Revisiting summer infertility in the boar: impact of heat stress on the quality and DNA integrity of spermatozoa, and its mitigation by antioxidant therapy

Thesis submitted by

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In January 2018

for the degree of Doctor of Philosophy

College of Public Health, Medical and Veterinary Sciences

James Cook University

ACKNOWLEDGMENTS

It hasn't been an easy journey as everyone may know but one thing for sure, with the right people, appropriate resources and a whole lot of grit, this PhD journey has its meaningful end. I truly and sincerely appreciate and best of thanks to my advisory committee headed by Dr. Damien Paris, who has painstakingly taken me on as his first PhD student and yet have exemplified utmost professionalism and proficiency in all our undertakings every step of the way. My electronic calendar hasn't failed to prompt me of our weekly meetings that have been sustained in the past four years which has not only been effective in monitoring outputs and performance assessments but served as a building block in establishing good relationships and collaborative perspective for future research initiatives. Prof. Bruce Gummow and A/Prof. Anthony Parker have been very close at providing respective hands-on assistance either in providing expert advice in the research framework as well as in the care and support of experimental animals when their respective expertise are called for.

Pertinent organizations which have been indispensable to this endeavor include 1) The Australian Awards Scholarship (AAS), 2) The College of Public Health Medical and Veterinary Sciences, James Cook University (CPHMVS, JCU) 3) The College of Veterinary Medicine, Visayas State University (CVM, VSU), and 4) The Philippines Australia Human Resource Organization and Development Facility (PAHRODF). I wish to express my heartfelt thanks for the financial aids either in the form of a student stipend and living allowance, grants and research funds; expert support and for simply being there at all times I needed to identify myself with. To you, I am greatly indebted.

Special mention goes to Dr. Roby Jose and Dr. Lynn Woodward for training and access to FACS facilities which played a key role so we can actually evaluate and analyse boar sperm DNA damage. Thanks to Felicity Stone who assisted in the initial establishment of sperm DNA assay, flow cytometry and general laboratory procedures. J. Penny, N. Breen, R. Jack, S. Blyth, V. Simpson, A. Blyth, J. Palpratt and I. Benu extended their hands and time assisting in the handling and care of boars sourced from the kind owners of Glenley Piggery farm. A/Prof J. Cavalieri is recognized for his initial assistance in the operation of the IceCube sperm freezer; Dr. S. Watts and Dr. K. Gerger on blood examination/evaluation; and Dr. L. Foyle's expert assistance in the humane disposition of our boars at the conclusion of the project. Also, special thanks to F. Stone in the early establishment of the TUNEL assay and flow cytometry. A. Salvador and K. Elliot of the Australia Awards Scholarship- JCU have been an all-time ally.

The constant support and encouragement from heaven-poured friends like Mama Myrna R., Dee C., Nemaria, Lofranco, Tabiling, Knight and Brana families and many other friends who have come to pitch in and share their lives and opened their homes for us. The family at Northreach Baptist Church has been a refuge when we seek a church to join in. Weir and Annandale State Schools, my girls will never forget your love and companionship. My Aussie-born son will carry in his life the kindest services received from the JCU Health and the Townsville Hospital and Health Services.

This PhD journey has become a one rare Australian experience for me and my family. In saying so, my PhD journey wouldn't have been one of the most cherished chapters in my life without the ever-loving support and lively presence of my dear wife Angelica, daughters

Hadassah (9), Olive (7) and son Timothy (2). You have made far greater sacrifices for my success.

To ever-loving God, how can I ever forget You. You deserve all the credits. I am humbled and grateful.

STATEMENT OF SOURCES

DECLARATION

I, the undersigned, declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education.

Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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STATEMENT OF THE CONTRIBUTION OF OTHERS

Dr. Damien Paris assisted with experimental design, establishment of sperm quality assays, acquisition of research equipment and consumables, provision of funds for the study, assistance with data analysis and interpretation and assistance with writing and revision of manuscript.

Prof. Bruce Gummow provided feedback on experimental design, assistance on statistical analyses and interpretation, support in animal health monitoring and care, formulation of antioxidant supplementation and feedback on manuscript preparation.

A/Prof. Anthony Parker contributed in the acquisition of experimental boars, improvement in pig facilities and equipment and formulation of antioxidant supplementation.

Felicity Stone assisted in the establishment of sperm DNA assay, flow cytometry and general laboratory procedures.

This PhD project was funded by a JCU Development Grant to Dr. Damien Paris, and the College of Public Health, Medical and Veterinary Sciences PhD Research Funds to Santiago Peña Jr. The Australia Awards Scholarship provided financial assistance for Santiago's full tuition fees and contribution to living expenses among others.

DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics*. *Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Animal Ethics Committee (Approval Number A1998).

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ABSTRACT

Summer infertility in the pig continues to affect both productivity and profitability among pig producers in tropical and subtropical regions, with losses due to heat stress amounting to at least $300 million per year in the US swine industry alone. Given the wide scale production of pork globally, with at least five tropical countries among the top producers in the world, there is a need to revisit the problem of summer infertility in the pig and identify putative boar factors likely to contribute to poor reproductive performance during periods of heat stress. An important endpoint with this approach is to improve boar management practices and develop strategies to mitigate summer infertility in the pig.

While the scrotum, pampiniform plexus, and cremaster and dartos muscles in mammals are specific adaptations to ensure sperm production in a regulated environment 4-6 °C below body temperature, the boar's inefficient capacity to sweat, non-pendulous scrotum, and low antioxidant activity in the semen, can make the it particularly vulnerable to the effects of heat stress. In Chapter 2, we demonstrated for the first time the link between summer heat stress and sperm DNA damage in Large White boars. Boars raised in the dry tropics of Townsville, Queensland, Australia during summer (peak wet) showed 16-fold more sperm DNA damage than early dry (cool and dry), and nearly 9-fold more than the late dry (warm and humid) season, respectively. Sperm concentration also decreased significantly in the peak wet. Sperm DNA damage has been previously demonstrated to contribute to early embryonic death in the mouse, and this magnitude of fragmentation is known to cause a reduction in litter size in sows. These findings provide impetus for the evaluation of sperm DNA integrity in commercial boar herds housed in the tropics as a putative contributing factor to seasonal infertility in the sow.

While cryopreservation of boar spermatozoa is not widely practiced in commercial pig production, mostly due to reduced viability and fertilising capacity of post-thawed spermatozoa, it has a greater potential in advancing studies related to seasonal effects of heat stress on boar sperm fertility using *in vitro* fertilisation. Since the freeze-thaw process can led to increased sperm DNA damage and subsequently contribute to early embryo loss, in Chapter 3 we tried to address the limited information about the protective effects of the more common cryoprotectant glycerol on sperm DNA integrity during boar sperm cryopreservation. We aimed to determine the optimal concentration of glycerol to protect sperm DNA integrity, without the deleterious effect of high concentrations negatively affecting sperm motility. We deemed this work particularly important to permit us to freeze boar sperm collected during summer, for downstream use to fertilise eggs *in vitro* during winter when oocyte quality is high. Our study revealed that 3%, 6% or 8% glycerol could be safely used to cryopreserve boar spermatozoa without inducing additional DNA damage compared to fresh spermatozoa. We deemed a concentration of 6% glycerol provided the best DNA protection, while maintaining sufficient levels of sperm motility.

In chapter 4, we aimed to develop reliable heat stress models that could be used at any time of the year, to advance the study of seasonal infertility in the pig by overcoming the variation and limitations associated with seasonal studies. We have successfully induced biologically meaningful levels of DNA damage in boar spermatozoa using either a whole animal *in vivo* model (hot room) or by direct exposure of semen to heat *in vitro* (heat shock model). However, we were only able to induce levels of damage observed during natural tropical summer (Chapter 2) using extreme *in vitro* temperatures that rendered boar spermatozoa completely immotile or dead. Here, our results suggest that boar sperm is vulnerable to heat-

induced DNA damage, but individual factors may also contribute to a boar's overall susceptibility to heat stress.

Given the limited endogenous levels of antioxidants in boar semen and the insufficient DNA repair mechanisms these cells have, Chapter 5 aimed to formulate and evaluate antioxidant therapy as a strategy to mitigate the effect of heat stress on boar sperm DNA integrity. We proposed that an exogenous multi-compound antioxidant supplementation could effectively combat heat stress induced oxidative damage and prevent the build-up of DNA fragmentation in boar spermatozoa. Supplementing boar diets with 100 g/day custom-mixed antioxidant during summer effectively reduced sperm DNA damage by as much as 55% after 42 and 84 days treatment, respectively. This implies that antioxidant supplementation during tropical summer could provide a measurable solution to the problem of boar-mediated summer infertility in the pig.

Overall, boar sperm DNA integrity can be compromised during tropical summer and this can be induced experimentally using our *in vivo* or *in vitro* heat stress models; with response particularly affected by individual boar variability. Exogenous antioxidant supplementation in feed could provide an effective means to mitigate the problem of summer infertility. Apparently, neither seasonal heat stress or heat stress models negatively affected sperm motility, suggesting that traditional evaluation of sperm motility in boars may not detect inherently compromised DNA damage spermatozoa. Antioxidant supplementation only appears to mitigate DNA damage since it did not improve sperm motility or concentration after 42 or 84 days treatment. Future studies are needed to measure the beneficial impact of antioxidant supplementation under tropical farm conditions, in terms of improved sperm DNA integrity and increased litter size following artificial insemination.

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AI	artificial insemination
ALH	Amplitude of lateral head displacement
ANOVA	Analysis of variance
ATP	Adenosine Triphosphate
BCF	Beat cross frequency
BTS	Beltsville thawing solution
CWE	Carcass weight equivalent
CASA	Computer assisted sperm analysis
DAPI	4′, 6-diamidino-2-phenylindole dihydrochloride
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELONG	Elongation
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
GSH	Glutathione peroxidase
ICM	Inner cell mass
LIN	Linearity
PBS	Phosphate buffered saline
TBARS	Thiobarbituric acid reactive substances
ROS	reactive oxygen species
SCSA®	Sperm Chromatin Structure Assay
STR	Straightness
THI	Temperature-humidity indices
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VAP	Average-path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity

CHAPTER 1

GENERAL INTRODUCTION

Published: Peña, S.T.Jr., Gummow, B., Parker, A.J. & Paris, D.B.B.P. 2017. Revisiting summer infertility in the pig: could heat stress-induced sperm DNA damage negatively affect early embryo development? *Animal Production Science,* 57**,** 1975-1983. http://dx.doi.org/10.1071/AN16079

1.1 Introduction

Pork production is a major contributor to the agricultural economy, with global production as high as 112 million tons carcass weight equivalent (CWE) compared to beef and veal at 59.2 million tons CWE and broiler meat at 89.3 million tons ready to cook equivalent, respectively (FAS, 2015). A 120 kg pig yields about 91 kg of carcass, providing 371 servings of high quality meat for human consumption (Snelson 2010; National Pork Board 2014). Pigs also contribute many other by-products while providing extensive employment opportunities due to rising production, consumption, and import and export demands. The demand for food continues to grow as the current population increases exponentially. Average global meat consumption is currently 100 g per person per day, providing at least 16% of the total calories and 34% of the total proteins in the human diet (Mcmichael *et al.*, 2007). While the latest FAO estimates show a positive trend at reducing global hunger as compared to the previous two decades (FAO, IFAD, WFP 2014), meeting the current and projected demands for food still poses enormous challenges considering that the human population is predicted to rise to 8.9 billion in 2050 (Cohen, 2003). The demand for food has been projected to increase significantly to at least 3050 kcal/person/day in 2050 from an average global food consumption of 2940 kcal/person/day in 2015 (Fao, 2009). Therefore, research efforts should continue to focus on improving the production potential and efficiency of the pig industry.

1.2 Summer Infertility: The Problem

Seasonal or summer infertility in the pig is a syndrome characterised by an over-all reduction in the reproductive performance of the breeding herd that usually occurs in summer when pigs are exposed to a combination of environmental stressors including heat in particular, as well as photoperiod, humidity, genetic background, and management practices among others (Love 1978, 1981; Hennessy and Williamson 1984; Quesnel *et al*. 2005; Auvigne *et al*. 2010). Summer infertility primarily manifests as either 1) difficulty in coming into oestrus,

expressed as delayed puberty in gilts, extended weaning-to-oestrus interval in sows, or increased anoestrus in both gilts and sows; or 2) higher rates of pregnancy failure with irregular returns to service, which may be attributed to untimely ovulation or early embryonic loss (Paterson 1978; Hughes and van Wettere 2010); and/or 3) reduced fertility potential in the male (Wettemann *et al.*, 1976; Wettemann *et al.*, 1979; Cameron and Blackshaw, 1980; Britt *et al.*, 1983; Hennessy and Williamson, 1984; Wettemann and Bazer, 1985; Boma and Bilkei, 2006; Auvigne *et al.*, 2010). Although the domestic pig may breed throughout the year, the seasonal reproductive activity of wild boars/sows (*Sus scrofa ferus*) is attributed to either decreasing day length, summer rainfall and/or the availability of food (Ahmad *et al*. 1995; Rosell *et al*. 2012).

Several tropical countries are among the top 10 pig producers in the world including Brazil, Vietnam, The Philippines, and Mexico (National Pork Board 2014). While different genetic lines/breeds of boars and sows show different tolerance to heat stress reflected in their reproductive performance (Bloemhof *et al*. 2008; Flowers 2008), the use of high-yield exotic white breeds from temperate countries have become commonplace in the tropics. As such, commercial farm animals particularly in these regions can inadvertently suffer from summer infertility when ambient temperatures rise beyond the animal's zone of thermal comfort (St-Pierre *et al.*, 2003). The negative impact of heat stress on productivity is becoming increasingly important to developed and developing nations due to decreasing profit margins. On average, 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). Longer weaning-to-conception intervals and reduced over-all reproductive performance in sows have been reported in large confinement units during hotter months from June to October in North Carolina (Britt *et al.* 1983). In a five-year study in France, season was shown to clearly impact the fertility rate of

pigs; with the lowest mean fertlity of 81.2% occuring during the end of August (end of summer), compared to the highest mean fertility of 86.8% during the end of March (end of winter; Auvigne *et al.* 2010). However, it is pig producers particularly in equatorial countries that are likely to be the most sensitive to the impacts of summer infertility. Reproductive problems associated with heat stress and other concomitant factors have been reported involving small, medium and large commercial pig farms in The Philippines. Small to medium farms are most severely affected, particularly in relation to the weaning to conception interval, farrowing index, farrowing interval and non-productive days (Vega *et al.*, 2010). Seasonal variation in the reproductive performance of sows has also been observed in Thailand (Suriyasomboon *et al*. 2006) along with negative effects of high temperature and high humidity on the sperm production of Duroc boars (Suriyasomboon *et al*. 2004). Several strategies can be adopted to minimise the effects of heat stress on the animal's reproductive performance. These include modification of the diet, breed selection, provision of floor and roof cooling systems, and varying building orientation, among others (Gourdine *et al.*, 2006; Silva *et al.*, 2006; Gholami *et al.*, 2011).

1.3 Effect of Heat Stress on Boar Fertility

The cycle of spermatogenesis in the boar is divided into four cycles and eight stages (Fig.1; Franca and Cardoso, 1998). Overall, it takes about 30-34 days in the boar to complete the process of spermatogenesis in the testis followed by a further 10-12 days for epididymal transit, maturation and storage in the cauda; yielding a total of about 42 days to complete one entire cycle (Franca and Cardoso, 1998; Almeida *et al*., 2006). While the duration of spermatogenic events and the relative stage frequencies in Piau boars appears to differ slightly from more advanced swine breeds (Franca and Cardoso, 1998), Yorkshire and Lacombe boars have previously been shown to be similar (Swiestra, 1968).

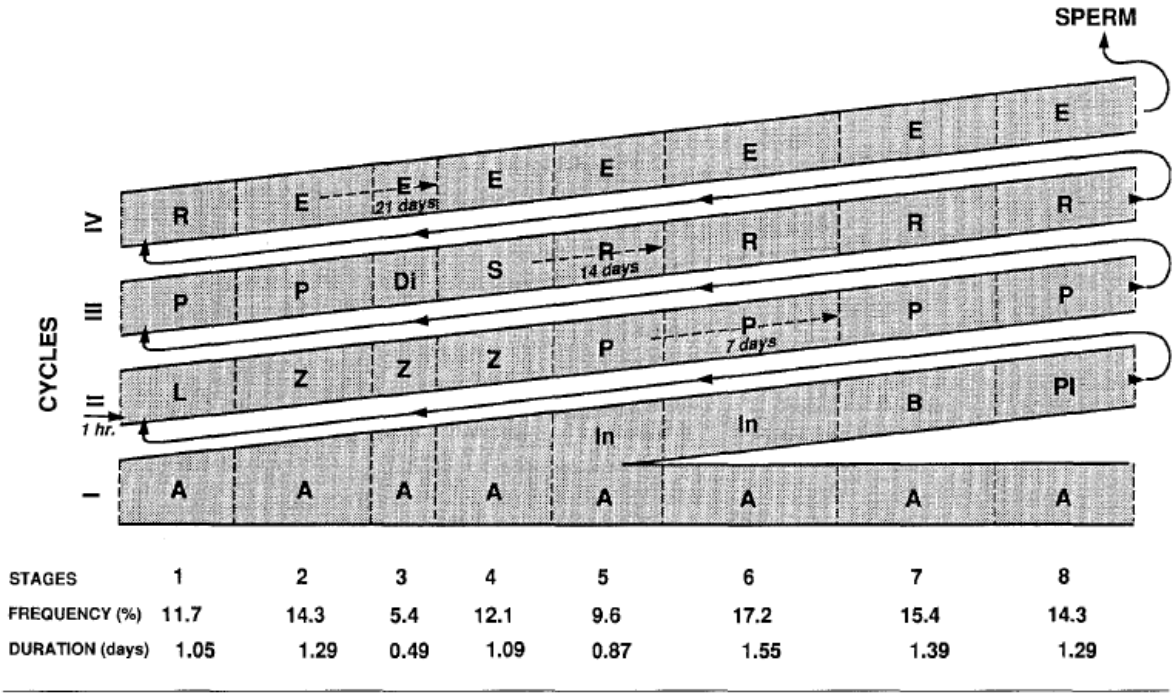

Figure 1-1: Diagram showing the most advanced germ cell labelled by thymidine injection at different times during the eight stages of the cycle of boar seminiferous epithelium.

Roman numerals indicate each of four spermatogenic cycles. The frequency (%) and duration in days for each stage is shown, with spacing proportional to each stage's duration. Letters indicate germ cell types at each stage of the cycle: Type A spermatogonia (A); intermediate spermatogonia (In); type B spermatogonia (B); preleptotene spermatocytes (Pl); leptotene (L); zygotene (Z); pachytene (P); and diplotene spermatocytes (Di); secondary spermatocytes (S); round spermatids (R); elongate spermatids (E). Adapted from Franca and Cardoso, 1998.

This processes of spermatogenesis and subsequent sperm maturation however are highly sensitive to temperature. In fact, the scrotum, pampiniform plexus, and cremaster and dartos muscles in mammals are specific adaptations to ensure sperm production in a regulated environment 4-6 °C below internal body temperature (Nakamura *et al.*, 1987; Setchell, 2006). Pigs are known to be inefficient at using sweat to cool their body during high ambient temperatures. While cutaneous water-loss over the general body surface appears to be similar to man and domestic species, the pig's ability to sweat is considerably limited (Ingram 1964,

1965; Einarsson *et al*. 2008). Stone (1981) reported that the thermal characteristics of the testis and epididymis of conscious boars ranged from 35.0-36.6 °C and 35.0-37.0 °C, respectively. These temperatures were 2.5 °C to 1.9 °C lower than the animal's rectal temperature of 38.2 °C. Moreover, Stone (1982) found that an ambient temperature of 29 °C appears to be the critical limit above which Large White boars are unable to produce normal numbers of motile spermatozoa. Specific breeds and/or genetic backgrounds also tend to influence normal sperm production (Huang *et al*. 2000; Flowers 2008). Landrace boars tend to have better semen quality than Yorkshire and Duroc boars during hot seasons (Huang *et al*. 2000). Unlike in rams and bulls, the boar scrotum is non-pendulous and is much closer to the body wall which could limit its ability to regulate testicular temperature and thus potentially make this species sensitive to the effects of environmental heat-stress on semen production (Knox 2003). Prolonged exposure of testes to high temperature (i.e. testicular temperature at 38 °C) can predispose boars to significantly reduced basal concentrations of peripheral testosterone along with hypertrophy and impaired function of the Leydig cells (Stone and Seamark 1984).

The effect of heat stress on semen production and reproductive efficiency has been extensively studied as early as the 1950's and 60's in various farm animals, including rams (Moule and Waites, 1963), bulls (Casady *et al.*, 1953) and boars. In the boar, the detrimental effects of heat stress on sperm quality and boar fertility can manifest several days or weeks post-heat treatment. These include decreased volume in seminal plasma (Cameron and Blackshaw 1980), decreased sperm concentration (Egbunike and Dede 1980), decreased motility and increased abnormal sperm (McNitt and First 1970; Wettemann *et al.* 1979; Heitman *et al.* 1984; Malmgren 1989; Huang *et al*. 2000), disturbance in androgen biosynthesis (Wettemann and Desjardins 1979; Stone and Seamark 1984), prolonged

ejaculation time (Egbunike and Dede 1980) and reduced libido (Flowers 1997). Recently, Zasiadczyk *et al*. (2015) reported the effect of seasonal variations (autumn-winter *vs* spring-summer) on the quality of ejaculates collected from individual boars. Ejaculates collected during spring-summer had significantly lower volume, sperm concentration and number of spermatozoa with functional mitochondria and intact plasma membrane (Zasiadczyk *et al*. 2015). By contrast, Petrocelli *et al*. (2015) observed photoperiod to be more important than housing temperature in affecting most boar semen characteristics.

Despite this extensive focus on classical parameters of sperm quality, there is a growing body of evidence that suggests damage to sperm DNA could invariably reduce male fertility and subsequent embryo survival (Evenson 1999; Paul *et al.* 2008b; Perez-Crespo *et al.* 2008; Didion *et al.* 2009; Evenson *et al.* 2009). That is, sperm may swim and fertilize eggs normally but embryos that have acquired a damaged paternal genome could die *in utero*. Thus, new approaches to breeding soundness evaluation in the boar (i.e. DNA fragmentation analysis and biomarkers for normal sperm phenotypes) may yield a better understanding of the underlying factors causing poor reproductive performance, thereby leading to a robust solution to the problem of summer infertility (Sutovsky, 2015).

1.4 Impact of Heat Stress on Sperm DNA Integrity

The DNA of mature sperm is uniquely condensed and tightly packaged primarily with protamines and to a lesser extent with histone-bound chromatin attached to a nuclear matrix (Wykes and Krawetz 2003; Ward 2010). This unique framework allows structural protection to spermatozoa but also includes molecular regulatory factors and several gene clusters that are important to successful embryo development (Hammoud *et al*. 2009). In boars, this DNA transitions from a weak structure at the late spermatid stage in the testis, to a very rigid structure in mature spermatozoa from the caudal epididymis; suggesting significant change in

histone-to-protamine content during sperm development (Ashikawa *et al*. 1987; Fortes *et al*. 2014). Protamine deficiency in bull sperm is closely associated with higher DNA fragmentation index as determined by Sperm Chromatin Structure Assay (SCSA; Fortes *et al*. 2014). Moreover, scrotal heat stress can significantly reduce protamine disulphide bonding in stallion sperm resulting in sperm DNA with higher susceptibility to denaturation (Love and Kenney 1999).

In general, mammalian spermatozoa are particularly sensitive to oxidative damage due to the limited endogenous antioxidant systems inherent in these cells, which is compounded by the presence of large amounts of unsaturated fatty acids in the plasma membrane that are exposed to free radical attack (lipid peroxidation; Aitken and De Iuliis, 2010). Furthermore, the loss of cytosolic machinery from these sperm cells during spermatogenesis makes them transcriptionally and translationally inactive, and results in a deficiency of repair mechanisms once such damage has occurred (Henkel *et al.*, 2004; Lewis and Aitken, 2005; Paul *et al.*, 2008a; Aitken et al. , 2012). By comparison, the epididymis secretes both intra-luminal free radical scavengers and extracellular antioxidant enzymes to help protect spermatozoa during the 12-14 days of epididymal transit and maturation, but these are absent during manufacture in the testis (Vernet *et al.*, 1996; Aitken and De Iuliis, 2010).

Spermatozoa immersed in caudal fluid further mix with secretions from the accessory sex glands, collectively called the seminal plasma, upon ejaculation. Unlike other species, the boar ejaculates large volumes of semen reaching up to 200-400 ml/ejaculate. Many studies have reported that seminal plasma contains lectin-like molecules belonging to the spermadhesin group of proteins. These proteins coat the plasma membrane of the sperm head during ejaculation and act as receptors to carbohydrate ligands present on the oviduct

epithelium (Sanz *et al.*, 1993; Dostalova *et al.*, 1994; Dostalova *et al.*, 1995; Ekhlasi-Hundrieser *et al.*, 2005), thus facilitating the sperm reservoir in the oviduct. Seminal plasma also contains several biochemical components which may facilitate over-all fertility of boar sperm (Lopez Rodriguez *et al*. 2013; Sancho and Vilagran, 2013). One of which is glutathione peroxidase (GPX5) that protects sperm membranes from oxidative stress. Novak *et al*. (2010) found that fertility index and farrowing rate appear to correlate with the presence of GPX5 in the sperm-rich fraction of the boar ejaculate. Moreover, there was a significant improvement in conception rates and litter size when seminal plasma was added to thawed epididymal spermatozoa during artificial insemination (Okazaki *et al.* 2012).

Exposure of the scrotum to 40-42 °C for 30 min in the mouse causes damage to spermatogonia, spermatocytes, spermatids or spermatozoa resulting in a significant increase in DNA damage and a distortion in sex-ratio of offspring born (Paul *et al.*, 2008b; Perez-Crespo *et al.*, 2008). Moreover, embryo development is blocked between the 4-cell and blastocyst stages, resulting in abnormal embryo development and loss (Paul *et al.*, 2008b). This detrimental effect might be attributed to heat stress causing testicular germ cell loss and abnormal gene expression (Rockett *et al.*, 2001) as well as dissociation in the chromosomes leading to chromosomally unbalanced gametes (Van Zelst *et al.*, 1995). Rockett *et al.* (2001) showed that heat stress down-regulates the expression of a number of DNA repair genes such as *Ogg1* (involved in base excision repair), *Xpg* (involved in nucleotide excision repair) and *Rad54* (involved in double-strand break repair), as well as polyADP ribose polymerase that is responsible for detection and signalling of strand breaks (Tramontano *et al.*, 2000). Moreover, a reduction in the expression of oxidative stress-induced antioxidants due to heat stress (Rockett *et al.* 2001), may lead to increased susceptibility of spermatozoa to oxidative damage.

In humans, sperm DNA damage is significantly higher in infertile men, with about 20-30% DNA damage (depending upon the test) used as the demarcation between infertile and fertile groups (Gandini *et al.*, 2000; Evenson and Wixon, 2006; Schulte *et al.*, 2010). In addition, *in vitro* fertilisation by human spermatozoa with greater than 8% DNA damage results in reduced blastocyst development (Ahmadi and Ng, 1999) and lower pregnancy rates (Henkel *et al.*, 2004). Fertilisation using DNA-damaged sperm reduces the rate or completely blocks blastocyst formation in cattle (Fatehi *et al.*, 2006; Fernandes *et al.*, 2008), and causes embryonic loss in the mouse (Paul *et al.*, 2008b).

Studies examining sperm DNA integrity in boars highlight the potential for using sperm DNA tests for boar fertility assessment. The percent DNA Fragmentation Index (%DFI) of boar spermatozoa showed a significant negative correlation to farrowing rate and average total number of pigs born (Didion *et al.*, 2009). Similarly, there was a strong relationship whereby fertility of boars that are used for AI can be attested upon evaluation of both sperm morphology and DNA integrity (Tsakmakidis *et al*. 2010). In other studies, DNA fragmentation in undiluted boar semen maintained at 37 °C was significantly higher and occurred much earlier (as early as 2 days) than semen maintained at 16 °C (Pérez-Llano *et al.*, 2010), whereas storage of extended liquid boar semen at 18 °C for 3 days resulted in reduced sperm DNA integrity (Boe-Hansen *et al*. 2005). Interestingly, a subsequent study by Boe-Hansen *et al*. (2008) reported a reduction in litter size by as much as 0.5-0.9 piglets per litter if the %DFI of semen is greater than 2.1%. Other studies suggest that a sperm sample with greater than 6% DFI results in decreased farrowing rate and average number of pigs born (Didion *et al.* 2009; Evenson *et al.* 2009).

Exposure to heat treatments (i.e. testicular insulation, scrotal heating, dipping of testes into hot water, heated incubation of spermatozoa, etc.) have been shown to cause significant fragmentation of sperm DNA in animals (Karabinus *et al.*, 1997; Fatehi *et al.*, 2006; Fernandes *et al.*, 2008; Paul *et al.*, 2008b). Boars that have been exposed to a controlled hot-room environment, direct sunlight or ambient temperatures ranging from 30-40 °C for between 3-90 days (Mcnitt and First, 1970; Wettemann *et al.*, 1976; Cameron and Blackshaw, 1980; Stone, 1982) have demonstrated a significant decrease in sperm motility, normal morphology, and sperm concentration; with one study reporting more than 1.5 times fewer embryos surviving the first month of pregnancy in gilts bred with semen from heat-stressed boars than gilts bred with semen from control boars (Wettemann *et al.*, 1976). Despite this work, the important link between heat stress and sperm DNA damage is still missing in the pig. Using TUNEL assay and flow cytometry techniques, preliminary results in our laboratory show a significant increase in the mean percentage of DNA damage in boar sperm from less than 2% during spring and winter to over 16% during summer in the dry tropics of Townsville, Queensland, Australia (Peña *et al*. 2016). This supports an earlier study by Zasiadczyk *et al.* (2015) in which sperm DNA fragmentation is markedly higher in spring-summer than in autumn-winter in fractionated ejaculates (particularly F1 & F2) using neutral comet assay. Moreover, results by Petrocelli *et al*. (2015) suggest possible seasonal DNA damage to boar spermatozoa.

While sperm DNA assays have their limitations (Barratt *et al*. 2010), the above studies suggest that examination of sperm DNA integrity may provide important answers to male-factor causes of summer infertility in the pig that would otherwise go undetected by routine sperm assessment (Evenson *et al.*, 1994; Enciso *et al.*, 2006).

1.5 Impact of Sperm Freezing on Sperm DNA Integrity

Sperm freezing is a valuable tool for long-term storage of genetic material while enabling faster distribution of desirable genes. However, the processes involved in freezing and thawing can be deleterious to some extent, causing damage to the structural integrity and function of the sperm by affecting sperm membranes, mitochondrial architecture and motility. This can lead to reduced fertilisation success (Watson, 1995; Thurston et al., 2001; Roca et al., 2006). Moreover, sperm freezing may also cause damage to sperm DNA as demonstrated in several species including boars (Hamamah et al., 1990), men (De Paula et al., 2006), rams (Peris et al., 2004), and mice (Yildiz et al., 2007). More specifically, cryopreservation of boar and human spermatozoa has been associated with the reduction in the sperm nuclear surface and Feulgen-DNA content leading to a state of 'overcondensation' (Royere et al., 1988; Hamamah et al., 1990), which could potentially affect the fertility of frozen spermatozoa (Royere et al., 1991).

The use cryoprotective agents has been extensively used in well-established sperm freezing protocols (Pursel and Johnson, 1975; Aricultural Research Service - US Department of Agriculture, 2007). Glycerol for example is known to improve motility and plasma membrane integrity of frozen-thawed sperm (Almlid and Johnson, 1988; Fiser and Fairfull, 1990; Das *et al.*, 2016) as it protects the cells from ice crytal formation during freezing. However, there is still limited information about the optimal concentration of glycerol to protect DNA integrity during cryopreservation of boar spermatozoa. Moreover, boar spermatozoa appear to be more sensitive to the standard concentrations of glycerol used in sperm cryopreservation protocols of other domestic species (Almlid and Johnson, 1988). An earlier report suggests that examination of sperm DNA structural damage in cryopreserved extended boar semen was able to correctly predict between potentially high and low fertility

boars based on DNA integrity (Evenson *et al.*, 2009). Thus, it is important to evaluate optimal levels of glycerol that are protective to sperm DNA integrity without compromising sperm motility.

1.6 Mechanisms by which Heat Stressed Spermatozoa Can Affect Blastocyst Formation and Early Embryo Loss

Apart from identifying male factor causes of summer infertility in the pig, it is equally important to understand the downstream mechanism by which heat-stressed sperm may cause early embryo loss. The formation of the blastocyst is an essential step in embryo development that facilitates proper implantation and establishment of pregnancy (Bruce and Zernicka-Goetz, 2010). It involves the formation of three distinct cell lineages that include the pluripotent epiblast that forms the embryo itself, and the trophectoderm and primitive endoderm that comprise the extra-embryonic tissues supporting the development of the embryo (Cockburn and Rossant, 2010). While our understanding of the mechanisms involved during these pre-implantation events are still limited, it is believed that a number of factors and signalling events including transcriptional regulation, epigenetic regulation (such as DNA methylation, histone modifications and chromatin modelling; Shi and Wu 2009), cell position and cell polarity, and cell-cell contact/positional relationships precede the eventual segregation of these three distinct populations of cells (reviewed in Zernicka-Goetz *et al.*, 2009; Bruce and Zernicka-Goetz, 2010; Gasperowicz and Natale, 2011; Oron and Ivanova, 2012).

In vitro and cytogenetic studies in humans demonstrate that about 30% of embryos fail to complete implantation, with anomalies in the DNA of gametes or embryos being the main reasons for this failure (reviewed in Macklon *et al.*, 2002). Despite the high fertilisation rates in the pig (90-100%), prenatal mortality of 30-40% can significantly limit the litter size and

dramatically impact economic profitability. The majority of these losses (20-30%) occur during the pre-implantation period of development (Anderson 1978; Bolet 1986; Geisert and Schmitt 2002) at a time when the embryo is forming a blastocyst and secreting maternal recognition of pregnancy (MRP) signals. Embryonic oestradiol (E_2) plays a crucial role in porcine MRP signalling by shifting the secretion of prostaglandin $F_2\alpha$ into the uterine lumen were it rapidly deteriorates; thus preventing transport to, and luteolysis of the corpus luteum via uterine vein-ovarian artery counter-current exchange (Bazer and Thatcher, 1977)

Blastocyst formation is regulated by specific genes that directly influence the organisation and differentiation processes. *Oct4* expression in internally-positioned populations of cells in the morula-stage murine embryo specifies differentiation of the inner cell mass (ICM), while *Cdx2* in externally-positioned cells specifies differentiation of the trophectoderm. *Nanog* and *Gata6* are responsible for the formation of the epiblast (from ICM cells) and primitive endoderm respectively (Ralston & Rossant 2005; 2010). Identifying the timing and expression patterns of these genes is important as this appears to differ among species, indicating a different role for such genes in other mammals. Kuijk *et al.* (2008) have demonstrated significant differences in expression patterns of these genes in porcine and bovine embryos compared to that of the mouse. While expression of *CDX2* and *GATA6* were similar, variation existed in the expression of *NANOG* and *OCT4* between species and during different stages of development. In the pig and cow, *OCT4* is not present in morulae but can be detected in both the trophectoderm and inner cell mass (ICM) of the blastocyst. *NANOG* expression is completely absent in porcine embryos during blastocyst formation.

However, in subsequent studies, *NANOG* was found to be expressed in the ICM and epiblast of porcine embryos at 7.5 embryonic days (E7.5) by which time, the embryos have already

arrived in the uterus. Moreover, expression of *NANOG* by the epiblast appears to be extended for a few days after the blastocyst has formed (Hall *et al.*, 2009; Du Puy *et al.*, 2011). These findings differ considerably to the timing of *NANOG* expression in the mouse (i.e., early stage of mouse blastocyst; around E3.5; Chazaud *et al.*, 2006). This early expression is believed to be indispensable for proper differentiation of the murine ICM leading to epiblast and primitive endoderm formation (Silva *et al.*, 2009; Messerschmidt and Kemler, 2010). In the pig however, primitive endoderm appears to have already formed before *NANOG* is expressed. Recently, Wolf *et al.* (2011) found what appears to be a sequential expression of *OCT4* and *NANOG* in the pig. *OCT4* but not *NANOG* appears initially in the ICM and is followed later by co-localised expression of both of these genes in the epiblast; with subsequent down-regulation of *NANOG* by the time the primitive streak develops.

Interestingly, while a seemingly healthy-looking sperm according to classical measures of sperm quality, may swim and fertilise an oocyte normally (Ahmadi and Ng, 1999; Fernandes *et al.*, 2008), structural abnormalities in its DNA can lead to serious problems during pronuclear formation, embryonic genome activation, and early embryo development (Evenson, 1999). Sperm DNA damage may manifest itself at the time of embryonic genome activation (EGA), in the form of altered or arrested expression of important developmental genes that lie in regions where damage is present. Understanding of the normal pattern of expression of these key developmental genes can serve as a guide to investigating altered expression in developing embryos fertilised *in vitro* using artificially heat-stressed spermatozoa and/or semen collected from boars exposed to environmental heat stress. In fact, one study in the mouse has demonstrated the link between heat stress, sperm DNA damage and arrested embryo development consistent with aberrant expression of key genes involved in blastocyst formation (Paul *et al.* 2008b). Compared to control blastocysts, OCT3/4

immunostaining of embryos retrieved from females mated to 42 °C heated males showed aberrant staining patterns associated with grossly abnormal embryos that lacked a blastocoel and had fragmented nuclei. Several embryos from females mated to 40 °C heated males were also developmentally delayed, lacking a blastocoel and still expressed OCT3/4 staining in all cell nuclei (Paul *et al.* 2008b).

Furthermore, the impact of heat stress may not only be limited to disturbing the integrity of paternal genomic DNA but could broadly alter epigenetic constituents, activation factors and a host of messenger RNAs and microRNAs. These factors appear to influence the survival of the embryo post-fertilisation through participation in various molecular functions, such as signal transduction, cell proliferation and transcriptional proliferation (Krawetz, 2005; Yamauchi *et al*. 2011; Kumar *et al*. 2013).

Normal and timely formation of the blastocyst is paramount not only to subsequent development of the embryo but in preparing the maternal environment to recognise the impending pregnancy (Leibfiied-Rutledge, 1996; Latham 1999; Latham & Schultz 2001; Bettegowda and Smith, 2007; Minami *et al.*, 2007; Jeanblanc *et al.*, 2008). Any delay or arrest of embryo development will result in the delay or absence of properly timed MRP signalling by the trophectoderm. In porcine embryos, major morphological transformation occurs between 12-16 days of gestation when blastocysts elongate and reach their final length of about 800 mm to 1100 mm at day 16 of pregnancy (80 to 100 cm; Spencer, 2013; Tur, 2013). At this time, the trophectoderm secretes significant amounts of E_2 along with interferons gamma and delta (Spencer, 2013). This is essential for preventing luteolysis of the corpus luteum, as this structure is the primary source of progesterone production necessary to support pregnancy for the entire period of gestation in the pig (Meyer, 1994). Moreover, the

surge of E_2 from the trophectoderm is believed to influence gene expression in the endometrium and is responsible for promoting uterine receptivity and elongation of the conceptus (Johnson *et al.*, 2009). In this regard, fertilisation of oocytes with DNA-damaged sperm may disrupt the organisation of genes required in the formation of cell lineages (trophectoderm among others), distorting the sequence of events leading to the formation of the blastocyst (Ralston and Rossant, 2005; 2010). As a consequence, embryonic development may be delayed and/or arrested resulting in disrupted implantation, the loss of properly timed MRP signals and subsequent loss of the corpus luteum, and ultimately pregnancy failure. Using an *in vitro* fertilization system, ongoing research in our laboratory seeks to demonstrate the definitive link between heat stress in the boar and summer infertility in the sow; warranting a closer look at boar management strategies during periods of elevated ambient temperature.

1.7 Antioxidant Therapy to Mitigate Sperm DNA Damage

The low antioxidant activity in boar seminal plasma (Brzezińska-Ślebodzińska *et al.*, 1995) and the high proportion of easily oxidised long chain polyunsaturated fatty acids in the sperm plasma membrane could likely increase the risk of boar sperm DNA damage (Fraga *et al*., 1996). However, despite the widespread use of exogenous feed supplements including vitamin-mineral premixes and antioxidants particularly among commercial pig production, there have been no substantial reports to support the potential benefits of antioxidant supplementation on boar sperm DNA protection. Considering the extensive benefits of antioxidant supplementation on boar sperm including improvements in various sperm quality parameters such as motility, viability, survivability, acrosome integrity and storage among others (Pena *et al*., 2003; Strzezek *et al*., 2004; Funahashi and Sano, 2005; Chanapiwat *et al*., 2009), antioxidant therapy could be an effective strategy to mitigate the effects of environmental heat stress on boar sperm DNA damage.

1.8 Conclusion

While a number of sow-specific factors play a crucial role in sustaining embryo development in the pig, there is a strong case for the hypothesis that reduced fertility and embryo survival associated with summer infertility in the sow may be due in significant part to a reduction in DNA integrity of spermatozoa in the boar. If oocytes are fertilized by heat stress-induced, DNA-damaged sperm, it is highly probable that subsequent embryo development will be affected. This may include decreased cleavage rates; decreased blastocyst formation due to the disruption of specific genes responsible for early lineage formation and eventually delayed embryo development or early embryonic death, disrupted implantation and pregnancy loss. This has important implications for the proper management of boars from housing conditions to nutritional requirements including antioxidant supplementation during summer.

1.9 Hypothesis and Aims

We hypothesise that heat-stress causes sperm DNA damage that leads to poor fertilisation & early embryonic death, which can be mitigated by antioxidant therapy. The purpose of this research therefore is to determine the effect of heat stress on the quality and DNA integrity of boar sperm, then evaluate the therapeutic effect of antioxidant supplements. This aim will be achieved via a number of specific objectives:

1. Determine the effect of tropical summer on boar sperm quality and DNA integrity.
2. Optimise a boar semen freezing protocol that protects sperm DNA integrity.
3. Develop *in vivo* and *in vitro* heat stress models that can induce natural rates of sperm DNA damage in the boar.
4. Determine an antioxidant formula as potential intervention to mitigate effects of heat stress on sperm quality.

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CHAPTER 2

TROPICAL SUMMER INDUCES DNA FRAGMENTATION IN BOAR SPERMATOZOA: IMPLICATIONS FOR THE MANAGEMENT OF SEASONAL INFERTILITY

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Accepted: Peña, S.T.Jr., Gummow, B., Parker, A.J. & Paris, D.B.B.P. 2018. Tropical Summer Induces DNA Fragmentation in Boar Spermatozoa: Implications for Evaluating Seasonal Infertility**.** *Reproduction, Fertility and Development*

2.1 Abstract

While pork is the most widely eaten meat in the world, the problem of summer infertility continues to undermine 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. In mice, studies show that heat stress causes sperm DNA damage, which in turn contributes to embryo loss. However, the link between summer heat stress and sperm DNA damage has not been demonstrated in the pig. Semen from five Large White boars, raised in an open gable roof-type facility in the dry tropics of Townsville, North Queensland, Australia, was collected and evaluated during the early dry (cool and dry), late dry (warm and humid) and peak wet (hot and wet) seasons to determine the effect of seasonal heat stress on the quality and DNA integrity of boar spermatozoa. Sperm concentration was 1.6-fold lower in the peak wet than early dry but did not differ to the late dry season (221.8 ± 20.2 x 106 vs 354.1 ± 44.0 x 106 vs 268.0 ± 30.6 x 106 sperm/mL respectively, $P \leq 0.05$). Moreover, the peak wet showed 16-fold more DNA damage in spermatozoa than early dry and nearly 9-fold more than the late dry season (16.1 ± 4.8 vs 1.0 ± 0.2 vs 1.9 ± 0.5% respectively, $P \leq 0.05$). However, motility of spermatozoa in the peak wet did not differ to early or late dry seasons (total motility: 71.3 ± 8.1 vs 90.2 ± 4.2 vs 70.8 ± 5.5% respectively, $P > 0.05$; progressive motility: 35.4 ± 7.0 vs 46.6 ± 4.0 vs 41.7 ± 2.8% respectively, $P \geq 0.05$). Furthermore, no difference was found across several other motility parameters as determined by CASA. These results demonstrate that tropical summer (characterised by the peak wet season) induces DNA damage and reduces concentration, without depressing motility in boar spermatozoa; suggesting that traditional evaluation of sperm motility may not detect inherently compromised spermatozoa. Moreover, given the detrimental link between sperm DNA damage and embryo loss, boar management strategies need to be developed to mitigate this problem. Such strategies may include better housing conditions with efficient cooling systems, use of more heat-tolerant pig breeds, or nutritional supplements that could bolster an animal's resilience to heat stress.

Keywords: pig, heat stress, sperm DNA damage, tropics, pork

2.2 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 (Board, 2011). 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 is caused by a number of factors including: ambient temperatures greater than the animal's thermal comfort zone (i.e. 18-20º C) (Stone, 1982; Prunier *et al.*, 1997), humidity, photoperiod, genetic background, and management practices (Love, 1981; Hennessy and Williamson, 1984; Sonderman and Luebbe, 2008; Auvigne *et al.*, 2010), causing significant reduction in 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 it's 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 *et al.*, 2010 and Vega *et al.*, 2010).

The boar is particularly vulnerable to the effects of heat stress due to its inefficient capacity to sweat; its non-pendulous scrotum; and the high susceptibility of boar spermatozoa to temperature shock (Ingram, 1965; Mount, 1968; Einarsson *et al.*, 2008; Ford and Wise, 2011). Stone (Stone, 1982) demonstrated that spermatogenesis in boars is impaired when ambient temperatures rise above 29º 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), 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.*, 2016). 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). In pigs, Didion *et al.* (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.* (Petrocelli *et al.*, 2015) reported that neither season, photoperiod or 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.

2.3 Materials and Methods

Boars and Location

Five 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, 2017). 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) for Townsville were obtained from the Australian Bureau of Meteorology (2011). 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 by the (National Weather Service, 2016), and interpreted using a Temperature-Humidity Index chart (Thom, 1959; Hahn *et al.*, 2009). Mean values were calculated spaning 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 and epididymal maturation (Franca and Cardoso, 1998; França *et al.*, 2005).

Seasonal Semen Collection and Processing

One ejaculate per boar was collected from the same n=5 boars 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. Boars were sexually mature (20 - 28 months old) at the time of collection and were regularly collected by the same person 2-3 times every two weeks prior to experimental sampling to maintain training to the dummy and regular turnover of epididymal sperm populations, without causing sperm depletion. To qualify for the study, each boar met minimum standards of sperm quality that included 70% motility, 65% morphologically normal spermatozoa and an ejaculate volume of at least 100 ml prior to the experiment. 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 38º C water and immediately brought to the laboratory for processing. Raw semen from each boar was diluted 1:3 with 38º 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 x 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 µl of 20 x 10^6 sperm/mL semen was loaded into each chamber of 38º C pre-warmed Leja Standard Count 4 Chamber Slides (Leja Products, Nieuw-Vennep, Netherlands) and loaded into the CASA machine where at least five random fields were examined per sample. Motility characteristics of spermatozoa were analysed as previously described (Peña Jr *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 µm/sec; medium VAP cut-off, 22.0 µm/sec; low VSL cut-off, 11.0 µm/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, 38º C.

The following characteristics were evaluated: total motility, progressive motility, average-path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN), and elongation (ELO) 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 x 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 (Takeda *et al.*, 2015). 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. These were used to accurately gate different populations of spermatozoa in the flow cytometer before experimental samples were analysed (Fig. 2-1).

One mL (5 x 10^6 sperm) of each sample was used for TUNEL labelling and centrifuged 720 x g for 5 min. Each sperm pellet was washed twice in 200 µl of Phosphate Buffered Saline (PBS) by centrifugation at 720 x g for 5 min. The final pellet was resuspended in 100 µl PBS to which 100 µl of 4% (w/v) paraformaldehyde in PBS was added to fix spermatozoa for 60 min at room temperature. Thereafter, samples were centrifuged at 720 x g for 5 min and the pellet resuspended in 200 µl PBS and stored at 4^0 C overnight.

The next day, samples were centrifuged 720 x g for 5 min and pellets resuspended in 100 µL of 0.5% Triton X-100 in 0.1% sodium citrate permeabilisation solution then incubated for 30 min at 37º C. Samples were washed twice and resuspended in 200 µL PBS except for positive controls (P1 and P2) which were resuspended in 100 µl of 1000 U/mL DNase 1 in Roche Buffer 2 and incubated for 30 min at 37° C to induce doubled-stranded DNA breaks. 1000 U/mL DNase 1 in Roche Buffer 2 is comprised of 20 µl of 10 U/µl Roche DNase 1 stock solution (500 µL 40mM Tris-HCl 2mM $MgCl_2$ solution plus 10000 U lyophilized DNase 1 and 500 µL glycerol) plus 180 µL of Roche Buffer 2 (0.058 g NaCl, 0.099 g $MnCl_24H_2O$, 0.0011 g $CaCl_2$ 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.

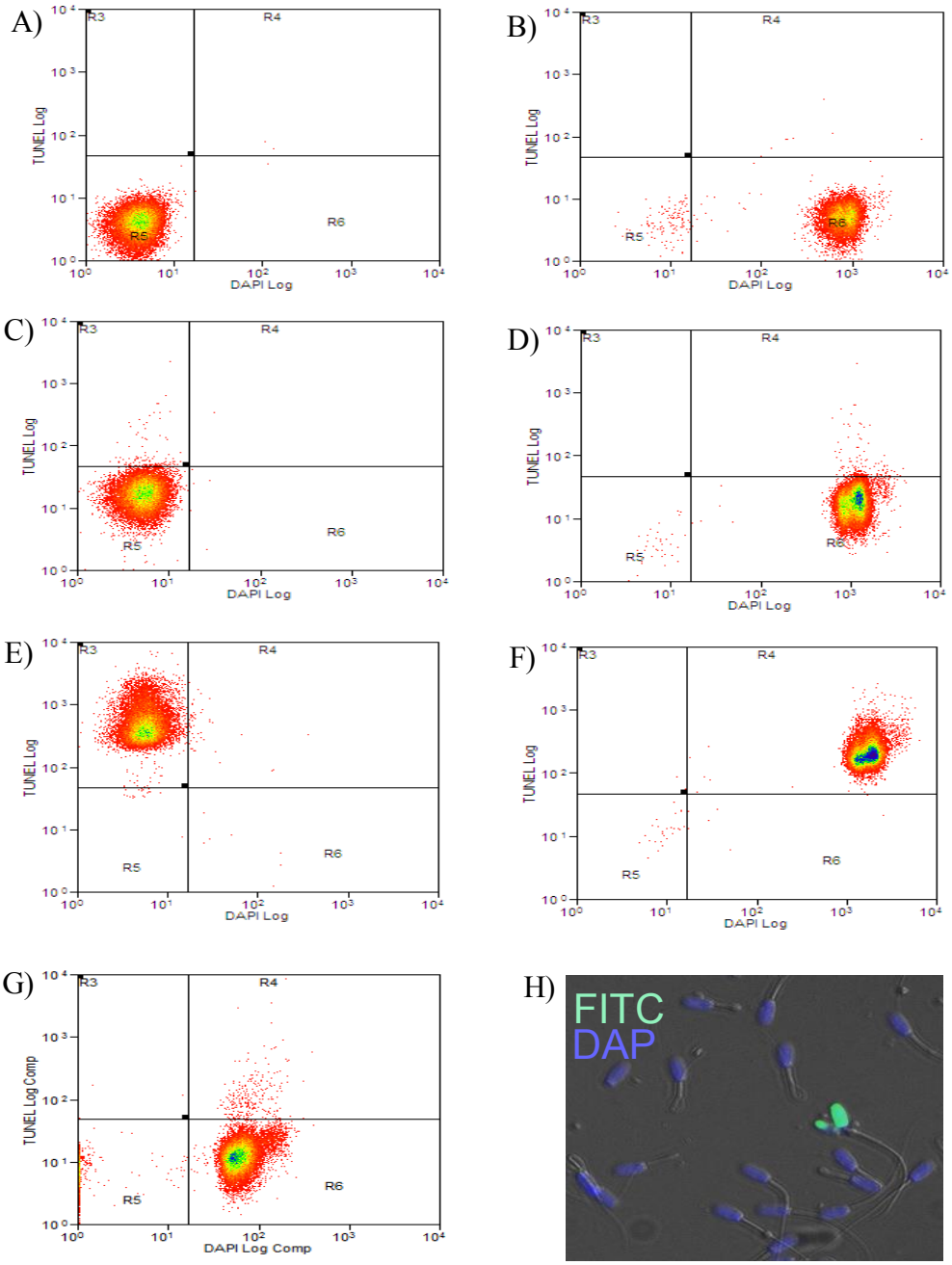

Figure 2-1: Calibration of flow cytometer for boar spermatozoa subjected to different staining treatments for FITC (TUNEL) and DAPI.

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).

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 37º 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 (Fig 2-1H).

Flow Cytometry Analysis

All samples were washed twice and resuspended in 2 mM EDTA in PBS and evaluated using a CyanADP flow cytometer (Dako Cytomation, Glostrup, Denmark). Samples were first passed through a 60 µm 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. 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 (Figs. 2-1A-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 (Fig. 2-1G).

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 was performed to normalise the data before an ANOVA or either the Greenhouse-Geisser or Huynd-Feldt correction test were carried out to look for differences between groups. Data were analysed by single-group or one-way repeated measures ANOVA using IBM SPSS Statistics version 22 (IBM Corporation, NY, USA), along with pairwise comparisons based on marginal means with Bonferroni adjustments applied. Graphs were plotted using Microsoft Excel 2016. $P \leq 0.05$ was considered statistically significant.

2.4 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 2-1. 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 2-1). 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 2-1). 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 2-1).

Semen collected in the peak wet season had significantly lower sperm concentration than early dry but did not differ to the late dry season ($P \leq 0.05$; Fig. 2-2A). 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 ($P \leq 0.05$; Fig. 2-2B). Both total and progressive motility of

spermatozoa collected in the peak wet season did not differ to that in early or late dry seasons ($P > 0.05$; Figs. 2-2C and 2-2D, respectively).

Table 2-1: Mean (± SEM) ambient temperature, relative humidity and temperature-humidity index 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 (°C)			
Daily Mean	24.2 ± 0.4^{b}	23.0 ± 0.2^{b}	29.2 ± 0.2^{a}
Mean Minimum	18.4 ± 0.5^{b}	17.7 ± 0.3^{b}	24.8 ± 0.3^{a}
Mean Maximum	**29.6 ± 0.2^{b}**	**28.2 ± 0.1^{c}**	**33.4 ± 0.2^{a}**
Relative Humidity (%)			
Daily Mean	61.9 ± 2.1^{c}	67.6 ± 0.7^{b}	71.4 ± 1.2^{a}
Mean 6 am	75.7 ± 3.3^{ab}	70.8 ± 1.4^{bc}	82.5 ± 1.2^{a}
Mean 3 pm	**45.7 ± 2.0^{b}**	**60.7 ± 2.4^{a}**	**59.2 ± 2.0^{a}**
Temperature-Humidity Index (THI)			
Daily Mean	75.9 ± 0.9^{b}	73.6 ± 0.4^{b}	92.9 ± 1.1^{a}
Mean Minimum (6 am)	64.9 ± 1.0^{b}	63.3 ± 0.7^{b}	79.3 ± 1.0^{a}
Mean Maximum (3 pm)	**86.8 ± 0.8^{b}**	**86.5 ± 0.6^{b}**	**106.3 ± 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.

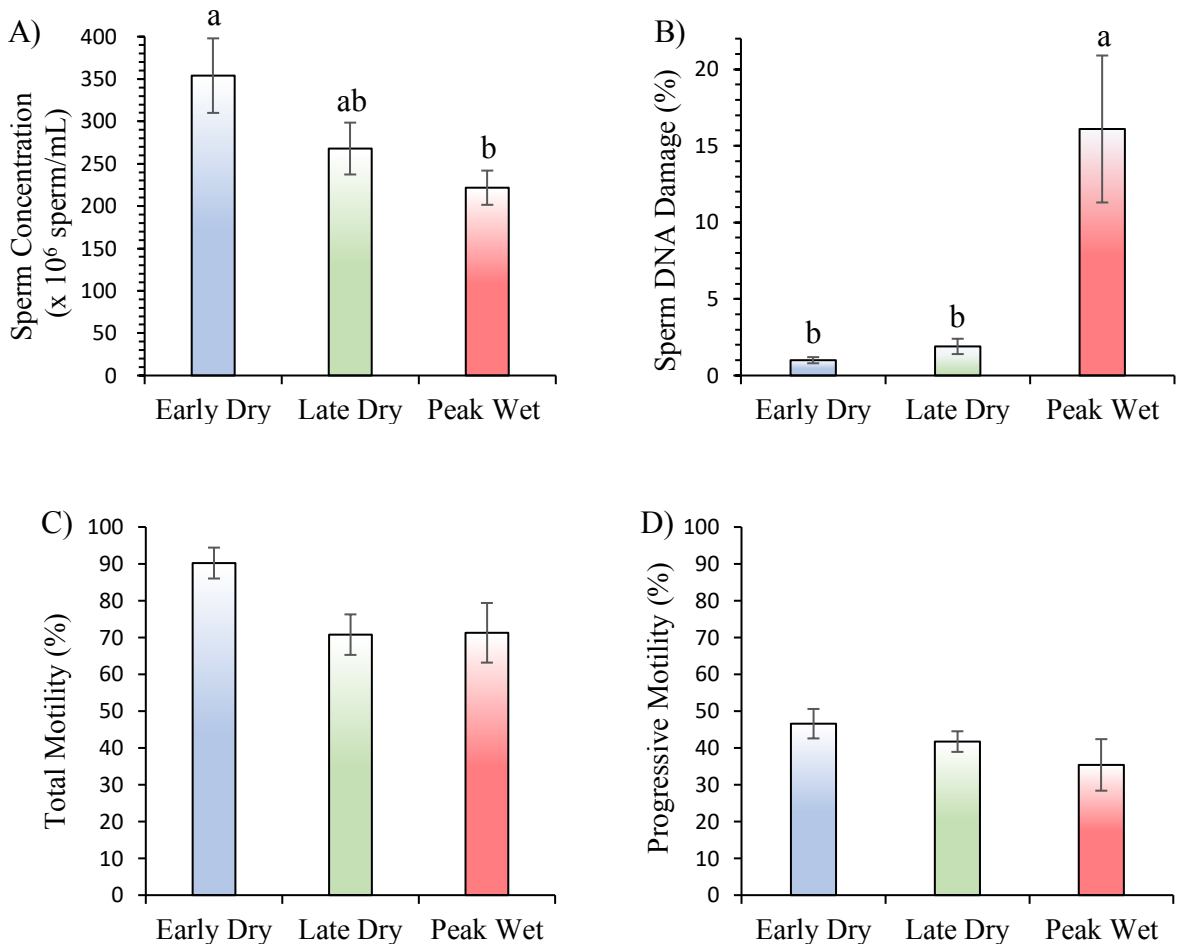

Figure 2-2: Mean (±SEM) concentration (A), percentage of DNA damage (B), percentage of total motility (C), and percentage of progressive motility (D) of boar spermatozoa collected during the early dry, late dry and peak wet seasons.

Different letters indicate a significant difference between seasons ($P \leq 0.05$).

Detailed sperm motility and head shape characteristics determined by CASA are shown in Table 2-2. 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, these were not statistically different from values obtained during the peak wet or late dry seasons ($P > 0.05$; Table 2-2).

Table 2-2: Mean (± 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 ± 7.0	54.2 ± 5.7	46.0 ± 4.0
VSL	30.7 ± 3.3	26.9 ± 1.7	22.1 ± 2.4
VAP	38.8 ± 4.5	32.1 ± 2.7	26.7 ± 2.7
ALH	3.4 ± 0.3	2.6 ± 0.3	2.3 ± 0.2
BCF	19.1 ± 1.5	21.2 ± 0.9	21.1 ± 0.6
STR	74.1 ± 1.3	80.2 ± 2.3	76.9 ± 2.2
LIN	44.8 ± 1.2	50.7 ± 2.7	47.3 ± 2.1
ELONG	78.3 ± 1.4	79.3 ± 2.2	80.3 ± 1.2

No significant difference between seasons for all parameters ($P > 0.05$). Numbers in parentheses indicate sample size. VCL, curvilinear velocity (μm/sec); VSL, straight-line velocity (μm/sec); VAP, average-path velocity (μm/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).

2.5 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 evaluation of sperm motility may not detect inherently compromised

spermatozoa, which has important implications for the management of boars during periods of seasonal infertility.

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). 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; Ahmadi and Ng, 1999; Henkel *et al.*, 2004; Paul *et al.*, 2008). 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 this species (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 may currently go undetected by the pig industry. Moreover, they could significantly contribute to the high rates of embryo loss and pregnancy failure observed in sows during summer infertility.

Didion *et al.* (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.* (Brahem *et al.*, 2011) in men with history of recurrent pregnancy loss, but with the fertile group showing much lower damage (approximately 10%). 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). Given the level of DNA damage observed in boar spermatozoa by TUNEL 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.* (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.

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). 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 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). 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.*, 2016).

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 (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 - between *caution* and *extreme caution* zones of the NSW Heat Index chart 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 a reduction in semen volume, 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; Broekhuijse *et al.*, 2012a). 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 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 can cause a significant decrease in sperm motility (Mcnitt and First, 1970; Wettemann *et al.*, 1979; Heitman *et al.*, 1984). 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 measures of sperm motility in these early studies, compared to more precise quantitative measures using CASA in our study. On this basis, we postulate that even objective measures of sperm motility as determined by CASA, may not detect DNA-compromised spermatozoa. 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).

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.

2.6 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.

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CHAPTER 3

BOAR SPERMATOZOA MAINTAINS DNA INTEGRITY AFTER CRYOPRESERVATION USING DIFFERENT CONCENTRATIONS OF GLYCEROL

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3.1 Abstract

Despite its limited application in commercial pig production, cryopreservation of boar spermatozoa can provide enormous benefits to facilitate faster distribution of desirable genes, to control disease transmission and to facilitate gene banking. Nevertheless, its broad use is currently hindered by a reduction in viability and fertilising capacity of post-thawed spermatozoa. Moreover, the freeze-thaw process has been observed to increase sperm DNA damage, which could subsequently contribute to early embryo loss. The aim of this study was to determine the cryoprotective effect of different concentrations of glycerol on DNA integrity and motility of frozen-thawed boar spermatozoa. TUNEL and flow cytometry of 20,000 spermatozoa/boar/treatment revealed no significant difference in the percentage of sperm DNA damage between fresh or frozen-thawed sperm cryopreserved at 3%, 6% and 8% glycerol (1.9 ± 0.4 *vs.* 3.5 ± 0.8 *vs.* 2.8 ± 0.5 *vs.* 3.0 ± 0.8% respectively; $P > 0.05$) Computer assisted sperm analysis of 20 x 10^6 sperm/ml at 38 °C demonstrated that both total and progressively motile spermatozoa were higher in fresh than frozen-thawed samples (total motility: 72.1 ± 2.4 fresh *vs.* 35.27 ± 4.1 3% *vs.* 26.8 ± 2.5 6% *vs.* 28.6 ± 3.0 8% glycerol; progressive motility: 39.5 ± 2.2 fresh *vs.* 23.8 ± 3.2 3% *vs.* 19.5 ± 2.7 6% *vs.* 18.1 ± 2.2 8% glycerol, both $P \leq 0.05$, respectively). Other CASA motion parameters such as straight-line velocity, average path velocity, straightness and linearity were generally better in frozen than fresh samples ($P \leq 0.05$). Our study confirms that boar semen can be safely cryopreserved using glycerol without affecting sperm DNA integrity using standard protocols for boar sperm freezing. Moreover, we suggest that a glycerol concentration of 6% is sufficient to maximize DNA protection, while being low enough to prevent major declines in sperm motility due to cell toxicity.

3.2 Introduction

Despite advantages such as faster distribution of desirable genes, control of disease transmission and gene banking, the use of frozen-thawed semen still lags behind traditional chilled boar semen in artificial insemination (AI) operations (Johnson *et al.*, 2000; Bailey *et al.*, 2008). This is mainly due to the complex processing and expensive equipment required for freezing, along with significantly reduced survival and fertility of frozen-thawed spermatozoa. Damage to the structural integrity and function of the sperm (including sperm membranes, mitochondrial architecture, motility and possibly DNA integrity) during freezing and thawing procedures can lead to greatly reduced fertilisation success (Watson, 1995; Thurston *et al.*, 2001; Roca *et al.*, 2006). Moreover, up to 70% of the variability in pig sperm cryosurvival is due to individual boar effects (Holt, 2000; Roca *et al.*, 2006).

While standard protocols for boar sperm cryopreservation have been used extensively (Pursel and Johnson, 1975; Aricultural Research Service - US Department of Agriculture, 2007), cellular stress occurs when the temperature drops below 0 ^{o}C. The damage is mainly due to osmotic dehydration of the cell and crystallisation of the extracellular components (Watson, 1995). In order to overcome this, freezing media typically includes cryoprotective agents (Fuller, 2004). Glycerol (1,2,3-propanetriol or glycerine), which is the main by-product upon transesterification of vegetable oils or animals fats, is an organic molecule abundant in nature as a structural component of many lipids (Pagliaro *et al.*, 2007; Da Silva *et al.*, 2009). Glycerol has been commonly used as a cryoprotectant at different concentrations ranging from 2-4% in many different freezing protocols across species, to improve the motility and plasma membrane integrity of sperm after thawing (Almlid and Johnson, 1988; Fiser and Fairfull, 1990; Das *et al.*, 2016). When spermatozoa are mixed with hypertonic medium

containing glycerol, water leaves the cells and is replaced by glycerol. This mechanism protects the cells from ice crystal formation during freezing (Fowler and Toner, 2005). Moreover, glycerol has proven to be superior to other cryoprotectants in preserving boar spermatozoa (Watson, 1995; Kim *et al.*, 2011), but boar spermatozoa appears to be more sensitive to the standard concentrations of glycerol used in sperm cryopreservation protocols of other domestic species (Almlid and Johnson, 1988). This high sensitivity has been attributed to boar sperm's hypersensitivity to cold shock including the high levels of unsaturated phospholipids and low levels of cholesterol on the boar sperm plasma membrane; thus increasing the likelihood of oxidative damage (Rath *et al.*, 2009).

Despite the critical role glycerol plays in sperm cryosurvival, high concentrations can be toxic to cells (Buhr *et al.*, 2001; Macias Garcia *et al.*, 2012), affecting sperm motility and acrosomal integrity. The freezing process itself may also cause damage to sperm DNA, which could potentially compromise early embryo survival. Sperm DNA fragmentation has been observed in the boar (Hamamah *et al.*, 1990), human (De Paula *et al.*, 2006), ram (Peris *et al.*, 2004), and mouse (Yildiz *et al.*, 2007) as a result of cryopreservation. Boar and human spermatozoa, had significantly reduced Feulgen-DNA content and sperm nuclear surface area as a result of freeze-thawing; leading to a state of 'overcondensation' (Royere *et al.*, 1988; Hamamah *et al.*, 1990), which may explain the reduced fertilising potential of frozen spermatozoa (Royere *et al.*, 1991).

Currently, there is still limited information about the optimal concentration of glycerol to protect DNA integrity during cryopreservation of boar spermatozoa. It has been reported that 2-4% glycerol results in better sperm motility and acrosome integrity, while 8% reduction reduces motility and normal acrosome morphology (Almlid and Johnson, 1988; Buhr *et al.*,

2001; Kim *et al.*, 2011). Moreover, 3% glycerol (in either lactose-hen egg yolk or extender with lactose, lyophilized lipoprotein fractions isolated from ostrich egg yolk) significantly reduced DNA damage (determined by comet assay) in frozen-thawed boar spermatozoa than those cryopreserved without glycerol (Fraser and Strzeżek, 2007). However, terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) has been reported to provide more robust predictive results of DNA damage than neutral comet or other similar assays (Ribas-Maynou *et al.*, 2013; Cui *et al.*, 2015). As such, based on the limited information above, our study aimed to investigate which glycerol concentration between 3, 6 and 8% would provide the best DNA protection (determined by TUNEL assay), without inducing toxic effects on function as measured by sperm motility.

3.3 Materials and Methods

Boars and Location

Six 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.

Semen Collection and Processing

The procedures used for semen collection and processing were according to Pena *et al*. (2018). Briefly, sexually mature boars were collected using the gloved hand technique (Hancock and Hovell, 1959) by the same person at regular intervals prior to experimental sampling. This was necessary to maintain regular turnover of mature epididymal sperm populations while maintaining the boars training to the dummy mount (Minitube, USA). For inclusion in the study, boars were selected that produced semen with minimum standards: having spermatozoa of at least 70% motility, 65% normal morphology and an ejaculate volume of at least 100 ml. Semen samples from the time of collection to dilution in 1:3 Beltsville Thawing Solution (BTS; pH 7.2; Pursel and Johnson, 1975) were maintained at 38 ºC in a water bath during processing. Concentration of sperm was determined using a Neubauer haemocytometer following standard protocols (WHO, 2010).

Freezing and Thawing of Semen Samples

Semen was frozen following procedures adapted from Pursel and Johnson (1975), Agricultural Research Service - US Department of Agriculture (2007) and Purdy (2008). Initially, each 1:3 BTS-diluted boar ejaculate was split into 3 cryopreservation treatments and equilibrated at room temperature for 1 h before storage at 15 °C for not more than 5 h until needed. Thereafter, samples were centrifuged and resuspended in 8 mL BF5 cooling extender (CE; 52 mM TES, 16.5 mM Tris(hydroxymethyl)aminomethane, 178 mM glucose, 20% egg yolk; ~300 × 10^6 sperm/mL), and cooled to 5 °C over 2.5 h. Samples were then further diluted drop-wise with 4 mL BF5 freezing extender (FE; containing 2.5% Equex Paste - Minitube, Tiefenbach, Germany, and either 3, 6 or 8% glycerol in BF5 CE). Sperm samples of ~200 × 10^6 sperm/mL were then manually loaded into 0.5 mL CBS straws (IMV Corporation, Minneapolis, MN, USA) using a modified sterile syringe (Braun) with a pipette tip attached into it. Straws were then frozen in liquid nitrogen vapour using an IceCube

programmable freezer (Minitube, Tiefenbach, Germany) at the following freeze rate: -20 °C/min from 5 to -8 °C; -69 °C/min from -8 to -120 °C; -20 °C/min from -120 to -140 °C. Straws were grouped in goblets according to treatment then stored in liquid nitrogen for approximately three months. Prior to downstream analysis of sperm motility and DNA integrity, samples were thawed by submerging two 0.5 mL semen straws in a 38 °C water bath while gently agitating for 30 s (Buranaamnuay *et al.*, 2011) and gently mixed into 9 ml of BTS. Thereafter, the straws were maintained at 38 °C and processed immediately for analysis.

Determination of motility characteristics by CASA

About 3 µl of 20 x 10^6 sperm/mL of fresh or frozen-thawed semen in BTS was loaded into each chamber of 38 ºC pre-warmed Leja Standard Count 4 Chamber Slides (Leja Products, Nieuw-Vennep, Netherlands) and loaded into a computer-assisted sperm analyser (CASA; IVOS version 10, Hamilton Thorne Research. Beverly, MA, USA). At least five random fields of approximately 40 spermatozoa each were examined per sample. Motility characteristics of spermatozoa were analysed as previously described by Peña *et al.* (2015).

Sperm DNA Integrity Assay and Flow Cytometric Analysis

The procedures used for sperm DNA evaluation were according to Pena *et al*. (2018) 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. The TUNEL reaction labels DNA damaged cells positive for Fluorescein isothiocyanate (FITC). Multiple control and reaction treatments were prepared including: Unlabelled controls (U1 and U2) resuspended in 50 µL PBS; Negative controls (N1 and N2) resuspended in 50 µL TUNEL

labelling solution without the enzyme; and Positive controls (P1 and P2) and test samples resuspended in 50 µL TUNEL reaction mixture containing enzyme. In addition, U2, N2, P2 and all test samples were counter-stained with 5 µg/mL of the nucleic acid stain 4', 6-diamidino-2-phenylindole (DAPI) to ensure that only nucleated TUNEL-positive spermatozoa were accounted for as DNA damaged cells during analysis by FACS. Fluorescent microscopy of stained boar sperm was conducted to confirmed specificity of the staining technique showing FITC/DAPI positive DNA damaged sperm in green alongside DAPI positive intact nucleated boar sperm in blue (Peña *et al.*, 2017).

Twenty-thousand cells per sample at about 150 events/second were evaluated using a CyanADP flow cytometer (Dako Cytomation, Glostrup, Denmark) after filtering samples through to a 60 µm nylon woven net filter into 5 mL round-bottom polystyrene tubes. Before the treatment samples were analysed, 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. Sample N2 (Negative control in Label Solution with DAPI) was used to set a 0.5% threshold cut-off before running all test samples while cells in R4 were considered as nucleated DNA damaged spermatozoa, expressed as a percentage of the total number of cells analysed within the gated area.

Data Presentation and Statistical Analyses

Data were analysed using IBM SPSS Statistics version 22 (IBM Corporation, NY, USA). Graphs were plotted using Microsoft Excel 2016. The Shapiro-Wilk test was used to evaluate normality of the data while Levene's test was used to determine if variation between groups

was homogeneous. Data were Log_{10} transformed when the distribution was not found to be normal and the variance was heterogeneous. Statistical comparisons were carried out using one-way ANOVA followed by a post-hoc Tukey's HSD test to determine significant differences in sperm DNA damage. A Kruskal Wallis test was used to determine differences in sperm quality (total motility, progressive motility, and motion parameters determined by CASA). If the Kruskal Wallis test showed one or more means differed then this was followed by the post hoc Mann-Whitney U test to determine which means differed significantly. The level of significant difference was set at $P \leq 0.05$.

3.4 Results

While DNA damage appeared to be slightly lower in fresh spermatozoa, there was no significant difference between fresh or frozen-thawed spermatozoa at each concentration of glycerol (Fig. 3-1).

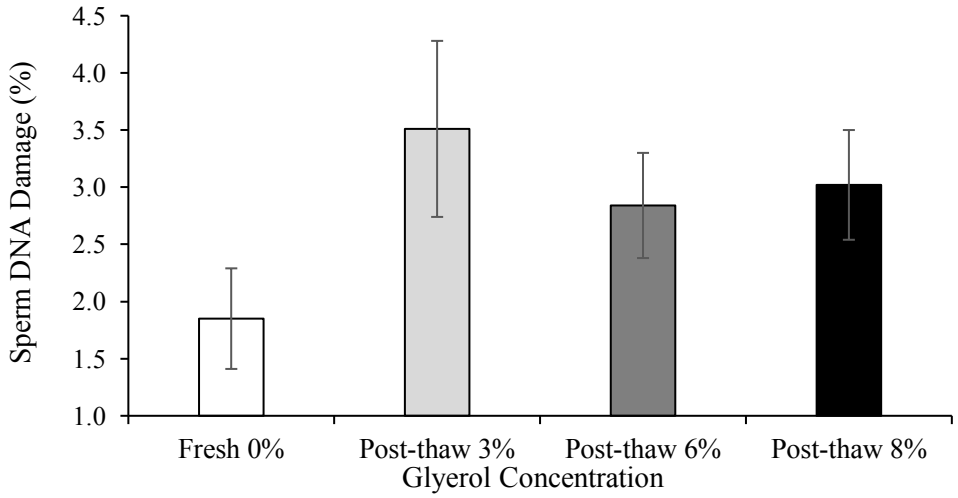

Figure 3-1: Mean (± SEM) percentage of DNA damage in fresh and frozen-thawed boar spermatozoa cryopreserved using different concentrations of glycerol.

No significant difference observed ($P > 0.05$); n=6 boars.

Both total and progressively motile spermatozoa were higher in fresh than frozen-thawed spermatozoa ($P \leq 0.05$). There was no difference observed in either motility parameter between frozen-thawed samples cryopreserved in either 3, 6 or 8% glycerol ($P > 0.05$; Figs. 3-2a and 3-2b).

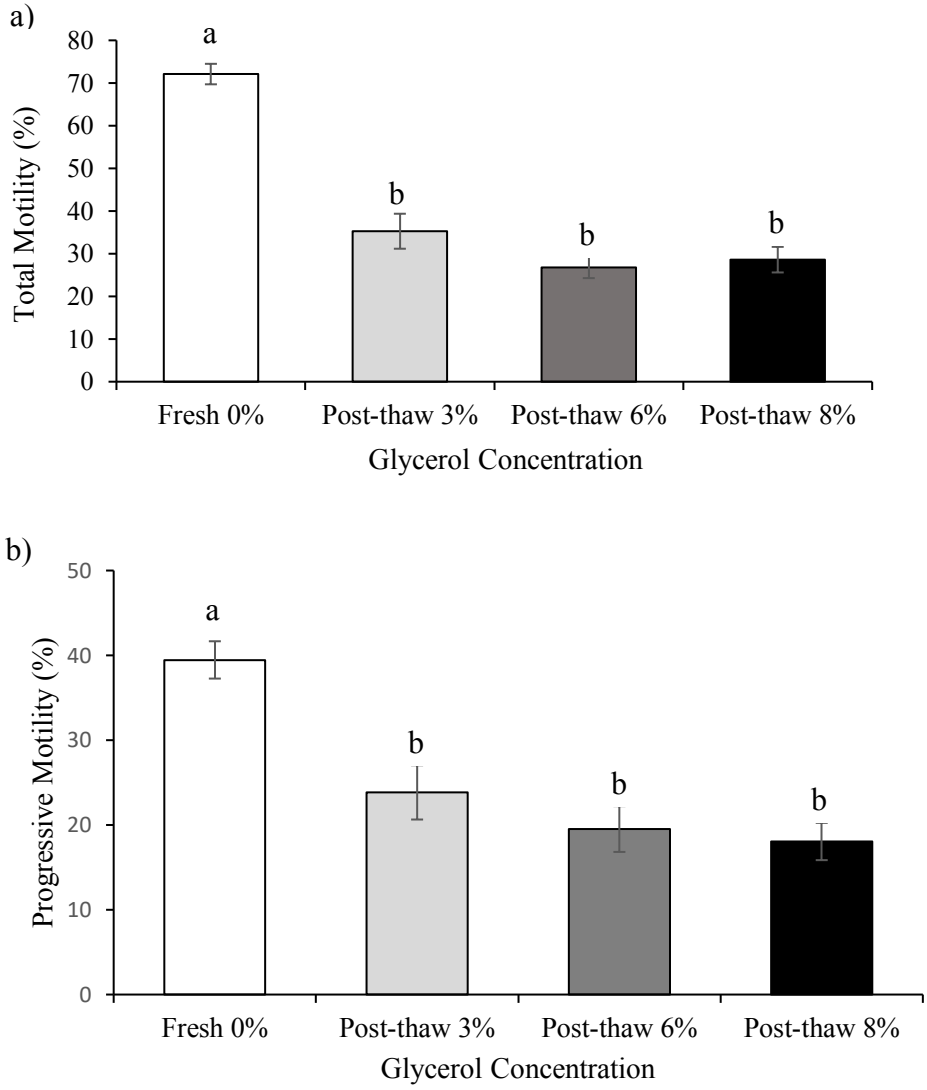

Figure 3-2: Mean (± SEM) percentage of total (a) and progressive (b) motility in fresh and frozen-thawed boar spermatozoa cryopreserved using different concentrations of glycerol.

Values not sharing the same letter are significantly different ($P \leq 0.05$); n=6 boars.

Sperm motility and head shape characteristics determined by CASA are shown in Table 3-1. There was no difference observed in curvilinear velocity, lateral head displacement, beat cross frequency, and elongation between fresh and cryopreserved samples ($P > 0.05$). By contrast, straight-line velocity, average path velocity, straightness and linearity were significantly higher after cryopreservation using 3 and 6% glycerol (as well as 8% glycerol for straight-line and average path velocities) compared to fresh samples ($P \leq 0.05$), but these parameters did not differ significantly between glycerol treatments ($P > 0.05$; Table 3-1).

Table 3-1: Mean (± SEM) sperm motility and head shape characteristics between fresh and frozen-thawed boar spermatozoa cryopreserved using different concentrations of glycerol.

CASA Parameter	Fresh (n=6)	Post-thaw		
		3% Glycerol (n=6)	6% Glycerol (n=6)	8% Glycerol (n=6)
VCL	51.7 ± 5.2	55.6 ± 3.0	63.7 ± 5.0	65.9 ± 6.5
VSL	25.3 ± 2.1^{b}	33.0 ± 2.3^{a}	35.8 ± 2.7^{a}	35.6 ± 3.2^{a}
VAP	30.2 ± 2.9^{b}	37.7 ± 2.3^{a}	41.8 ± 3.0^{a}	40.9 ± 3.5^{a}
ALH	2.6 ± 0.2	2.5 ± 0.2	2.7 ± 0.2	2.7 ± 0.2
BCF	21.4 ± 0.9	18.1 ± 1.8	16.0 ± 2.0	16.6 ± 1.9
STR	79.9 ± 1.9^{b}	83.4 ± 2.3^{a}	83.9 ± 2.6^{a}	83.9 ± 2.2^{ab}
LIN	49.6 ± 2.5^{b}	58.6 ± 3.1^{a}	57.8 ± 2.7^{a}	54.7 ± 2.5^{ab}
ELONG	79.0 ± 1.8	79.2 ± 3.3	81.0 ± 3.9	82.9 ± 3.7

Values with different letters differ significantly between treatments for each parameter ($P \leq 0.05$); Numbers in parentheses indicate sample size. VCL, curvilinear velocity (μm/sec); VSL, straight-line velocity (μm/sec); VAP, average-path velocity (μm/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)

3.5 Discussion

Cryopreservation using boar semen is not new, however our study demonstrates that despite the reduction in total and progressive motility, boar spermatozoa can be safely frozen in 3-8% glycerol-containing medium without significantly damaging its DNA integrity or other motility parameters. It is yet to be determined whether such frozen-thawed sperm is of sufficient quality for artificial insemination, but at the very least is suitable for *in vitro* fertilization, without the induction of additional DNA damage caused by the cryopreservation process itself. It is important to select a glycerol concentration that is sufficiently adequate to protect DNA integrity while being low enough to prevent cell toxicity. As such, our study suggests a glycerol concentration of 6% is sufficient to achieve both outcomes.

Sperm DNA integrity can be compromised during freezing (Yildiz *et al.*, 2007) and boar sperm appears to be highly susceptible to cryoinjury mainly due to its hypersensitivity to cold shock along with the elevated levels of unsaturated phospholipids and low levels of cholesterol on the plasma membrane; predisposing sperm to oxidative damage (Rath *et al.*, 2009). The freeze-thaw process can cause reduction of glutathione (GSH) content (Gadea *et al.*, 2004), tyrosine phosphorylation associated with capacitation (Kumaresan *et al.*, 2012), calcium imbalance, and acrosome damage among others (reviewed by Yeste, 2015). Damage to sperm DNA due to freezing could potentially affect fertilisation and/or blastocysts formation rates *in vitro* (Royere *et al.*, 1988; Hamamah *et al.*, 1990; Royere *et al.*, 1991; Watson, 1995; Johnson *et al.*, 2000), cause early embryonic loss, interrupted embryo development, genetic abnormalities in offspring, lower pregnancy rates (Ahmadi and Ng, 1999; Henkel *et al.*, 2004; Paul *et al.*, 2008), and ultimately negatively impact breeding efficiency (Evenson, 1999). In fact, boar sperm with greater than 6% DNA fragmentation was found to cause both decreased farrowing rates and average number of piglets born (Didion *et*

al., 2009); with up to 0.5 to 0.9 fewer piglets born per litter when sperm DNA fragmentation was above 2.1% (Boe-Hansen *et al.*, 2008). Moreover, freeze-thawing may lead to overcondensation of sperm chromatin which can lead to poor conception rates in cryopreserved boar and human sperm (Hamamah *et al.*, 1990). Hence, it is important to maintain sperm DNA integrity during sperm cryopreservation. In addition, evaluating sperm DNA integrity before and after freezing could provide valuable information about individual boar susceptibility to the freeze-thaw process (Holt *et al.*, 2005). Our study found no difference in the proportion of DNA damage between fresh and frozen spermatozoa at 3, 6 and 8% glycerol concentration, respectively. Our study appears to support earlier results (Fraser and Strzeżek, 2007) as measured by neutral comet assay, using 3% glycerol in either a lactose-hen egg yolk (lactose-HEY-G) extender or a lactose-lyophilized lipoprotein fractions extracted from ostrich egg yolk (lactose-LPFo-G) extender. Although not significant, in our study 6% glycerol appeared to provide the lowest rate of sperm DNA damage across the n=6 boars tested; a concentration consistent with that recommended in standard freezing protocols by the Agricultural Research Service - US Department of Agriculture (2007) and several others (Purdy, 2008; Rath *et al.*, 2009). Nonetheless, our study re-affirms the protective effect of glycerol in maintaining sperm DNA integrity during freezing.

The motility of spermatozoa before and after freezing in different concentrations of glycerol was also evaluated using CASA. Sperm motility is an important parameter to detect semen of poor fertility potential both in farm (Holt *et al.*, 1997; Vyt *et al.*, 2008) and laboratory use (Tardif *et al.*, 1999). Moreover, it can also be used to determine any possible toxic effect high concentrations of glycerol has during the freeze-thaw process. Previous studies found that motility and acrosomal integrity of boar spermatozoa frozen with 0 and 8% glycerol were

significantly lower than those frozen with 2 and 4% glycerol (Almlid and Johnson, 1988; Buhr *et al.*, 2001); while 5% glycerol exhibits maximum toxicity in stallion sperm due to osmotic and non-osmotic effects (Macias Garcia *et al.*, 2012). When compared with other cryoprotectants such as dimethylacetamide (DMA) and dimethyl sulfoxide (DMSO), glycerol at 3% yielded better motility and intact plasma membrane integrity than either DMA or DMSO (Kim *et al.*, 2011). However, in another recent study glycerol was replaced with trehalose, a non-permeable cryoprotectant, resulting in much better post-thaw sperm quality (Athurupana *et al.*, 2015).

In our study, there was a significant drop to less than 35% in both total and progressively motile spermatozoa post-thaw at each glycerol concentration. While utilisation of frozen-thawed semen in pig AI is very limited and far from the quality used in cattle, this level of motility is still suitable for use in pig IVF, where average post-thaw motility of 38% has been used (Daigneault *et al.*, 2014). Our highest post-thaw total motility of 35% was achieved with 3% glycerol and lowest of 27% with 6% glycerol, suggesting these rates of motility should still be sufficient for porcine IVF. Nevertheless, post-thaw motility of cryopreserved boar spermatozoa does not appear to predict penetration rates nor IVF success (Martinez *et al.*, 1993; Suzuki *et al.*, 1996); although other studies argue that motility along with other sperm quality parameters correlate with oocyte penetration rates (Xu *et al.*, 1996; Gadea and Matas, 2000). In humans, a minimal 30% motility is sufficient for successful IVF while ICSI can facilitate fertilisation even with immotile and/or morphologically abnormal spermatozoa (Michelmann, 1995). Surprisingly, straight-line velocity, average path velocity, linearity and straightness parameters were better in our frozen-thawed than fresh boar semen samples. While this was unexpected, it is possible this may be due to the fact that processing of our frozen-thawed spermatozoa removed the seminal plasma compared to fresh samples, despite

both fresh and frozen samples being diluted in BTS medium. Prolonged storage of sperm in seminal plasma has been shown to result in reduced motility without affecting viability in stallion spermatozoa (Mata-Campuzano *et al.*, 2015). Moreover, it is known that motility of frozen-thawed boar spermatozoa can be improved by supplementing freezing media with various forms of enrichments such as Vitamin E analogue Trolox and glutathione (Pena *et al.*, 2003; Gadea *et al.*, 2005), suggesting that additives in our freeze media may have enhanced some motility parameters. We did not examine capacitation status in our frozen-thawed boar spermatozoa. However, the freeze-thaw process may also initiate physiological changes to the plasma membrane of spermatozoa leading to capacitation (Gillan *et al.*, 1997; Thundathil *et al.*, 1999), and by so-doing, trigger the beginning of hyperactivated motility.

The quality of frozen boar spermatozoa is still insufficient for extensive use in commercial pig production, despite its potential to facilitate gene banking for easy and faster distribution of desirable genes while helping control transmission of diseases (Bailey *et al.*, 2008; Knox, 2011). In this respect, our results demonstrate that semen can be successfully cryopreserved using glycerol as cryoprotectant at 3-8% in boar freezing medium, without inducing sperm DNA damage. A glycerol concentration of 6% appears to provide slightly better levels of DNA protection but has slightly lower total motility than 3 and 8% glycerol. Being able to cryopreserve boar spermatozoa without inducing additional DNA damage could be highly beneficial to *in vitro* heat stress studies, in which higher quality pig oocytes harvested in winter can be used to assess developmental competence of embryos fertilized by heat stressed sperm obtained and frozen in summer.

3.6 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 and cryopreservation; 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.

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CHAPTER 4

SUSCEPTIBILITY OF BOAR SPERMATOZOA TO HEAT STRESS USING *IN VIVO* AND *IN VITRO* EXPERIMENTAL MODELS

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4.1 Abstract

Induction of heat stress as an experimental procedure in animals is commonly used to examine heat-related impacts on sperm quality. Heat stress is widely known to negatively affect sperm motility, morphology, viability and storage among others. We recently demonstrated that ambient environmental heat stress encountered by boars during tropical summer led to increased sperm DNA damage compared to other seasons of the year. This study aimed to develop reliable 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 inside a temperature-controlled room at elevated temperatures (hot room) for 42 days, or by directly exposing boar semen to heat *in vitro* (heat shock) at various temperatures. Our *in vivo* hot room boars were exposed to temperatures and temperature humidity index that exceeded both the dry winter season control and those observed during Townsville's peak wet summer season; resulting in a significant rise in core body temperature each afternoon. This was sufficient to induce biologically meaningful levels of DNA damage in boar spermatozoa (10.1 ± 1.9 hot room *vs*. 6.7 ± 1.7% control; $P > 0.05$), but this was not statistically significant from controls largely due to individual boar variability. Similar results were observed with the *in vitro* heat shock model, but we were only able to mimic levels of sperm DNA damage (10.8 ± 4.0, 19.4 ± 7.8 and 16.9 ± 6.3%) observed during the natural tropical summer of Townsville, using extreme *in vitro* temperatures (46, 50 and 54 °C, respectively) that rendered boar spermatozoa completely immotile or dead. Neither sperm concentration nor multiple motility parameters were affected by the *in vivo* hot room compared to control. By contrast, most motility parameters declined rapidly to zero above 40 or 42 °C for the *in vitro* heat shock model. While boar spermatozoa is vulnerable to heat-induced DNA damage, our results suggest that temperature combined with individual factors may contribute to a boar's overall susceptibility to heat stress. The

development of reliable heat stress models, particularly *in vitro* models, is a priority to overcome environmental variability, reduce whole animal experiments, and provide a putative diagnostic fertility screening tool to evaluate heat-tolerance in the boar.

4.2 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 worldwide (National Pork Board, 2017). Ambient temperatures beyond the animal's thermal comfort zone (i.e. 18-20º 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 1950's and 60's 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; Wettemann and Bazer, 1985). 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 *et al.*, 1976; Wettemann and Desjardins, 1979; Cameron and Blackshaw, 1980; Egbunike and Dede, 1980; Greer, 1983; Heitman *et al.*, 1984; Wettemann and Bazer, 1985; Flowers, 1997; Huang *et al.*, 2000; 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 minimise 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.*, 2017a). Recently, we have demonstrated using TUNEL that tropical summer induces 16% sperm DNA damage; which can be mitigated by antioxidant supplementation (Peña *et al.*, 2017b; Chapter 5). 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.*, 2008a; 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 structual 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 viabilty, and may partly explain reduced litter size observed in sows during seasonal infertilty (Peña *et al.*, 2017a).

Further work is needed to evaluate boar factors important 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, could accelerate progress. In previous studies, boars were exposed to a controlled hot room environment, direct sunlight or ambient temperatures ranging from 30 °C to 40 °C for between 3-90 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, the thermo-neutral zone may have increased 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.*, 2017b).

4.3 Materials and Methods

Boars and Location

Prior to the experiment, n = 6 Large White boars between 2.5 - 2.8 years of age were housed and maintained 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 2.3 - 2.5 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.

Induction of Heat Stress

Hot Room Model

All n = 6 boars were used in the 'hot room' experiment using a standard 3 x 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. 4-1).

The hot room was maintained at approximately 30 ^{0}C between 9am - 5pm, mimicking the ambient temperature experienced in Townsville during the peak wet season (Chapter 2), and a relative humidity between 55-65% (Fig. 4-1). Moreover, 12 h artificial light was automatically provided daily from 6am to 6pm. Boars inside the hot room were fed and hosed once every day before 9am. Water was provided *ad libitum* as previously described.

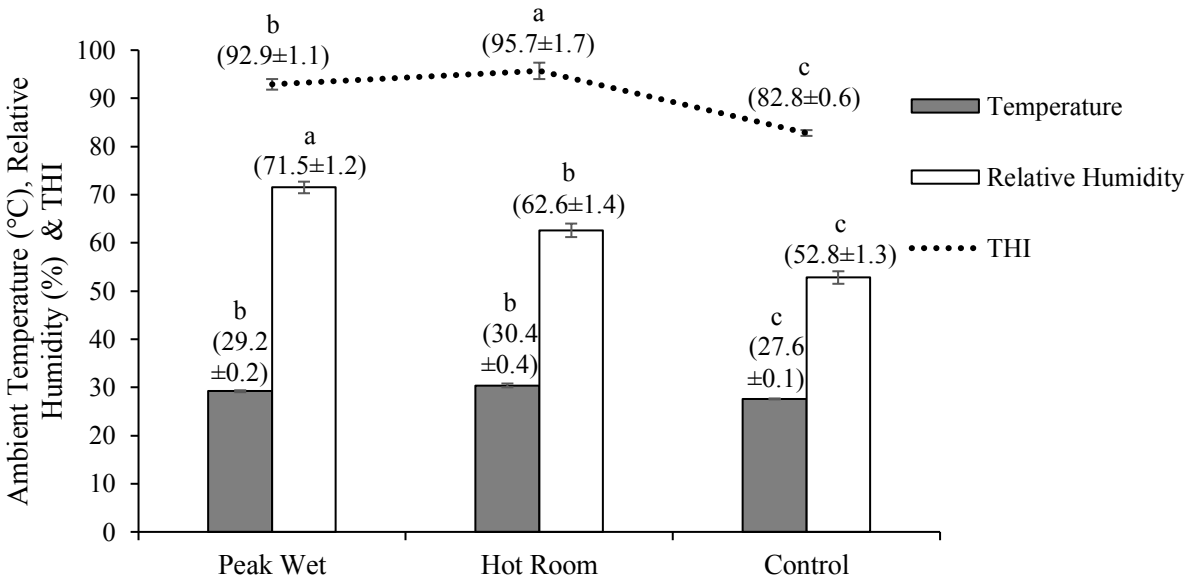

Figure 4-1: Mean (± SEM) daily ambient temperature, relative humidity and temperature-humidity index between peak wet, hot room and control treatments in Townsville, North Queensland, Australia.

Different letters indicate a significant difference between treatment groups ($P \leq 0.05$).

Moreover, rectal temperatures were collected twice daily throughout the treatment period using a digital thermometer during feeding time in the morning and at 5pm. 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 was compared to data collected during extreme ambient conditions experienced by boars in the peak wet season as described previously (Peña *et al*. 2017b).

Heat Shock Model

On a separate occasion, semen was collected once over a two-day period from each of these n = 5 boars and used in an *in vitro* heat shock experiment. Individual semen samples were aliquoted in an 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 and 54 °C) temperature treatments. Temperatures at the lower range were based upon the boar's core and peripheral temperatures (Stone, 1981), while other temperatures rose by 4 °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 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 ºC water and immediately brought to the laboratory for processing as previously described (Peña *et al.*, 2017b). Semen samples were analysed for sperm concentration using a Neubauer haemocytometer (WHO, 2010), before dilution to 20 x 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 x 10^6 sperm/mL in BTS to evaluate sperm DNA damage by TUNEL assay (Peña *et al.*, 2017b).

Sperm DNA Integrity and Flow Cytometry Analysis

Sperm DNA integrity and flow cytometry analysis was performed as described in Chapter 2. Percoll purified boar spermatozoa at a final concentration of 5 x 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 (Peña *et al.*, 2017b). 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 ranks 2-sample related test (sperm DNA damage between heat shock temperatures, ambient temperatures, THI, rectal temperatures) was used to determine if values were significantly different ($P \leq 0.05$). Graphs were plotted using Microsoft Excel 2016.

4.4 Results

Daily mean temperature, relative humidity and THI differed between peak wet, hot room and control ($P \leq 0.05$, Fig. 4-1). 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 were higher at 5pm than 9am in both the hot room and control treatments ($P \leq 0.05$; Fig. 4-2). Moreover, rectal temperatures of hot room boars were consistently higher than control boars in both the morning and afternoon ($P \leq 0.05$).

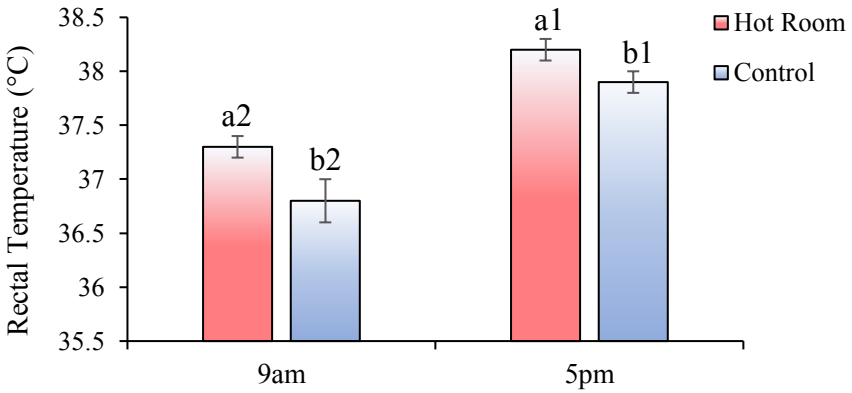

Figure 4-2: Mean (± SEM) rectal temperature at 9am and 5pm in boars subjected to *in vivo* hot room and control treatments.

Different letters indicate a significant difference between treatment groups while different numbers denote significant difference between time of day within treatment group ($P \leq 0.05$).

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 was not sufficiently different from controls ($P > 0.05$; Fig. 4-3A). By contrast, DNA damage in the peak wet was higher than control ($P \leq 0.05$). Sperm concentration did not differ between *in vivo* treatments ($P > 0.05$; Fig. 4-3B). Similarly, the percentage of both total and progressively motile sperm did not differ between *in vivo* treatments ($P > 0.05$, Fig. 4-3C and 4-4D).

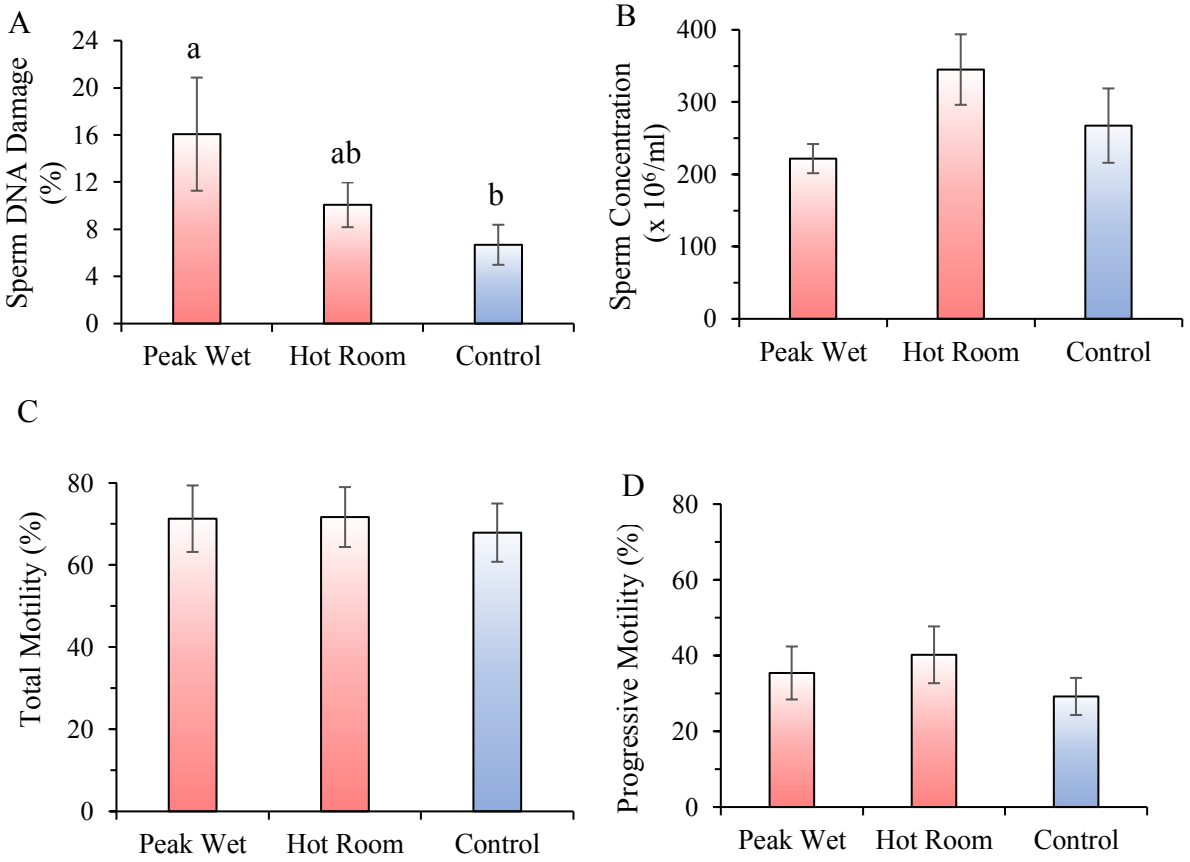

Figure 4-3: Mean (± SEM) percentage of DNA damage (A), concentration (B) and percentage of total (C) and progressive motility (D) of boar spermatozoa subjected to in vivo peak wet, hot room and control treatments.

Different letters indicate a significant difference between treatment groups ($P \leq 0.05$).

Detailed sperm motility and head shape characteristics for *in vivo* treatments determined by CASA are shown in Table 4-1. 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 curvilinear velocity (VCL) and average path velocity (VAP; $P > 0.05$).

Table 4-1: 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/sec); VSL, straight-line velocity (μm/sec); VAP, average-path velocity (μm/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).

For the *in vitro* heat shock experiment, the level of sperm DNA damage exceeded the 6% limit for normal farrowing rates (Didion *et al.*, 2009) when spermatozoa were exposed to temperatures higher than 42 °C, although no significant difference was observed between temperature treatments due to the high variability observed in pooled boar data at these elevated temperatures ($P > 0.05$; Fig. 4-4). Interestingly, spermatozoa from boars B-327 and B-303 appeared to be more temperature stable across all *in vitro* temperatures, and thus, contributed to this variability (Fig. 4-5). All CASA parameters were highest at 35.5 °C and did not differ to values at 38.8 °C except for VCL and VAP ($P > 0.05$; Table 4-2). 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.

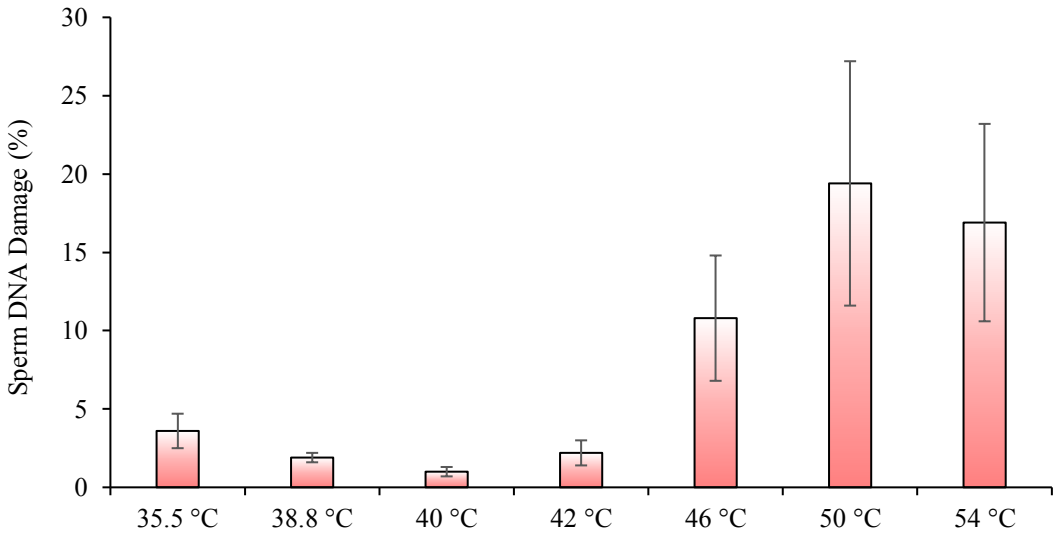

Figure 4-4: Mean (± SEM) percentage of DNA damage in boar spermatozoa subjected to different *in vitro* heat shock temperatures.

No significant difference between treatment groups ($P > 0.05$).

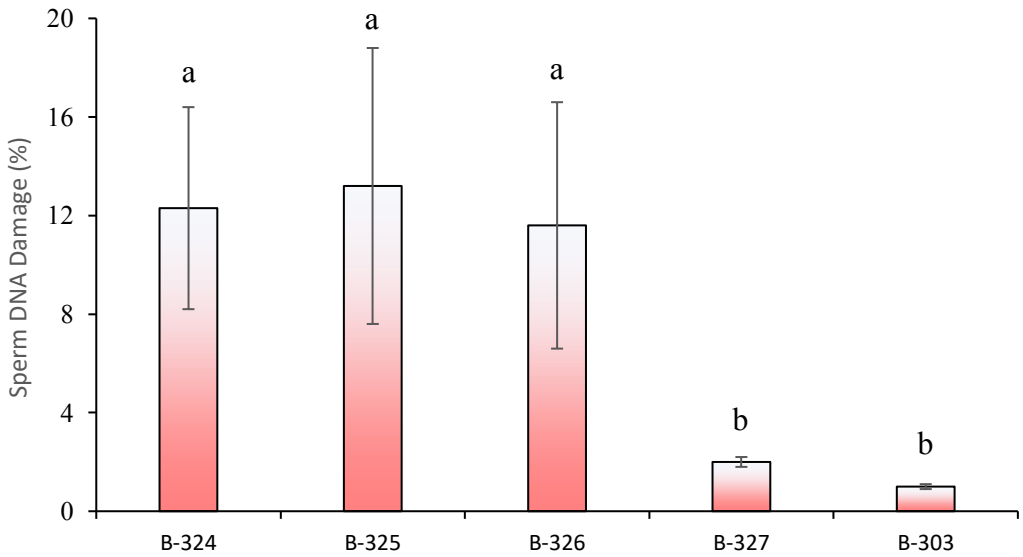

Figure 4-5: Mean (± SEM) percentage of sperm DNA damage between boars across different *in vitro* heat shock temperatures.

Different letters denote significant difference between boars ($P \leq 0.05$).

Table 4-2: Mean (± SEM) sperm motility and head shape characteristics in boar spermatozoa collected after exposure to different in vitro heat shock temperatures.

CASA Parameter	35.5 °C (n=5)	38.8 °C (n=5)	40 °C (n=5)	42 °C (n=5)	46 °C (n=5)	50 °C (n=5)	54 °C (n=5)
VCL	56.8 ± 6.2^{a}	30.2 ± 8.2^{b}	18.0 ± 5.8^{bc}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
VSL	25.6 ± 3.3^{a}	13.5 ± 4.3^{ab}	5.5 ± 2.2^{bc}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
VAP	32.8 ± 4.1^{a}	16.5 ± 5.0^{b}	7.1 ± 2.4^{bc}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
ALH	2.7 ± 0.2^{a}	1.7 ± 0.4^{ab}	1.4 ± 0.8^{bc}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
BCF	20.9 ± 1.5^{a}	17.6 ± 5.0^{ab}	10.1 ± 4.1^{ab}	2.0 ± 2.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
STR	70.2 ± 1.5^{a}	63.0 ± 6.5^{ab}	31.4 ± 11.3^{bc}	1.7 ± 1.7^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
LIN	44.7 ± 1.1^{a}	41.0 ± 3.6^{a}	15.0 ± 6.0^{b}	0.8 ± 0.8^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}
ELONG	83.5 ± 0.9^{a}	73.3 ± 9.2^{a}	41.2 ± 15.7^{ab}	4.0 ± 4.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}

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

4.5 Discussion

The development of reliable heat stress models is important to advance the study of seasonal infertility in the pig. Our study demonstrated that both *in vivo* and *in vitro* heat stress models are able to induce biologically meaningful levels of DNA damage in boar spermatozoa, but this is not significantly different from controls due to individual boar variability. Moreover, 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 (Parrish *et al.*, 2017). For example, thermal sweating appears to be functionally nil from the apocrine-like glands in the pig (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.,* 2017b). During this time, hot room boars were exposed to temperatures and temperature humidity index that exceeded those observed during Townsville's peak wet season (Fig. 4-1); 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 (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 was 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. 4-1). 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-damage 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 °C 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 possibly due to individual boar variability. Interestingly, this was caused by spermatozoa from n=2 boars which appeared to consistently show resistance to heat-induced DNA damage at these higher temperatures (Fig. 4-5). Moreover, 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 °C to 54 °C may in fact be due to immotile, plasma membrane-damaged or dead spermatozoa. 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 and epididymal maturation 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 (~7%) was also higher than previously reported for the same control group in Chapter 2 (1%-2%). This may partly be explained by the fact that semen from n=3 control boars was collected after a 42 days 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 days 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 to near 1-2%. In addition, these same boars used in Chapter 2 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 which 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 lead 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 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 show 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.

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 and antioxidant studies (Pena *et al.*, 2018a and 2018b). Collectively, these results suggest that DNA damaged boar spermatozoa are likely to swim and potentially fertilise oocytes normally (Ahmadi and Ng, 1999) (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; Wettemann *et al.*, 1979; 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 in 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 note 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, (Pena, *et al*., 2018a), in our current study we found no difference between the hot room, peak wet and control treatments. 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 days 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 two months after the heat stress treatment (Parrish *et al.*, 2017).

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, 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.

4.6 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 was funded by a JCU Development Grant to DP, and College of Public Health, Medical and Veterinary Sciences PhD Research Funds to SP. SP was also supported by the Australia Awards Scholarship.

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CHAPTER 5

ANTIOXIDANT SUPPLEMENTATION MITIGATES DNA DAMAGE IN BOAR SPERMATOZOA INDUCED BY TROPICAL SUMMER

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5.1 Abstract

Heat stress-induced sperm DNA damage has recently been demonstrated in boars during tropical summer; which could negatively impact early embryo survival and litter size in sows. This can be attributed to the boar's peculiar characteristics such as non-pendulous scrotum, inefficient capacity to sweat and low antioxidant activity in seminal plasma. Elevated endogenous levels of antioxidants are needed to combat reactive oxygen species induced during periods of heat stress, and thus, should act to prevent the build-up of pathological levels of DNA damage in boar spermatozoa. Our aim was to investigate whether a combined antioxidant supplement could mitigate sperm DNA damage in boars exposed to tropical summer conditions. TUNEL and flow cytometry of 20,000 spermatozoa/boar/treatment revealed that boar diets supplemented with 100 g/day custom-mixed antioxidant during peak wet summer effectively reduced sperm DNA damage by as much as 55% after 42 and 84 days treatment respectively (16.1 ± 4.9 peak wet control *vs.* 9.9 ± 4.5 42 day *vs.* 7.2 ± 1.6% 84 day treatments; $P \leq 0.05$). While sperm concentration was lower in the peak wet compared to early dry winter control (221.8 ± 20.2 *vs.* 354.1 ± 44.0 sperm/mL respectively; $P \leq 0.05$), supplementation did not improve sperm concentration beyond control levels for either season ($P > 0.05$). Computer assisted sperm analysis of 20 x 10^6 sperm/ml at 38 °C demonstrated that total and progressive motility were not altered by the supplement (total motility: 71.3 ± 8.1 peak wet control *vs.* 72.9 ± 8.9 42 day *vs.* 81.0 ± 3.2% 84 day treatments; progressive motility: 35.4 ± 7.0 peak wet control *vs.* 35.2 ± 5.9 42 day *vs.* 34.5 ± 1.8% 84 day treatments, both $P > 0.05$). Moreover, most other motion characteristics measured by CASA were not altered by the supplement in either season, except for sperm elongation; which was higher for all 84-day treatments over controls ($P \leq 0.05$). Antioxidant supplementation during tropical summer appears to mitigate the negative impact of heat stress on DNA integrity but not

concentration nor motility of boar spermatozoa; which may provide one solution to the problem of summer infertility in the pig.

5.2 Introduction

Tropical countries such as Brazil, Vietnam, The Philippines and Mexico are among the top 10 pork producers globally (National Pork Board, 2011). Pig production during summer in the tropics can be impacted considerably by the phenomenon of seasonal or summer infertility. Summer temperature and humidity can predispose pigs to heat stress when ambient temperatures rise beyond than the animal's thermal comfort zone (i.e. 18-20º C; Stone, 1982; Prunier *et al.*, 1997). This consequently affects food and water consumption, general comfort and reproductive performance, causing significant reduction in profitability. In pigs, poor reproductive performance due to summer infertility has been associated with reduced expression of oestrus and increased pregnancy failure in females (Paterson *et al.*, 1978; Hughes and Van Wettere, 2010), and decreased breeding efficiency in males (Wettemann *et al.*, 1976; Boma and Bilkei, 2006).

While the sow plays a central role in overall reproductive success, the inefficient capacity to sweat, non-pendulous scrotum, and the high susceptibility of spermatozoa to temperature shock (Ingram, 1965; Mount, 1968; Einarsson *et al.*, 2008; Ford and Wise, 2011), makes the boar particularly vulnerable to the effects of heat stress. Moreover, ambient temperatures above 29 ºC causes impaired spermatogenesis in Large White boars (Stone, 1982). Overall, fertility of heat stressed boars is known to be affected by multi-faceted declines in sperm concentration (Egbunike and Dede, 1980), motility and morphology (Mcnitt and First, 1970; Heitman *et al.*, 1984), testosterone production (Stone and Seamark, 1984), ejaculate volume (Egbunike and Dede, 1980) and libido (Flowers, 1997).

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 stress which can lead to sperm DNA damage during periods of heat stress (Peña *et al.*, 2017a). Studies in mice show that heat stress induces sperm DNA damage, leading to arrested embryo development and ultimately foetal loss (Paul *et al.*, 2008). Our group has recently demonstrated that tropical summer induces 16% DNA damage and reduces concentration of boar spermatozoa without depressing motility (Peña *et al.*, 2017b). Sperm with greater than 6% DNA fragmentation results in decreased farrowing rates (Didion *et al.*, 2009); and, in another study, reduced litter size when sperm DNA fragmentation was greater than 2.1% (Boe-Hansen *et al.*, 2008). Thus, heat stress-induced DNA damaged boar spermatozoa may contribute significantly to early embryo loss in sows.

Antioxidant supplementation is a common practice geared towards combating oxidative stress and optimising the overall health conditions of many animals but more so particularly in commercial animal production when the demands for growth and reproduction are high (Matte *et al.*, 1993; Miller *et al.*, 1993; Pena *et al.*, 2004; Zhu *et al.*, 2012; Sejian *et al.*, 2014). In boars specifically, several antioxidants have been identified that improve various sperm quality parameters including Vitamin C (Lin *et al.*, 1985; Audet *et al.*, 2004; Lechowski, 2009), zinc (Liao *et al.*, 1985), selenium and Vitamin E (Marin-Guzman *et al.*, 2000b; Echeverria-Alonzo *et al.*, 2009; Horky *et al.*, 2012), glutathione (Funahashi and Sano, 2005), and garlic powder (Park *et al.*, 2010) among others. Nevertheless, there appears to be no substantial reports demonstrating the benefit of antioxidant supplementation on boar sperm DNA integrity; except for one *in vitro* experimental study in which the antioxidant was

directly added to the semen extender (Szczesniak-Fabianczyk *et al.*, 2003). By contrast, another study demonstrated negligible or detrimental effects of the antioxidants selenium and α-tocopherol on sperm health (Marin-Guzman *et al.*, 2000b). In humans, oral administration of 1 g vitamin C and 1 g vitamin E daily for two months (Greco *et al.*, 2005) or a cocktail of various antioxidants for three months (Tunc *et al.*, 2009), has resulted in improved sperm DNA integrity in men with unexplained infertility and elevated levels of sperm DNA damage. By contrast, another study demonstrated decondensation of sperm DNA after antioxidant supplementation, making it vulnerable to damage, ultimately causing a negative impact on male fertility (Ménézo *et al.*, 2007).

Exogenous antioxidant supplementation has been used previously in commercial piggeries to improve overall productivity. In the boar, antioxidants have been shown to improve sperm motility, sperm membrane lipid architecture, mitochondrial membrane potential, viability, survivability and storage, acrosome integrity and functional status, among others (Liao *et al.*, 1985; Pena *et al.*, 2003; Pena *et al.*, 2004; Strzezek *et al.*, 2004; Chanapiwat *et al.*, 2009; Echeverria-Alonzo *et al.*, 2009). While other studies conclude that antioxidants provide little or no value to boar sperm health (Marin-Guzman *et al.* 2000). Conclusive evidence regarding the effectiveness of antioxidant supplementation to protect boar sperm DNA integrity are limited or at times conflicting; and appear to be related to the specific antioxidant and dosage used, or boar-specific factors (Szczesniak-Fabianczyk *et al.*, 2003; Chanapiwat *et al.*, 2010). Supplementing anti-lipid peroxidases to thawing and incubation media of frozen-thawed boar spermatozoa protects against DNA fragmentation (Casey *et al.*, 2011), while the opposite occurs in the presence of glutathione (GSH; Whitaker *et al.*, 2008). Nevertheless, improvements in sperm DNA after antioxidant supplementation has been demonstrated in other species such as cattle (Bucak *et al.*, 2010), cats (Thuwanut *et al.*, 2008) and humans

(Greco *et al.*, 2005; Tunc *et al.*, 2009). More specifically, 3 months ingestion of a commercial oral multi-antioxidant supplement comprised of folic acid, zinc, selenium, Vitamins C & E and garlic resulted in improved sperm DNA integrity, protamine packaging and reduction in seminal reactive oxygen species (ROS) production in infertile men (Tunc *et al.*, 2009). Such a cocktail of antioxidants are known to either directly neutralize ROS and/or bolster sperm DNA synthesis and protamine packaging (Brewer *et al.*, 2002; Hodge *et al.*, 2002; Ebisch *et al.*, 2007; Surai and Fisinin, 2015). To date however, there are no substantial reports validating the potential benefits of antioxidant supplementation on boar sperm DNA integrity. Moreover, it is known that heat stress is associated with reduced expression of oxidative stress-induced antioxidants (Rockett *et al.*, 2001). As such, we hypothesize that a multi-antioxidant supplement might act synergistically to bolster boar sperm DNA more effectively during periods of heat stress. Therefore, the aim of this study was to investigate whether a combined antioxidant supplement could mitigate sperm DNA damage in boars exposed to tropical summer conditions.

5.3 Materials and Methods

Boars and Location

Five Large White boars between 3-3.5 years of age were housed and maintained 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 2.3 - 2.8 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

Temperature and relative humidity in Townsville spanning the 42-day period immediately before semen was collected were obtained from the Australian Bureau of Meteorology (2011). This period corresponds to one complete cycle of spermatogenesis and epididymal maturation in this species (Franca and Cardoso, 1998; França *et al.*, 2005) during which boars where exposed to ambient environmental conditions. Townsville's weather, climatic conditions and the procedures by which values for temperature, humidity and temperature-humidity index (THI) were generated were as described in Chapter 2.

Antioxidant Supplementation

Boars were fed 100 g per boar per day custom-mixed multi-antioxidant supplement (PG581 JCU) for 42 and 84 days, respectively during the peak wet (hot and wet; January to April 2016) and early dry (cool and dry; May to August 2016) seasons, and semen samples collected and compared to those from the same boars exposed to the peak wet and early dry seasons of the previous year without supplement (February and end of May 2015 respectively). One boar was excluded from the study in the early dry season during the 42-day treatment and a second during the 84-day treatment due to illness. The antioxidant was prepared by a commercial animal feed manufacturer (Rabar Pty Ltd, Queensland, Australia) and contained multiple ingredients mixed with a suitable carrier (pollard) for easy handling, as specified in Table 5-1. The ingredients of the antioxidant supplement were based on previous studies showing relevant improvements in the quality of boar or human sperm after supplementation (Liao *et al.*, 1985; Pena *et al.*, 2003; Pena *et al.*, 2004; Strzezek *et al.*, 2004; Chanapiwat *et al.*, 2009; Echeverria-Alonzo *et al.*, 2009; Tunc et al. 2009). At the time of feeding, 100 g of antioxidant was thoroughly mixed into the first half of the basal feed and

given to each boar. The second half of the basal ration was given once the boar had fully consumed the first half to ensure the full antioxidant dose was taken each day.

Table 5-1: Ingredients and active level per kg premix of custom-made antioxidant supplement PG581 JCU

Ingredient	Active level per kg premix (mg)
Vitamin E	3,250
Vitamin C	25,000
Folic Acid	330
B carotene	2,250
Zinc	250
Selenium	6
Garlic Powder	75,000
Pollard	*

* acts as carrier

Semen Collection and Processing

At the end of each treatment and from controls, semen was collected from the same n=5 boars using a dummy sow (Minitube, USA) and gloved hand technique (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 ºC water and immediately brought to the laboratory for processing. Raw semen from each boar was diluted 1:3 with 38 ºC 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 (World Health Organization, 2010), a second aliquot adjusted to 20 x 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 µl of 20 x 10^6 sperm/mL semen in BTS was loaded into each chamber of 38 ºC pre-warmed Leja Standard Count 4 Chamber Slides (Leja Products, Nieuw-Vennep, Netherlands) and loaded into the CASA machine and motility characteristics of spermatozoa were analysed as previously described (Pena *et al*., 2015).

Sperm DNA Integrity Assay and Flow Cytometry Analysis

The procedures used for sperm DNA integrity analysis were according to (Pena *et al.* 2018). Briefly, BTS-diluted semen samples were purified by Percoll gradient centrifugation to remove seminal plasma and possibly dead and damaged spermatozoa (Grant *et al.*, 1994). The final sperm pellet was adjusted to 5 x 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. Six control samples (2 positive, 2 negative, and 2 unlabelled) were prepared in parallel using pooled semen. These were used to accurately gate different populations of spermatozoa in the flow cytometer before experimental samples were analysed as previously described (Chapter 2). The TUNEL reaction labels DNA damaged cells positive for Fluorescein isothiocyanate (FITC). Positive controls (P1 and P2) and all test samples were

incubated in 50 µL 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, U2, N2, P2 and all test samples were subsequently 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 validated using fluorescent microscopy as described in Chapter 2, and showed FITC/DAPI positive DNA damaged sperm heads in green alongside DAPI positive DNA intact boar sperm heads in blue.

All samples were evaluated using a CyanADP flow cytometer (Dako Cytomation, Glostrup, Denmark). 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 20,000 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 (Peña *et al.*, 2017b). 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.

Data Presentation and Statistical Analyses

Standard tests to check for normality and variance in the data were performed using the Shapiro-Wilk test and Levene's test, respectively and data were transformed using Log_{10} where necessary before any statistical analysis was done. Differences in test parameters were analysed using the parametric paired sample tests (sperm DNA damage, sperm concentration and most CASA parameters) or independent sample T-tests (involving the 42 and 84 days antioxidant supplementation in winter) in SPSS (SPSS Statistics version 22, IBM Corporation, NY, USA). Where a parametric test was inappropriate (i.e. assumptions for parametric tests were not met), a 2-sample related test (mean maximum, mean minimum and daily mean temperatures, humidity and THI values) or Mann-Witney test (CASA parameters for VSL & ALH) was used to determine if values were significantly different ($P \leq 0.05$). Data were tabulated and graphs plotted using Microsoft Excel 2016.

5.4 Results

Daily mean temperatures spanning the 42-day period immediately prior to semen collection were consistently hotter during peak wet than early dry season ($P \leq 0.05$, Table 5-2). Moreover, daily mean temperatures were identical for the control and 42-day supplement groups during either the peak wet or early dry seasons. Daily mean relative humidity was generally similar for most treatments, ranging from 70 – 73%. However, the 84-day supplement group during the peak wet was more humid while the early dry control was dryer. Daily mean temperature-humidity index was consistently higher during the peak wet than early dry season ($P \leq 0.05$), although values started to decline in the 84-day supplement groups during the peak wet, but was lowest for the early dry season ($P \leq 0.05$).

Antioxidant supplementation of boars during the peak wet resulted in more than a 1.6 and 2.2-fold reduction of DNA-damaged spermatozoa after both 42 and 84 days treatment,

respectively ($P \leq 0.05$; Fig. 5-1). Peak wet supplementation did not reduce DNA damage to basal levels observed during the early dry season, but values were similar to those observed during supplementation in the early dry.

While sperm concentration was lower in the peak wet compared to early dry control ($P \leq 0.05$; Fig. 5-2), antioxidant supplementation did not improve sperm concentration beyond control levels for either season ($P > 0.05$).

Table 5-2: Mean (± SEM) ambient temperature, relative humidity and temperature-humidity index in Townsville, North Queensland, Australia spanning the 42 day treatment period immediately preceding semen collection during the peak wet and early dry season

	Peak Wet Control (Feb 2015)	Peak Wet + 42 day Antiox (Feb 2016)	Peak Wet + 84 day Antiox (Apr 2016)	Early Dry Control (May 2015)	Early Dry + 42 day Antiox (Jun 2016)	Early Dry + 84 day Antiox (Aug 2016)
Ambient Temperature (°C)						
Daily Mean	29.2 ± 0.2^{a}	29.3 ± 0.2^{a}	27.3 ± 0.2^{b}	24.2 ± 0.4^{c}	23.7 ± 0.3^{c}	21.1 ± 0.3^{d}
Relative Humidity (%)						
Daily Mean	71.4 ± 1.2^{bc}	72.4 ± 1.0^{bc}	77.1 ± 1.3^{a}	61.9 ± 2.1^{d}	73.0 ± 1.4^{ab}	70.0 ± 2.3^{c}
Temperature-Humidity Index (THI)						
Daily Mean	92.9 ± 1.1^{a}	93.4 ± 1.2^{a}	86.3 ± 0.7^{b}	75.8 ± 0.9^{c}	75.5 ± 0.6^{c}	70.2 ± 0.7^{d}

Different letters indicate a significant difference between treatments ($P \leq 0.05$).

Total sperm motility was similar in the peak wet and early dry and this was not altered by 42 or 84-day treatment with antioxidants during either season ($P > 0.05$; Fig. 5-3). Similarly, the number of progressively motile spermatozoa were similar in the peak wet and early dry and this was not altered by 42 or 84 day treatment with antioxidants during either season ($P \geq 0.05$; Fig. 5-4). However, there were more progressively motile spermatozoa after 84 days antioxidant supplementation during early dry than peak wet season ($P \leq 0.05$).

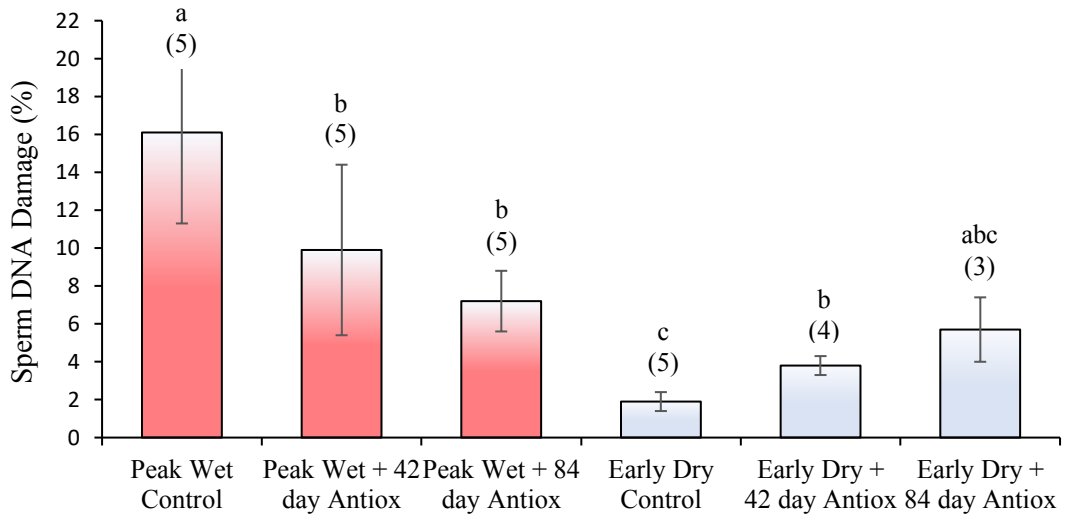

Figure 5-1: Mean (± SEM) percentage of DNA damage in boar spermatozoa collected after no (control), 42 or 84 days antioxidant supplementation during peak wet and early dry seasons.

Different letters indicate significant difference between treatment groups ($P \leq 0.05$); numbers in parenthesis indicate sample size.

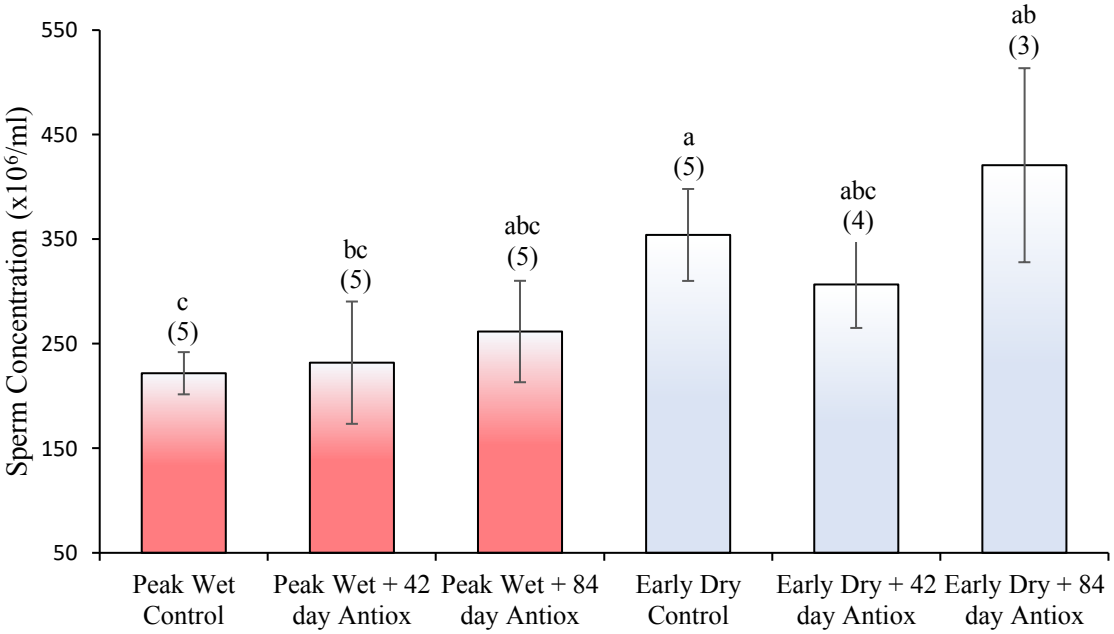

Figure 5-2: Mean (± SEM) concentration of boar spermatozoa collected after no (control), 42 or 84 days antioxidant supplementation during peak wet and early dry seasons.

Different letters indicate a significant difference between treatment groups ($P \leq 0.05$); number in parenthesis indicate sample size.

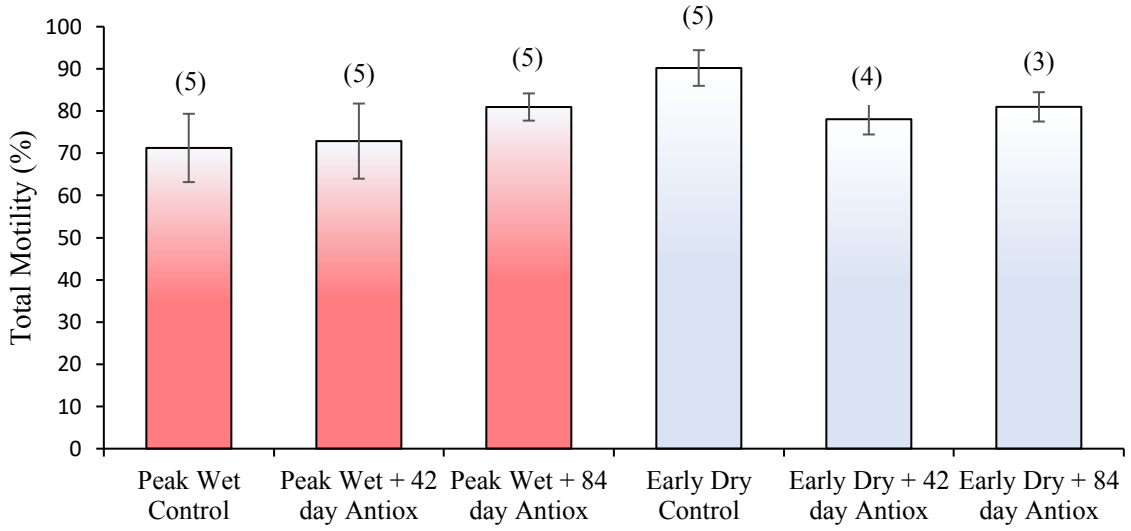

Figure 5-3: Mean (± SEM) percentage of total motility of boar spermatozoa collected after no (control), 42 or 84 days antioxidant supplementation during peak wet and early dry seasons.

No significant difference between treatment groups ($P > 0.05$); numbers in parenthesis indicate sample size.

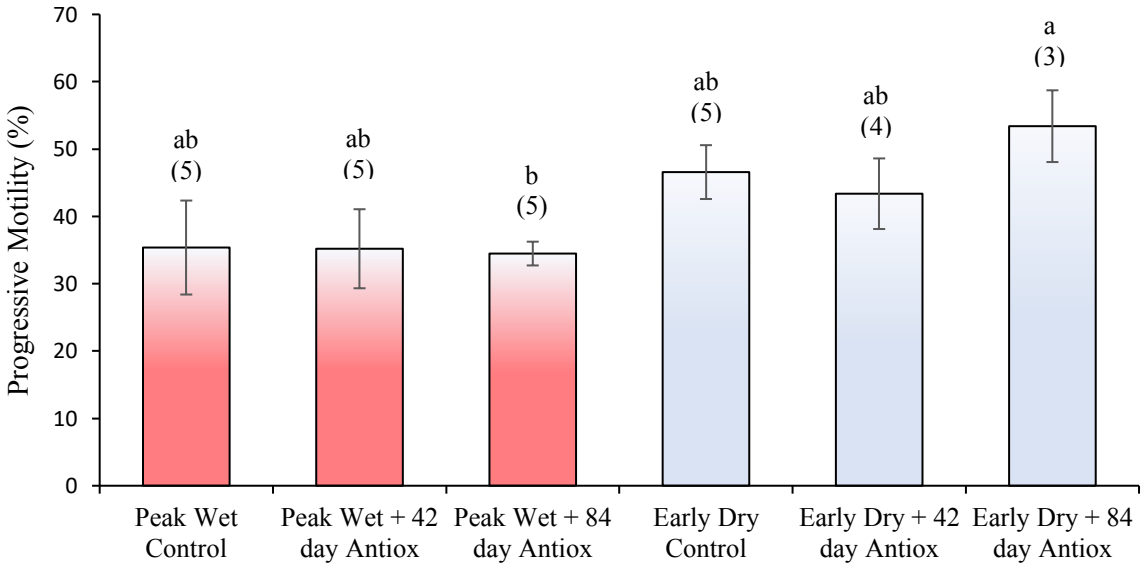

Figure 5-4: Mean (± SEM) percentage of progressively motile boar spermatozoa collected after no (control), 42 or 84 days antioxidant supplementation during peak wet and early dry seasons.

Different letters indicate a significant difference between treatment groups ($P \leq 0.05$); numbers in parenthesis indicate sample size.

Detailed sperm motility and head shape characteristics determined by CASA are shown in Table 5-3. Average path velocity, straight-line velocity, curvilinear velocity, amplitude of lateral head displacement and beat cross frequency were similar in the peak wet and early dry and this was not altered by 42 or 84-day treatment with antioxidants during either season ($P > 0.05$). Sperm elongation was higher after 42 days antioxidant supplementation in the early dry but also after 84 days treatment in both early dry and peak wet seasons, respectively ($P \leq 0.05$). Straightness and linearity of spermatozoa only increased compared to control after 84 days supplementation during the early dry season ($P \leq 0.05$).

Table 5-3: Mean (± SEM) sperm motility and head shape characteristics in boar ejaculates collected after no (control), 42 days or 84 days antioxidant supplementation during peak wet and early dry seasons in Townsville, North Queensland, Australia.

CASA Parameters	Peak Wet Control (n=5)	Peak Wet + 42 day Antiox (n=5)	Peak Wet + 84 day Antiox (n=5)	Early Dry Control (n=5)	Early Dry + 42 day Antiox (n=4)	Early Dry + 84 day Antiox (n=3)
VAP	26.7 ± 2.7	31.9 ± 2.7	32.5 ± 2.7	38.8 ± 4.5	33.8 ± 1.7	35.6 ± 2.1
VSL	22.2 ± 2.4	25.8 ± 2.5	26.8 ± 2.5	30.7 ± 3.5	28.9 ± 1.2	31.3 ± 2.1
VCL	45.9 ± 4.1	55.9 ± 4.5	52.7 ± 3.6	68.3 ± 7.0	56.2 ± 2.3	59.0 ± 2.3
ALH	2.3 ± 0.2	2.7 ± 0.2	2.5 ± 0.2	3.4 ± 0.3	2.7 ± 0.1	2.8 ± 0.1
BCF	21.1 ± 0.6	17.3 ± 0.6	16.9 ± 1.2	19.1 ± 1.5	18.3 ± 1.2	20.2 ± 1.9
STR	76.9 ± 2.2^{ab}	76.1 ± 2.5^{ab}	76.4 ± 1.2^{b}	74.1 ± 1.3^{b}	80.6 ± 2.1^{ab}	83.2 ± 2.8^{a}
LIN	47.3 ± 2.1^{ab}	46.4 ± 2.7^{ab}	47.9 ± 1.6^{ab}	44.8 ± 1.2^{b}	51.2 ± 3.0^{ab}	52.0 ± 3.1^{a}
ELONG	80.3 ± 1.2^{b}	86.9 ± 3.1^{ab}	87.7 ± 2.3^{a}	78.3 ± 1.3^{b}	87.8 ± 1.0^{a}	88.0 ± 0.7^{a}

Different letters indicate a significant difference between treatment groups ($P \leq 0.05$).VAP, average-path velocity (μm/sec); VSL, straight-line velocity (μm/sec); VCL, curvilinear velocity (μm/sec); ALH, amplitude of lateral head displacement; BCF, beat cross frequency (Hz); STR, straightness; LIN, linearity; ELONG, elongation.

5.5 Discussion

The negative impact of heat stress on sperm DNA integrity coupled with its downstream effect on early embryo development (Peña *et al.*, 2017a), presents a new challenge to

maintaining seasonal sperm quality in boars (Szczesniak-Fabianczyk *et al.*, 2003; Sutovsky, 2015). Here, we demonstrate for the first time the beneficial effect of a multi-antioxidant supplement in reducing DNA damage in boar spermatozoa during periods of tropical heat stress. Supplementation of boars at 100 g/day using a custom-made antioxidant formula resulted in 38% to more than 55% reduction in sperm DNA damage after for 42 and 84 days, respectively.

Baseline levels of sperm DNA damage occur naturally in the final stages of spermiogenesis (Aitken and Koppers, 2011). Physiologically, it helps to relieve torsional stress during the DNA packaging process into the compact nucleus of the sperm head. For example, our study has shown that the baseline level of sperm DNA damage in boars raised under tropical conditions during the early dry (when environmental temperature is cool) is about 1%. There are however, several additional causes of sperm DNA damage including environmental stress, toxicants, pollution, infection, poor nutrition and low antioxidant activity in the seminal plasma (Aitken and De Iuliis, 2007; Aitken and Koppers, 2011). Oxidative stress-induced antioxidants are reduced in cells during heat stress (Rockett *et al.*, 2001), predisposing them to DNA attack by reactive oxygen species. Spermatozoa are specifically vulnerable to oxidative damage due their inherent high level of polyunsaturated fatty acids (PUFAs) in the plasma membrane (Sheweita *et al.*, 2005; Ahmadi *et al.*, 2016). Excessive production of reactive oxygen species (ROS) increases rates of cellular damage (Halliwell, 2007), and in sperm increase the rate of sperm ATP depletion; which in turn leads to insufficient axonemal phosphorylation, lipid peroxidation, and loss of motility and viability (Bansal and Bilaspuri, 2011). As such, tropical heat stress encountered by boars during the peak wet season when the ambient temperature, humidity and THI are high appears to be the major contributor to the substantial DNA strand breakages that occur in boar sperm (Peña *et*

al., 2017b) during this time. Given that spermatozoa lack DNA repair machinery, some could be released from the germinal epithelium still carrying their broken DNA (Aitken and Koppers, 2011). However, results found in this study during periods of heat stress appear to support the role of antioxidants in neutralizing free radical activity and protecting sperm DNA from ROS that are already produced (Tremellen, 2008).

Our study tested a multi-antioxidant formulation, an approach that can increase the putative synergistic effect each compound has on sperm quality, as observed in other studies using a mixed formula (Marin-Guzman *et al.*, 2000b; El-Masry and Nasr, 2010; Ahmadi *et al.*, 2016). Our antioxidant formula given at 100 g/day resulted in a 1.6 to 2.2-fold reduction in sperm DNA damage after 42 and 84 days, respectively. While the beneficial compound(s) and mechanism by which this antioxidant cocktail functions in protecting sperm DNA is still unclear, the reduction in sperm DNA damage can be related to other positive effects of antioxidants in boar sperm biology. Selenium, a crucial component in swine nutrition, serves as a raw material in the synthesis of selenoprotein. Selenoprotein plays a significant role in antioxidant system regulation in the body (Surai and Fisinin, 2015), from which a popular Se-dependent enzyme glutathione peroxidase (GSH-Px) depends. Glutathione and vitamin E increase sperm production but also protect against lipid peroxidation (Brezezinska-Slebodzinska *et al.*, 1995). In fact lipid peroxidation, as measured by the levels of ascorbate-induced thiobarbituric acid reactive substances (TBARS), was inhibited by as much as 62% and 57% using water-soluble vitamin E analog (TROLOX) and GSH, respectively (Brezezinska-Slebodzinska *et al.*, 1995). Moreover, garlic, which is also part of our antioxidant cocktail, is able to regulate leukocyte cell proliferation and cytokine production (Hodge *et al.*, 2002) and this anti-inflammatory effect could potentially reduce ROS production by seminal leukocytes.

Where pigs are reared in groups/herds, administration of a multi-antioxidant supplement via their feed is both convenient and has been shown to have synergistic effects. For example, selenium and Vitamin E tend to produce better results in improving boar sperm motility, concentration and/or morphology when given together (Echeverria-Alonzo *et al.*, 2009). Similarly, Vitamin B12 and folic acids tend to produce better results on folate and homocysteine metabolism in pigs during early pregnancy (Guay *et al.*, 2002). Overall, our work and the above studies suggest a cocktail of antioxidants in a supplement formula appears to be more beneficial than a single antioxidant approach to treating boars.

Nevertheless, not all antioxidants are guaranteed to protect boar sperm against DNA damage. While survival of boar sperm improved, adding magnesium fumarate to Biosolwens extender increased the proportion of sperm DNA damage (Szczesniak-Fabianczyk *et al.*, 2003). Moreover, zinc in the form of zinc-methionate at 200 ppm adversely affected boar sperm quality including increased sperm DNA damage (García-Contreras *et al.*, 2011). It is not known whether antioxidant supplementation in our study has led to accumulated levels of zinc in the testis or spermatozoa of our boars, but in our case zinc was administered as zinc sulphate at a recommended dose of ~100 ppm (Liao *et al.*, 1985). Perhaps this might partly explain the increase in sperm DNA damage compared to control after 42 days treatment during the much cooler early dry season (Fig. 5-1). Given these levels were similar to those observed in antioxidant treated groups during the peak wet but both were significantly lower than control at this time, suggests zinc may be a beneficial antioxidant during periods of tropical heat stress but may be detrimental as a long-term general supplement.

Interestingly, despite sperm concentration in the peak wet control being significantly lower that the early dry control, we did not observe any significant improvement in sperm

concentration nor sperm motility after antioxidant supplementation. Several previous studies also showed no improvement in sperm motility (Pena *et al.* 2003; Foote *et al.* 2008; Tunc *et al.* 2009), and selenium has been reported to reduce sperm motility *in vitro* when added to extender (Marin-Guzman *et al.*, 2000b). However, in other studies (Marin-Guzman *et al.*, 2000a; Echeverria-Alonzo *et al.*, 2009; Lechowski, 2009; Park *et al.*, 2010; Horky *et al.*, 2012) improved sperm motility, concentration and/or morphology were the primary consequences of antioxidant supplementation; with one paper specifically highlighting the beneficial effect of antioxidants Selenium and Vitamin E during the warm season (Echeverria-Alonzo *et al.*, 2009). These papers were the basis upon which we selected compounds for inclusion in our antioxidant formula. However, the mechanisms by which antioxidants support DNA structural integrity is still not clear and may not necessarily be linked to pathways that enhance sperm motility and increased spermatogenesis during periods of heat stress. Our previous study showed that tropical heat stress does not affect sperm motility in boars (Peña *et al.*, 2017b), suggesting more detailed studies are needed on the mechanism by which heat stress acts on sperm physiology and the protective role antioxidants play across the different sperm quality parameters.

In conclusion, antioxidant supplementation appears to be an effective measure to mitigate the negative impact of heat stress on sperm DNA integrity but not sperm concentration nor motility during tropical summer. While further research is needed to identify which specific antioxidant(s) in the formula confer this DNA protection and their precise mechanism of action, our study provides a practical solution to improving boar fertility during periods of heat stress, which may greatly improve pig production during summer in tropical and sub-tropical environments.

5.6 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 was funded by a JCU Development Grant to DP, and College of Public Health, Medical and Veterinary Sciences PhD Research Funds to SP. SP was also supported by the Australia Awards Scholarship.

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CHAPTER 6

GENERAL DISCUSSION

Sperm DNA damage is inevitable, both from the perspective of a natural physiological event during sperm head DNA packaging, and as a pathological outcome of constant exposure of mammalian spermatozoa to various environmental stressors, toxic agents, poor nutrition, diseases and increased susceptibility to health challenges (Aitken and Koppers, 2011). The loss of DNA repair mechanisms during latter stages of sperm development, makes these cells highly prone to accumulated DNA fragmentation, which gives rise to a build-up of subpopulations of DNA damaged sperm within the ejaculate. Given the strong link between sperm DNA damage and early embryo loss (Paul *et al.,* 2008), this presents a unique challenge in the re-assessment of male fertility; making evaluation of sperm DNA integrity an important priority (Sutovsky, 2015).

The key thrust of our research initially focused on establishing the link between heat stress and boar sperm DNA damage; geared towards mitigating its impact using antioxidant therapy. Summer infertility due to heat stress is a major impediment to efficient, sustainable and profitable pig production among top pork producing tropical countries in the world such as Brazil, Vietnam, The Philippines and Mexico (National Pork Board, 2011). In effect, loss of production associated with summer infertility can have a massive impact on the sustainability of food production in these regions as well as the global economy where import/export activities are important. The pig's thermal comfort zone stands between 18-20º C (Stone, 1982; Prunier *et al.*, 1997), and rising temperatures particularly during the summer season in tropical and subtropical regions can significantly impact an animal's reproductive performance. Reduced expression of oestrus and increased pregnancy failure in females (Paterson *et al.*, 1978; Hughes and Van Wettere, 2010), and decreased breeding efficiency in males (Wettemann *et al.*, 1976; Boma and Bilkei, 2006) are considered hallmarks of summer infertility. Other production-related problems that beset the summer season further exacerbate

poor growth and subfertility, such as reduced voluntary feed intake, lower feed quality and feed contamination (Vega *et al.*, 2010; Vega *et al.*, 2010). Moreover, reduced reproductive performance relative to season has also been observed in non-tropical countries like France (Auvigne *et al.*, 2010), while millions are lost annually in the U.S. swine industry due to heat stress (St-Pierre *et al.*, 2003).

The crucial role environmental heat stress has on boar sperm DNA integrity and its relationship to boar fertility has been extensively reviewed in Chapter 1. Peculiar characteristics of the boar that include 1) inefficient capacity to sweat (Ingram, 1965; Mount, 1968; Einarsson *et al.*, 2008; Ford and Wise, 2011); 2) non-pendulous scrotum; 3) high content of polyunsaturated fatty acids in the sperm plasma membrane (Cerolini *et al.*, 2001); and 4) low antioxidant activity in the seminal plasma (Brzezińska-Ślebodzińska *et al.*, 1995) can all contribute to boar sperm's high sensitivity to peroxidative stress, which can lead to sperm DNA damage during periods of heat stress (Peña *et al.*, 2017a). Interestingly, Large White boars, which make up a major proportion of breeds used in commercial scale production in tropical countries like The Philippines, shown impaired spermatogenesis in temperatures above 29º C (Stone, 1982).

Our results obtained in Chapter 2 substantiated our hypothesis postulated in Chapter 1 that increased ambient temperatures (such as can be observed during summer in the tropical Townsville, North Queensland, Australia) could compromise boar sperm DNA integrity. Boars in tropical summer (hot peak wet) season exhibited 16 times more sperm DNA damage than early dry (cool and dry), and about 9 times more damage than the late dry (warm and humid) season. How this would impact key production parameters on the farm is yet to be known, however, sperm DNA damage in mice causes arrested embryo development and

eventually death of the embryo (Paul *et al.*, 2008). Moreover, unrelated studies have shown that > 6% DNA fragmentation in boar spermatozoa causes a decline in both farrowing rates and litter size (Boe-Hansen *et al.*, 2008; Didion *et al.*, 2009). Despite a marked reduction in sperm concentration during the peak wet, interestingly, sperm motility determined by CASA remained relatively unaffected across seasons. Overall, these findings highlight the importance of evaluating sperm DNA integrity in routine commercial assessment of boar fertility. Moreover, it is apparent that traditional measures of sperm motility in boars is insufficient to detect inherently compromised, DNA-damaged spermatozoa. This is likely to redefine the way pig producers view summer infertility from being predominantly a sow problem, to one in which the boar can make a significant contribution. Moreover, they will need to adopt strategies to screen for heat tolerant *vs.* susceptible boars in terms of sperm DNA integrity and develop strategies to appropriately manage boars during periods of heat stress.

Damage to sperm DNA due to freezing has been well-documented in various species including humans (De Paula *et al.*, 2006), rams (Peris *et al.*, 2004), and mice (Yildiz *et al.*, 2007) and is likely to cause impaired early embryonic development. Thus, the ability to freeze boar semen collected across different seasons while maintaining sperm DNA integrity was the core objective of Chapter 3. The freezing of boar spermatozoa also faces various challenges including (i) higher susceptibility to the toxic effect of glycerol (Almlid and Johnson, 1988); (ii) a tendency to 'overcondensate' resulting in a reduced nuclear surface area (Royere *et al.*, 1988; Hamamah *et al.*, 1990) that affects fertility (Royere *et al.*, 1991); and (iii) greater variability in sperm cryosurvival due to individual boar effects (Holt, 2000; Roca *et al.*, 2006). We hypothesised that glycerol, a commonly used cryoprotectant (Yang *et al.*, 2016; De Oliveira *et al.*, 2017), effectively protects boar sperm DNA during

cryopreservation but causes serious toxicity to sperm motility at high concentration. Our results demonstrate that 3%, 6% or 8% glycerol can be safely used to cryopreserve boar spermatozoa without inducing additional DNA damage compared to fresh spermatozoa. This correlates with the 2-4% standard inclusion rates for glycerol in many different freezing protocols used across species (Almlid and Johnson, 1988; Fiser and Fairfull, 1990; Das *et al.*, 2016). Moreover, sufficient levels of sperm motility are maintained using these glycerol concentrations. This validated protocol now permits us to freeze semen collected during summer in order to study heat stress effects on embryo survival using *in vitro* fertilisation techniques in winter, when oocyte quality is high.

Now that a suitable protocol for a long-term storage of boar semen has been validated, in Chapter 4 we tried to develop both *in vivo* and *in vitro* heat stress models to induce sperm DNA damage in the boar. This experiment was geared towards reducing seasonal variability associated with natural ambient temperatures and would advance the study of summer infertility by permitting experiments to be conducted at any time of the year. Our *in vivo* model involved holding boars inside a temperature-controlled environment (hot room) for 42 days, while the *in vitro* model directly exposed boar semen to heat using a heat block set to various elevated temperatures. Unlike in other large farm animals, our greatest challenge with the hot room model was to induce sufficient heat stress while maintaining normal boar condition. In fact, while different heat stress treatments have been successfully used in boars in the past (Mcnitt and First, 1970; Wettemann *et al.*, 1976; Cameron and Blackshaw, 1980; Stone, 1982), today's lean fast growing pigs may be more susceptible to heat stress than pigs several decades ago, due to changes their thermo-neutral zone associated with genetic improvement (Parrish *et al.*, 2017). While we were able to induce biologically meaningful levels of sperm DNA damage (6%) using both models, we failed to replicate the high levels

of damage comparable to those observed during the peak wet season (Chapter 2), without exposing spermatozoa to extreme temperatures *in vitro* that rendered boar sperm completely immotile or dead. Concurrently, we also observed considerable individual boar variability in the susceptibility of sperm to heat stress-induced DNA damage. This suggests that with optimisation, the *in vitro* heat shock model could be used as a diagnostic test to screen for heat-tolerant boars for use in breeding herds in the tropics.

Finally, in Chapter 5, we demonstrated for the first time the beneficial effect of antioxidant therapy in reducing boar sperm DNA damage due to heat stress during tropical summer (Chapter 2). Considering the low antioxidant activity in the seminal plasma (Brzezińska-Ślebodzińska *et al.*, 1995) of boar semen and that low antioxidant levels appear to correlate with increased sperm DNA damage (Fraga *et al.*, 1996), Chapter 5 was strategically aimed to evaluate a multi-antioxidant supplement to mitigate the effects of environmental heat stress on boar sperm DNA damage. The use of feed supplements in the pig industry has become the gold standard to meet the higher demands of production particularly in commercial farms (Matte *et al.*, 1993; Miller *et al.*, 1993; Pena *et al.*, 2004; Zhu *et al.*, 2012; Sejian *et al.*, 2014). These exogenous feed supplements, including vitamin premixes and antioxidants, were formulated to boost growth, reproduction and general wellbeing of production animals. Furthermore, boar sperm has a high proportion of easily oxidised long chain polyunsaturated fatty acids in the plasma membrane, meaning effective antioxidant defence systems are crucial for boar sperm survival and maintenance of functional integrity (Agarwal *et al.*, 2016). Several antioxidants including Vitamin C (Lin *et al.*, 1985; Audet *et al.*, 2004; Lechowski, 2009), zinc (Liao *et al.*, 1985), selenium and Vitamin E (Marin-Guzman *et al.*, 2000; Echeverria-Alonzo *et al.*, 2009; Horky *et al.*, 2012), glutathione (Funahashi and Sano, 2005) and garlic powder (Park *et al.*, 2010), have been shown to improve various sperm

quality parameters such as motility, viability, survivability, acrosome integrity and storage among others (Pena *et al.*, 2003; Pena *et al.*, 2004; Strzezek *et al.*, 2004; Funahashi and Sano, 2005; Chanapiwat *et al.*, 2009). Other studies have demonstrated that administering a combination of antioxidants could act synergistically to improve overall sperm quality (Ahmadi *et al.*, 2016). Using 100 g per day of a custom-made multi-antioxidant supplement we were able to reduce boar sperm DNA damage in the peak wet season from 16% without supplement to 10% then 7% after 42 and 84 days treatment respectively. We believe we have developed a practical solution to mitigate one aspect of summer infertility in pigs. Further work is required to identify the specific compound(s) responsible for this protective effect, as well as the optimum duration of supplementation. Perhaps more importantly, future trials are needed to determine the magnitude of sperm DNA protection under tropical farm conditions, as well as downstream improvements in litter size following artificial insemination.

Other means of combating oxidative damage during periods of heat stress are possible in pig production, including the use of more heat-tolerant breeds (Gourdine *et al.*, 2006) and installation of air-conditioning and related evaporative cooling systems in pens (Lucas *et al.*, 2000). However, the use of exogenous antioxidant supplements may be more feasible/cost effective, particularly in developing tropical countries, due to their ease of application compared to high infrastructure and running costs associated with temperature control systems.

Overall, assessment of sperm DNA integrity during periods of increased environmental temperature can provide greater insight into boar (in)fertility. Our findings will redefine the traditional view of pig producers that summer infertility is a sow problem, and will hopefully change the way they manage boars and screen for infertility during tropical summer.

Antioxidant supplementation presents a potential practical strategy to mitigate sperm DNA damage in boars and alleviate summer infertility, thereby improving production efficiency and profitability in the pig industry.

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