Susceptibility of boar spermatozoa to heat stress using in vivo and in vitro experimental models

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Abstract

Induction of heat stress as an experimental procedure in animals is commonly used to examine heat-related impacts on sperm quality. This study aimed to develop potential heat stress models that could be used at any time of the year, to advance the study of seasonal infertility in the pig under controlled conditions. Heat stress was induced by either housing boars ($n = 6$) at 30 °C inside a hot room for 42 days (55–65% humidity; LD 12:12 h; in vivo), or by heating boar semen ($n = 7$) for 30 min at various temperatures (35.5, 38.8, 40, 42, 46, 50, 54 and 60 °C; in vitro). Sperm motility was then characterized by computer-assisted sperm analysis (CASA; IVOS version 10: Hamilton Thorne, USA), and DNA integrity was evaluated by terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) and flow cytometry. Our in vivo hot room model induced biologically meaningful levels of DNA damage in boar spermatozoa (10.1 ± 1.9 hot room vs. $6.7 \pm 1.7\%$ control; $P > 0.05$), although not statistically significant from controls. Moreover, sperm concentration and motility parameters did not differ between treatments ($P > 0.05$). Compared to the 38.8 °C control, our in vitro heat shock model significantly increased sperm DNA damage after incubation at 54 and 60 °C (3.0 ± 1.0, 2.9 ± 1.0, 1.2 ± 0.3, 2.5 ± 0.7, 9.0 ± 3.7, 16.2 ± 7.1, 14.2 ± 5.8 and $41.8 \pm 18.6\%$ respectively; $P \leq 0.05$). However, these temperatures rendered sperm completely immotile or dead, with most motility parameters declining rapidly to zero above 40 or 42 °C. In conclusion, our results suggest that temperature combined with individual factors may contribute to a boar's overall susceptibility to heat stress. Refinement of these models particularly of the in vitro heat shock model could be further pursued to overcome environmental variability, reduce whole animal experiments and provide a putative diagnostic fertility screening tool to evaluate heat tolerance in the boar.

Keywords: Sperm DNA damage; Sperm motility; Heat stress; Hot room; Boar; Pig

Introduction

Summer infertility associated with heat stress is still a major problem in pig reproduction particularly in the tropics. Reduced reproductive output in pigs during warmer months results in significant losses in profitability and have broader economic implications, given the high demand for pork products worldwide (National Pork Board 2017). Ambient temperatures beyond the animal's thermal comfort zone (i.e. 18–20 °C; Stone 1982; Prunier et al. 1997) can predispose pigs to a wide range of heat stress-related problems, affecting food and water consumption, general discomfort and reduced reproductive performance. While heat stress can affect both males and females, the effect on semen production and reproductive efficiency has been extensively studied as early as the 1950s and 1960s in various farm animals including rams (Moule and Waites 1963), bulls (Casady et al. 1953) and boars (McNitt and First 1970; Wettemann et al. 1976; Egbunike and Dede 1980; Stone 1982). Spermatogenesis is highly sensitive to temperature, and in boars, heat stress causes a decline in sperm motility, concentration, volume, morphology and overall fertility/fecundity (Thibault et al. 1966; McNitt and First 1970; Wettemann and Desjardins 1979; Cameron and Blackshaw 1980; Egbunike and Dede 1980; Greer 1983; Boma and Bilkei 2006; Rahman et al. 2011). Furthermore, given the extensive use of lean, fast-growing genotypes in commercial production, it is unclear how today's modern temperate pigs tolerate extreme environmental temperatures found in tropical production systems (Parrish et al. 2017). As such, pig producers need to consider serious investment in mitigation strategies to cool boars during periods of heat stress to minimize productivity losses associated with summer infertility.

The negative impact of heat stress on sperm DNA damage and the downstream reduction in embryo viability in the pig has been discussed previously (Peña et al. 2017). Recently, we have demonstrated using TUNEL that tropical summer induces 16% sperm DNA damage, which can be mitigated by antioxidant supplementation (Peña et al. 2019a, b). The downstream effect on embryo viability and litter size is yet to be determined, but in mice, 30 min scrotal heat stress-induced sperm DNA damage, which consequently resulted in arrested embryo development, reduced pregnancy rates and litter size (Paul et al. 2008; Paul et al. 2009). What we do know in pigs is that sperm with greater than 2.1% or 6% DNA fragmentation, as determined by SCSA, result in reduced litter size (Boe-Hansen et al. 2008) and decreased farrowing rates (Didion et al. 2009), respectively. Potential litter size is correlated with good vs poor structural chromatin in spermatozoa after artificial insemination (Waberski et al. 2011). Thus, we can conclude that 16% DNA-damaged sperm induced by tropical summer is likely to have a considerable negative impact on embryo viability and may partly explain reduced litter size observed in sows during seasonal infertility (Peña et al. 2017).

Further work is needed to evaluate the importance of boar factors in seasonal infertility in the sow, particularly sperm DNA damage; however, this research is limited by the annual nature and variability of extreme ambient temperatures. The development of suitable in vivo and in vitro heat stress models for the boar that can be used at any time of the year may help advance the study of seasonal infertility in the pig under controlled conditions. In previous studies, boars were exposed to a controlled hot room environment, direct sunlight or ambient temperatures ranging from 30 to 40 °C for between 3 and 90 days (McNitt and First 1970; Wettemann et al. 1976; Cameron and Blackshaw 1980; Stone 1982). Sperm DNA damage was not assessed in any of these studies; however, in one study, at least 1.5 times fewer embryos survived the first month of pregnancy in gilts impregnated with semen from heat

stressed boars (Wettemann et al. 1976). It is possible that the thermo-neutral zone may have changed by as much as 5 °C in modern pigs, compared to those three to four decades ago (Parrish et al. 2017), warranting further research using current lines.

While scrotal insulation may provide a good testicular model for heat stress (Parrish et al. 2017), the use of a temperature-controlled hot room may be a more appropriate whole animal heat stress model to induce systemic physiological responses (including basal body temperature changes and alterations to the hypothalamic-pituitary-testicular hormone regulatory axis) as the animal attempts to cope with the stressor (Setchell 1998). Conversely, a low-cost, welfare-friendly model might involve direct exposure of boar ejaculates to temperature extremes. Such an approach only requires fresh-chilled boar semen, which can be readily purchased from commercial boar studs to rapidly test susceptibility of individual boars without having to induce heat stress on the animal itself. The aim of this study was to evaluate the use of in vivo (hot room) and in vitro (heat shock) models to mimic levels of sperm DNA damage found in boars exposed to tropical summer temperatures in Townsville, North Queensland, Australia (Peña et al. 2019a).

Materials and methods

Boars and location

Prior to the experiment, $n = 7$ large white boars between 2.5 and 2.8 years of age were housed and maintained in an open, gable roof-type facility within individual 3 × 3 m 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 2.3–2.5 kg/day of a commercial pelleted diet (Barastoc, Ridley AgriProducts, Victoria, Australia) to maintain a body score between 3 and 3.5. Water was provided ad libitum via an automatic pig nipple waterer. Boars used in the study were not proven sires but were selected on 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 (Peña et al. 2019a).

Induction of heat stress

Hot room model

A total of $n = 6$ boars were used in the 'hot room' experiment using a standard 3 × 2 matched cross-over design in which 3 boars act as parallel controls for 3 treatment boars followed by a reversal of roles during a 2nd treatment cycle after a 42-day recovery period. The first batch of boars were introduced to a temperature-controlled facility (hot room) containing individual stalls during the early dry (July 2015) and the second batch in late dry season (September 2015), both of which are cooler than Townsville's peak wet season (Fig. 1).
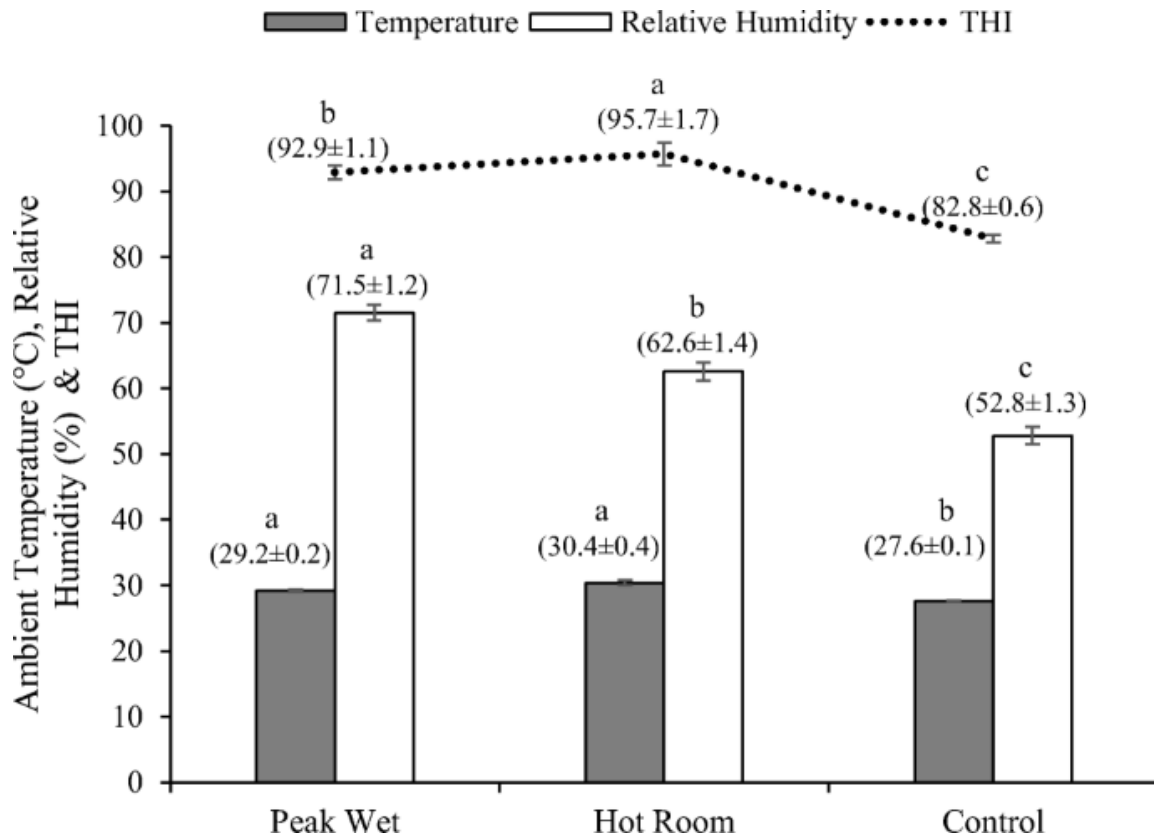
Fig. 1. Mean (± SEM) daily ambient temperature, relative humidity and temperature-humidity index between peak wet, hot room and control treatments in Townsville, North Queensland, Australia. Different letters indicate a significant difference between treatment groups ($P \leq 0.05$). Peak wet, ambient temperature during the hotter wet season of Townsville (February; Peña et al. 2019a); hot room, approximately 30 °C between 9 am and 5 pm and relative humidity between 55 and 65%; control, ambient temperature during the cooler dry season (July–September; Peña et al. 2019a)

The hot room is maintained at approximately 30 °C between 9 am and 5 pm, mimicking the ambient temperature experienced in Townsville during the peak wet season (Peña et al. 2019a), and a relative humidity between 55 and 65% (Fig. 1). Moreover, 12-h artificial light was automatically provided daily from 6 am to 6 pm. Boars inside the hot room were fed and hosed once every day before 9 am. Water was provided ad libitum as previously described.

Moreover, rectal temperatures were collected twice daily throughout the treatment period using a digital thermometer during feeding time in the morning and at 5 pm. Control boars were housed in parallel within the adjacent pig facilities (open, gable roof-type shed) described earlier and were managed the same as hot room boars. A temperature and relative humidity tracking device was installed in both facilities to monitor ambient conditions experienced by boars during the treatment period. Environmental data collected during these two treatments were compared to data collected during extreme ambient conditions experienced by boars in the peak wet season as described previously (Peña et al. 2019a).

Heat shock model

On a separate occasion, semen was collected once over a 2-day period from each of $n = 7$ boars to ensure at least $n = 6$ semen samples were subjected to different incubation

temperatures in an in vitro heat shock experiment. Thus $n = 5$ boars were used across all 8 temperature treatments, while boar 101 was used for 35.5, 46, 50 and 54 °C, and boar 305 was used for 38.8, 40, 42 and 60 °C. Individual semen samples were aliquoted in 1.5 mL micro-centrifuge tubes (Eppendorf™ tube) and inserted into a temperature adjustable heat block. Heat stress was induced by exposing individual semen samples for 30 min (Paul et al. 2008) to testicular (35.5 °C); body (38.8 °C); and several different elevated (40, 42, 46, 50, 54 and 60 °C) temperature treatments. Temperatures at the lower range were based upon the boar's core and peripheral temperatures (Stone 1981), while other temperatures rose in 2–6 °C increments in an effort to artificially induce DNA damage in mature highly DNA-condensed boar spermatozoa. After exposure, semen samples were evaluated for motility and DNA integrity as described below.

Semen collection and processing

After the hot room treatment, boars were returned to their original pens, and semen was collected 1–2 days later using a dummy sow (Minitube, USA) and the gloved hand technique as previously described (Hancock and Hovell 1959). Briefly, the boar's penis was directed 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 as previously described (Peña et al. 2019a). Semen samples were analysed for sperm concentration using a Neubauer haemocytometer (WHO 2010), before dilution to 20×10^6 sperm/mL in BTS to evaluate sperm motility characteristics by computer-assisted sperm analysis (CASA; IVOS version 10, Hamilton Thorne Research. Beverly, MA, USA), and dilution to 5×10^6 sperm/mL in BTS to evaluate sperm DNA damage by TUNEL assay (Peña et al. 2019a).

Sperm DNA integrity and flow cytometry analysis

Sperm DNA integrity and flow cytometry analysis were performed as previously described (Peña et al. 2019a). Percoll purified boar spermatozoa at a final concentration of 5×10^6 sperm/mL in BTS was stained following 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. Six control samples (2 positive, 2 negative and 2 unlabelled) were prepared in parallel using pooled semen and used for gating sub-populations of spermatozoa in the flow cytometer before experimental samples were analysed. Positive controls (P1 and P2) and all test samples were incubated in TUNEL reaction mixture containing enzyme, while the negative controls (N1 and N2) were incubated in TUNEL labelling solution without the enzyme. Unlabelled controls (U1 and U2) were incubated in PBS. Moreover, all experimental samples including the U2, N2 and P2 controls were subsequently incubated with 5 μg/mL of the nucleic acid stain 4′, 6-diamidino-2-phenylindole (DAPI) in PBS. This ensured that only nucleated TUNEL-positive spermatozoa were accounted for as DNA-damaged cells during analysis by FACS.

Using the CyanADP flow cytometer and Summit 4.3 software (Dako Cytomation, Glostrup, Denmark), boar sperm were identified by their forward and side scatter profiles following a scatter-area vs. scatter-height gate previously calibrated specifically for boar spermatozoa. Control samples were used to define different cell staining populations into four distinct quadrants: (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. 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 counted as nucleated DNA damage spermatozoa, expressed as a percentage of the total number of cells analysed within the gated area.

Data presentation and statistical analyses

The Shapiro-Wilk test was used to evaluate normality of the data, and Levene's test was used to determine if variances were equal. If these assumptions were not met, a Log_{10} transformation of the data was performed before data were analysed using the parametric paired-sample test (relative humidity) or ANOVA with a post-hoc Tukey's HSD test (total and progressive motility, sperm concentration, CASA parameters, sperm DNA damage between boars), to determine significant differences (SPSS version 22, IBM Corporation, NY, USA). Where the assumptions for parametric tests were not met, a Wilcoxon signed-rank 2-sample related test (ambient temperatures, THI or temperature humidity index, rectal temperatures) and Kruskal Wallis test followed by pairwise comparison using the Mann-Whitney test (sperm DNA damage between heat shock temperatures) were used to determine if values were significantly different ($P \leq 0.05$). Specifically for evaluating the effect of in vitro heat shock on sperm DNA integrity, normalized relative levels of DNA damage were calculated by dividing every boar's individual value across all treatment temperatures by the mean baseline level of DNA damage measured at testicular (35.5 °C) and core body (38.8 °C) temperatures for that boar (Van den Berghe et al. 2019). By so doing, this controlled for potential individual animal differences in baseline DNA damage, allowing us to measure fold changes from baseline for each boar. Normalized relative data were then grouped by temperature treatment and evaluated as described above. Graphs were plotted using Microsoft Excel 2016.

Results

Temperature, relative humidity and THI

Daily mean temperature, relative humidity and THI differ between peak wet, hot room and control ($P \leq 0.05$, Fig. 1). The daily mean temperature was hottest (exceeding the 29 °C limit for normal spermatogenesis; Stone 1982) and THI highest in the hot room, while the control treatment was coolest and had the lowest THI. By contrast, the peak wet season was more humid and the control was the driest ($P \leq 0.05$). Rectal temperatures of all boars are higher at 5 pm than 9 am in both the hot room and control treatments ($P \leq 0.05$; Fig. 2). Moreover, rectal temperatures of hot room boars were consistently higher than control boars in both the morning and afternoon ($P \leq 0.05$).
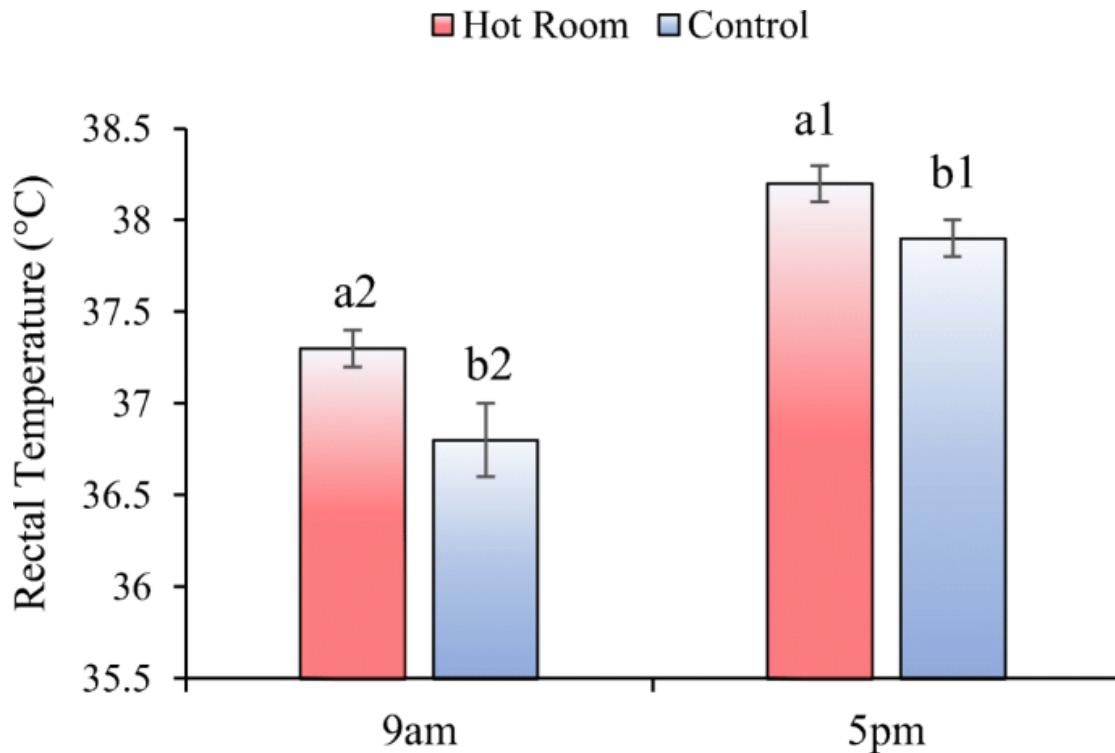
Fig. 2. Mean (± SEM) rectal temperature at 9 am and 5 pm in boars subjected to in vivo hot room and control treatments. Different letters indicate a significant difference between treatment groups while different numbers denote significant difference between time of day within treatment group (P ≤ 0.05). $n = 6$ boars total following standard 3 × 2 matched cross-over design; hot room, approximately 30 °C between 9 am and 5 pm and relative humidity between 55 and 65%; control, ambient temperature during the cooler dry season (July–September; Peña et al. 2019a)

Sperm DNA damage, concentration, motility and head shape characteristics in vivo

For the in vivo hot room experiment, the level of sperm DNA damage induced by the hot room exceeded the 6% limit for normal farrowing rates (Didion et al. 2009), being similar to that experienced during the peak wet season. However, the level of damage is not sufficiently different from controls ($P > 0.05$; Fig. 3A). By contrast, DNA damage in the peak wet was higher than control ($P ≤ 0.05$). Sperm concentration did not differ between in vivo treatments ($P > 0.05$; Fig. 3B). Similarly, the percentage of both total and progressively motile sperm did not differ between in vivo treatments ($P > 0.05$, Fig. 3C and D).
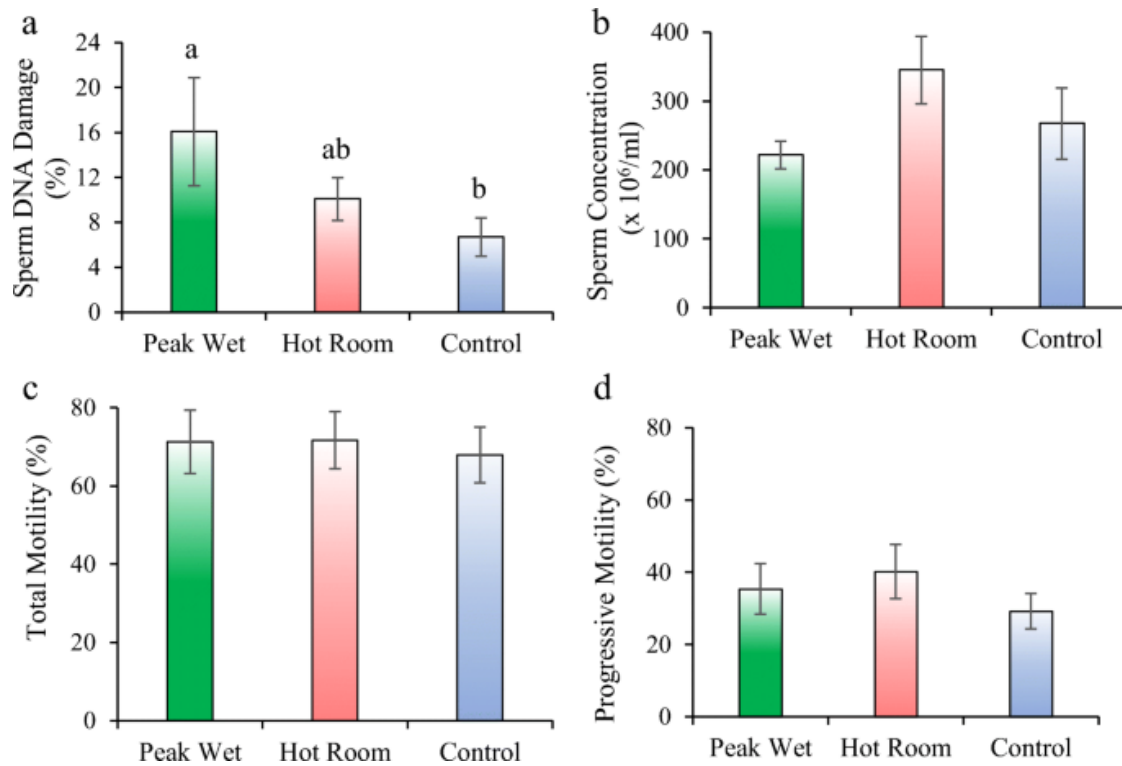
Fig. 3. Mean (± SEM) percentage of DNA damage (A), concentration (B) and percentage of total (C) and progressive motility (D) of boar spermatozoa subjected to in vivo peak wet, hot room and control treatments. Different letters indicate a significant difference between treatment groups ($P \leq 0.05$); $n = 6$ boars total following standard 3×2 matched cross-over design; peak wet, ambient temperature during the hotter wet season of Townsville (February; Peña et al. 2019a); hot room, approximately 30 °C between 9 am and 5 pm and relative humidity between 55 and 65%; control, ambient temperature during the cooler dry season (July–September; Peña et al. 2019a)

curvilinear velocity (VCL) and average path velocity (VAP; $P > 0.05$).

Table 1. Mean (± SEM) sperm motility and head shape characteristics in boar spermatozoa collected after in vivo peak wet, hot room and control treatments

| CASA parameter	Peak wet ($n = 5$)	Hot room ($n = 6$)	Control ($n = 6$)
VCL	46.0 ± 4.0	59.3 ± 5.7	53.2 ± 7.1
VSL	22.1 ± 2.4	29.5 ± 3.2	23.4 ± 2.8
VAP	26.7 ± 2.7	35.8 ± 3.8	30.1 ± 3.9
ALH	2.3 ± 0.2	2.8 ± 0.2	2.6 ± 0.3
BCF	21.1 ± 0.6	18.2 ± 1.3	18.6 ± 1.2
STR	76.9 ± 2.2	76.4 ± 2.1	73.7 ± 2.8
LIN	47.3 ± 2.1	48.8 ± 3.0	44.5 ± 3.1
ELONG	80.3 ± 1.2	80.2 ± 2.3	81.2 ± 2.3

No significant difference between treatment groups for all parameters ($P > 0.05$). Numbers in parentheses indicate sample size. *VCL*, curvilinear velocity (μm/s); *VSL*, straight line velocity (μm/s); *VAP*, average path velocity (μm/s); *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 for in vivo treatments determined by CASA are shown in Table 1. There was no difference between treatments for any CASA sperm parameter ($P > 0.05$), despite boars in the peak wet showing a trend for lower

Sperm DNA damage, motility and head shape characteristics in vitro

Compared to the 38.8 °C control, our in vitro heat shock model increased sperm DNA damage after incubation at 46 and 50 °C. However, due to considerable variation between boar semen at these elevated temperatures, this is only significant at 54 and 60 °C (3.0 ± 1.0, 2.9 ± 1.0, 1.2 ± 0.3, 2.5 ± 0.7, 9.0 ± 3.7, 16.2 ± 7.1, 14.2 ± 5.8 and $41.8 \pm 18.6\%$ respectively; $P \leq 0.05$; Fig. 4). Interestingly, spermatozoa from boars B-303 and B-101 appear to be more stable across all in vitro temperatures and, thus, contributed to this variability (Fig. 5). All CASA parameters are highest at 35.5 °C and did not differ to values at 38.8 °C except for VAP and VCL ($P > 0.05$; Table 2). A significant reduction in most motility parameters occurred after boar spermatozoa were incubated at 40 or 42 °C, beyond which boar spermatozoa were largely immotile and/or dead.
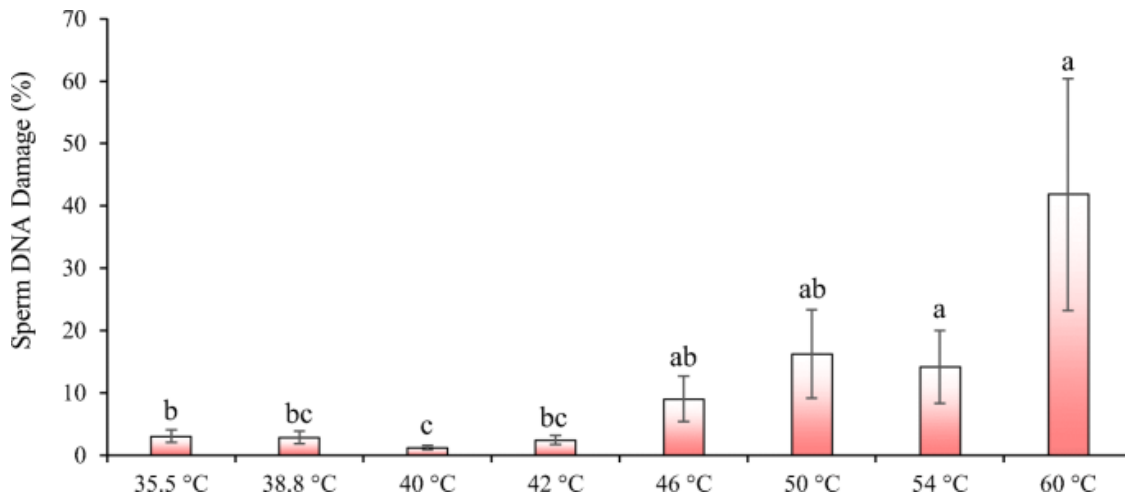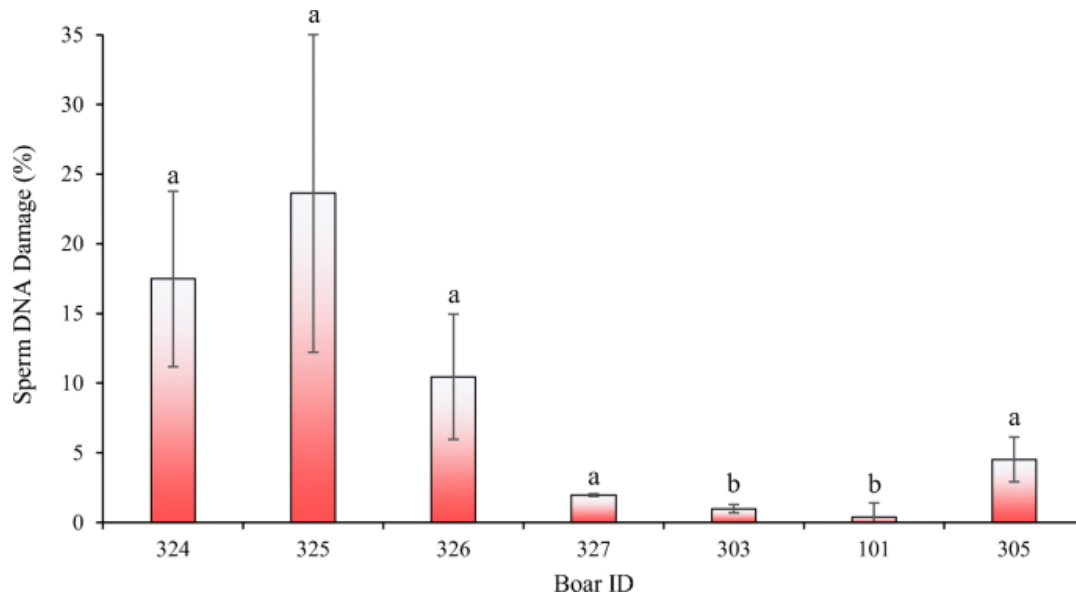

Fig. 4. Mean (± SEM) percentage of DNA damage in boar spermatozoa subjected to different in vitro heat shock temperatures. Different letters indicate a significant difference between treatment groups ($P \leq 0.05$); $n = 6$ boars per treatment

Table 2. Mean (± SEM) sperm motility and head shape characteristics in boar spermatozoa collected after exposure to different in vitro heat shock temperatures

Parameter	35.5 °C (n=6)	38.8 °C (n=6)	40 °C (n=6)	42 °C (n=6)	46 °C (n=6)	50 °C (n=6)	54 °C (n=6)	60 °C (n=6)
VAP	29.8 ± 4.5^a	14.0 ± 4.7^{ab}	6.6 ± 2.0	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.9 ± 0.9^b
VSL	22.0 ± 4.4^a	11.5 ± 4.1^{ab}	5.1 ± 1.9	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.6 ± 0.6^b
VCL	55.6 ± 5.2^a	25.6 ± 8.1^{ab}	17.0 ± 4.8	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	2.4 ± 2.4^b
ALH	2.8 ± 0.2^a	1.4 ± 0.4^{ab}	0.8 ± 0.2	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.1 ± 0.1^b
BCF	23.9 ± 3.3^a	16.3 ± 4.3	11.3 ± 3.6	1.2 ± 1.2^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	4.6 ± 4.6
STR	63.0 ± 7.3^a	55.2 ± 9.5^{ab}	33.6 ± 9.5	1.4 ± 1.4^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	10.5 ± 10.5^b
LIN	39.5 ± 5.3^a	35.4 ± 6.3^{ab}	16.8 ± 5.8	0.6 ± 0.6^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	5.4 ± 5.4^b
ELONG	79.8 ± 3.7^a	64.4 ± 11.6^{ab}	41.6 ± 12.8	3.3 ± 3.3^b	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	13.3 ± 13.3^b

Different letters indicate a significant difference between treatment groups ($P \leq 0.05$). Numbers in parentheses indicate sample size. *VCL*, curvilinear velocity (µm/s); *VSL*, straight line velocity (µm/s); *VAP*, average path velocity (µm/s); *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)

Fig. 5. Mean (± SEM) percentage of sperm DNA damage between boars across different in vitro heat shock temperatures. Different letters denote significant difference between boars ($P \leq 0.05$); $n = 8$ ejaculates per boar except for boar 101 ($n = 4$ ejaculates) and boar 305 ($n = 4$ ejaculates)

Discussion

The development of reliable heat stress models is important to advance the study of seasonal infertility in the pig, while also needed to overcome environmental variability, reduce whole animal experiments, and provide a putative diagnostic fertility screening tool to evaluate heat tolerance in boars. Our study demonstrated that both in vivo and in vitro heat stress models were able to induce biologically meaningful levels of DNA damage in boar spermatozoa. However, we were only able to mimic levels of damage observed during the natural tropical summer of Townsville, using extreme in vitro temperatures that rendered boar spermatozoa completely immotile or dead. As such, our results suggest that temperature alone may not be sufficient to induce damage and that individual factors may also contribute to a boar's overall susceptibility to heat stress (Pérez-Llano et al. 2010; Renaudeau et al. 2011; Parrish et al. 2017).

Induction of heat stress using an in vivo whole animal model can be challenging and laborious. We deemed it necessary, however, to activate the animal's complete physiological response (including temperature regulation mechanisms and the hypothalamic-pituitary-gonadal axis) to the stressor (Baldwin and Ingram 1967). Such responses are often missing in strictly controlled scrotal insulation models. For example, thermal sweating appears to be functionally nil from the apocrine-like glands in the pig (Baldwin and Ingram 1967), resulting in inefficient temperature regulation during periods of heat stress.

Our in vivo hot room model was conducted during the early and late dry seasons, when ambient temperatures for control animals are cooler and drier (Peña et al. 2019a). During this time, hot room boars are exposed to temperatures and temperature humidity indices that exceeded those observed during Townsville's peak wet season (Fig. 1), which were sufficient to induce a significant and consistently elevated core body temperature compared to control boars. While this was sufficient to induce more than 10% DNA damage in spermatozoa

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(above the 6% limit for normal farrowing rates; Didion et al. 2009), we could not achieve 16% observed during the peak wet season nor could we induce levels significantly higher than controls (Fig. 3A). We note that relative humidity is significantly lower in the hot room than peak wet season, but this did not negatively affect overall THI, which was highest in the hot room (Fig. 1). Thus, temperature/THI alone may not be sufficient to induce very high levels of DNA damage. It is possible that 42 days in the hot room may not be enough time to accumulate significantly higher amounts of DNA-damaged boar spermatozoa in the epididymis. Individual boar factors may also contribute to their susceptibility to heat stress, with some modern genetic lines of boars that appear more heat tolerant and produce 10% more sperm during warmer weather (Flowers 2008; Parrish et al. 2017), while other modern boars appear more susceptible to heat stress than older genotypes (Renaudeau et al. 2011).

In our in vitro heat shock experiment, DNA damage of boar spermatozoa was relatively low ($<4\%$) between the 35.5 and 42 °C treatments. Biologically deleterious levels ($>6\%$; Didion et al. 2009) of DNA damage were only achieved from 46 °C and only exceeded levels ($>16\%$) observed during peak wet summer conditions from 50 °C onward, although these were not statistically significant until 54 and 60 °C (Fig. 4) possibly due to individual boar variability. Interestingly, this could have been largely influenced by spermatozoa from $n=2$ boars which appeared to consistently show resistance to heat-induced DNA damage at these higher temperatures (Fig. 5). It also suggests that fully mature spermatozoa are resilient to heat stress-induced DNA damage, with early spermatogenic stages being more vulnerable.

Moreover, based on CASA motility data, increased DNA damage seen in mature sperm from 46 to 60 °C may in fact be due to immotile, plasma membrane-damaged or dead spermatozoa. Specifically, the presence of non-zero CASA parameters at 60 °C must be solely due to the inclusion of boar 305 at this temperature. Figure 5 clearly shows individual animal variation in tolerance to heat stress-induced DNA damage. It is likely the same individual variation operates on sperm motility as well. However, on close examination of the data in Table 2, you will note that accepted forward progressive motility parameters (VAP, VSL and VCL) are in fact very low/near zero, suggesting these sperm are merely beating in a near stationary position. This might explain why some parameters are slightly elevated (BCF, STR, LIN and ELONG) despite little to no progressive motility. In short, the sperm from this boar appears to have survived 60 °C heat treatment, but have negligible progressive motility. Scrotal heat stress in mice at 40–42 °C for 30 min resulted to DNA damage across multiple stages of sperm development with spermatocytes and round spermatids being predominantly affected (Paul et al. 2008) rather than pre-meiotic spermatogonia. Research by Perez-Crespo et al. (2008) further clarified that heat stress-induced DNA damage in mouse spermatozoa was more pronounced among spermatozoa that developed from spermatids present in the testis at the height of heat stress. Apparently, post-meiotic spermatids have limited capacity to induce apoptosis or DNA repair as they are both translationally and transcriptionally inactive (Sotomayor and Sega 2000), making these cells the most sensitive to heat stress (Setchell 2006).

The in vivo hot room trial was conducted for 42 days in order to span the complete cycle of spermatogenesis in this species (Franca and Cardoso 1998; França et al. 2005). While this was able to increase sperm DNA damage to over 10%, (i) this period of time may have been too short to reach levels seen during the peak wet, and (ii) damage to more sensitive early spermatogenic stages may still not have had time to fully reach maturity. Interestingly, the level of sperm DNA damage in the control group ($\sim 7\%$) was also higher than for previously reported experiment using the same animals as controls (1–2%; Peña et al. 2019a). This may

partly be explained by the fact that semen from $n = 3$ control boars was collected after a 42-day recovery period (after exiting the hot room where they too were a treatment group) following the standard cross-over design outlined in the methods. Although 42-day recovery corresponds to the duration of spermatogenesis to produce one fresh batch of 'unaffected' sperm in the boar (Franca and Cardoso 1998), it is possible that this recovery window was insufficient for new rounds of spermatogenesis to reduce the level of sperm DNA damage in the epididymis to near 1–2%. In addition, these same boars used previously (Peña et al. 2019a) were now more than 1 year older in this study. Studies particularly in humans demonstrate that age is associated with an increase in sperm DNA damage (Wyrobek et al. 2006; Vagnini et al. 2007).

In terms of the structural integrity of mammalian spermatozoa, boar and bull sperm only contain one type of active protamine (P1); unlike primates, most rodents and perissodactyla contain both P1 and P2 protamines (Lee and Cho 1999; Balhorn 2007). Protamines are small arginine-rich proteins synthesized towards the final stages of spermatogenesis that bind DNA, replacing histones and condensing the spermatid genome to become genetically inactive and more compact (Balhorn 2007). Normally, upon synthesis, P1 and P2 are phosphorylated, but most phosphate groups are removed after binding to DNA which leads to oxidation of cysteine residues. This then allows the formation of disulphide bridges that serve to link sperm protamines together forming a more stable sperm chromatin complex, both mechanically and chemically (Jager 1990). Thus, any defect in protamine structure or cross-linking may cause ultrastructural anomalies in sperm DNA which could eventually affect male fertility (de Yebra et al. 1993; Iranpour 2014). In fact, a defect in P2 has been demonstrated to cause sperm DNA damage and embryo death in the mice (Cho et al. 2003). It is unclear whether the absence of P2 in boar spermatozoa makes its DNA more stable to damage. However, the fact that P2 protamine is low in cysteine residues, which translates to a lower concentration of disulphide bridges, strongly implies that bull and boar sperm DNA are more stable than that of mice or human (Jager 1990).

The amount and type of protamines as well as the concentration of disulphide bonds appear to correlate with the rate by which sperm chromatin decondenses (Brewer et al. 1999). Dithiothreitrol-induced sperm decondensation in vitro shows that human sperm nuclei decondense faster than mouse and hamster with bull sperm being more stable (Perreault et al. 1988). Interestingly, the lack of disulphide bonds in the nuclei of rooster, tilapia and those immature mammalian sperm from hamster and mouse shows greater susceptibility to decondensation during heat treatment than from mature mammalian spermatozoa (Yanagida et al. 1991). This suggests that thermostability of sperm nuclei is determined by the amount of disulphide cross-linking in protamines, which is related to sperm maturation and is species-dependent. However, these studies exposed sperm samples to temperatures as high as 60–125 °C for 20–120 min, well beyond the range use in our study to maintain viable motile spermatozoa. Thus, there appears to be an interplay between species, state of sperm maturity and level of heat treatment which determines the degree of sperm DNA damage induced. Mature boar spermatozoa exposed to heat shock appear to be highly DNA stable during treatment with viable temperatures up to 42 °C. Moreover, in the context of our study, the results should be interpreted with caution and should not be considered as direct comparison between the two heat stress models. Specifically, the use of more stable mature spermatozoa in the in vitro heat shock treatment is not comparable to the in vivo heat stress on developing spermatozoa in the testis.

Interestingly, heat stress induced by the in vivo hot room did not appear to significantly affect sperm motility, similar to results we found in our previous seasonality study (Peña et al. 2019a). This suggests that DNA-damaged boar spermatozoa are likely to swim and potentially fertilize oocytes normally (Ahmadi and Ng 1999). These motility results however contrast to those reported in other studies using either the whole animal model (McNitt and First 1970; Wettemann et al. 1976; Cameron and Blackshaw 1980) or scrotal insulation (Parrish et al. 2017). While this is quite difficult to explain, our study was conducted over a much longer duration of 42 days, and our boars were pre-screened for high sperm motility before they qualified for the study. In this respect, motility and morphology of spermatozoa from boars with average ejaculate quality were not significantly affected by heat stress compared to boars with below-average ejaculate quality (Pribilova et al. 2016). Despite this, it must be noted that it is still possible to produce boar spermatozoa with superior motility even during summer (Gorski et al. 2017).

In contrast to our previous study in which tropical summer caused a significant decline in sperm concentration (Peña et al. 2019a), we found no difference between the hot room, peak wet and control treatments in our current study. Exposure of our boars to elevated temperatures for 42 days in the hot room was to ensure that the stressor was present for at least one complete cycle of spermatogenesis (Franca and Cardoso 1998; França et al. 2005). Given the peak wet summer season in Townsville spans a period of up to 4 months, it is possible that 42-day treatment was insufficient to cause significant levels of apoptosis, measurable as reduced sperm concentration in the ejaculate. That said, boars subjected to about 4 days of scrotal insulation still suffered from poor quality sperm up to 2 months after the heat stress treatment (Parrish et al. 2017).

Conclusions

Using both in vivo and in vitro models, our study confirms that boar sperm DNA is susceptible to heat stressed-induced damage. However, greater sample size and longer exposure times are needed to generate significant effects. Moreover, results from the in vitro heat shock treatment using more stable and mature spermatozoa should not be considered as direct comparison to the in vivo heat stress results using developing spermatozoa in the testis. We consider it imperative to optimize in vitro heat shock models that induce significant, biologically meaningful levels of sperm DNA damage without deleterious effects on motility. This will reduce the need for whole animal experiments from a welfare perspective but could also provide a valuable diagnostic tool to screen the ejaculates of individual boars for heat tolerance as a means to select breeding stock for animal production industries based in the tropics.

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Ethics declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval

Experiments were approved by the James Cook University Animal Ethics Committee (Approval Number 1998).

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