysed (Figure 1).

One mL (5×10^6 sperm) of each sample was used for TUNEL labelling and centrifuged $720 \times g$ for 5 min. Each sperm pellet was washed twice in 200 μ l of Phosphate Buffered Saline (PBS) by centrifugation at $720 \times g$ for 5 min. The final pellet was resuspended in 100 μ l PBS to which 100 μ l of 4% (w/v) paraformaldehyde in PBS was added to fix spermatozoa for 60 min at room temperature. Thereafter, samples were centrifuged at $720 \times g$ for 5 min and the pellet resuspended in 200 μ l PBS and stored at $4^{\circ} C$ overnight.

The next day, samples were centrifuged $720 \times g$ for 5 min and pellets resuspended in 100 μ L of 0.5% Triton X-100 in 0.1% sodium citrate permeabilisation solution then incubated for 30 min at $37^{\circ} C$. Samples were washed twice and resuspended in 200 μ L PBS except for positive controls (P1 and P2) which were resuspended in 100 μ l of 1000 U/mL DNase 1 in Roche Buffer 2 and incubated for 30 min at $37^{\circ} C$ to induce double-stranded DNA breaks. 1000 U/mL DNase 1 in Roche Buffer 2 is comprised of 20 μ l of 10 U/ μ L Roche DNase 1 stock solution (500 μ L 40 mM Tris-HCl 2 mM $MgCl_2$ solution plus 10000 U lyophilized DNase 1 and 500 μ L glycerol) plus 180 μ L of Roche Buffer 2 (0.058 g NaCl, 0.099 g

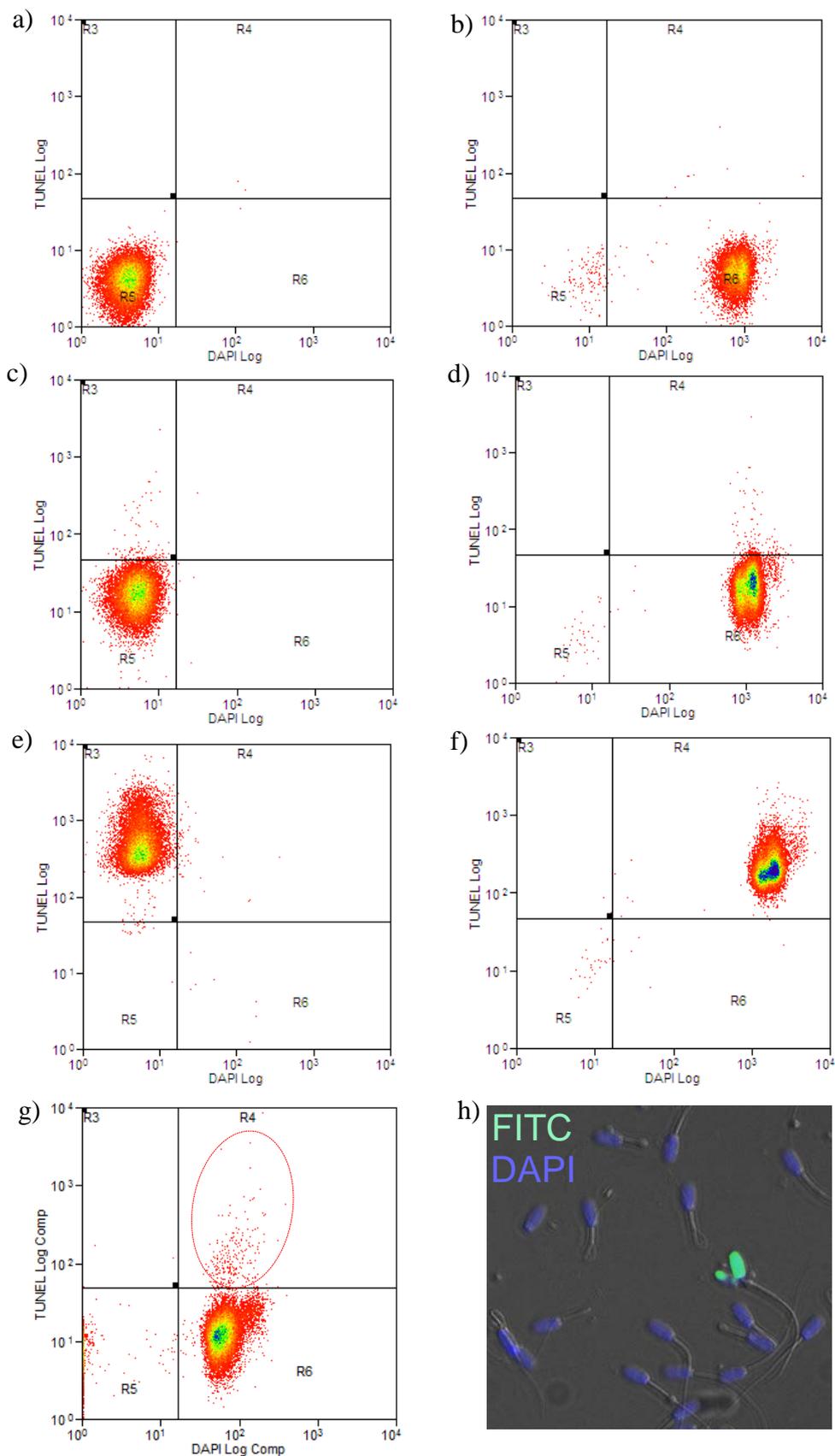


Figure 1: Calibration of flow cytometer for boar spermatozoa subjected to different staining treatments for FITC (TUNEL) and DAPI. TUNEL Log vs. DAPI Log scatter plots for unlabelled control, U1 (a); unlabelled control with DAPI, U2 (b); negative control in Label Solution, N1 (c); negative control in Label Solution with DAPI, N2 (d); DNase-treated FITC positive control, P1 (e); DNase-treated FITC positive control with DAPI, P2 (f); test sample showing DNA damaged sperm sub-population encircled by dotted line (g); and microscopic validation of DNA damaged (green; FITC) and intact (blue; DAPI) boar spermatozoa labelled by TUNEL (h).

MnCl₂·4H₂O, 0.0011 g CaCl₂ and 0.1864 g KCl in 100 mL 10 mM Tris-HCl solution). P1 and P2 controls were subsequently washed twice and resuspended in 200 µL PBS prior to TUNEL labelling.

The TUNEL reaction labels DNA damaged cells positive for Fluorescein isothiocyanate (FITC). All samples were centrifuged 720 x g for 5 min and their sperm pellets subjected to different treatments: Unlabelled controls (U1 and U2) were resuspended in 50 µL PBS; Negative controls (N1 and N2) were resuspended in 50 µL TUNEL labelling solution without the enzyme; while Positive controls (P1 and P2) and all test samples were resuspended in 50 µL TUNEL reaction mixture containing enzyme. All samples were then incubated for 90 min at 37⁰ C before washing twice in PBS. Thereafter, U2, N2, P2 and all test samples were incubated with 5 µg/mL of the nucleic acid stain 4', 6-diamidino-2-phenylindole (DAPI) in PBS for 20 min at room temperature to ensure that only nucleated TUNEL-positive spermatozoa were accounted for as DNA damaged cells during analysis by FACS. The specificity of sperm staining was further validated using fluorescent microscopy, which showed FITC/DAPI positive DNA damaged sperm in green alongside DAPI positive intact nucleated boar sperm in blue (Figure 1h).

Flow Cytometry Analysis

All samples were washed twice and resuspended in 2 mM EDTA in PBS and evaluated using a CyanADP flow cytometer (serial number 389; manufactured July 2005; Dako Cytomation, Fort Collins, Colorado, USA). The instrument was not altered and consisted of a fixed-alignment quartz cuvette flow cell and used the following light source/filter combinations: (i) 488 nm Coherent Sapphire solid state; 20 mW for detector FL1 with 95/5 mirror, 530/40 nm filter for FITC; and (ii) 405 nm Coherent semiconductor; 50 mW for detector FL6 with

450/50 nm filter for DAPI. Detector voltages were set to: FSC=220V, SSC=330V, FL1=500V and FL6=500V. Samples were first passed through a 60 μ m nylon woven net filter before being loaded onto the machine in 5 mL round-bottom polystyrene tubes. Spermatozoa were identified by their forward and side scatter profiles using a scatter-area vs. scatter-height gate previously calibrated specifically for boar spermatozoa. Data were analysed using Summit 4.3 software (Dako Cytomation). The flow cytometer was set to analyse 20000 cells per sample at about 150 events/second. Prior to evaluating test samples, control samples were used to accurately define the different cell staining populations delineated into four distinct quadrants by adjusting both vertical and horizontal thresholds: (i) R3, FITC-positive cells only; (ii) R4, both FITC and DAPI-positive cells; (iii) R5, unstained cells; and (iv) R6, DAPI-positive cells only (Figure 1a-f). Sample N2 (Negative control in Label Solution with DAPI) was used to set a 0.5% threshold cut-off before running all test samples. Cells in R4 were designated as nucleated DNA damaged spermatozoa, expressed as a percentage of the total number of cells analysed within the gated area (Figure 1g).

Data Presentation and Statistical Analyses

The Shapiro-Wilk test was used to evaluate normality of the data, while Mauchly's test of sphericity was used to determine if variances of the difference scores between each within-subject variable were equal. If these assumptions were not met, a Log_{10} transformation of the data (sperm DNA damage, daily mean temperatures, relative humidity) was performed before ANOVA or either the Greenhouse-Geisser or Huynh-Feldt correction was used to interpret significant difference. Given the same boars were used across the 3 sample time points, data were analysed by single-group or one-way repeated measures ANOVA to reduce random variance and improve statistical sensitivity, along with pairwise comparisons based on marginal means with Bonferroni

adjustments applied (IBM SPSS version 22; IBM Corporation, NY, USA). Graphs were plotted using Microsoft Excel 2016. $P \leq 0.05$ was considered statistically significant.

Results

Daily mean, mean minimum and mean maximum temperatures spanning the 42-day period immediately before semen was collected at each time point are shown in Table 1. The peak wet season was significantly hotter for all three temperature measures than early and late dry seasons ($P \leq 0.05$; Table 1). Similarly, the daily mean relative humidity spanning the 42-day period immediately before semen collection differed across seasons; with the peak wet season being more humid than early or late dry season ($P \leq 0.05$; Table 1). It was typically more humid at 6 am during the coolest part of the day, than 3 pm that was the hottest for all seasons. In this regard, the peak wet season had more humid mornings than the late dry and more humid afternoons than the early dry season ($P \leq 0.05$; Table 1). Moreover, temperature-humidity index was also highest during the peak wet season for all three mean measures than early or late dry seasons ($P \leq 0.05$; Table 1).

Strikingly, spermatozoa collected during the peak wet season had more than 16-fold higher DNA damage than early dry, and nearly 9-fold higher DNA damage than in the late dry season ($P \leq 0.05$; Figure 2a). Moreover, semen collected in the peak wet season had significantly lower sperm concentration than early dry but did not differ to the late dry season ($P \leq 0.05$; Figure 2b). Both total and progressive motility of spermatozoa collected in the peak wet season did not differ to that in early or late dry seasons ($P > 0.05$; Figures 2c and 2d respectively).

Table 1: Mean (\pm SEM) ambient temperature, relative humidity and temperature-humidity indices in Townsville, North Queensland, Australia spanning the 42-day period immediately before semen collection during the early dry, late dry and peak wet seasons.

	Early Dry	Late Dry	Peak Wet
Ambient Temperature ($^{\circ}$ C)			
Daily Mean	24.2 \pm 0.4 ^b	23.0 \pm 0.2 ^b	29.2 \pm 0.2 ^a
Mean Minimum	18.4 \pm 0.5 ^b	17.7 \pm 0.3 ^b	24.8 \pm 0.3 ^a
Mean Maximum	29.6 \pm 0.2^b	28.2 \pm 0.1^c	33.4 \pm 0.2^a
Relative Humidity (%)			
Daily Mean	61.9 \pm 2.1 ^c	67.6 \pm 0.7 ^b	71.4 \pm 1.2 ^a
Mean 6 am	75.7 \pm 3.3 ^{ab}	70.8 \pm 1.4 ^{bc}	82.5 \pm 1.2 ^a
Mean 3 pm	45.7 \pm 2.0^b	60.7 \pm 2.4^a	59.2 \pm 2.0^a
Temperature-Humidity Index (THI)			
Daily Mean	75.9 \pm 0.9 ^b	73.6 \pm 0.4 ^b	92.9 \pm 1.1 ^a
Mean Minimum (6 am)	64.9 \pm 1.0 ^b	63.3 \pm 0.7 ^b	79.3 \pm 1.0 ^a
Mean Maximum (3 pm)	86.8 \pm 0.8^b	86.5 \pm 0.6^b	106.3 \pm 2.1^a

Different letters indicate a significant difference between seasons ($P \leq 0.05$); bold indicates environmental extremes to which boars were exposed during each 42-day period of the study.

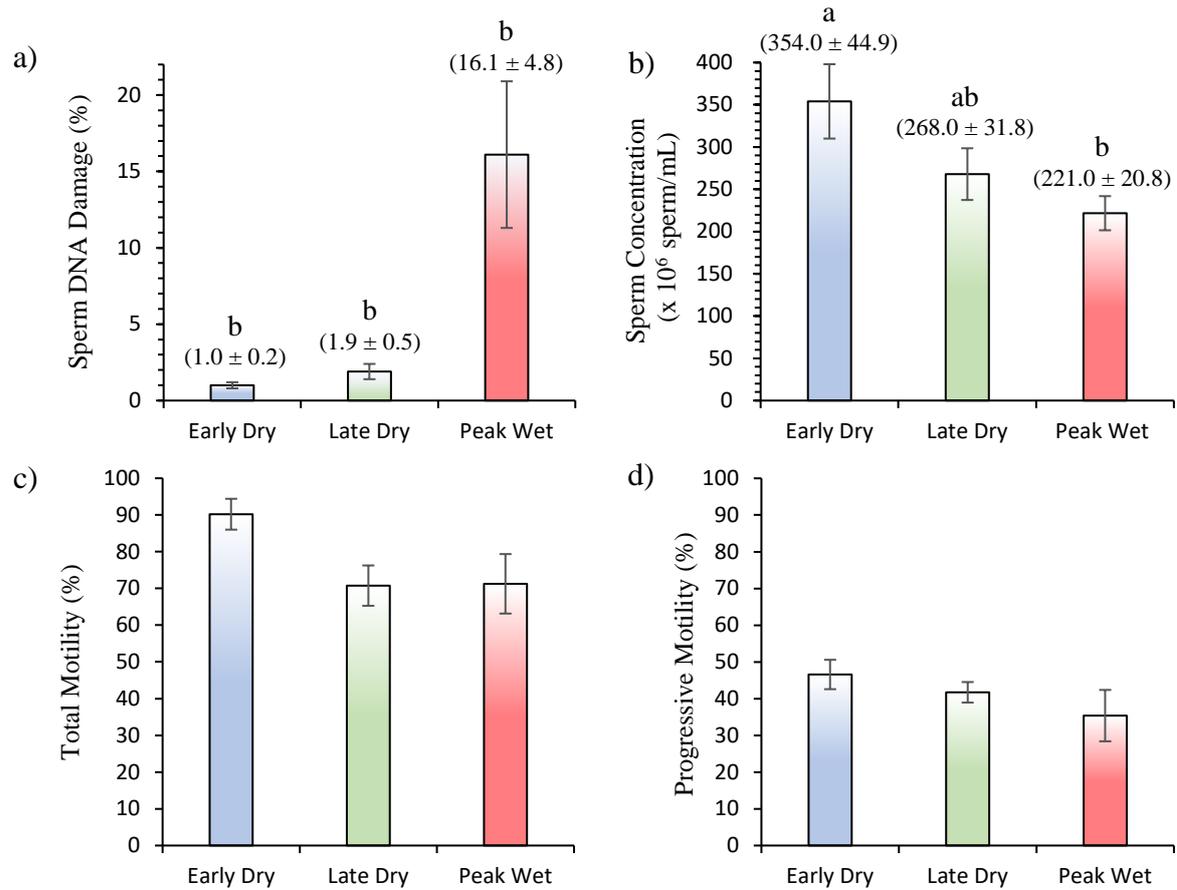


Figure 2: Mean (±SEM) percentage of DNA-damage (a); concentration (b); percentage of total motility (c); and percentage of progressive motility (d) of boar spermatozoa collected during the early dry, late dry and peak wet seasons. Different letters indicate a significant difference between seasons ($P \leq 0.05$).

Table 2. Mean (\pm SEM) sperm motility and head shape characteristics in boar ejaculates collected during the early dry, late dry and peak wet seasons in Townsville, North Queensland, Australia.

CASA Parameter	Early Dry (n=5)	Late Dry (n=5)	Peak Wet (n=5)
VCL	68.3 \pm 7.0	54.2 \pm 5.7	46.0 \pm 4.0
VSL	30.7 \pm 3.3	26.9 \pm 1.7	22.1 \pm 2.4
VAP	38.8 \pm 4.5	32.1 \pm 2.7	26.7 \pm 2.7
ALH	3.4 \pm 0.3	2.6 \pm 0.3	2.3 \pm 0.2
BCF	19.1 \pm 1.5	21.2 \pm 0.9	21.1 \pm 0.6
STR	74.1 \pm 1.3	80.2 \pm 2.3	76.9 \pm 2.2
LIN	44.8 \pm 1.2	50.7 \pm 2.7	47.3 \pm 2.1
ELONG	78.3 \pm 1.4	79.3 \pm 2.2	80.3 \pm 1.2

No significant difference between seasons for all parameters ($P > 0.05$). Numbers in parentheses indicate sample size. VCL, curvilinear velocity ($\mu\text{m}/\text{sec}$); VSL, straight-line velocity ($\mu\text{m}/\text{sec}$); VAP, average-path velocity ($\mu\text{m}/\text{sec}$); ALH, amplitude of lateral head displacement (μm); BCF, beat cross frequency (Hertz); STR, straightness (ratio of VSL/VAP); LIN, linearity (ratio of VSL/VCL); ELONG, elongation (ratio in % of head width to head length).

Detailed sperm motility and head shape characteristics determined by CASA are shown in Table 2. There was no significant difference observed between seasons for any CASA parameter ($P > 0.05$). While spermatozoa collected in the early dry appeared to have better curvilinear, straight line and average path velocities ($P = 0.08, 0.09, \text{ and } 0.09$ respectively), these were not statistically different from values obtained during the peak wet or late dry seasons (Table 2).

Discussion

Heat stress has been widely shown to impede proper growth and reproductive function in domestic animals. Moreover, the negative effect sperm DNA damage can have on male fertility has been extensively studied in many species, including humans. However, to our knowledge, this is the first study that significantly demonstrates the critical link between ambient environmental heat stress and sperm DNA damage in a domestic production animal (Pacey, 2010). Interestingly, our results show that the peak wet tropical summer season found in Townsville, North Queensland, Australia, induces DNA damage and reduces concentration of boar spermatozoa without depressing its motility. This suggests that traditional methods to evaluate sperm motility may not detect inherently compromised spermatozoa, which has important implications for the management and evaluation of seasonal infertility in boars during periods of heat stress.

Predicting overall sperm quality using conventional established laboratory guidelines for semen analyses (i.e. sperm motility, morphology, viability, concentration, etc.) has proven to be controversial and/or insufficient in determining fertility outcomes in both animals and humans (Love and Kenney, 1998; Carrell *et al.*, 2003; Garcia-Macias *et al.*, 2007). Semen known to be normal may in fact carry a sub-population of DNA damaged spermatozoa

(Dobrinski *et al.*, 1994; Kishikawa *et al.*, 1999). Moreover, DNA damaged spermatozoa may actually swim and fertilize an oocyte normally (Ahmadi and Ng, 1999; Simon and Lewis, 2011). However, nuclear damage to spermatozoa can negatively impact breeding efficiency (Evenson, 1999) along with early embryonic loss, interrupted embryo development, genetic abnormalities in the offspring, and lower pregnancy rates (Sailer *et al.*, 1997; Henkel *et al.*, 2004; Paul *et al.*, 2008; Simon and Lewis, 2011). It is likely that the true impact of sperm DNA fragmentation would only manifest as arrested embryo development from the 4-cell stage onward; a period corresponding to genome activation in the pig (Oestrup *et al.*, 2009; Deshmukh *et al.*, 2011). Of concern is the fact that the high rates of sperm DNA fragmentation observed during the peak wet season in our study (without noticeable effect on sperm motility) may currently go undetected by pig industries in tropical and subtropical climates. Moreover, they could significantly contribute to the high rates of embryo loss and pregnancy failure observed in sows during summer infertility (Peña *et al.*, 2017).

Didion *et al.* (2009) reported that a sperm sample with greater than 6% DNA fragmentation could result in decreased farrowing rates and average number of piglets born. In another study, 0.5 to 0.9 fewer piglets were born per litter when sperm DNA fragmentation was above 2.1% (Boe-Hansen *et al.*, 2008). In humans, 30.3% appears to be the threshold to discriminate between fertile and infertile men (Venkatesh *et al.*, 2011). A similar threshold was reported by Brahem *et al.* (2011) in men with history of recurrent pregnancy loss, but with the fertile group showing much lower damage (approximately 10%). From a conservative perspective, the overall threshold appears to be about 8% in boars, 10-20% in bulls and 30% in humans (Rybar *et al.*, 2004; Evenson and Wixon, 2006). However, utmost care should be taken when comparing levels of DNA fragmentation determined by the Sperm Chromatin Structure Assay (SCSA[®]) and TUNEL assay (Evenson, 2016). While pioneering

authors are convinced that both assays are correlated in terms of detecting and measuring the same existing DNA strand breaks (Gorczyca *et al.*, 1993), the two techniques fundamentally differ in that TUNEL detects 'real' DNA damage and SCSA detects abnormal chromatin structure and 'potential' DNA damage that depends on the susceptibility of DNA to denaturation (Henkel *et al.*, 2010). If the level of DNA damage observed in boar spermatozoa in our study represents 'real' DNA damage at over 16%, it is highly likely that pregnancy rates and litter sizes in sows fertilized by such spermatozoa will be significantly reduced. Collectively however, these studies suggest that sperm DNA fragmentation could be a valuable prognostic tool to predict final fertility outcomes in pigs (Simon *et al.*, 2013; Roca *et al.*, 2015).

In one study, Tsakmakidis *et al.* (2010) found that live morphologically normal spermatozoa and intact sperm DNA in boars accounted for 62.2% and 81.7% respectively of the variability in farrowing rates following artificial insemination. However, such findings appear to present limited value to indicate subfertility in fresh or stored semen from normospermic boars (Waberski *et al.*, 2011). High standards of screening and maintaining boars used in large scale commercial artificial insemination centres may preclude less fertile boars, since up to 95.5% of semen samples collected from Pietrain boars used in such centres have < 5% sperm DNA fragmentation (Waberski *et al.*, 2011). Nevertheless, early detection of boars with consistently low sperm DNA damage and good fertilizing capacity could prove economically beneficial especially in overcoming individual variations in boar fertility (Roca *et al.*, 2015). Our boars were pre-screened based on classical semen quality parameters before they qualified for the study, but were not tested for fertility by artificial insemination nor natural mating. Such a scenario is likely to reflect current practices in boar selection in small to medium farms in developing countries of the tropics. Moreover, the 16-fold increase in DNA

fragmentation observed in our study from 1% in the early dry to over 16% in the peak wet season suggests that even carefully selected commercial AI boars may be prone to considerable sperm DNA damage and reduced fertility if exposed to elevated temperatures. This can be aggravated by the fact that the activity of free radical scavengers such as glutathione peroxidase does not appear to increase in spermatozoa during summer (Argenti *et al.*, 2018).

The same five boars were experimentally subjected to identical environmental, husbandry and sample collection conditions at each time point of our study, in order to demonstrate seasonal effects were not caused by using different batches of boars. Chronologically these boars first exhibited 2% sperm DNA damage in October 2014 (late dry), then 16% damage in February 2015 (peak wet), followed by 1% damage in May 2015 (early dry season). Thus, DNA damage in our boars is clearly not cumulative, but caused by a cyclical rise in damage during hotter, and subsequent recovery during cooler times of the year. Damage is most likely induced during spermatogenesis since it is manifest in all spermatogenic populations during heat stress (Paul *et al.*, 2008). Compared to other species, the DNA of mature boar spermatozoa is highly stable with 10 cysteine groups bound to each protamine molecule (Gosálvez *et al.*, 2011); suggesting that heat stress must interfere during DNA - protamine complexing. Our boars ejaculated regularly prior to each experimental sample collection and all ejaculates analysed were Percoll purified, to avoid measuring possible DNA damage in senescent/degenerating spermatozoa associated with prolonged storage in the epididymis due to sexual rest or pathological anejaculation (Qiu *et al.*, 2012; Serafini *et al.*, 2016). Furthermore, given that the TUNEL assay directly detects 'real' DNA damage (Henkel *et al.*, 2010) and we examined at least 20000 spermatozoa per boar per time point by FACS,

suggest the 16-fold increase in sperm DNA damage induced by tropical summer in our study should be regarded as conservative at the very least.

Heat has previously been shown to induce sperm DNA fragmentation in mice. Immersion of the scrotum in 40-42°C water for 30 min causes DNA damage to spermatogonia, spermatocytes, spermatids and spermatozoa; resulting in a disruption to blastocyst formation, implantation failure, pregnancy loss, and a distortion in sex-ratio of offspring born (Paul *et al.*, 2008; Perez-Crespo *et al.*, 2008). In addition to disrupted DNA-protamine complexing mentioned above, the underlying mechanism by which heat causes sperm DNA fragmentation may be attributed to a number of putative factors. For example, it has been observed that heat stress causes apoptosis and testicular germ cell loss, abnormal expression of a number of DNA repair genes such as *Ogg1*, *Xpg* and *Rad54* as well as reduction in the expression of oxidative stress-induced antioxidants (Rockett *et al.*, 2001; Parrish *et al.* 2017). Moreover, polyADP ribose polymerase that helps in detection and signalling of DNA strand breaks may also be reduced (Tramontano *et al.*, 2000). Heat stress induced by scrotal immersion in 42°C water for 20 min also causes dissociation in X-Y chromosomes of mice and rats, leading to chromosomally unbalanced gametes, even in heat-adapted animals (van Zelst *et al.*, 1995). We postulate that the above mechanisms may play a significant role in inducing DNA damage in boar spermatozoa during periods of heat stress (Peña *et al.*, 2017).

Mean maximum (33.4 ± 0.2 °C) daily temperatures observed during the peak wet in Townsville appear to exceed the 29 °C threshold identified by Stone (1982) as the upper critical air temperature in which Large White boars are able to produce normal numbers of spermatozoa. Moreover, even a daily mean temperature of 29.2 ± 0.2 °C combined with a daily mean relative humidity of 71.4 ± 1.2 during the peak wet season results in a

temperature-humidity index of 92.9 ± 1.1 – at *extreme caution* zone of the heat index chart (National Weather Service, 2016b) or between *danger* and *emergency* zones for grower-finisher pigs (Hahn *et al.*, 2009). By comparison, the daily mean THI for the early dry (75.9 ± 0.9) and late dry (73.6 ± 0.4) seasons fall safely outside the *alert*, let alone the *danger* zone. Consistent with this, our results show that the concentration of boar spermatozoa decreased significantly in the peak wet season compared to early dry, but was similar to late dry. This is further supported by previous studies (Egbunike and Dede, 1980; Sarlós *et al.*, 2011) that show reduced concentration and total number of boar spermatozoa during the summer-spring period (Zasiadczyk *et al.*, 2015). Collectively, these studies suggest that seasonal heat stress causes disrupted spermatogenesis. Sperm concentration is an important aspect in pig production particularly with artificial insemination operations. Highly concentrated semen of sufficient volume can be economically beneficial as it can be extended into a large number of commercial doses to inseminate many females. Sperm concentration declined by only 1.6-fold in our study. However, compared to the 16-fold increase in sperm DNA damage, it is not clear whether such a reduction in sperm concentration, if left uncompensated, would have a major impact on litter size in sows.

Evaluation of sperm motility by CASA permits the identification of ejaculates that are below optimal standards set by the boar stud, which could otherwise result in lower fertility outcomes in commercial farm production (Holt *et al.*, 1997; Gadea *et al.*, 2004; Vyt *et al.*, 2008). An extensive comparison of insemination records with semen parameters from 45,532 boar ejaculates over a 3-year period revealed that progressive motility, curvilinear velocity, and beat cross frequency highly influenced farrowing rate, while total motility, average path velocity, straight line velocity, and amplitude of lateral head displacement correlated with the total number of piglets born (Broekhuijse *et al.*, 2012a). Other factors that affect over-all

fertility include boar related sources of variation (direct boar effect) such as genetic line, technician and AI centre, age of the boar, and days between ejaculation (Broekhuijse *et al.*, 2012b; a). Accordingly, sperm motion characteristics obtained from CASA accounted for 9% of the boar and semen-related variation in farrowing rate and 10% for total number of piglets born (Broekhuijse *et al.*, 2012a). Nevertheless, when viewed on an individual level, the predictive value of motility parameters on conception and farrowing rates was not found to be significant and only became obvious when associated with other parameters (Vyt *et al.*, 2008). Given that sperm DNA integrity was found to account for nearly 82% of the variation in farrowing rates after artificial insemination in one study (Tsakmakidis *et al.*, 2010), it would seem that motility parameters in selected highly fertile boars may have a relatively minimal influence on downstream fertility compared to DNA damage. At the very least, this suggests greater attention be placed on the evaluation of DNA integrity of boar spermatozoa; something which the industry is yet to widely adopt.

Heat stress has been reported to significant decrease sperm motility (McNitt and First, 1970; Wettemann *et al.*, 1979; Heitman *et al.*, 1984; Barranco *et al.*, 2013). However, mean total motility across seasons among our boars was greater than 70%, the cut-off point for sperm motility used in artificial insemination (Holt *et al.*, 1997; Eriksson and Rodriguez-Martinez, 2000). Moreover, the motility of spermatozoa collected in the peak wet season did not differ to early or late dry seasons across all CASA parameters we evaluated, despite a 16-fold increase in DNA damage. The difference in results may reflect the use of subjective estimates of sperm motility particularly in early studies ($\pm 10\%$), compared to more precise quantitative measures using CASA in our study. Moreover, the margin reported by Barranco *et al.* (2013) differed by only 3% total motility between summer and winter collections. Media containing >5 mM bicarbonate has been used to rapidly activate motility in sperm subpopulations, as a

more sensitive method to detect differences in fertility between individual boars (Satake *et al.*, 2006). Our study measured motility in BTS media already containing 15 mM sodium bicarbonate, without detecting noticeable differences between treatment groups. On this basis, we postulate that even objective measures of sperm motility as determined by CASA may not detect DNA-compromised spermatozoa; a view supported by observations that DNA damaged spermatozoa may actually swim and fertilize oocytes normally (Ahmadi and Ng, 1999). As such, evaluation of sperm DNA fragmentation may provide greater insight into potential contributing factors causing poor reproductive performance in the sow during summer infertility (Sutovsky, 2015). While, evaporative cooling, air conditioning, dripping and fogging systems have been slowly introduced to tropical pig farms in South-East Asia, they are not yet commonplace especially among older facilities that require extensive investment and redesign (Tantasuparuk and Kunavongkrit, 2014). Finding economically affordable strategies to mitigate the effects of heat stress in these countries is a big challenge considering that 70% of pig farms in The Philippines, Vietnam, Laos and Cambodia are small-scale with very limited capital and labour resources (Huynh *et al.*, 2007).

In conclusion, summer heat stress significantly increases sperm DNA damage in boars housed in tropical environments and causes a significant decline in sperm concentration. Sperm motility does not appear to be affected by season and, as such, measurement of this parameter alone may mask inherent deficiencies found in DNA damaged boar spermatozoa. Evaluation of sperm DNA integrity could provide an important diagnostic tool to further discriminate spermatozoa of low and high quality during summer.

Acknowledgment:

We thank J. Penny, N. Breen, R. Jack, S. Blyth, V. Simpson, A. Blyth, J. Palpratt and I. Benu for assistance with the boars; R. Jose and L. Woodward for training and access to FACS facilities; Glenley Piggery for animals; and Roche for TUNEL assays. The project is funded by a JCU Development Grant to DP, and College of Public Health, Medical and Veterinary Sciences PhD Research Funds to SP. SP is also supported by the Australia Awards Scholarship.

Conflict of Interest:

The authors declare no conflicts of interest.

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