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Consequences of Testicular Heat Stress and Potential Amelioration Strategies

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Consequences of Testicular Heat Stress and Potential Amelioration Strategies

by

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Abstract

Heat stress (HS) is a worldwide problem due to temperature elevations associated with global warming. Monitoring temperature changes in testes and scrotum over time is important to understand thermoregulatory capacity of testes and scrotum. Temperature data loggers (DLs) are reliable devices that can be implanted in scrotal subcutaneous tissues to serially record intrascrotal temperature without altering animal behavior. Testicular HS causes a variety of outcomes, depending on the degree and duration of testicular heating. Some effects of testicular HS on testicular tissues, blood flow and sperm quality and attempts to prevent them, by treating animals or semen extender, have been reported. The overall objective of this study was to investigate impacts of HS on sperm quality and use various ameliorative strategies to mitigate these detrimental effects, using bull and ram models. We validated data loggers as a reliable, less invasive and serial method to measure intrascrotal temperature. Scrotal subcutaneous temperature was significantly increased by scrotal insulation or whole-body heating, but not by scrotal neck insulation; however, all three heat-stress models decreased sperm motility and morphology in bulls and rams. In addition, our hypothesis that melatonin or L-arginine improve quality of frozen-thawed sperm from HS rams was supported; 1 mM of either gave best results, except 0.5 mM minimized DNA Fragmentation Index (DFI%). Moreover, during hot weather, bulls voluntarily accessed shade, significantly lowering scrotal subcutaneous temperatures and improving sperm quality. In rams exposed to testicular HS, slow-release melatonin significantly improved testicular blood flow, protected sperm motility and morphology, and lessened HS-induced reductions in post-thaw sperm quality. Therefore, melatonin has potential for mitigating effects of testicular HS under field conditions.

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Dedication

To my family (Mom, brother and my two sisters), for their immense love and support, for always believing in me and my dreams, for being so present in my life, despite the physical distance.

To the soul of my Dad.

To the soul of my Professor Dr. Mohamed Moustafa Ayoub, Professor Dr. Abdel Raof Ghallab, Professor Dr. Adel Moawad, and Professor Dr. Khaled El-Shahat for their kindness and support.

To Salwa my wife, for her love, support and patience, for helping me every single day to reach our goals and dreams, for our little kids Yassin and Aqsa.

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List of symbols, abbreviations, and nomenclature

Symbol	Definition
DLs	Data loggers
IRT	Infrared thermography
VAP	Average path velocity
VSL	Straight line velocity
VCL	Curvilinear velocities
STR	Straightness coefficient
LIN	Linearity coefficient
WOB	Wobble coefficient
SOD	Superoxide dismutase
CAT	Catalase
DFI%	DNA fragmentation index%
PI	Pulsatility index
RI	Resistive index

Chapter 1: Literature review; modified from: AM Shahat, G Rizzoto, JP Kastelic. (2020). Amelioration of heat stress-induced damage to testes and sperm quality. *Theriogenology*, 158:84-96.

1.1 Abstract

Heat stress (HS) occurs when temperatures exceed a physiological range, overwhelming compensatory mechanisms. Most mammalian testes are ~ 4 to 5 °C cooler than core body temperature. Systemic HS or localized warming of the testes affects all types of testicular cells, although germ cells are more sensitive than either Sertoli or Leydig cells. Increased testicular temperature has deleterious effects on sperm motility, morphology and fertility, with effects related to extent and duration of the increase. The major consequence of HS on testis is destruction of germ cells by apoptosis, with pachytene spermatocytes, spermatids and epididymal sperm being the most susceptible. In addition to involvement of various transcription factors, HS triggers production of reactive oxygen species (ROS) that cause apoptosis of germ cells and DNA damage. Effects of HS on testes can be placed in three categories: testicular cells, sperm quality, and ability of sperm to fertilize oocytes and support development. Various substances have been given to animals, or added to semen, in attempts to ameliorate heat stress-induced damage to testes and sperm. According to their composition or activity, they have been divided into various groups, as follows: amino acids, antibiotics, antioxidant cocktails, enzyme inhibitors, hormones, minerals, naturally produced substances, phenolic compounds, traditional herbal medicines, and vitamins. Herein, we summarized those substances according to their actions to mitigate HS' three main mechanisms: oxidative stress, germ cell apoptosis, and sperm

quality deterioration and testicular damage. The most promising approaches are to use substances that overcome these mechanisms, namely reducing testicular oxidative stress, reducing or preventing apoptosis, and promoting recovery of testicular tissue and restoring sperm quality. Although some of these products have considerable promise, further studies are needed to clarify their ability to preserve or restore fertility following HS; these may include more advanced sperm analysis techniques, e.g., sperm epigenome or proteome, or direct assessment of fertilization and development, including *in vitro* fertilization or breeding data (either natural service or artificial insemination).

1.2 Introduction

Most mammalian testes migrate from their embryonic origin in the abdomen to the scrotum, which has a lower temperature. This migration is not complete, however, and the testes' final location varies widely among species. For bulls and rams, descended scrotal testes (that have descended into a well-developed extra-abdominal scrotal sack) are present [1]. Mammalian testes are divided into two major interconnected compartments: interstitial/intertubular compartment and seminiferous tubular compartment. Blood and lymphatic vessels, testosterone-secreting Leydig cells, macrophages, and several other somatic cells are located in the former. Seminiferous tubules are lined with many layers of growing germ cells and Sertoli cells; the latter support developing germ cells structurally [2], secrete tubular fluid to nourish germ cells [3], function as macrophages, secrete inhibin and androgen binding proteins, facilitate germ cell movement and release [4], and maintain the blood-testis barrier (BTB) [5]. The BTB is located in the basal third of the seminiferous epithelium, dividing the tubule into basal, intermediate, and adluminal compartments. The basal compartment contains spermatogonia, preleptotene and

leptotene spermatocytes, the intermediate zone transports germ cells between the basal compartment and the tubular lumen, and the adluminal compartment contains spermatocytes, round spermatids, and elongated spermatids. BTB works as an immunogenic barrier to protect germ cells while also preventing the diffusion of chemicals and/or fluid into the tubular lumen [6]. Any breach in the integrity of this barrier increases fluid permeability into the tubular lumen and widens intercellular gaps between the Sertoli cells.

Spermatogenesis is a complex and delicate process that takes approximately 61 d to complete in bulls and 49 d in rams, beginning with spermatogonia and ending with elongated spermatids. Two major processes, spermatocytogenesis and spermiogenesis, characterize this transformation. Spermatocytogenesis takes 44 d and includes the mitotic and meiotic divisions of spermatogonia to primary and secondary spermatocytes, and ultimately to round spermatids. Spermiogenesis is a 17-d process that involves the morphological transition of a round spermatid into an elongated mature spermatozoon. During spermiation, sperm are discharged into the lumen of seminiferous tubules [7].

1.3 Scrotal and testicular thermoregulation

In many mammals, spermatogenesis requires that testicular temperature be cooler than body temperature [8]. Various local mechanisms have important roles in controlling testicular thermoregulation in animals and can be divided into two components: non-vascular and vascular. Nonvascular thermoregulation includes physiological responses such as sweat, changes in the location of testes and other behavioral approaches, e.g., seeking shade. Vascular reactions include countercurrent heat exchange and peripheral vasodilation [9,10].

1.3.1 Nonvascular thermoregulatory mechanisms

There are many phenotypic and genotypical characteristics that confer adaptive capacity, allowing animals to cope under adverse conditions. These adaptive mechanisms enable animals to thrive in specific environments [11]. In animals with a scrotum, the tunica dartos, cremaster muscles and sweat glands all contribute to cooling the testes [12]. The tunica dartos is smooth muscle in the scrotal wall that contracts and relaxes to lift or lower the testes; it is an important regulatory factor in ambient temperatures up to 30 ℃. In addition, the striated cremaster muscle, attached to the inner abdominal oblique muscle, contracts in cold weather to draw testes closer to the body, but relaxes to allow testes to shift away from the body in warm weather [12].

Respiratory rates and water consumption of bulls exposed to elevated ambient temperatures were greater than those of control bulls throughout an 8-wk treatment interval and were regarded as compensatory mechanisms [13]. It is well established that warming the testes in scrotal mammals results in heat loss and inhibits heat output [14,15]. When local effectors are overloaded and scrotal temperature rises to levels where spermatogenesis is impaired, central thermoregulatory mechanisms (e.g., panting) are activated and whole-body cooling is initiated [16] .

Scrotal sweat glands respond to heat stress by pulsating releases of sweat that evaporate to cool the testes [17]**.** In rams, scrotal skin temperatures > 35 ℃ trigger synchronous discharges of apocrine sweat glands (at intervals of 2 to 14 min). In bulls, scrotal sweat glands are more widely distributed, larger and produce more sweat than those elsewhere in the body [17,18].

1.3.2 Vascular thermoregulatory mechanisms

Regardless of the presence or absence of a scrotum, heat exchange in the testicular vascular cone (TVC) seems to be critical to decrease testicular temperature [19]. The TVC is situated

dorsal to the testis and consists of the tightly coiled testicular artery, surrounded by the venous pampiniform plexus, with blood circulating in opposite directions in those vessels. Heat exchange inside the vascular cone functions by facilitating transfer of heat from the warm arterial blood to the cooler venous blood returning from the testis [9,20,21]. Furthermore, the TVC has an important role in heat radiation from the scrotum, as the scrotal skin above the TVC is normally the warmest portion of the scrotum [22].

In bulls, development of the testicular artery is rapid between 6 and 12 mo of age; its length, diameter and distance in the spermatic cord between the vein and the artery all increase with age, whereas the thickness of the artery wall decreases [20]. In the course of monthly ultrasonography of testes in bulls, TVC diameter increased until 13.5 mo [23]. After puberty, all of these features promoted increased heat exchange in the TVC, consistent with the need to keep testes cooler than core body temperature [10].

1.4 Methods to assess scrotal and testicular temperatures

Mammalian testes must be ~ 4 to 5 °C cooler than body temperature for production of normal and motile sperm [8], highlighting the importance of scrotal and testicular temperatures. Several techniques have been used for many years to assess scrotal/testicular temperature in animals. Infrared thermography (IRT) has been used to measure scrotal surface temperature (SST), whereas needle thermocouples have been used to measure scrotal subcutaneous (SQT) or intratesticular (ITT) temperature; all require the animal to be restrained [24]. However, to monitor intrascrotal temperature in unrestrained animals, small data loggers (DLs) have been used in bulls [25] and rams [26] to continuously monitor intrascrotal temperature.

1.4.1 Infrared thermography

Non-invasive infrared thermography (IRT) cameras have been widely used to evaluate physiological and metabolic parameters and their effects on animal welfare from environmental conditions [27]. Furthermore, since the 1980s, IRT has been used to estimate temperature of the scrotal surface in bulls as a noninvasive method, establishing temperature patterns in clinically normal bulls and detecting abnormal temperature patterns in some bulls [22,24,28].

Infrared scrotum thermograms from bulls with relatively normal scrotal/testicular thermoregulation have left-to-right symmetry and a decrease in temperature (typical range, 4 to 6 °C) from the top of the testis (ventral to testicular vascular cone) to the bottom. More irregular temperature patterns, often missing left-to-right symmetry and having localized areas of elevated temperature (“hot spots”) were thought to be indicative of abnormal thermoregulation of the underlying testes or epididymis; these bulls typically had poor quality semen [29]. Whereas almost every bull with an abnormal thermogram had decreased semen quality [29], not every bull with poor semen quality had an abnormal thermogram. As a result, although infrared thermography is a useful tool for evaluating bulls' breeding soundness, it does not replace collection and evaluation of semen [30].

1.4.2 Needle thermocouples

To estimate subcutaneous scrotal and intratesticular temperatures in bulls and rams, needle thermocouples (18 gauge, 1.5 inch injection needles containing copper and copper-constant wires) as reported in Kastelic et al (1995) [24], have been used in many studies [22,24,28,31,32]. Using these thermocouples, the authors reported a positive temperature gradient (top warmer than the bottom) in scrotal subcutaneous tissues, but inside the testicular parenchyma there was a slight negative temperature gradient (top cooler than the bottom). The positive temperature

gradient on the scrotal surface and to a lesser extent in the subcutaneous tissue is consistent with an expected proximal to distal pattern of the scrotum being supplied with arterial blood. Furthermore, the slightly negative temperature gradient in the testicular parenchyma indicates that the testicular vasculature helps to cool the testes as blood flows up the branching arteries on the caudal-lateral surface towards the dorsal pole of the testis. However, these experiments required that the animals were restrained and under some form of analgesia, thereby not allowing them to exhibit natural behaviors during measurements. Furthermore, insertion of thermistors, especially into the testicular parenchyma, can cause varying degrees of hemorrhage that could alter testicular temperature.

1.4.3 Data loggers

Small data loggers (DL) are relatively inexpensive and have internal data storage that reduces the risk of data loss, although they need to be surgically inserted and removed [33]. Furthermore, DLs offered a reliable serial source of data for synchronous intrascrotal temperature measurement in which animals can express natural behaviors and are not restricted to handling facilities. Wallage et al 2017 [25] reported that there was no evidence of testicular adhesion with the use of DLs and spermatogenesis in bulls was not affected. In rams, DL reliably assessed intrascrotal temperatures as a minimally invasive method, with no indications of adhesions or inflammations at the site of implantation in subcutaneous scrotum [26,34].

1.5 Consequences of increased testicular temperature on male fertility

Heat stress (HS) occurs when an animal is subjected to temperatures that exceed its physiological range and compensatory ability; it usually involves the entire body, although it may be confined to a particular organ or anatomic area [35]. Regarding effects of HS on

reproductive functions in mammals, spermatogenesis is particularly susceptible, with varying degrees of reduced fertility [36].

Air temperature, humidity, air movement, solar radiation, and precipitation all have impacts on heat stress [37]. The temperature-humidity index (THI) is a single statistic that depicts the combined effects of air temperature and humidity on the amount of heat stress. The THI takes into account both temperature and relative humidity and is often used to assess the degree of heat stress in dairy and beef cattle. This indicator was created as a weather safety measure to control and reduce heat stress-related losses [38]. THI is a heat load parameter computed using the equation: THI = 0.8DBT+ RH x (DBT-14.4) + 46.4, where DBT is dry bulb temperature ℃ and RH is relative humidity in decimal notation. A THI of 74 or less is regarded normal, a THI of 75 to 78 is deemed alert, a THI of 79 to 83 is considered dangerous, and a THI of 84 or more is considered an emergency [39]. Because THI includes effects of both ambient temperature and relative humidity in a single index, it is widely used globally as a realistic indicator for the degree of stress on dairy and beef cattle caused by weather conditions.

Temperature elevations associated with global warming refer to increasing temperatures of the earth's surface, attributed to increasing concentrations of greenhouse gases (including CO_2 and methane) in the atmosphere. Greenhouse gas emissions may cause a temperature rise of 1 to 3.5 ℃ over the next century [40]. A warming environment is predicted to have a strong influence on reproductive capacity of living organisms, contributing to selection of species better adapted to new climatic conditions, with concomitant reductions in numbers of less-adaptable species [41]. Whereas the overall trend is for increased temperatures, it is well known that there are substantial regional variations in the extent to which ambient temperatures are increased and also

large differences among species and individuals in their susceptibility to HS. Therefore, effects of climate change on reproduction will be highly variable.

Here we describe impacts of HS as a result of climatic changes, or various experimental models, on spermatogenesis and male fertility. In addition, it also describes several categories of compounds used to mitigate harmful effects of HS on testicular tissues, sperm and male fertility.

1.5.1 Consequences of increased testicular temperature due to climate change

Cattle use sweating and panting to remove excessive heat from their bodies; however, if those mechanisms are insufficient for heat transfer to the environment, this is the threshold at which the ambient temperature is considered to be HS [42]. It is noteworthy that the patterns of testicular HS (proportion of body involved, extent and duration) vary markedly under natural conditions e.g., cryptorchidism, varicocele, and ambient heat exposure [43].

Several studies reported effects of increased environmental temperatures on male reproduction. Bulls exposed to elevated ambient temperatures (40 ℃) had lower sperm quality, namely reduced percentage of motile sperm and increased percentage of abnormal sperm. Approximately 8 wk were required before semen quality returned to normal [13,44,45]. In rams exposed to 5 h of high ambient temperature (40 °C), the tunica dartos muscle relaxed and scrotal sweat glands were activated; nevertheless, scrotal temperature rose and the gradient between body and scrotal temperatures decreased, indicating that thermoregulatory mechanisms were insufficient to maintain scrotal temperature during exposure to extreme heat [46].

Although increases in ambient temperature due to global climate change are highly variable, in many locations, ambient conditions are already causing testicular HS, with an expectation that this will become more prevalent as global temperatures rise. Simmental bulls

(*Bos taurus*) had slightly higher rates of major sperm defects in summer than in winter, with younger bulls more sensitive to elevated ambient temperatures. With lower glutathione peroxidase (GPx) activity in Simmental vs Nelore (*Bos indicus*) bulls, lipid peroxidation in the former was high, consistent with higher percentages of defective sperm [47,48]. Greater heat resistance in *B. indicus* versus *B. taurus* bulls and quality of frozen-thawed semen from *B. taurus* bulls differs throughout the year, with a high proportion of ejaculates discarded during summer [49]. Furthermore, in rams, there is a marked decrease in testicular blood flow, with a remarkable increase in both resistive index (RI) and the pulsatility index (PI) during hot months, with deleterious effects on seminal plasma enzymatic activity and other aspects of semen quality [50].

1.5.2 Consequences of increased testicular temperature in various experimental models

Although there are various HS models, they can generally be divided into two major categories: whole-body heating or local heating of the testes. Arguably, the most realistic model of HS is to subject the entire animal to a hot environment. However, there are two important considerations. First, the body responds in several ways to heat stress and there are important physiological, metabolic and endocrinological changes that could have indirect effects on the testis. Second, the ability of the scrotal skin to produce sweat can be affected by an animal's prior exposure to heat, and thus, testicular temperature can be quite variable in response to a given heat exposure [51]. Furthermore, climate chambers are expensive, not widely available and animals are typically closely confined, which can reduce animal welfare and wellbeing.

The second major model, local heating of testes, is typically accomplished in one of three ways: induced cryptorchidism, scrotal insulation or short-term heating (the latter generally by immersion in a water bath [51]). In farm animals, scrotal insulation (e.g. with a cloth bag and

insulating material) is the most common model to simulate increased testicular temperature, by retaining heat and also by interfering with scrotal sweating [52]. There are several advantages, including being easy to do, inexpensive and no need to confine animals after the insulation is applied. Regardless, it also has several limitations, including continuous, relatively high heat applied to the testes, in the absence of whole-body warming and systemic responses, reducing its relevance and usefulness as a model for climate change. Furthermore, although this model has been widely used, in most cases, the extent of testicular warming was not determined, making it difficult to compare outcomes among studies.

Impacts of heat load on scrotal thermoregulation have been done by monitoring scrotal temperature. Most of these investigations were done as one-time measurements, or at most, done two or more times over a short interval, e.g., 1 d [8,23,24,53]. Based on such studies, it is well established that testes are kept ~4 to 5 °C below core body temperature by means of special vascular arrangements and other factors.

Consequences of increasing testicular temperature depend on degree and duration of testicular heating. Although a brief, mild increase in testicular temperature may only cause a temporary reduction in sperm quality, sustained and/or severe testicular heating is likely to induce infertility. Moreover, a prolonged, serious thermal insult may cause spermatogenesis to cease permanently [10]. Increases in testicular temperature do not immediately affect sperm characteristics, as damaged spermatogenic cells typically do not enter ejaculates for some time after HS. For instance, in bulls, spermatogenesis takes ~61 d and changes in sperm morphology and motility are apparent ~2 wk after HS and do not return to normal for as long as 8 wk after HS terminates [13].

In the following, we categorize consequences of testicular HS as a result of various experimental models into three components: **(1.5.2.1)** Effects of HS on testicular cells; **(1.5.2.2)** Effects of HS on sperm quality; and **(1.5.2.3)** Effects of HS on ability of sperm to fertilize oocytes and support development.

1.5.2.1 Effects of HS on testicular cells

Increased testicular temperature, due to scrotal insulation or other HS models, affects all types of testicular cells, although germ cells are more sensitive than either Sertoli or Leydig cells [54]. Pachytene spermatocytes, spermatids and epididymal sperm are the most heat-sensitive germ cells [51,55,56]. Scrotal insulation in bulls affects both spermatids and immature sperm in the caput epididymis, although selective sperm resorption in rete testis and excurrent ducts may prevent or reduce the number of affected sperm appearing in the ejaculate [57].

The major consequence of HS on testis is destruction of germ cells by apoptosis [36]. As early as 1 or 2 d after mouse testes are immersed in 42 °C water (HS), there is apoptosis of germ cells, primarily at early (I–IV) and late (XII–XIV) stages. Pachytene spermatocytes in stages I–IV and IX–XII, diplotene and dividing spermatocytes in stages XIII–XIV, and early (stages I–IV) spermatocytes are cell types most frequently affected [58]. This increased apoptosis of germ cells reduces testis size [59].

Based on genomic studies, some apoptotic genes including BCL-2 Antagonist X (*BAX*; alpha isoform), BCL-2 Antagonist/Killer (*BAK*) and *CASPASE-2* have low expression under normal conditions [56,60]. In recent studies, *BCL-2* was downregulated ~30% ($P < 0.05$) in bulls [61] and mice [62] 24 h after heat exposure, resulting in a molecular environment that promotes apoptosis. *CASPASE -9* and *CASPASE -3*, two hallmarks of apoptosis, are activated after HS in

rat germ cells, whereas their pharmacological inhibition prevents germ cell death, implicating caspases in HS-induced death of germ cells [63,64]. Similarly, in a murine model, at 14 d after heat exposure, there was approximately four-fold upregulation ($P<0.05$) of *CASP 8* (initiator), plus reductions ($P<0.05$) in paired testes weight and seminiferous tubules diameter [62], with the latter two endpoints attributed to apoptosis [65]. One of the main factors involved in this pathway is the Heat Shock Factor 1 (*HSF1*) gene and its product, HSF1 protein; the latter regulates development of heat shock proteins (HSPs) that confer protection to cells after HS [66]. *HSP 70* and *90* are the most common *HSPs* in various tissues [67]. In response to HS, there is upregulation of *HSF1* and *HSP* genes, with increased expression of their protein products, with a goal of preventing protein denaturation and thus mitigating deleterious effects of HS [66]. To detect rapid activation of the chaperone system, testes were recovered from *B. indicus* bulls after 12 h of testicular insulation; there was a significant increase (~40 fold, $P<0.05$) in expression of *HSP 70* [61]. Additionally, *HSF1* induces apoptosis of germ cells when over-expressed in mice, but prevents heat-induced apoptosis of sperm in mice lacking *HSF1* [68–70].

Tumour suppressor *p53* is a potential heat-responsive inducer of germ cell apoptosis. Activity of *p53* in testes is elevated after HS and associated with loss of germ cells [71,72]. Following induction of cryptorchidism in *p53* knockout mice, death of male germ cells was delayed, but still occurred, indicating that both p53-dependent and p53-independent pathways mediate germ cell apoptosis [73]. Recent studies confirmed the involvement of the p53-dependent pathway, leading to activation of two distinct mechanisms, namely intrinsic and extrinsic, with activation of downstream factors leading to apoptosis (Fig. 1). In murine models of testicular warming [62], *p53* was upregulated (approximately eight-fold, $P < 0.05$) 14 d after

heat exposure. Furthermore, Fas (FAS cell surface; CD95/Apo-1) may be responsible for p53-independent germ cell apoptosis [74].

Hormonal imbalances, including down-regulation of *StAR* (Steroidogenic Acute Regulatory Protein) gene and protein, often occur in response to HS [75]. Reduced activity of this enzyme, an essential step in steroidogenesis, will reduce blood concentrations of testosterone, a steroid hormone with critical roles in promoting spermatogenesis and maintaining testicular integrity [76]. There were reductions in *StAR* gene expression and testicular testosterone concentration (~10 fold decrease for each) after 48 h of scrotal insulation in *B. indicus* bulls [61], indicating the severity of the impact of HS on steroidogenesis and the resulting hormonal milieu.

As well as involvement of transcription factors, production of ROS in apoptosis initiation seems to be of utmost importance for apoptosis of germ cells and DNA damage [77]. ROS are molecules with at least one unpaired electron, making them highly unstable and extremely reactive for lipids, amino acids and nucleic acids [78]. Heat stress induces upregulation of heme oxygenase 1 (*HMOX1*) and antioxidant enzymes, namely glutathione peroxidase 1 (*GPX1*), glutathione S-transferase alpha and superoxide dismutase 1 (*SOD1*), indicating a robust oxidative stress response. Furthermore, using *GPX1* as representative of the antioxidant response under heat stress, mice exposed to 40 ºC for 20 min had an approximately seven-fold increase in *GPX1* mRNA ($P < 0.05$) at 12 h after exposure, indicating a prompt and intense response [62]. Thus, there is a complex network of gene regulation in mammalian testes to regulate HS-induced apoptosis of germ cells [79].

Degree of HS regulates timing of apoptosis in germ cells. For instance, in mice, number of apoptotic germ cells begins to rise 6-7 d after experimentally induced cryptorchidism [80],

whereas with more severe HS, namely placing the rear end of a mouse (including scrotum) in 43 °C water for 20 min, apoptotic germ cells are first apparent 8 h after exposure. In contrast, brief exposure of testes to 39-40 °C does not accelerate germ cell death [56]. Therefore, it was concluded that male germ cells have a time and temperature exposure threshold to induce apoptosis [81].

Male germ cells are not the only cell type affected by heat, as somatic testicular cells also respond to HS. Sertoli and Leydig cells, whose main functions are to support germ cells and produce testosterone, respectively, provide an appropriate environment for germ cell development. Consequently, impairments in their functions may interfere with production of male germ cells. Decreased testicular androgen binding protein (ABP) production following experimental cryptorchidism is evidence that heat adversely affects Sertoli cells [82,83]. Moreover, expression of intermediate filaments in Sertoli cells was disrupted in cryptorchid monkeys [84]. Heat treatment caused de-differentiation of adult Sertoli cells in pubertal monkeys [85], perhaps by disrupting junctions of cells in seminiferous epithelium and dramatically affecting expression of junction-associated molecules in Sertoli cells. These changes disrupt the spermatogenesis-supporting role and may induce germ cell apoptosis [86]. Furthermore, exposure of Sertoli cells to 42 °C heat may threaten their survival and connexin-43 overexpression due to HS may cause Sertoli cells to malfunction [87].

Although Leydig cells were historically regarded as unaffected by HS [88], Leydig cell morphology was disrupted in rat testes exposed to heat for 30 min daily for 6 d [89,90]. Moreover, there is evidence that in Leydig cells, hypoxia-inducible factor 1 alpha (*HIF1A*) and *HMOX1* are up-regulated, indicating that heat induces hypoxia and oxidative stress in these cells

[91,92]. Additionally, testicular heat leads to hyperplasia of Leydig cells, mediated by stimulating cyclin proteins activity and reducing testosterone biosynthesis in adult rat testes [93]. Thus, heat may indirectly damage germ cells by modifying somatic cell functions [36].

Few studies have investigated pathophysiological pathways associated with impacts of HS on testicular function and sperm quality [56,61,94]. However, very recent results improved understanding of factors involved in the testicular response to heat stress in cattle [61] and mice [62]. Confirmation of the involvement of the apoptotic p53 dependent-intrinsic and extrinsic pathways (Fig. 1.1) supported the information described in this section.

The chaperone and antioxidant systems are defense mechanisms promptly activated after exposure to HS; under elevated temperatures, *HSF1* induces increases in *Hsp70* and *90* [61,67] that work in conjunction with the chaperones to reduce activity of the *p53* gene [61,94]. However, when the *p53* gene is upregulated due to thermal exposure, this leads to the activation of two important apoptotic pathways, namely p53-dependent intrinsic and extrinsic apoptotic pathways. In the extrinsic pathway, *p53* stimulates the association between *FAS* and *FADD* to form the so-called Death receptor complex, which leads to activation of initiator caspases (*CASP 8* and *10*) [94]. In addition, in the intrinsic pathway, *p53* impairs the balance of *BAX* and *BCL-2* in mitochondria [61,94], leading to the release of agents (*APAF1+Cyt C*) that induce activation of another group of initiator caspases (*CASP 2* and *9*). Lastly, both groups of initiator caspases (from both the intrinsic and extrinsic pathways) will lead to activation of the executioner caspases (*CASP 3*, *6* and *7*), leading to the final steps that induce apoptosis (Figure 1.1).

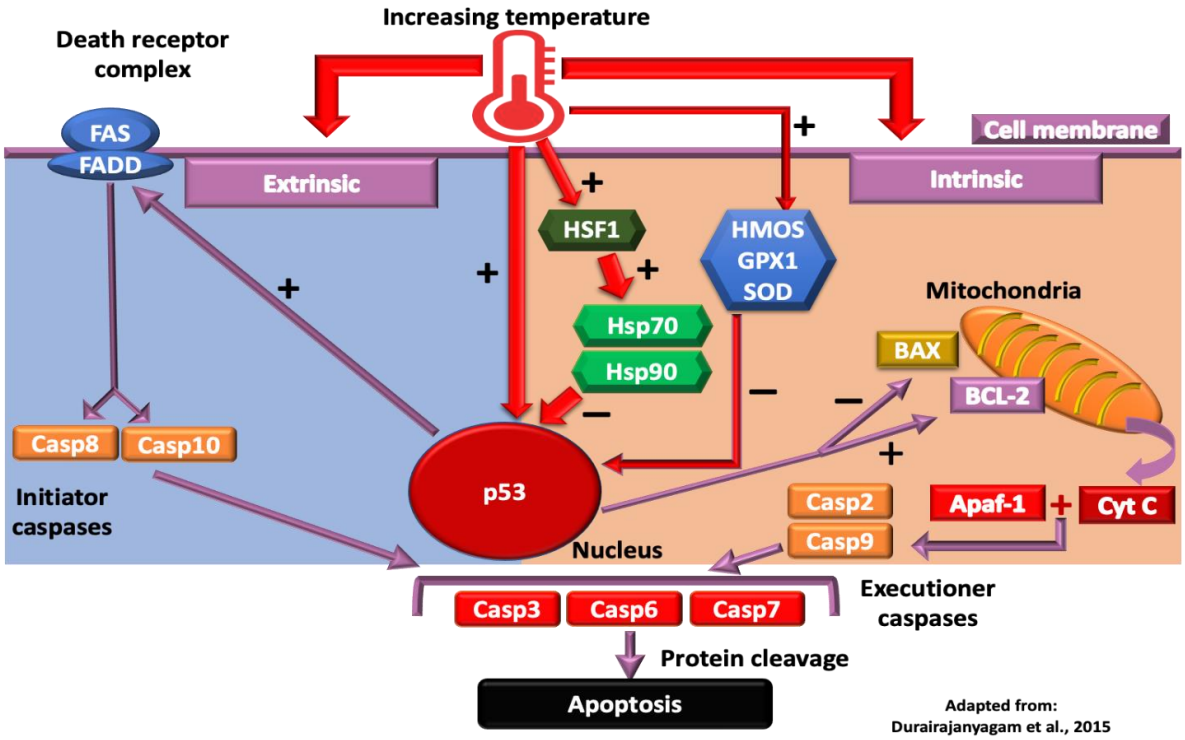


Figure 1.1 Suggested pathways of heat stress impacts and responses in testes (adapted from [94]).

1.5.2.2 Effects of HS on sperm quality

In addition to direct effects on sperm production, HS alters sperm quality, with effects dependent on extent and duration of testicular heating.

When testicular temperature rises, sperm morphology often remains normal for a few days (if sperm in the epididymis are minimally affected), followed by appearance of morphologically abnormal sperm [95]. However, in some studies, epididymal sperm were affected by higher testicular temperatures [28,96–98]. For example, after 48 h scrotal insulation in bulls, epididymal sperm were adversely affected, manifested by their diminished capacity to

retain motility and acrosomal integrity after cryopreservation [97,98]. They had various morphological defects, e.g. distal midpiece reflexes, proximal and distal droplets and knobbed acrosomes, that peaked after 11 to 18 d post-insulation, whereas there were microcephalic and teratoid sperm, nuclear vacuoles and pyriform heads, as well as coiled tails present in the ejaculates collected 21 to 23 d after scrotal insulation [98,99].

Scrotal insulation for 96 h and dexamethasone treatment (20 mg daily for 7 d) were used as models to compare effects of testicular heating and stress, respectively, on spermatogenesis in bulls. Remarkably, for these two insults, the kinds of morphological defects and the temporal relationships of rises and declines of these defects were very similar [99].

Mild HS in bulls (8 h scrotal insulation) adversely affected quality of frozen-thawed sperm [100]. Furthermore, insulation of scrotal neck in *B. taurus* bulls increased scrotal/testicular temperatures and reduced morphologically normal sperm, with increased percentage of abnormalities affecting sperm head and midpiece [28]. In contrast, scrotal neck insulation in *B. taurus* x *B. indicus* (crossbred) bulls did not have a major impact on semen quality [101]. Whole-scrotum insulation for 4 d reduced sperm production and quality in both *B. indicus* × *B. taurus* as well as *B. indicus* bulls, although testicular echotexture changes were not correlated with degree of change in the spermiogram [101]. In addition, 14 and 21 d after 5 d whole-scrotum insulation of *B. indicus* (Nellore) bulls, there was a decrease in normal sperm with higher incidence of head defects, especially nuclear vacuoles and chromatin defects [102], with similar effects in Holstein and Belgian Blue bulls after only 48 h insulation [103] and in Brangus beef bulls [104,105] after 72 h insulation. In Brahman (*B. indicus*) bulls, 48 h scrotal insulation decreased sperm motility and increased percentage of head defects and droplets from 11 d post-insulation to 41 d post-

insulation, whereas percentage of protamine-deficient sperm increased 14 d post-insulation in comparison to a control group [106].

1.5.2.3 Effects of HS on ability of sperm to fertilize oocytes and support development

It is well known that paternal HS affects fertilization and embryonic development. When sperm from males with heated testes are used to sire embryos, embryo mortality increases and embryonic development is often retarded. Furthermore, when *in vitro* fertilization (IVF) is done using sperm from males that had been exposed to heat, rates of sperm penetration, fertilization and pronuclear formation decrease, with increased abnormalities of early embryonic development [36]. Sperm collected from scrotal-insulated bulls decreased embryonic development rates after IVF, due to a high percentage of morphologically abnormal sperm [107]. In another study [108], frozen-thawed semen samples from bulls with 48-h scrotal insulation, caused failure in normal pronuclear formation, associated with an absence of normal decondensation of the penetrating spermatozoon (attributed to a high percentage of morphologically abnormal sperm).

Five days of whole-scrotum insulation of Nellore (*B. indicus)* bulls reduced frequency of cleaved oocytes and decreased blastocyst rates, following the use of semen collected 21 or 14 d, respectively, after insulation [102]. In another study using Nellore bulls, 3-d scrotal insulation decreased percentage of cleaved oocytes for semen collected from 28 to 42 d post-insulation. Furthermore, blastocyst formation and cleavage rate decreased at 14 d post-insulation and remained decreased until 56 d post-insulation [109]. Moreover, HS affects bovine embryos by lowering rate of development, increasing percentage of apoptotic blastomeres and decreasing

expression of developmentally important genes, with effects more apparent in embryos derived from *B. taurus* versus *B. indicus* females [110].

1.6 Effect of HS on testicular blood flow

There has been a long-standing dogma that testes operate on the edge of hypoxia and although rising testicular temperatures cause an increase in O_2 demand, there is a lack of concomitant increase in testicular blood flow, leading to hypoxia that is the underlying cause of abnormal spermatogenesis [111]. However, in a recent study in bulls, there were significant increases in testicular blood flow following increased testicular temperatures [112]. In addition, testes preserved O_2 delivery and absorption under acute hypoxic conditions (13% O_2 in inspired air) with increased testicular blood flow and O_2 extraction, without any sign of a change to anaerobic metabolism in rams [113,114]. Moreover, *B. taurus* and *B. indicus* bulls responded to testicular warming by significantly increasing blood flow, providing enough O_2 to meet increased metabolic testicular needs and prevent hypoxia [115].

1.7 Natural responses to HS

There are many phenotypic and genotypical characteristics that confer adaptive capacity, allowing animals to cope under adverse conditions. These adaptive mechanisms enable animals to thrive in specific environments [11]. In animals with a scrotum, the tunica dartos, cremaster muscles and sweat glands all contribute to cooling the testes [12]. Furthermore, the tunica dartos is smooth muscle in the scrotal wall that contracts and relaxes to lift or lower the testes; it is an important regulatory factor in ambient temperatures up to 30 ℃. In addition, the striated cremaster muscle, attached to the inner abdominal oblique muscle, contracts in cold weather to

draw testes closer to the body, but relaxes to allow tests to shift away from the body in warm weather [12].

Respiratory rates and water consumption of bulls exposed to elevated ambient temperatures were greater than those of control bulls throughout an 8-wk treatment period and were regarded as compensatory mechanisms [13]. It is well established that warming testes in scrotal mammals causes heat loss and inhibits heat output [14,15]. When local effectors are overloaded and scrotal temperature rises to levels where spermatogenesis is impaired, central thermoregulatory mechanisms such as panting are activated and whole-body cooling is initiated [16] .

Scrotal sweat glands respond to heat stress by pulsating sweat release, with sweat evaporation cooling the testes [17]**.** In rams, scrotal skin temperatures >35 ℃ trigger synchronous discharges of apocrine sweat glands (at intervals of 2 to 14 min). In bulls, scrotal sweat glands are more widely distributed, larger and produce more sweat than those elsewhere in the body [17,18].

1.7.1 Shade access as a response to HS

Seeking shade is a natural behavior for bulls in pasture and is considered an efficient way of helping them to maintain temperature control in a hot climate and reducing incoming thermal radiation [116]. Shade affects the radiation balance of the animal [117]. In general, animals avoid discomfort and most European cattle search for shade when they have direct solar radiation (350 W/m^2) and when they have air temperatures > 28 °C [118]. Furthermore, the use of shade seems to provide improvement in some reproductive parameters and also provide some protection for

breeding bulls from extreme summer temperatures [119,120]. However, effects on scrotal subcutaneous temperature and sperm quality are not well characterized.

1.8 Therapeutic approaches to mitigate testicular damage and semen quality deterioration

As oxidative stress is the main cause of damage after testicular HS, in some animal models, antioxidants were given to prevent apoptosis of germ cells and reduce sperm damage [79]. Antioxidants are only one of a wide variety of substances have been given to animals, or added to semen, with an intention to ameliorate heat stress-induced damage to testes and sperm quality. In the following sections, a wide variety of substances are described. For convenience, they have been divided into various groups according to their chemical nature, as follows: amino acids, antibiotics, antioxidants, enzyme inhibitors, hormones, minerals, natural substances, phenolic compounds, vitamins, and traditional herbal medicines, and presented in detail in Table 1.1 (Appendix A), with a brief summary in Figure 1.2.

1.8.1 Melatonin use either *in vivo* or *in vitro*

Melatonin (N-acetyl-5-methoxytryptamine) is a substituted indoleamine derived from tryptophan, isolated 60 y ago [121]. Originally discovered as a secretory pineal gland product, it is now known to be present in many, perhaps all, organs of the body [122,123]. Furthermore, melatonin has many physiological functions; it has a synchronous circadian rhythm and is involved in sleep control, seasonal reproduction, reduction of blood pressure, immune stimulation, inflammatory suppression and GIT protection (reviewed by [124]). Additionally, it is well known that melatonin is a free-radical scavenger and antioxidant [125].

In addition to its function in controlling hormone release from the hypothalamus and pituitary, melatonin can also have a protective role in testicular development. Compounds with antioxidant properties, like melatonin, protect the testes from environmental damage, side effects of cancer therapy, and other toxic molecules [126,127]. In a rat model of testicular torsion, 50 mg/kg of melatonin reduced oxidative stress markers and lipid peroxidation [128,129]. Additionally, in artificially induced varicocele, melatonin therapy decreased the damage sustained by the epithelium and seminiferous tubules, while increasing production of antioxidant enzymes and nitric oxide (NO), which could promote sperm function. There are limited studies to demonstrate the role of melatonin in the testicular response to HS. In rats, 20 mg/kg BW (i/p) melatonin given 2 h before 42 ℃ treatment, reduced vacuolization of seminiferous tubules; there were more tubules with regular seminiferous epithelium and multiple layers of germ cells post-HS. Furthermore, significantly decreased MDA and H_2O_2 generation (markers of ROS) but increased SOD and CAT activity, implied melatonin alleviated heat-induced oxidative stress in mouse testis [130]. Moreover, suppressed activation of JNK and p38 mitogen-activated protein kinase (MAPK) (apoptosis-related signaling pathway) and upregulated HSPA2 and anti-apoptotic BCL-2 in testes (protected against heat-induced damage). Melatonin treatment (same dose and route for 14 d immediately after 42 ℃ treatment) hastened recovery of spermatogenesis and preserved integrity of Sertoli cell tight junction [130].

In multiple studies, melatonin had strong beneficial effects on sperm. Humans with high concentrations of endogenous melatonin had better quality sperm. In vitro melatonin treatment improved human sperm motility [131], as well as several quality parameters of ram [132] and pig [133] sperm, with reduced oxidative cell damage and intracellular ROS and NO rates [133,134], reduced membrane lipid peroxidation [134,135], markers of apoptosis [136] and DNA

degradation [137]. As semen extender additive, melatonin (0.05, 0.1, 0.2, or 1 mM) were beneficial to preservation of ram semen during liquid storage at 4°C, mainly through antioxidative actions [138,139], whereas in stallions, 1.5 mM improved sperm quality during 48 h of cool storage. Moreover, adding melatonin to the semen freezing media has been done in several species, including cattle (0.1, 2 or 3 mM) [135,140], horses (1 mM) [141], chickens (10^{-3}, 10^{-6}, or 10^{-9} M) [136,142], and rabbits (0.1 mM) [143] which enhanced post-thaw sperm quality by decreasing oxidative stress level, lipid peroxidation, DNA fragmentation, and apoptotic-like changes after thawing. However, there are apparently no reports regarding effects of adding melatonin to semen extender on post-thaw semen quality of animals exposed to testicular HS.

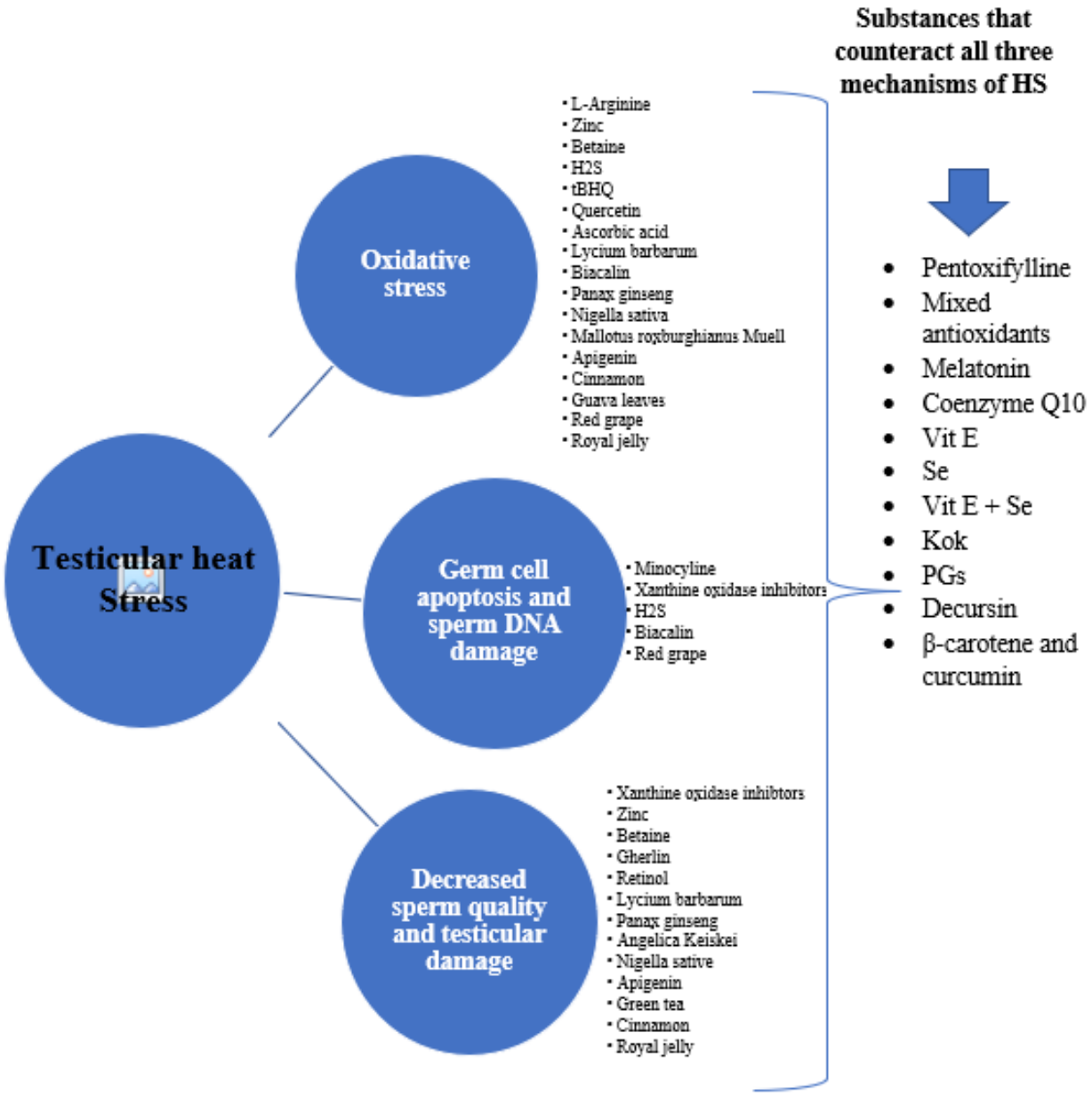


Figure 1.2 Summary of three testicular HS mechanisms, substances that counteract each mechanism, and those that counteract all three.

Testicular blood flow increased as measured by a colour-pulsed Doppler ultrasound (through reduction of resistive index (RI) and pulsatility index (PI) values of the testicular arteries) in Shiba goats given slow-release melatonin [144]. In addition, increasing doses of melatonin improved blood flow in ovarian and middle uterine arteries of fat tailed ewes [145] and maternal administration of melatonin ameliorated intrauterine oxidative stress-induced maternal placental malperfusion, fetal cardiovascular impairment, and fetal neuroinflammation [146]. Moreover, dietary supplementation of melatonin in pregnant heifers improved uterine blood flow, attributed to an antioxidant effect [147]. However, there are apparently no reports regarding effects of melatonin supplementation on testicular blood flow in heat-stressed males.

1.9 How good are laboratory assessments of sperm quality as a predictor of fertility?

In this article, a critical goal was to review various substances used to mitigate effects of testicular HS on male fertility. To interpret these data, it is important to understand the reliability of laboratory tests, commonly used for semen quality analysis, for predicting fertility.

Fertility varies widely among individual males. Conventional tools for predicting fertility are usually better for identifying low-fertility bulls than for rating bulls with reasonable to excellent fertility [30]. Normally, fertility increases with sperm dose until it reaches a threshold level, based on the definition of sperm features which are compensable and non-compensable [148]. Compensable features for male fertility can be compensated by increasing the insemination dose, based on the assumption that a threshold amount of competent sperm (with appropriate motility, normal morphology, and capacity to undergo capacitation and induce an acrosome reaction, e.g. 70% morphologically normal sperm) is needed for normal fertilisation [149–151]. Nevertheless, infertility due to non-compensable characteristics cannot be resolved

by increasing numbers, because sperm with these deficiencies are usually unable to complete fertilisation and support ongoing embryo development [151]. Using more advanced techniques, e.g. Computer-Assisted Semen Analysis (CASA), sperm DNA, RNA, proteomics and epigenome are expected to improve our ability to determine compensable and non-compensable traits in semen that based on more conventional assessments, appeared to be normal, but had variations in fertility, due to failure to continue beyond various stages of fertilisation and early embryo development [152].

It is noteworthy that fertility tests obtained from a sperm sample assay are generally applicable only to the analysed sample. Given the inherent variability among ejaculates and the potential of male fertility to differ over time, it seems unlikely or indeed impossible that male fertility could be predicted correctly using only *in vitro* assessments. Nonetheless, IVF has been used to assess sperm fertilizing ability. Used on their own, or more commonly in conjunction with other tests, IVF enables prospective semen donors to be screened and insemination doses or semen preservation methods assessed, although it is not a complete replacement for *in vivo* testing [153]. Ultimately, the best test of fertility is breeding, either natural service or artificial insemination, and determining pregnancy rate at various stages of gestation, as well as birth of live offspring. However, this approach is generally time consuming and expensive, and it is often difficult to generate large amounts of reliable data.

1.10 Conclusions

In conclusion, by inducing apoptotic and oxidative pathways in testes, HS using various experimental models suppressed spermatogenesis and detrimentally affected germ cells, as well as Leydig and Sertoli cells, resulting in deleterious effects on sperm quality, affecting sperm

motility, morphology and fertility. In addition, if fertilization was initiated, there were negative effects on developing embryos. Fortunately, there are various therapeutic agent categories that can be used to mitigate testicular HS actions. As most studies involve only one or a limited number of therapeutic approaches, it is difficult to make valid comparisons. Regardless, perhaps the most promising substances are Vitamin E, either alone or in combination with selenium, zinc or melatonin. These substances share a similar mechanism of action, namely reducing testicular oxidative stress, aiding in rapid recovery of testicular tissues and restoring sperm quality characteristics. In addition, various traditional herbal compounds also had good impacts in ameliorating HS effects; however, many of these are not broadly available. Although some of these products have considerable promise, further studies are needed to clarify their suitability for preventing or minimizing deleterious effects of HS on sperm quality, fertility and development.

Aims, hypotheses and objectives

Aim 1: Develop a method of monitoring intrascrotal temperatures and validate that method with needle thermocouples (**Chapter 2**).

Objective: Validate the use of temperature data loggers as a substitute for needle thermocouples as a gold standard measurement for intrascrotal temperatures.

Hypothesis: Measurement of intrascrotal temperature with a data logger is directly correlated with testicular temperature.

Aim 2: Determine consequences of testicular HS on sperm quality and relate that to data logger temperatures (**Chapter 3**).

Objective: Determine outcomes of testicular HS on sperm quality and relate those changes to data logger temperatures.

Hypothesis: Testicular HS has detrimental effects on sperm quality, which are related to intrascrotal temperatures recorded by data loggers.

Aim 3: Determine effects of melatonin or l-arginine supplementation in semen extender on post-thaw sperm quality in rams under testicular HS (**Chapter 4**).

Objective: Determine effects of melatonin or l-arginine supplementation on post-thaw sperm quality in rams exposed to testicular HS.

Hypothesis: Melatonin or l-arginine supplementation enhance post-thaw sperm quality in rams exposed to testicular HS.

Aim 4: Determine associations between bull location within shaded or non-shaded areas, intrascrotal and ambient temperatures and sperm quality in bulls (**Chapter 5**)

Objective: Relate intra-scrotal and ambient temperatures, bull location either in shaded or non-shaded areas to sperm quality in bulls.

Hypothesis: In hot ambient temperatures, bulls seek shade and consequently reduce intrascrotal temperature and improve semen quality.

Aim 5: Determine effects of melatonin on testes function, testicular blood flow, and sperm quality after testicular HS (**Chapter 6**).

Objective: Determine effects of melatonin on testes function, testicular blood flow, and sperm quality after testicular HS.

Hypothesis: Heat stress reduces sperm quality, whereas melatonin supplementation protects testes function, enhances testicular blood flow and lessens reductions in sperm quality after testicular HS in rams.

Aim 6: Determine effects of melatonin injection on post-thaw sperm quality after mild testicular HS in rams (**Chapter 7**).

Objective: Determine effects of melatonin post-thaw sperm quality in rams exposed to mild testicular HS.

Hypothesis: Heat stress reduces post-thaw sperm quality, whereas melatonin supplementation protects testes function, lessens reductions in post-thaw sperm quality after mild testicular HS in rams.

Chapter 1 – Published in *Theriogenology*:

Shahat AM, Rizzoto G, Kastelic JP. (2020). Amelioration of heat stress-induced damage to testes and sperm quality. *Theriogenology* 158:84-96.

Chapter 2 – Published in *Small Ruminant Research*:

Shahat AM, Thundathil JC, Kastelic (2020). Data loggers in scrotal subcutaneous tissues reliably assess intrascrotal temperatures in rams. *Small Ruminant Research*, 193:106247

Chapter 3 – Published in *Journal of Thermal Biology*:

Shahat, A. M., Thundathil, J. C., & Kastelic, J. P. (2021). Scrotal subcutaneous temperature is increased by scrotal insulation or whole-body heating, but not by scrotal neck insulation; however, all three heat-stress models decrease sperm quality in bulls and rams. *Journal of Thermal Biology*, 100:103064.

Chapter 4 – Published in *Animal Reproduction Science*:

Shahat, A. M., Thundathil, J. C., & Kastelic, J. P. (2022). Melatonin or L-arginine in semen extender mitigate reductions in quality of frozen-thawed sperm from heat-stressed rams. *Animal Reproduction Science*, 238:106934.

Chapter 5 – Published in *Canadian Journal of Veterinary Research*:

Shahat, A. M., Juan Castillo, Thundathil, J. C., & Kastelic, J. P. (2023). Angus bulls voluntarily access shade during hot weather, reducing scrotal subcutaneous temperatures and improving sperm quality. *Canadian Journal of Veterinary Research*, 87:17-22.

Chapter 6 – Published in *Theriogenology*:

Shahat, A. M., Thundathil, J. C., & Kastelic, J. P. (2022). Melatonin improves testicular hemodynamics and sperm quality in rams subjected to mild testicular heat stress. *Theriogenology*, 188:163-169.

Chapter 7 – **Shahat, A. M.,** Thundathil, J. C., & Kastelic, J. P. (2022). Melatonin improves post-thaw sperm quality after mild testicular heat stress in rams. **Accepted** in *Reproduction in Domestic Animals*.

Statement of contribution

Abdallah Mohamed, John Kastelic, and Jacob Thundathil designed the experiments. AMS and JPK performed animal studies. AMS conducted all laboratory work, data recording and analyses, and preparation of draft figures and manuscript and Juan Castillo helped in the laboratory work for Chapter 5. JPK and JCT revised the manuscripts. All authors provided intellectual feedback on the manuscripts and approved the final version.

Chapter 2: Data loggers in scrotal subcutaneous tissues reliably assess intrascrotal temperatures in rams; modified from: Abdallah M Shahat, Jacob C Thundathil, John P Kastelic (2020). Data loggers in scrotal subcutaneous tissues reliably assess intrascrotal temperatures in rams. *Small Ruminant Research*, 193:106247

2.1 Abstract

Ram testes must be ~ 4 to 5 ^{0}C cooler than core for normal spermatogenesis, emphasizing importance of intrascrotal temperatures. Our goal was to use implantable data loggers (DL; ~8 x 25 mm) to measure changes in intrascrotal temperature in rams and this was the first time to use this method in rams. We compared DL temperature (DLT) to expected and measured intrascrotal temperatures. In four Ile de France rams (11 to 12 mo of age), a DL was surgically implanted subcutaneously (ventral aspect of anterior scrotum) with minimal reactions. After healing, DLT was high from ~1900 to 0600 (when rams were recumbent), then decreased, with a smaller peak mid-day (recumbency and increased metabolism after feeding). After whole-scrotum insulation (two rams), DL temperature was increased compared to that in the other two rams in a cool (ambient <5 ℃) environment (mean ± SEM, 36.8 ± 0.4 vs 29.4 ± 0.6 ℃, respectively, $p < 0.05$). In conclusion, DL were minimally invasive and reliably assessed intrascrotal temperatures.

2.2 Introduction

That mammalian testes must be ~4 to 5 ℃ cooler than body temperature for normal and motile sperm production [8] that highlights importance of scrotal and testicular temperatures. Infrared thermography (IRT) has been used to measure scrotal surface temperature (SST),

whereas needle thermocouples have been used to measure scrotal subcutaneous (SQT) or intratesticular (ITT) temperature; all require restrained rams [154]. To monitor scrotal/testicular temperatures in unrestrained rams, we plan to implant small data loggers (DLs) to continuously monitor temperature [25] and thereby provide novel insights into thermoregulatory capacity of testes and scrotum in rams. Our objectives were to compare DL temperature (DLT) to expected and measured intrascrotal temperatures.

2.3 Materials and methods

Temperature data loggers (DL; DST Micro-T, Star-Oddi, Gardabaer, Iceland), 8.3×25.4 mm and 3.3 g and designed to be implanted, were used (Figure 2.1). The DLs were activated (using manufacturer's communication box and Mercury® software) and set to record temperature every 10 min, placed in a waterbath (28.0-40.5 °C). Thereafter, they were set to record at 15-min intervals, disinfected (2% glutaraldehyde for 10 h), rinsed with sterile water and implanted.

Four Ile de France rams, 11-12 mo of age, 55-65 kg, were group-housed indoors, at ~18-20 °C, with lights from 0700 to 1630. At 0800 and 1500, they were fed pellets and grass hay, with *ad libitum* water and salt. This study was approved by the University of Calgary Veterinary Sciences Animal Care Committee (AC19-0106) and conducted in accordance with Canadian Council on Animal Care guidelines.

Rams were given 8 μg/kg dexmedetomidine IM (Dexdomitor, Zoetis, Parsippany-Troy Hills, NJ, USA), lumbo-sacral epidural anesthesia (0.07 mg/kg xylazine in ~4 mL saline; Rompun, Bayer, Mississauga, ON, Canada), and local blocks with bupivacaine (Bupivacaine, 2.5 mg/ml, Hospira Inc., Lake Forest, IL, USA) at incision sites. A skin incision (~2 cm) was made

on the anterior scrotum, lateral to midline and ~6 cm from bottom of scrotum, blunt dissection used to create a subcutaneous 'pocket' ventral to surgery site, DL placed, and skin closed with staples (Figures 2.2). Implanting DLs on the anterior scrotum put the scrotal incision in apposition with the caudal abdomen during recumbency, to promote cleanliness. Rams were given 1 ml/10 kg oxytetracyline SQ (Bio-Mycin 200 LA, Boehringer Ingelheim, Burlington, ON, Canada), plus 0.5 mg/kg meloxicam SQ (Metacam, Boehringer Ingelheim) before surgery, with meloxicam repeated 24 h later.

For each ram, DLT for a 24-h interval on the second day after surgery was compared to average DLT for that ram over a 14-d interval, starting 11 d after surgery. In addition, the average of all four rams over the 14-d interval was calculated and inspected.

To alter scrotal and testicular temperatures, two rams were kept in a cool outdoor environment (approximately -4 to 6 ℃ for ~ 36 h), whereas the other two were kept inside, with their scrotum insulated (with disposable infant diapers) for ~36 h, with internal temperature measurements (side ipsilateral to the DL) done at those locations. After administering lumbosacral epidural anesthesia, rams were manually restrained in a vertical position and an infrared thermal imaging camera (FLIR E60 Thermal Imaging Camera; Flir, Billerica, MA, USA) used to image anterior scrotum (immediately after diaper removal), with manufacturer's software used to measure top and bottom SST in rectangles ~1.5 x 0.3 cm, ~3 cm from testis pole. Then, scrotal subcutaneous (SQT) and intratesticular (ITT) temperatures were assessed at the top and bottom of each side (~3 cm from testis pole), with a needle thermocouple (20-gauge × 2.5 cm), as described [154], with gradient equal to top minus bottom temperature. Thereafter, small incisions were made in scrotal skin and DLs recovered, and data retrieved.

A Student's *t*-test was done to compare corresponding temperature end points between cool environment versus insulation. The data expressed as mean ± SEM.

2.4 Results

In the waterbath, 42 of 64 (65.6%) of readings were ±0.3 ℃ of reference thermometer, whereas 28.1 and 6.2% were ±0.31- 0.60 and ±0.61 to 0.90 ℃, respectively (largest difference between DL and reference temperature was 0.82 ℃; Figure 2.3.). The SST and ITT (ºC) of top and bottom, SQT bottom location/s and data logger (DL) temperature were lower ($p < 0.05$) in outdoors non-insulated group than indoors insulated group (Table 2.1).

Regarding DLT on the second day after surgery, temperatures were ~1 ℃ warmer in Ram 1 (Figure 2.4) and Ram 4 (data not shown) than after healing or ~2 ℃ greater than DLT (Ram 2) after healing. For Ram 3, DLT was slightly warmer or similar on the second day after surgery than after healing.

Combined for all rams over the 14-d interval, DLT was elevated from ~1900 to ~0600, when rams were likely recumbent. There was a steep decline in association with lights on and feeding, followed by a secondary, smaller peak around noon, when rams were typically recumbent. There is a post prandial increase in SST in bulls at ~30 min to 3 h after feeding [154].

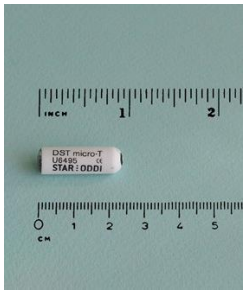


Figure 2.1. Temperature data logger (DST Micro-T, Star-Oddi, Gardabaer, Iceland).

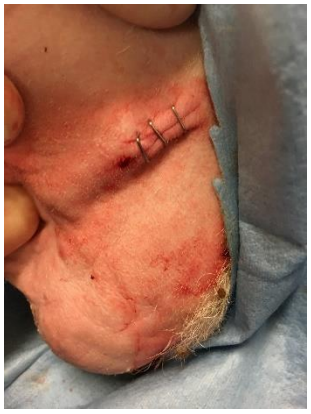


Figure 2.2. Incision closed after implantation of data logger.

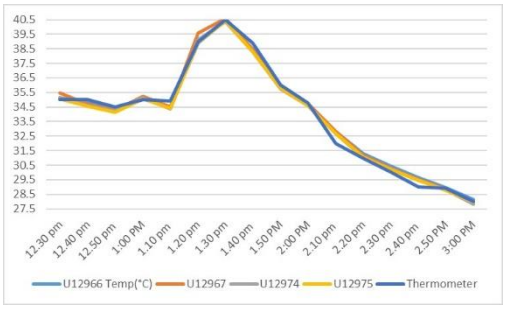


Figure 2.3. Validation of four data loggers in a waterbath.

Scrotal insulation versus a cool environment caused divergence in DLT (Figure 2.5). Insulated rams had very similar DLTs, whereas for the rams placed outside, DLT in Ram 3 was relatively stable, whereas DL in Ram 4 was much more variable; perhaps the former ram remained recumbent throughout the night, whereas the latter was up and down.

Table 2.1 Mean ± SEM scrotal surface (SST), intratesticular (ITT), scrotal subcutaneous (SQT) and data logger (DLT) temperatures and temperature gradients (°C) in two rams in a cool environment and two with scrotal insulation.

End point	**Cool**	**Insulated**	**Difference**
SST top	26.8 ± 0.2[a]	32.0 ± 0.6[b]	-5.2
SST bottom	25.2 ± 0.1[a]	31.3 ± 0.1[b]	-6.1
SST gradient	1.6 ± 0.03	0.7 ± 0.5	0.9
ITT top	32.9 ± 0.1[a]	36.2 ± 0.4[b]	-3.3
ITT bottom	33.3 ± 0.3[a]	37.0 ± 0.2[b]	-3.7
ITT gradient	-0.5 ± 0.4	-0.8 ± 0.2	0.3
SQT top	32.6 ± 0.6	33.8 ± 0.1	-1.2
SQT bottom	31.1 ± 0.02 [a]	34.9 ± 0.4[b]	-3.8
SQT gradient	1.5 ± 0.7	-1.1 ± 0.3	2.6
DLT	29.4 ± 0.6[a]	36.8 ± 0.4[b]	-7.3

[a,b]Within a row, means without a common superscript differed ($p < 0.05$).

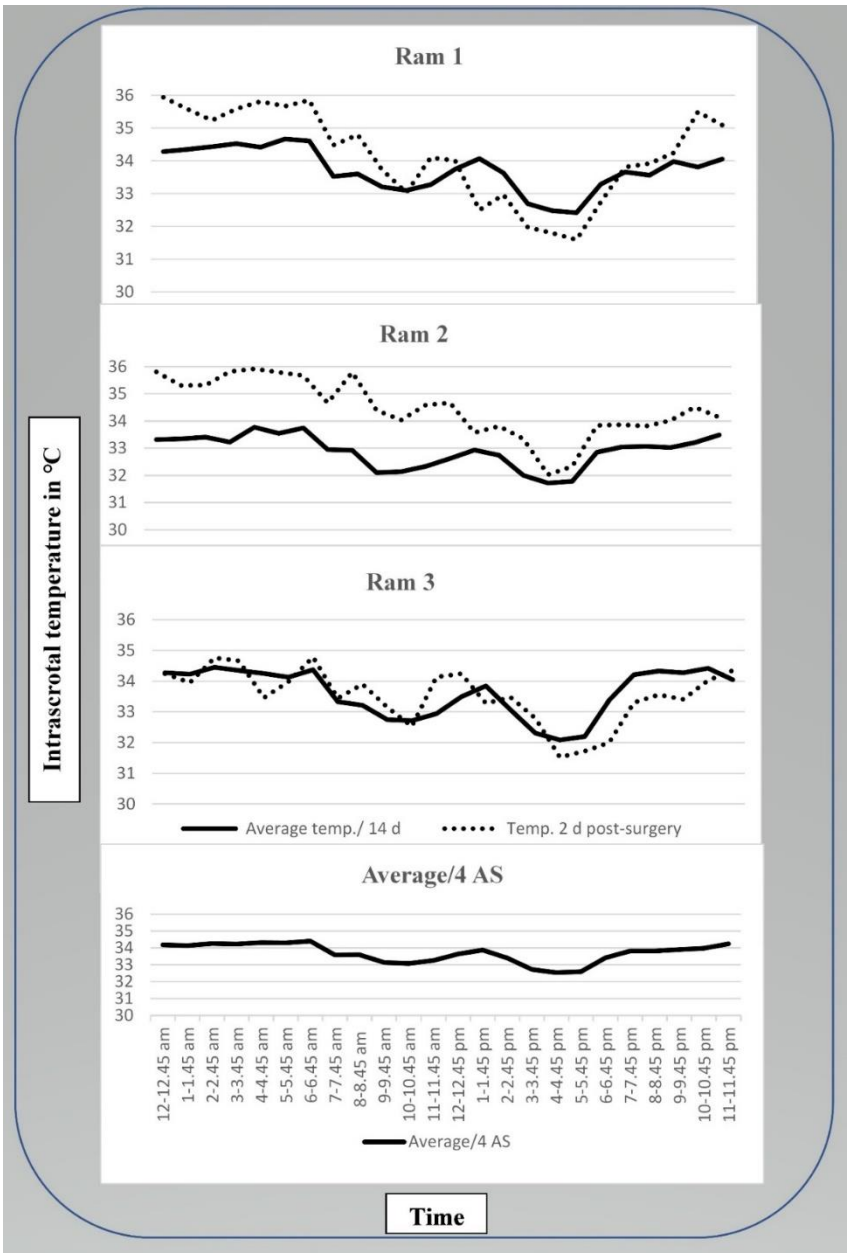


Figure 2.4. Data logger temperatures 2 d post-surgery in three rams compared to their average 14 d post-healing and average of all four rams 14 d post-healing.

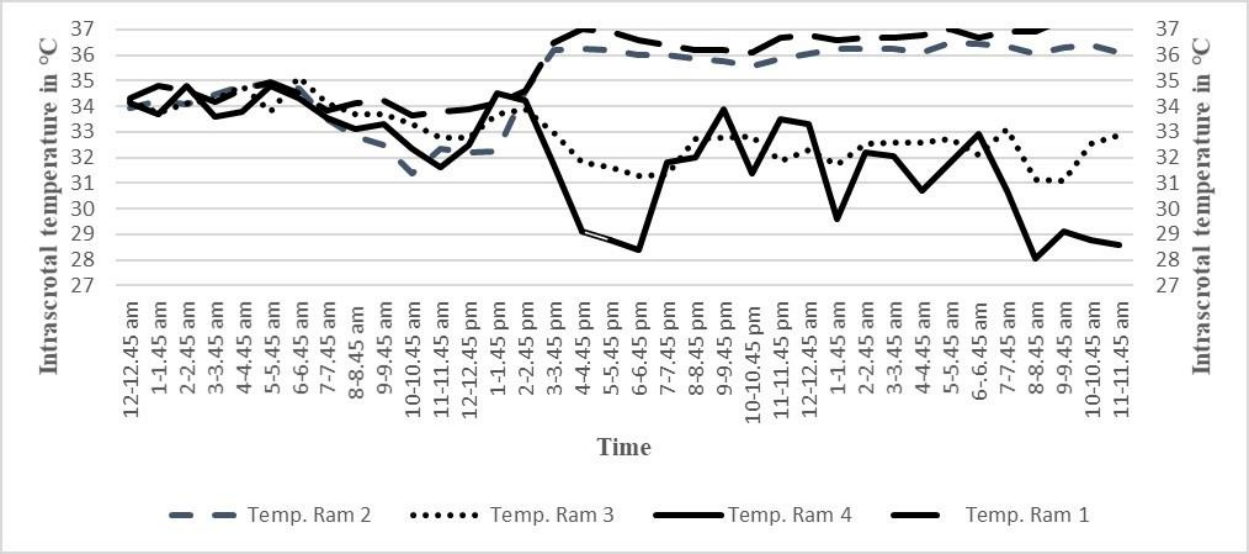


Figure 2.5. Data logger temperatures in Rams 1 & 2 after scrotal insulation and Rams 3 & 4 in a cool environment.

In both groups of rams, top SST was greater than bottom SST, resulting in positive gradient. In response to scrotal insulation, the increase in bottom SST was relatively greater than the increase in top SST, resulting in a numerical reduction in the SST gradient (Table 2.1).

2.5 Discussion

In this study, the intrascrotal temperature using DLs was significantly higher in insulated group rams in comparison with those placed in the cool environment. Moreover, both groups of rams, top SST was greater than bottom SST, resulting in positive gradient. In response to scrotal insulation, the increase in bottom SST was relatively greater than the increase in top SST, resulting in a numerical reduction in the SST gradient.

In a previous study in bulls, there were SST gradients of 3.3 and 1.0 ℃ at ambient temperatures of 5 and 35 ℃, respectively [112].

The SQT were warmer than SST, but cooler than ITT, consistent with a previous report [154] . In this study, in cooled rams the SQT gradient was positive, whereas in insulated rams, bottom SQT exceeded top SQT, resulting in a negative temperature gradient. Perhaps this was due to removing the insulation, capturing an image for SST and then measuring bottom SQT, followed by the top SQT, with the latter perhaps slightly cooling. The ITT at top and bottom had nearly similar increases after scrotal insulation (32.9 ± 0.08 and 33.3 ± 0.3 ^{0}C outside vs 36.2 ± 0.4 and 37.0 ± 0.2 insulated rams), with a negative gradient in both groups (-0.5 ± 0.4 and -0.8 ± 0.2 ^{0}C, respectively).

In this study, one of four rams had a small accumulation of clear fluid at DL removal. In six bulls with DL implanted inside the scrotal vaginal cavity, two developed scrotal hematomas that healed following antibiotic and anti-inflammatory treatments [25], with residual scar tissue but no testicular adhesions at DL removal.

2.6 Conclusion

Consistent with other temperature changes, DLT in insulated rams was much higher than rams in the cool environment. Based on those changes, as well as diurnal changes in DLT, we concluded that DL reliably assessed intrascrotal temperatures. Furthermore, that they are designated for implantation and minimally invasive makes them very well suited for our subsequent ram studies on relating intrascrotal temperature to sperm quality and fertility.

Chapter 3: Scrotal subcutaneous temperature is increased by scrotal insulation or whole-body heating, but not by scrotal neck insulation; however, all three heat-stress models decrease sperm quality in bulls and rams; modified from: Shahat, A. M., Thundathil, J. C., & Kastelic, J. P. (2021). Scrotal subcutaneous temperature is increased by scrotal insulation or whole-body heating, but not by scrotal neck insulation; however, all three heat-stress models decrease sperm quality in bulls and rams. *Journal of Thermal Biology*, 100:103064.

3.1 Abstract

Ruminant testes are ~2 to 6 ℃ below body temperature; increased testicular temperature reduces sperm motility and morphology. Models of testicular heat stress typically involve warming testes or increasing ambient temperature and monitoring sperm quality, with no or minimal monitoring of intrascrotal or testicular temperatures. Our objective was to serially monitor scrotal subcutaneous temperatures during testicular heat stress and relate those temperatures to changes in sperm quality. Two experiments were conducted, with temperature sensors surgically implanted in scrotal subcutaneous tissues recording temperatures every 15 min and semen collected and evaluated once weekly. After an initial control interval, testicular temperature was increased. In Experiment 1, in two Angus bulls, classical whole-scrotum insulation for 96 h, considered substantial testicular heat stress, increased scrotal subcutaneous temperatures by ~2.0-2.5 ℃ ($p < 0.05$). Total and progressive motility decreased ($p < 0.05$) and reached a nadir at Week 3 (by ~20 and 10%, respectively). Furthermore, morphologically normal sperm and acrosome integrity also decreased significantly, reaching nadirs at Weeks 3 (15%) and 4 (34%). In Experiment 2, 10 Dorset rams were subjected to moderate testicular heat stress.

Rams were allocated randomly into two equal groups and either: 1) exposed to 28 ℃ ambient temperature and 30-34% relative humidity (RH) for 8 h/d for 4 d; or 2) subjected to scrotal neck insulation that was applied and removed at the same time as the start and completion, respectively, of heat exposures in the other rams. Scrotal subcutaneous temperatures (monitored in three rams in each group) were increased in response to whole-body heating (~0.8 ^{0}C, $p <$ 0.05), but not significantly changed by scrotal neck insulation. Decreases in sperm quality in rams were generally similar between treatments, with the most profound changes evident 4 wk after heat stress, with ~10 percentage point reductions in both total and progressive motility and ~10 and 20 percentage point decreases in morphologically normal sperm in rams with whole-body heating versus scrotal neck insulation, respectively. In conclusion, scrotal subcutaneous temperature was significantly increased by scrotal insulation or whole-body heating, but not by scrotal neck insulation; however, all three heat-stress models decreased sperm motility and morphology in bulls and rams.

3.2 Introduction

Heat stress (HS) has deleterious effects on animal health and reproduction. As a consequence, climate change and frequent peaks of high ambient temperature in many countries are likely to reduce food-animal productivity [155,156]. Heat stress occurs when an animal is subjected to temperatures that exceed its physiological range and compensatory ability. Testes are especially vulnerable to HS because spermatogenesis requires mammalian testes to be ~2 to 6 ℃ below body temperature [157]. Consequently, increasing testicular temperature, either acute

or chronic, reduces sperm concentration, motility, viability, normal morphology, acrosome integrity, and chromatin stability, resulting in transitory infertility [51].

Numerous scrotal insulation studies have been done [101,103,105,106], but none monitored intrascrotal temperature. Scrotal or testicular temperatures have been monitored to assess effects of heat load on scrotal thermoregulation; however, most of these studies were one-time measurements, or at most, two or more times over a short interval, e.g., 1 d [8,23,24,53]. Only two studies used small, surgically implanted data loggers (DLs) to serially monitor intrascrotal temperatures in unrestrained bulls [25] and rams [26]. The latter study involved whole-scrotum insulation and was done to validate the measurement of scrotal subcutaneous temperature measurements with DLs, although semen collection and evaluation were not performed.

In general, models of testicular HS use one of two methods: heating the whole body or heating only the testes. Perhaps the most realistic model of HS is to expose animals to a warm environment. However, there are two crucial factors. First, the body reacts to heat stress in a variety of ways; important physiological, metabolic, and endocrinological changes may have indirect effects on the testis. Second, an animal's ability to produce sweat is influenced by its previous exposure to heat, so testicular temperature can vary significantly in response to given heat exposure [51].

Local testes heating is usually achieved in one of three ways: induced cryptorchidism, scrotal insulation, or short-term heating (the latter usually by immersion in a water bath). Scrotal insulation is the most common method in farm animals to simulate increased testicular temperature, both by retaining heat and interfering with scrotal sweating [52]. Although this

model has been widely used, the extent of testicular warming was usually not reported, making it difficult to compare results across studies [156]. Scrotal neck insulation has also been occasionally used and appears to cause a smaller increase in testicular temperature and less profound reductions in sperm quality [101,154].

Our objective was to continuously monitor scrotal subcutaneous temperatures during HS and relate those temperatures to changes in sperm quality. In Experiment 1, we applied whole-scrotum insulation (considered substantial HS) to bulls, whereas in Experiment 2, our objective was to compare two models of moderate HS in rams, namely scrotal neck insulation and whole-body heating. In both studies, DLs were used to serially monitor scrotal subcutaneous temperature and sperm quality and motility were assessed once weekly.

3.3 Materials and methods

3.3.1 General

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). These experiments were approved by the University of Calgary Veterinary Sciences Animal Care Committee (AC19-0106) and conducted in accordance with Canadian Council on Animal Care guidelines.

Temperature DL (DST Micro-T, Star-Oddi, Gardabaer, Iceland), 8.3 × 25.4 mm and 3.3 g and designed to be implanted, were used to continuously monitor scrotal subcutaneous temperatures. The DLs were activated (using the manufacturer's communication box and Mercury® software), set to record temperature every 10 min, and placed in a waterbath (28.0-

40.5 °C) to verify function. Thereafter, they were set to record at 15-min intervals, disinfected (2% glutaraldehyde for 10 h), rinsed with sterile water and surgically implanted.

3.3.2 Experiment 1: Bulls

Two adult Angus bulls (4 to 5 y), approximately 600 kg, were housed together in a small outdoor paddock, with *ad libitum* access to hay, water and salt and they were used during the breeding season. Immediately prior to surgical implantation of DLs, bulls were given 1 ml/10 kg oxytetracyline SQ (Bio-Mycin 200 LA, Boehringer Ingelheim, Burlington, ON, Canada), 0.5 mg/kg meloxicam SQ (Metacam, Boehringer Ingelheim), caudal epidural anesthesia (0.07 mg/kg xylazine in ~5 mL saline; Rompun, Bayer, Mississauga, ON, Canada) in the 1st intercoccygeal intervertebral space and local subcutaneous blocks with bupivacaine (Bupivacaine, 2.5 mg/ml, Hospira Inc., Lake Forest, IL, USA) at incision sites. Two skin incisions (~2 cm) were made on the posterior scrotum, one near the top of the testis and the second near the bottom (above the epididymis), lateral to the midline, with blunt dissection used to create a subcutaneous 'pocket' ventral to the surgery site, a DL placed in each site and skin closed with staples.

Starting 10 d after DLs were implanted, semen was collected weekly using an electro-ejaculator. After both bulls were collected for 2 wk as control samples, both bulls were subjected to whole-scrotum insulation (one layer each of disposable baby diapers and cloth taped around the scrotum) for 4 d. After heat treatment, semen was collected once weekly for 7 wk. Semen was transported to the laboratory and evaluated using CASA (Sperm Vision®), morphology using eosin-nigrosin, and acrosome integrity using FITC-PSA.

Once semen collection was completed, to enable retrieval of the DLs, small incisions were made in the scrotal skin using identical procedures as described above. Temperature data were

subsequently retrieved using the Mercury software. All DLs were working, there were no missing data and no signs of inflammation or reactions where DLs were located.

3.3.3 Experiment 2: Rams

Ten Dorset rams, 3 adults (3 to 4 y; 90-100 kg) and 7 juveniles (1 to 1.5 y; 65 to 80 kg) were housed together indoors, at ~18-20 °C, with lights going on at 7:00 am and going off at 4.30 pm. At approximately 8:00 am and 3:00 pm, they were fed pellets and grass hay, with *ad libitum* access to water and salt.

Six of the juvenile rams were given 8 μg/kg dexmedetomidine IM (Dexdomitor, Zoetis, Parsippany-Troy Hills, NJ, USA), lumbo-sacral epidural anesthesia (0.07 mg/kg xylazine in ~4 mL saline; Rompun, Bayer, Mississauga, ON, Canada), and local blocks with bupivacaine (Bupivacaine, 2.5 mg/ml, Hospira Inc.) at incision sites. A skin incision (~2 cm) was made on the anterior scrotum, lateral to the midline and ~6 cm from the bottom of the scrotum, blunt dissection used to create a subcutaneous 'pocket' ventral to the surgery site, a DL placed, and the incision closed with staples. Implanting DLs on the anterior scrotum put the scrotal incision in apposition with the caudal abdomen during recumbency, to promote cleanliness [26]. Rams were given 1 ml/10 kg oxytetracyline SQ (Bio-Mycin 200 LA), plus 0.5 mg/kg meloxicam SQ (Metacam) before surgery.

Semen samples were collected weekly using a mount ewe in estrus or, if that was not successful, an electroejaculator. At 10 d after implanting DLs, semen was collected and evaluated from the 10 rams for 1 wk as a control sample. Then, rams were allocated randomly into two groups, each with five rams; one group was exposed to whole-body heating at 28 °C ambient temperature and 30 to 34% relative humidity for 8 h daily (from 8:00 am to 4:00 pm) for

four consecutive days and we recorded the rectal temperature for those rams before and after heating time (table 3.1). For the other group, scrotal neck insulation (using approximately six layers of flannel cloth material encircling the scrotal neck and held in place with tape) was started concurrent with placement of the other group in the warm room for the first time and removed after 3.5 d, concurrent with removal of the other rams from the heated room on the last day of heat exposure. With the exception of when rams were exposed to whole-body heating, all rams were housed together in a single group.

After heat treatment, semen was collected once weekly from all rams for 5 wk, with evaluations for motility using CASA (Sperm Vision®), morphology using eosin-nigrosin, and acrosome integrity using FITC-PSA. Thereafter, DLs removal and data retrieval were as done in Experiment 1. All DLs were working, with no missing data and no signs of inflammation or swelling at the sites where DLs were implanted.

3.3.4 Statistical analyses

A Mixed Linear Model was done to compare scrotal subcutaneous temperatures before and during heat treatment in both experiments. In addition, the same statistical model was used to compare the two groups (whole body heating and scrotal neck insulation) in Experiment 2. A General Linear Model (univariate analysis) was done to compare corresponding semen evaluation end points among control and whole-scrotum insulation in Experiment 1 and whole-body heating and scrotal neck insulation groups in Experiment 2, with a *post-hoc* Bonferroni test used to locate differences.

3.4 Results

3.4.1 Experiment 1: Bulls

Scrotal subcutaneous temperatures were ~2-2.5 ℃ warmer ($p < 0.05$) during HS compared to before HS (Figure 3.1). Total and progressive motility decreased after whole-scrotum insulation, reaching a nadir at Week 3 (by ~20 and 10%, respectively); thereafter, both began to increase starting from Week 5 but had not returned to pre-treatment at the end of the experiment (Week 7, Figure 3.2). Similarly, morphologically normal sperm and acrosome integrity also decreased dramatically after whole-scrotum insulation, reaching nadirs at Weeks 3 (15%) and 4 (34%), respectively (Figure 3.2). All morphological abnormalities (Figure 3.3) began to increase after HS, with most peaking at 3 or 4 wk after HS, then started to decrease, reaching baselines at Weeks 6 or 7. However, midpiece defects increased until Week 2, with a minor reduction at Week 3 then a peak at Week 7 (end of the experiment).

3.4.2 Experiment 2: Rams

For scrotal subcutaneous temperatures, there were effects of treatment, time period (9:00 am-4:45 pm) and group, and interactions of time period*group, treatment*time period, and treatment*time period*group. In the whole-body heating group, there was a rise in temperature during HS (~0.8 ^{0}C, $p < 0.05$) compared to the same time frame prior to HS (Figure 3.4A). However, in rams with scrotal neck insulation, scrotal subcutaneous temperatures were not significantly different between prior to HS versus during HS (Figure 3.4B).

For acrosome integrity and total motility (Figure 3.5), there were effects of week ($p < 0.001$ and $p < 0.05$, respectively), whereas for total motility, there was an effect of group ($p <$

0.03) and for progressive motility, only an effect of group ($p < 0.003$). Regarding morphological abnormalities (Figure 3.6), proximal cytoplasmic droplets were increased in the whole-body heating group ($p < 0.05$), peaking at Week 2 (8%) compared to scrotum-neck insulation (3%). Distal cytoplasmic droplets were increased ($p < 0.05$) in scrotum-neck insulation, peaking at Week 4 (9%) compared to 7% at Week 2 for the whole-body heating group. Furthermore, midpiece defects peaked (9%) at Week 3 for whole-body heating and at Week 4 for the scrotum-neck insulation group. Moreover, ruffled acrosomes peaked (12%) at Week 5 in the whole-body heating group, whereas in the scrotum-neck insulation group, they peaked (8%) at Week 4.

3.5. Discussion

In this study, whole-scrotum insulation in bulls profoundly increased scrotal subcutaneous temperature; this decreased sperm quality parameters (reductions in total and progressive motility and in acrosome integrity and increases in various sperm morphological abnormalities). Our results were consistent with previous reports in which whole-scrotum insulation decreased sperm production and semen quality in bulls [100,101,105]. To our knowledge, this was the first report to describe serial recording of intrascrotal temperature in the bull during scrotal insulation, with concurrent collection of semen and assessment of sperm quality.

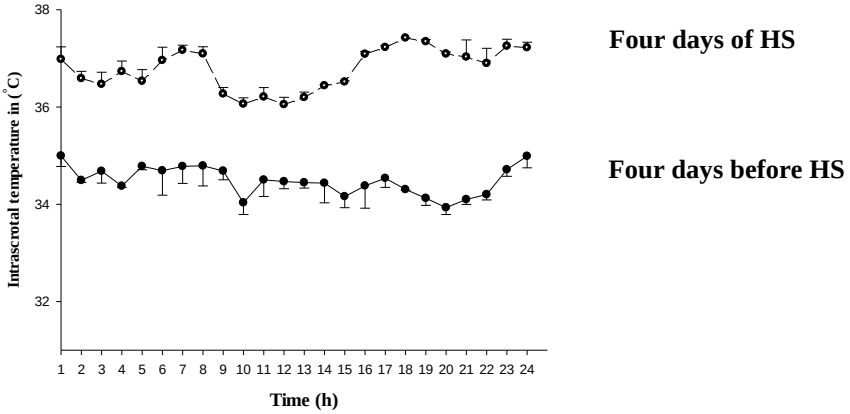


Figure 3.1. Mean and SEM scrotal subcutaneous temperatures in two bulls before and during whole-scrotum insulation. Difference ($p < 0.05$) during HS compared to before HS.

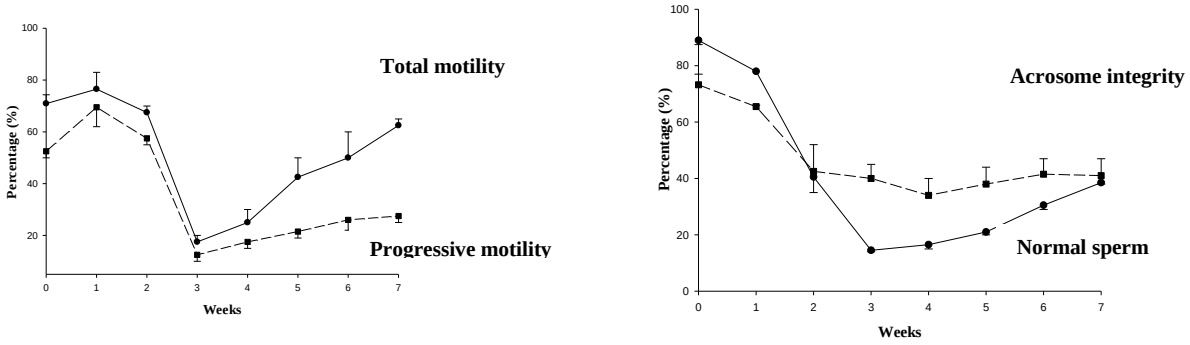


Figure 3.2. Mean and SEM motility and sperm characteristics before and after whole-scrotum insulation in two bulls. Difference ($p < 0.05$) during HS compared to before HS.

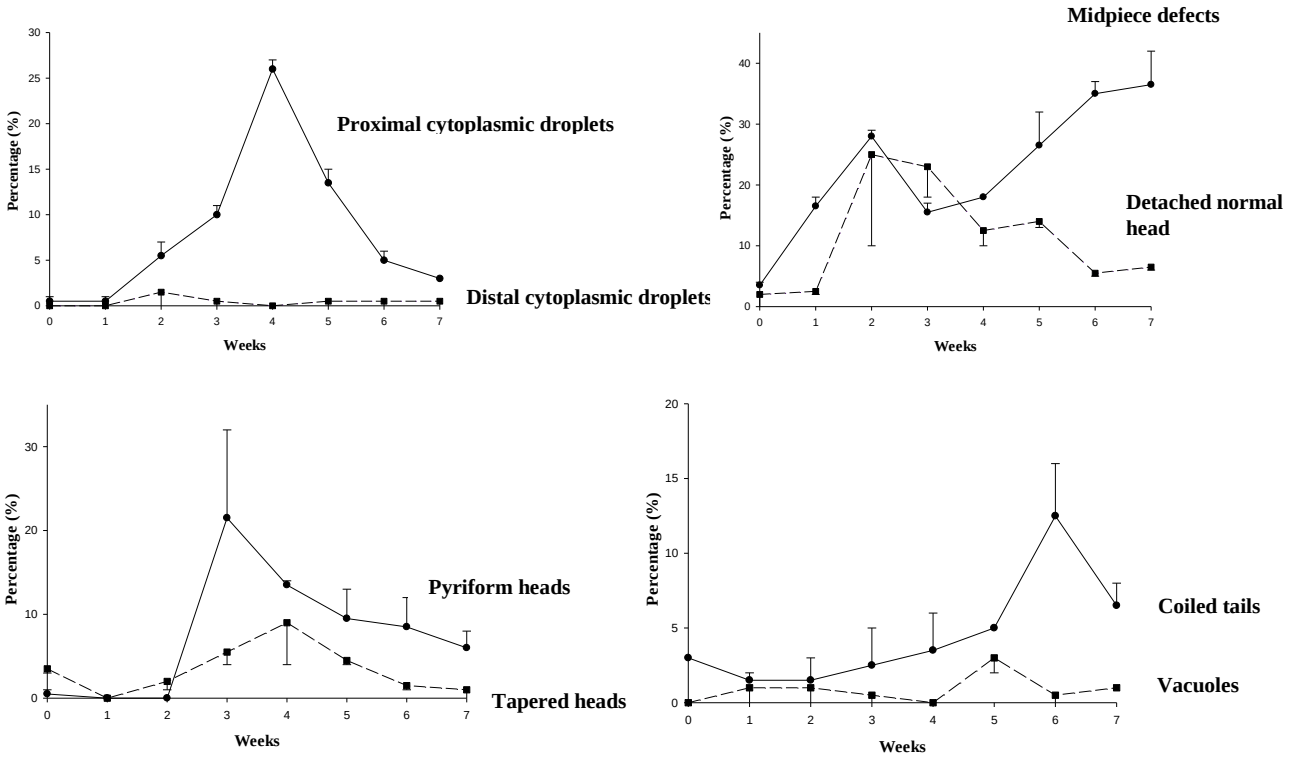


Figure 3.3. Mean and SEM of various sperm morphological abnormalities before and after whole-scrotum insulation in two bulls. Difference ($p < 0.05$) during HS compared to before HS.

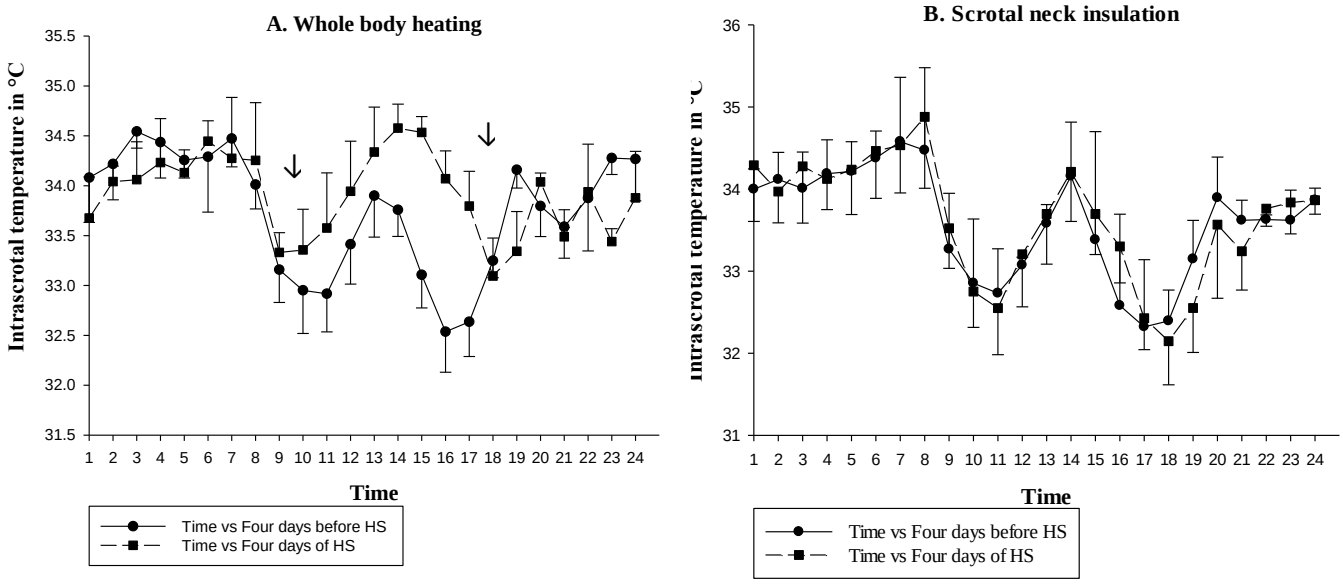


Figure 3.4A and 4B. Mean and SEM scrotal subcutaneous temperatures in rams before and during whole-body (WB; 5 rams) heating and scrotal neck insulation (SI; 5 rams). Arrows indicate the start and cessation of whole-body heating. There were effects of effects of treatment ($p < 0.015$), time period (9:00 am-4:45 pm; $p < 0.01$) and group ($p < 0.001$), and interactions of time period*group ($p < 0.038$), treatment*time period ($p < 0.001$), and treatment*time period*group ($p < 0.003$).

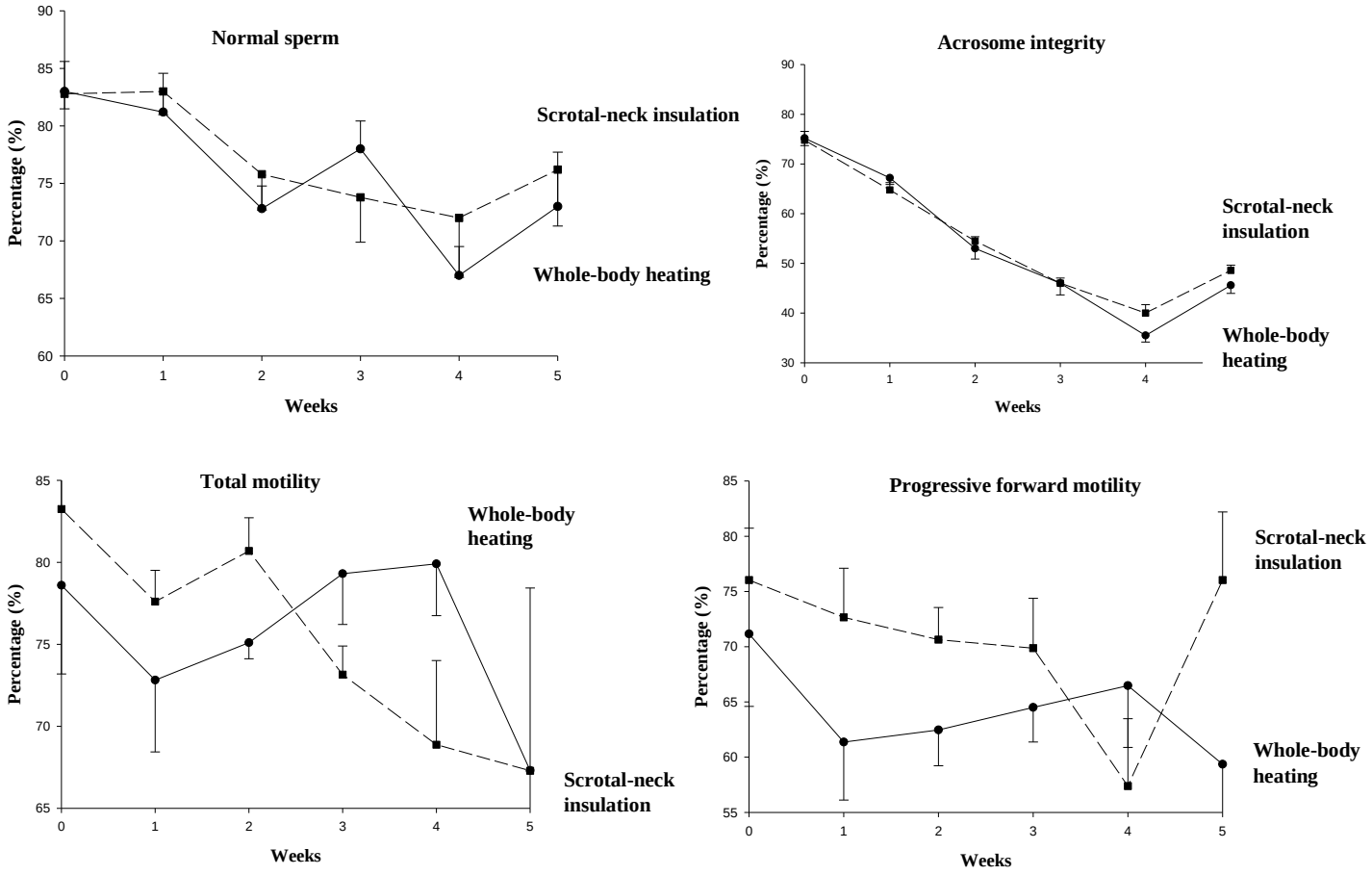


Figure 3.5. Mean and SEM sperm characteristics in rams before and during scrotal neck insulation and whole-body heating (five rams per group). For acrosome integrity and total motility, there were effects of week ($p < 0.001$ and $p < 0.05$, respectively), whereas for total motility, there was an effect of group ($p < 0.03$) and for progressive motility, only an effect of group ($p < 0.003$).

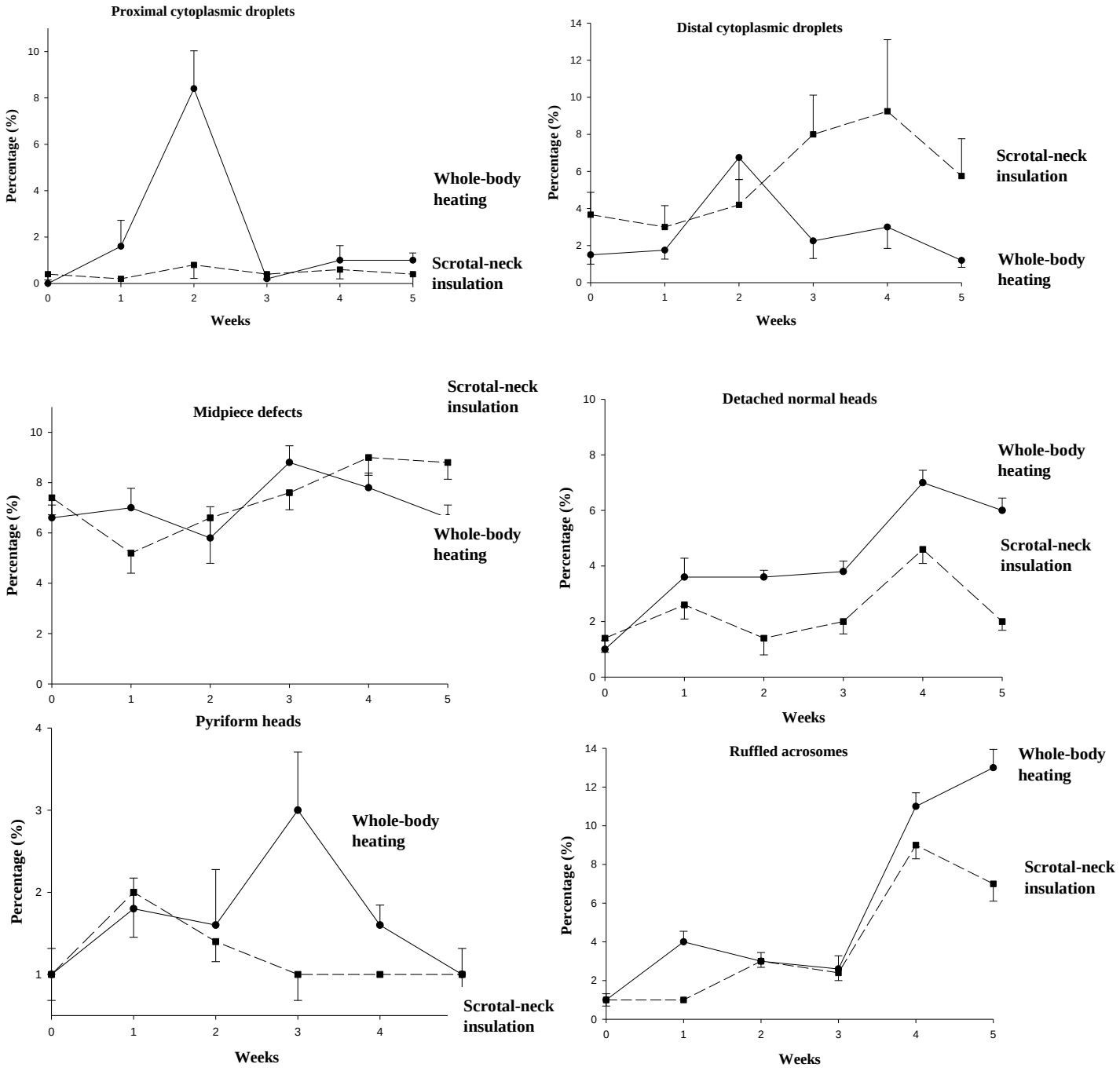


Figure 3.6: Mean and SEM of various sperm morphological abnormalities in rams before and during scrotal neck insulation and whole-body heating (five rams per group). Difference ($p < 0.05$) between whole-body heating group compared to scrotal neck insulation group.

Table 3.1: Rectal temperatures (°C) in five rams subjected to whole-body heating.

Ram	First day		Second day		Third day		Fourth day	
	Before HS	After HS	Before HS	After HS	Before HS	After HS	Before HS	After HS
1	39.0	40.0	38.8	39.5	38.8	39.3	40.0	40.2
Δ	1.0		0.7		0.5		0.2	
2	38.6	39.3	38.8	39.3	38.9	39.3	38.2	39
Δ	0.7		0.5		0.4		0.8	
3	39.3	40.2	39.3	39.9	39.1	39.5	39.3	39.7
Δ	0.9		0.6		0.4		0.4	
4	38.7	39.3	38.6	39.2	38.6	39.1	39.0	39.1
Δ	0.6		0.6		0.5		0.1	
5	38.8	39.9	38.9	40.0	38.6	39.6	38.9	39.5
Δ	1.1		1.1		1.0		0.6	

Δ = After HS – Before HS

Previous studies that involved serial monitoring of intrascrotal temperatures in bulls [25] and rams [26] did not concurrently assess sperm quality.

The proportion of total and progressive motile sperm in bulls declined starting from Week 2 and reach a nadir at Week 4, consistent with previous reports [98,99,101]. However, in the present study, it took ~50 d to reach pre-treatment values, compared to 30-42 d in those

previous studies. Furthermore, although acrosome integrity reached a nadir at Week 4, it was not fully restored by the end of the experiment (Week 7). In addition, morphologically normal sperm reached the lowest level at Week 3 but had not returned to pre-treatment levels at the end of the experiment. These differences between studies on the effect of HS on sperm quality or interval to recovery were attributed to differences in extent and duration of the HS model used [156]. An important limitation of this study was the limited sample size (only two bulls). Consequently, these preliminary results should be verified by additional studies involving more bulls.

Whole-body heating as a moderate HS model increased scrotal subcutaneous temperature during exposure to the warm room, with expected decreases in sperm quality after HS. However, for scrotal neck insulation, despite no changes in scrotal subcutaneous temperature, there was a similar decrease in sperm quality compared to whole-body heating. In a previous report [154], scrotal neck insulation in bulls did not change scrotal surface temperature but did increase scrotal subcutaneous and testicular temperatures. This difference could be attributed to species differences, as sheep are less sensitive to HS compared to cattle due to higher thermoregulatory capacity [158,159]. Furthermore, it could be due to differences in materials used to insulate the scrotal neck and as a consequence, differences in the extent of the HS and body response. Another possibility was that the scrotal neck insulation model used in our study did not impair testicular thermoregulatory ability. In that regard, in a study in bulls on fat accumulation around testicular vascular cones that resembled scrotal neck insulation, there were no significant correlations between vascular cone fat thickness and scrotal temperature, sperm quality, or sperm production [160]. Finally, perhaps scrotal neck insulation was stressful, increasing cortisol concentrations, decreasing LH and testosterone concentrations, and reducing sperm quality despite no changes in scrotal subcutaneous temperature, consistent with findings in bulls [99].

Both moderate HS models used in our ram study altered sperm quality parameters (total and progressive forward motility, morphological abnormalities, and acrosome integrity) with slight differences between the two methods, except for progressive forward motility that was significantly lower for whole-body warming compared to the scrotal neck insulation model.

3.6 Conclusion

In conclusion, scrotal subcutaneous temperature was significantly increased by scrotal insulation or whole-body heating, but not by scrotal neck insulation. Regardless, all three HS models decreased sperm motility and morphology in bulls and rams, with the extent of the decrease commensurate with the degree of testicular HS.

Chapter 4: Melatonin or L-arginine in semen extender mitigate reductions in quality of frozen-thawed sperm from heat-stressed rams; modified from: Shahat, A. M., Thundathil, J. C., & Kastelic, J. P. (2022). Melatonin or L-arginine in semen extender mitigate reductions in quality of frozen-thawed sperm from heat-stressed rams. *Animal Reproduction Science*, 238:106934.

4.1 Abstract

Our objective was to determine effects of melatonin or L-arginine on quality of frozen-thawed sperm from heat-stressed (HS) rams. Ten Dorset rams were randomly allocated to either scrotal neck insulation for 3.5 d or whole-body heating (28 °C and 30-34% relative humidity (RH) for 8 h/d for 4 consecutive days). Semen was collected before HS then once weekly for 1 to 5 wk, extended (Steridyl CSS One Step ®), and divided into five aliquots: control (no additive) or either 0.5- or 1-mM of either melatonin or L-arginine. For total and progressive motility (CASA), there were effects of group ($p = 0.023$ and $p = 0.008$, respectively); for morphological abnormalities (eosin-nigrosin), effects of group ($p = 0.01$) and a group*week interaction ($p = 0.03$); and for acrosome integrity (FITC-PSA), effects of group ($p = 0.046$) and week ($p = 0.001$). All four treatments improved motility (by ~5 to 10 percentage points), whereas 1 mM of either compound reduced abnormalities and improved acrosomal integrity (by ~7 and 12 percentage points, respectively better than control). For superoxide dismutase and catalase, there were effects of week ($p = 0.01$ and $p = 0.045$, respectively), with 1 mM of either additive yielding best results. For DNA fragmentation index (DFI%), there was an effect of week ($p = 0.01$), and a group*week interaction ($p = 0.05$), with all 4 treatments reducing DFI%. For total

ROS%, there was an effect of week (p = 0.044) and a group*week interaction (p = 0.037), with 1 mM melatonin or L-arginine being best. The hypothesis that melatonin or L-arginine improve quality of frozen-thawed sperm from HS rams was supported; 1 mM of either gave best results, except 0.5 mM minimized DFI%.

4.2 Introduction

Heat stress (HS) has detrimental effects on animal health and reproduction [155,161]; it occurs when an animal is exposed to temperatures exceeding its physiological range and compensatory ability. Typical models of testicular HS are either whole-body or localized testes heating. Although a warm environment may be the most realistic model of HS, in farm animals, scrotal insulation is the most common model used to increase testicular temperature [52]. Other approaches to localized testes heating include induced cryptorchidism or short-term heating (e.g., immersion in warm water).

The extent and duration of testicular heating determine changes induced by increased testicular temperature. A modest, transient increase in testicular temperature may cause a short-duration decrease in sperm quality; chronic and/or severe testicular heating is likely to cause infertility; and a prolonged, severe thermal insult may halt spermatogenesis [10]. When testicular temperature rises, sperm morphology often remains initially normal, followed by emergence of morphologically abnormal sperm [95]. Bovine epididymal sperm, for example, were negatively affected by 48 h of scrotal insulation, with reductions in sustained motility and acrosomal integrity apparent after cryopreservation [97,98].

The major pathways by which HS acts on germ cells, as well as Leydig and Sertoli cells, are induction of apoptosis and oxidative processes, with negative effects on sperm quality,

including motility, morphology, and fertility [10,61,156]. However, antioxidants have been protective [79].

Melatonin (N-acetyl-5-methoxytryptamine) is a small neurohormone produced and secreted primarily by the pineal gland [162] and also present in many other extra-pineal tissues including the gastrointestinal tract, thymus, immune system cells, spleen, liver, kidney, heart, ovary, placenta, and testes [163,164]. It has strong antioxidant capabilities, due to its remarkable efficacy as a hydroxyl radical (•OH) scavenger [165,166]. Melatonin can also detoxify various reactive oxygen and nitrogen species (ROS and RNS), including singlet oxygen (O2), nitrogen oxide (NO), and the ONOO anion, as well as associated metabolites, including peroxynitrous acid and hydrogen peroxide [167]. Furthermore, it stimulates enzymes involved in responses to oxidative stress and maintains cell membrane fluidity [168,169]. In addition, as a sperm cryoprotectant, melatonin had beneficial effects on sperm function and morphological characteristics by reducing excessive free radical generation [170,171]. *In vitro,* adding melatonin to semen extender improved total and progressive motility, viability, integrity of DNA and sperm membrane, and it also reduced membrane lipid peroxidation and modulated sperm capacitation [132,172,173].

L-arginine is a key amino acid for protein synthesis, regarded as a semi-essential or essential amino acid. As an NO precursor, it acts as a free radical scavenger and pro-oxidant enzyme inhibitor [174]. Adding L-arginine as an antioxidant was recently considered for sperm cryopreservation [175]. Furthermore, it has been added to the diet as a potential treatment for reducing seasonal infertility due to increased temperatures, particularly in boars [176].

Apparently neither melatonin nor L-arginine have been added to semen extender to improve quality of frozen-thawed sperm from HS males. Our objective was to determine effects of melatonin or L-arginine on the quality of frozen-thawed sperm from HS rams; we tested the hypothesis that either melatonin or l-arginine improve the quality of these sperm.

4.3 Materials and methods

4.3.1. Animals and chemicals

Unless otherwise indicated, all chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA). This experiment was approved by the University of Calgary Veterinary Sciences Animal Care Committee (AC19-0106) and conducted in accordance with Canadian Council on Animal Care guidelines. Ten Dorset rams, three adult and seven juvenile rams (3-4 y, 90 to 100 kg and ~1 y, 65 to 80 kg, respectively), were group-housed indoors, at ~18-20 °C, with lights on from 07:00 to 16:30. At approximately 08:00 and 15:00, they were fed pellets and grass hay, with *ad libitum* access to water and salt.

4.3.2. Experimental design

Semen samples were collected weekly using either a mount ewe and artificial vagina or an electroejaculator [177]. We compared seminal samples obtained by artificial vagina and electroejaculation and there was no difference in sperm quality (data not shown). Semen was collected from the 10 rams for 1 wk as a control sample (semen from each ram was kept separate, with no pooling of ejaculates). After semen was extended (Steridyl CSS One Step ®; Minitube Canada Ltd., Ingersoll, ON, Canada), it was put in a refrigerator for ~3 h until it reached + 5 °C, then loaded into 0.5 ml straws, held horizontally 5 cm above liquid nitrogen in a

Styrofoam box for 10 min, and finally plunged into liquid nitrogen (-196 ℃) and subsequently stored there [178].

Rams were allocated randomly into two groups, each with five rams; one group was exposed to whole-body heating at 28 ℃ ambient temperature and 30 to 34% relative humidity for 8 h daily (from 09:00 to 17:00) for 4 consecutive days. For the other group, scrotal neck insulation (using approximately six layers of flannel cloth material encircling the scrotal neck and fastened with medical tape) was started concurrent with placement of the other group in the warm room for the first time and was removed after ~3.5 d, concurrent with removal of the other rams from the heated room on the last day of heat exposure. Except for when rams were exposed to whole-body heating, all rams were housed together in a single group.

Following HS, semen was collected once weekly from all rams for 5 wk (semen from each ram was kept separate, with no sample pooling), evaluated for motility using CASA (Sperm Vision®), morphology using eosin-nigrosin, and acrosome integrity using FITC-PSA, then extended (Steridyl CSS One Step ®) and divided into five aliquots: no additives (Control) or 0.5 or 1 mM of either melatonin or L-arginine. Then, semen was cryopreserved as described above. Melatonin was dissolved in DMSO and PBS (the final concentration of DMSO in all samples was 0.1%; Succu et al., 2011), whereas L-arginine was dissolved in miliQ water [175].

4.3.3. Sperm evaluation

Straws were thawed at 37 ℃ for 35 s (2 straws/treatment/wk/ram) and immediate post-thaw motility (total and progressive forward) evaluated using CASA (Sperm Vision®; [179], morphology using eosin-nigrosin (200 sperm were evaluated), and acrosome integrity using FITC-PSA ([180]; 200 sperm were evaluated). Regarding morphological defects, we assessed

proximal and distal cytoplasmic droplets, distal midpiece reflex (DMR), bowed midpiece, pyriform and tapered heads, detached normal and abnormal heads, microcephalic head, coiled and double tails, knobbed and ruffled acrosomes.

4.3.3.1 Biochemical assays

For catalase (CAT) and superoxide dismutase (SOD), semen samples were centrifuged at 4 °C at 250 *g* for 10 min to isolate sperm. The pellet was washed using ~1 ml of ice-cold PBS. Then, cells were immediately homogenized by repeated pipetting on ice. After centrifugation for 15 min at 4 °C at 10,000 *g*, the supernatant was recovered and kept at -80 °C. Enzyme activities were determined using commercial kits (Catalase Activity Assay Kit, colorimetric, Abcam83464, CA and SOD Colorimetric Activity Kit, Invitrogen Life Technologies, Carlsbad, CA, USA) by spectrophotometry at 570 and 450 nm, respectively. All samples were done in duplicate.

4.3.3.2 Measurement of sperm characteristics using flow cytometry

For DNA integrity evaluation, a sperm chromatin structure assay (SCSA) was conducted using a BD™ LSR II flow cytometer (BD Biosciences, East Rutherford, NJ, USA). Flow rate was set to 200 events/s, with 10,000 events counted per sample and all samples done in duplicate. The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. Detectors 1 and 3 (FL1 and FL3) were used for detection of green (515-530 nm) and red fluorescence (>630 nm), respectively. Two straws of each semen sample were thawed (37 °C, 35 s) and pooled. Immediately after thawing, pooled samples were extended to a final concentration of 2×10^6 sperm per mL using TNE buffer (0.01 M TRIS-HCl, 0.15 M NaCl, and 1 mM EDTA; pH 7.4). For each sample, DNA fragmentation was evaluated using SCSA, as

described [181]. Acid-induced denaturation of DNA *in situ* was achieved by adding 400 µL of acid-detergent solution (0.1% v:v Triton X-100, 0.15 M NaCl, and 0.08 N HCl; pH 1.2) to 200 µL of extended semen sample. After 30 s, sperm were stained by adding 300 µL of acridine orange (AO) staining solution containing 10 mg purified AO (Acridine Orange, AO, chromatographically purified) per mL of buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, and 0.15 M NaCl; pH 6.0). Stained sperm were incubated on ice for 3 min before flow cytometric analysis. The mean extent of sperm DNA fragmentation (DNA fragmentation index; DFI), calculated by the ratio red/(red + green) fluorescence multiplied by 1000, and the percentage of sperm outside the main population (%DFI, %) in each sample were determined as described [182]. For each sample, there were two controls, both negative (control semen samples without any heat treatment) and positive (heat-treated sperm; sperm from HS males was heated to 95 ℃ then cooled suddenly to 5 ℃ on ice).

For total reactive oxygen species (ROS), ~3×10^6 sperm were centrifuged at 400 *g* for 5 min, yielding a working concentration of 1 x 10^5 sperm/sample. Then, an equal volume of the ROS Detection Solution (provided with the Total ROS Assay kit; Total ROS Assay Kit 520 nm, Invitrogen Life Technologies) was added, and the resulting solution incubated under standard tissue culture conditions (37 °C incubator with 5% CO_2) for 30 min to 1 h with periodic shaking. Thereafter, cells were centrifuged at 400 *g* for 5 min to remove ROS Detection Solution. Total ROS was analyzed using a Total ROS Assay Kit 520 nm (Invitrogen Life Technologies) on BD™ LSR II flow cytometer (BD Biosciences) at 520 nm using the FITC channel. All samples were done in duplicate. The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. Flow rate was set to 200 events/s, with 10,000 events counted per sample and all samples done in duplicate. The ROS Detection Solution contains a

fluorescent probe that stains ROS in sperm, with results expressed as a percentage of sperm that had high ROS activity.

4.3.4 Statistical analyses

Normality of data distributions was evaluated, and repeated measures ANOVA used to analyse data, including main effects (treatment, group, and week) and their interactions. General Linear Model (univariate analysis) was used to compare semen evaluation end points between whole-body heating and scrotal neck insulation groups, and between semen collected by artificial vagina versus electroejaculation. A Bonferroni *post-hoc* test was used to compare between groups within each week. Data variance was uniform (based on Levene's tests). Results are reported as mean ± standard error of the mean (SEM). For all analyses, $p < 0.05$ was considered significant. The IBM SPSS 27.0 Software Package was used to conduct statistical analyses (IBM Corp., New York, NY, USA).

4.4 Results

4.4.1 Characteristics of fresh sperm

Characteristics of fresh sperm before and after HS were described in detail in a previous report [34]. In brief, for acrosome integrity and total motility, there were effects of week ($p < 0.001$ and $p < 0.05$, respectively), whereas for total motility, there was an effect of group ($p < 0.03$) and for progressive motility, only an effect of group ($p < 0.003$). Regarding morphological abnormalities, proximal cytoplasmic droplets, distal cytoplasmic droplets, midpiece defects and ruffled acrosomes were the most common abnormalities present in both groups [34].

4.4.2 Total and progressive motility of frozen-thawed sperm

For total and progressive motility, there were effects of group ($p = 0.023$ and $p = 0.08$, respectively). Whole-body heating group had more pronounced reductions (by ~5 to 7% lower) in both total and progressive motility compared to the scrotal neck insulation group. Sperm treated with either 1 mM melatonin or L-arginine had significantly higher total and progressive motility (Table 4.1) than the no additives control group for both scrotal neck insulation (Weeks 2, 3, 5; and Weeks 2, 3, 4 and 5, respectively), while for whole-body heating (Week 3 only for 1 mM melatonin and for 0.5 mM L-arginine for total motility and Weeks 1, 2 and 5 for progressive motility). However, in Week 2 for the whole-body heating group, the effect was with 1 mM melatonin and with 0.5 mM arginine for total motility, whereas for progressive motility, the effect was only for 0.5 mM melatonin. For Week 3 in the whole-body heating group, there was only an effect with 1 mM melatonin for progressive motility. Regarding 0.5 mM melatonin or L-arginine, total motility was higher ($p < 0.05$) than in the no additives control group on Weeks 2, 3 and 5 for scrotal neck insulation. Progressive motility was higher ($p < 0.05$) after Week 1, in both groups, for scrotal neck insulation.

4.4.3 Total abnormalities and acrosome integrity

For total abnormalities, there were effects of group ($p = 0.01$) and a group*week interaction ($p = 0.03$) and for acrosome integrity, there were effects of group ($p = 0.046$) and week ($p = 0.001$). Total abnormalities were significantly higher in the no additives control than in all other treatments. Improvements in total abnormalities (Table 4.1) and acrosomal integrity (Table 4.1) were dose-dependent (greatest improvement with 1 mM melatonin or L-arginine). Total abnormalities and intact acrosomes were improved by ~7 and 12 percentage points,

respectively. Regarding specific sperm morphological abnormalities (Table 4.2), bowed midpiece, ruffled acrosome, detached normal heads and distal midpiece reflex were highest in the no additives control group. However, those abnormalities were reduced in treatment groups in a dose-dependent manner in both scrotal neck insulation and whole-body heating groups. For distal midpiece reflex, melatonin and L-arginine caused reductions in a dose-dependent manner in both whole-body heating (Week 2, 3 and 4) and scrotal-neck insulation groups (Week 1, 3 and 5). Distal cytoplasmic droplets increased from Weeks 1 to 2, then had a nearly constant level, with treatments conferring improvements following either whole-body heating (Weeks 2, 4 and 5) or scrotal neck insulation (Weeks 2 to 5). Concurrently, detached normal heads defect had nearly the same level of increase over the 5 wk, with 1 mM melatonin or L-arginine having the best results in both in both groups. Bowed midpiece defect increased up to Week 4 then started to decline at Week 5. In both HS groups, treatments conferred improvements in Weeks 3 to 5. Conversely, ruffled acrosome defect had a high level of increase until Week 3 then started to decline in Weeks 4 and 5, with treatments having improvement in Weeks 1 to 4 in whole-body heating group, and in Weeks 2, 3 and 5 in scrotal-neck insulation group. There were other abnormalities not described in detail as they have very low, non-significant numbers (proximal cytoplasmic droplets, pyriform and tapered heads, detached abnormal heads, microcephalic head, coiled and double tails, and knobbed acrosomes.

4.4.4 Sperm antioxidant enzymes (SOD and CAT)

Regarding superoxide dismutase (SOD) and catalase (CAT), there were effects of week ($p = 0.01$ and $p = 0.045$, respectively). Improvements in SOD and CAT were dose-dependent, with 1 mM melatonin or L-arginine yielding the best results (Table 4.3). For SOD, all four

treatments had significantly higher values compared to the no additives control group, whereas for CAT, there were no significant differences between no additives control and 0.5 mM melatonin or L-arginine. Values for SOD had an increasing pattern over the 5 wk in all treatment groups, including the no additives control. Whole body heating and scrotal neck insulation had nearly the same pattern over the 5 wk in all treatment groups for SOD and CAT enzymes.

4.4.5 DFI and total ROS

For DNA fragmentation index (DFI%), there was an effect of week ($p = 0.01$) and a group*week interaction ($p = 0.05$); all 4 treatment groups had reduced DFI% compared to the no additives control group. Results were nearly the same for all 4 treatments over the 5 wk (Table 4.3). The scrotal neck insulation group had a lower level of DFI% when compared to the whole-body heating group in all treatment groups, including the no additives control group. All treatment groups had increasing DFI over the 5 wk. For total ROS%, there were week ($p = 0.044$) and group*week interaction ($p = 0.037$) effects. All 4 treatment groups reduced total ROS% compared to the no additives control group. However, the higher concentration (1 mM) of either melatonin or L-arginine gave the best results (Table 4.3) over the 5 wk, followed by 0.5 mM melatonin or L-arginine. The scrotal neck insulation group had a lower level of total ROS% when compared to whole-body heating group in all treatment groups including the no additives control group in Week 1; otherwise, both groups had similar patterns.

Table 4.1: Mean and SEM of characteristics of frozen-thawed ram sperm in various sperm extender additives after scrotal-neck insulation (n=5) or whole-body heating (n=4).

		Whole-body heating (WB)					Scrotal neck insulation (SN)				
			Melatonin (mM)		L-Arginine (mM)			Melatonin (mM)		L-Arginine (mM)	
Before HS	**Weeks after HS**	**CONT**	**0.5**	**1**	**0.5**	**1**	**CONT**	**0.5**	**1**	**0.5**	**1**
Total motility (%) **WB:** 69.3 ± 0.9 **SN:** 75.0 ± 0.7	**1**	47.8 ± 2.8	46.1 ± 1.1	50.8 ± 2.8	48.4 ± 2.5	49.2 ± 2.1	59.7 ± 2.3	60.3 ± 2.1	55.6 ± 1.5	61.3 ± 1.9	60 ± 2.0
	2	51.7^{a} ± 1.2	52.4^{a} ± 2.4	59.8^{b} ± 2.8	64.1^{b} ± 2.1	56.4^{ab} ± 1.9	56.2^{a} ± 2.1	65.8^{b} ± 2.8	65.5^{b} ± 2.5	67.4^{b} ± 2.1	71.8^{b} ± 2.7
	3	47.1^{a} ± 1.2	49.5^{a} ± 2.0	54.6^{b} ± 2.8	54.5^{b} ± 2.8	53.5^{ab} ± 2.2	60.9^{a} ± 2.6	69.8^{b} ± 1.4	66.1^{b} ± 2.3	67.3^{b} ± 2.1	67.9^{b} ± 2.2
	4	42.0 ± 1.3	45.1 ± 2.8	44.9 ± 1.5	42.9 ± 2.2	48.6 ± 1.4	55.1 ± 2.6	55.5 ± 2.1	51.4 ± 1.9	55.8 ± 2.7	56.8 ± 3.5
	5	49.4 ± 1.0	54.3 ± 1.8	52.6 ± 1.8	53.8 ± 1.5	55.1 ± 2.0	32.6^{a} ± 2.5	51.7^{b} ± 1.2	55.1^{b} ± 2.7	56.7^{b} ± 1.7	55.8^{b} ± 2.6
Progressive motility (%) **WB:** 41.0 ± 0.9 **SN:** 44.6 ± 0.9	**1**	24.5^{a} ± 4.0	25.4^{a} ± 4.6	31.1^{b} ± 5.6	30.6^{b} ± 4.7	28.3^{b} ± 4.9	35 ± 3.4	35.7 ± 3.2	38.7 ± 4.0	37.5 ± 3.5	35.2 ± 1.8
	2	26.1^{a} ± 2.8	30.0^{ab} ± 4.2	30.9^{ab} ± 4.0	34.2^{b} ± 4.1	31.1^{ab} ± 4.0	31.4^{a} ± 1.3	45.6^{b} ± 4.7	41.6^{b} ± 4.2	43.6^{b} ± 4.4	48.1^{c} ± 5.1

	3	24.1^{a} ± 4.5	27.4^{ab} ± 4.8	29.6^{b} ± 4.5	28.4^{ab} ± 4.7	27.7^{ab} ± 4.1	36.9^{a} ± 4.8	46.7^{b} ± 5.0	43.4^{b} ± 3.9	46.0^{b} ± 3.5	44.8^{b} ± 4.2
	4	18.8 ± 1.3	21.3 ± 1.9	20.7 ± 2.3	22.4 ± 1.8	21.4 ± 1.7	27.7^{a} ± 4.4	33.3^{b} ± 4.1	31.7^{b} ± 4.0	32.9^{b} ± 4.9	33.4^{b} ± 4.1
	5	24.6^{a} ± 4.0	28.6^{b} ± 4.4	29.9^{b} ± 4.3	29.5^{b} ± 3.2	29.4^{b} ± 3.2	27.7^{a} ± 1.8	32.6^{b} ± 2.4	32.3^{b} ± 2.5	33.3^{b} ± 4.8	34.2^{b} ± 3.3
Total abnormalities (%) **WB:** 19.5 ± 1.1 **SN:** 21.0 ± 0.6	**1**	31.8 ± 2.3	29.6 ± 2.6	28.2 ± 2.7	30.2 ± 2.4	28.6 ± 2.9	27.6^{a} ± 1.8	24.4^{b} ± 1.9	22.4^{b} ± 1.9	23.8^{b} ± 1.7	22.4^{b} ± 2.1
	2	35.8^{a} ± 2.3	31.8^{b} ± 2.4	29.3^{b} ± 2.2	30.5^{b} ± 2.3	28.3^{b} ± 2.6	30.3^{a} ± 1.2	26.0^{b} ± 1.1	22.3^{c} ± 2.4	24.0^{bc} ± 2.6	22.0^{c} ± 1.0
	3	31.4^{a} ± 2.2	27.8^{ab} ± 2.0	24.8^{b} ± 1.8	25.8^{b} ± 2.0	24.2^{b} ± 1.7	35.0^{a} ± 1.7	29.8^{b} ± 1.7	28.2^{b} ± 1.6	30.6^{b} ± 1.5	28.4^{b} ± 3.3
	4	35.8^{a} ± 2.4	30.3^{b} ± 2.8	27.5^{b} ± 2.8	29.0^{b} ± 1.7	26.0^{b} ± 2.8	34.4^{a} ± 2.5	30.8^{b} ± 2.9	28.2^{b} ± 2.8	31.0^{b} ± 2.2	29.2^{b} ± 3.9
	5	29.2^{a} ± 2.0	25.4^{b} ± 1.7	22.4^{b} ± 1.6	24.0^{b} ± 1.5	22.0^{b} ± 1.7	32.6^{a} ± 2.4	27.4^{b} ± 2.1	23.2^{c} ± 2.1	27.6^{b} ± 2.2	23.4^{c} ± 1.9
Acrosome integrity (%) **WB:** 69.2 ± 1.0 **SN:** 67.6 ± 1.0	**1**	61.0^{a} ± 1.9	65.0^{b} ± 1.8	68.0^{c} ± 1.1	65.4^{b} ± 1.5	68.4^{c} ± 1.5	58.0^{a} ± 1.3	63.2^{b} ± 0.9	67.4^{c} ± 1.0	63.4^{b} ± 1.4	67.8^{c} ± 0.5
	2	47.0^{a} ± 2.2	54.0^{b} ± 1.9	59.8^{c} ± 1.2	54.3^{b} ± 1.9	60.3^{c} ± 1.2	48.3^{a} ± 1.2	55.3^{b} ± 1.3	60.3^{c} ± 1.7	54.8^{b} ± 0.8	60.5^{c} ± 1.4
	3	39.8^{a} ± 2.9	46.2^{b} ± 3.1	50.8^{c} ± 3.4	47.0^{b} ± 3.3	50.8^{c} ± 3.7	40.8^{a} ± 1.1	45.6^{b} ± 1.0	51.4^{c} ± 1.3	45.4^{b} ± 1.1	48.8^{c} ± 1.3

	4	$30.8^{a} \pm 1.2$	$37.5^{b} \pm 0.9$	$42.3^{c} \pm 0.7$	$37.8^{b} \pm 1.4$	$42.8^{c} \pm 1.3$	$34.8^{a} \pm 1.7$	$41.8^{b} \pm 1.2$	$45.8^{c} \pm 1.4$	$41.2^{b} \pm 1.7$	$47.0^{c} \pm 1.3$
	5	$39.8^{a} \pm 2.3$	$44.4^{b} \pm 2.2$	$49.0^{c} \pm 1.5$	$45.8^{b} \pm 1.7$	$49.0^{c} \pm 1.2$	$41.4^{a} \pm 1.1$	$46.4^{b} \pm 1.0$	$50.4^{c} \pm 0.5$	$45.8^{b} \pm 1.0$	$50.6^{c} \pm 1.4$

[a-c]Within each heat model and row, means without a common letter differed ($p \leq 0.05$). Data prior to heat stress are included in the first column. For total and progressive motility, there were effects of group ($p = 0.023$ and $p = 0.08$, respectively); for total abnormalities, there were effects of group ($p = 0.01$) and a group*week interaction ($p = 0.03$); and for acrosome integrity, there were effects of group ($p = 0.046$) and week ($p = 0.001$).

Table 4.2: Means and SEM of various morphological abnormalities in frozen-thawed ram sperm, with 0.5 or 1 mM of melatonin or l-arginine added to extender. Semen was collected after scrotal neck insulation (*n*=5) or whole-body heating (*n*=5).

		Whole-body heating (WB)					Scrotal neck insulation (SN)				
			Melatonin (mM)		L-Arginine (mM)			Melatonin (mM)		L-Arginine (mM)	
Before HS	**Weeks after HS**	**CONT**	**0.5**	**1**	**0.5**	**1**	**CONT**	**0.5**	**1**	**0.5**	**1**
Distal midpiece reflex (DMR) **WB: 5.0** **SN: 4.5**	**1**	8.2 ± 2.4	6.4 ± 1.5	7.6 ± 2.2	8.0 ± 1.4	7.4 ± 1.4	6.6^{a} ± 1.0	6.2^{a} ± 1.1	3.0^{b} ± 0.9	5.8^{a} ± 1.8	4.4^{ab} ± 1.7
	2	11.8^{a} ± 2.3	6.5^{b} ± 1.4	10.5^{a} ± 1.9	7.0^{b} ± 1.5	8.2^{b} ± 2.5	7.2 ± 1.1	9.7 ± 1.2	5.8 ± 0.2	6.8 ± 1.1	7.0 ± 0.9
	3	8.7^{a} ± 2.5	5.0^{b} ± 1.4	5.3^{b} ± 1.8	6.8^{b} ± 1.5	6.0^{b} ± 2.3	10.3^{a} ± 1.4	6.4^{b} ± 1.2	7.0^{b} ± 1.6	7.8^{b} ± 1.1	7.2^{b} ± 0.8
	4	8.5^{a} ± 2.3	6.0^{a} ± 1.2	6.8^{a} ± 0.7	4.2^{b} ± 1.8	4.5^{b} ± 1.9	10.8 ± 1.9	10.2 ± 1.6	11.0 ± 4.0	10.2 ± 2.7	10.4 ± 2.6
	5	9.0 ± 1.4	8.0 ± 0.3	7.0 ± 1.4	6.8 ± 2.0	6.8 ± 1.7	14.0^{a} ± 2.8	9.8^{b} ± 0.6	7.4^{b} ± 2.0	12.0^{a} ± 2.1	10.5^{ab} ± 1.9

Distal cytoplasmic droplets **WB: 1.5** **SN: 1.4**	**1**	2.0 ± 0.4	2.5 ± 0.6	4.5 ± 1.6	2.3 ± 0.7	2.0 ± 0.4	2.8 ± 1.3	4.0 ± 0.8	4.5 ± 1.3	3.0 ± 0.8	4.0 ± 1.2
	2	7.8^{a} ± 0.4	4.3^{b} ± 0.7	3.2^{b} ± 0.4	8.5^{a} ± 0.4	4.5^{b} ± 0.9	9.3^{a} ± 1.1	5.7^{b} ± 1.8	5.0^{b} ± 0.4	5.0^{b} ± 1.3	3.3^{b} ± 0.7
	3	4.5 ± 2.2	2.0 ± 0.3	4.5 ± 2.2	3.0 ± 0.5	3.3 ± 1.4	4.3^{a} ± 1.4	5.6^{a} ± 1.7	2.0^{b} ± 0.9	6.4^{ac} ± 2.4	8.0^{c} ± 2.4
	4	4.3^{a} ± 1.4	3.3^{a} ± 0.5	1.8^{b} ± 0.4	5.3^{a} ± 2.0	1.3^{b} ± 1.1	6.4^{a} ± 2.7	9.0^{b} ± 2.1	6.2^{a} ± 2.4	7.2^{a} ± 2.7	7.8^{a} ± 2.4
	5	6.0^{a} ± 1.6	6.3^{a} ± 1.7	4.3^{b} ± 0.7	6.2^{a} ± 1.2	4.0^{b} ± 1.2	8.2^{a} ± 2.3	8.0^{a} ± 2.3	7.2^{a} ± 2.2	8.4^{a} ± 1.7	5.8^{b} ± 1.4
Detached normal heads	**1**	5.0 ± 1.9	4.2 ± 1.7	3.8 ± 2.2	5.0 ± 2.7	4.5 ± 2.3	3.8^{a} ± 0.7	3.0^{a} ± 0.9	2.3^{a} ± 0.7	2.5^{a} ± 0.6	1.0^{b} ± 0.4
	2	3.3^{a} ± 0.9	2.9^{a} ± 0.6	1.0^{b} ± 0.3	2.5^{a} ± 0.9	6.0^{c} ± 0.6	2.2 ± 0.4	2.3 ± 0.7	2.0 ± 0.4	2.6 ± 0.5	3.0 ± 0.9
	3	4.7^{a} ± 1.3	5.0^{a} ± 1.3	3.5^{a} ± 1.6	3.5^{a} ± 0.9	1.7^{b} ± 0.7	3.7^{a} ± 0.5	2.8^{a} ± 0.6	1.3^{b} ± 0.3	3.0^{a} ± 0.4	1.3^{b} ± 0.3

WB: 1.8 **SN: 1.0**	**4**	$5.0^{a} \pm 1.8$	$2.7^{b} \pm 0.9$	$3.3^{b} \pm 1.1$	$6.5^{a} \pm 1.8$	$3.3^{b} \pm 1.8$	$3.0^{a} \pm 1.5$	$2.3^{a} \pm 0.6$	$1.7^{b} \pm 0.5$	$2.7^{a} \pm 1.3$	$1.8^{b} \pm 0.6$
	5	$5.5^{a} \pm 2.6$	$2.3^{b} \pm 1.4$	$6.5^{a} \pm 2.2$	$6.0^{a} \pm 2.2$	$6.5^{a} \pm 2.8$	1.3 ± 0.3	1.0 ± 0.3	1.0 ± 0.5	1.0 ± 0.3	1.7 ± 0.3
Bowed mid-piece **WB: 1.0** **SN: 1.4**	**1**	2.0 ± 0.4	2.2 ± 0.2	1.5 ± 0.3	1.2 ± 0.2	2.3 ± 0.7	2.3 ± 0.7	1.7 ± 0.3	1.0 ± 0.4	1.0 ± 0.4	2.3 ± 0.2
	2	2.7 ± 1.3	2.0 ± 0.4	1.8 ± 0.2	1.0 ± 0.2	1.3 ± 0.3	1.0 ± 0.5	1.5 ± 0.3	1.0 ± 0.3	1.5 ± 0.3	1.0 ± 0.2
	3	$3.0^{a} \pm 0.4$	$2.5^{a} \pm 0.3$	$3.5^{a} \pm 1.6$	$1.2^{b} \pm 0.4$	$1.0^{b} \pm 0.3$	$3.5^{a} \pm 0.3$	$4.7^{a} \pm 1.4$	$3.2^{a} \pm 0.6$	$2.4^{a} \pm 0.5$	$1.5^{b} \pm 0.3$
	4	$5.5^{a} \pm 0.2$	$3.2^{b} \pm 0.8$	$3.8^{b} \pm 0.4$	$3.2^{b} \pm 0.4$	$2.3^{b} \pm 0.7$	$7.0^{a} \pm 1.9$	$4.5^{b} \pm 2.0$	$3.3^{b} \pm 1.2$	$3.0^{b} \pm 1.9$	$3.7^{b} \pm 1.6$
	5	$4.4^{a} \pm 0.6$	$2.0^{b} \pm 1.2$	$2.2^{b} \pm 0.7$	$3.0^{ab} \pm 0.8$	$3.3^{ab} \pm 0.7$	$4.2^{a} \pm 1.7$	$5.2^{a} \pm 1.3$	$2.8^{b} \pm 1.1$	$3.0^{ab} \pm 0.9$	$4.2^{a} \pm 1.0$
Ruffled acrosomes **WB: 2.7** **SN: 3.6**	**1**	$14.4^{a} \pm 2.3$	$11.0^{b} \pm 1.5$	$11.6^{b} \pm 1.3$	$12.2^{b} \pm 1.9$	$10.8^{b} \pm 1.1$	12.8 ± 1.6	11.0 ± 1.6	10.8 ± 2.4	11.0 ± 2.3	11.8 ± 1.7
	2	$11.8^{a} \pm 1.9$	$12.0^{a} \pm 2.4$	$6.5^{b} \pm 1.1$	$8.2^{b} \pm 2.0$	$8.0^{b} \pm 2.0$	$9.8^{a} \pm 2.1$	$6.7^{b} \pm 1.3$	$9.2^{a} \pm 1.1$	$8.8^{a} \pm 2.8$	$7.0^{ab} \pm 1.5$
	3	$14.0^{a} \pm 1.5$	$14.7^{a} \pm 2.3$	$12.0^{b} \pm 1.9$	$11.5^{b} \pm 1.1$	$10.7^{b} \pm 0.5$	$13.0^{a} \pm 1.5$	$10.4^{b} \pm 2.4$	$10.8^{b} \pm 1.9$	$11.0^{b} \pm 1.4$	$11.5^{b} \pm 2.1$

	4	12.8[a] ± 1.3	13.2[a] ± 0.6	8.8[b] ± 0.9	11.2[a] ± 0.8	11.0[a] ± 1.0	7.2 ± 2.2	5.4 ± 1.9	6.2 ± 1.6	7.8 ± 1.2	6.0 ± 0.8
	5	5.4 ± 1.7	5.2 ± 0.9	3.8 ± 0.7	4.8 ± 1.0	6.3 ± 1.0	6.2[a] ± 0.8	6.0[a] ± 1.5	6.5[a] ± 0.3	5.7[a] ± 1.8	3.5[b] ± 0.9

[a-c]Within each heat model and row, means without a common letter differed ($p \leq 0.05$).

Table 4.3: Mean and SEM of characteristics of frozen-thawed ram sperm in various sperm extender additives after scrotal neck insulation (*n*=5) or whole-body heating (*n*=5) groups. Data prior to heat stress are included in the first column.

		Whole-body heating (WB)					**Scrotal neck insulation (SN)**				
			Melatonin (mM)		**L-Arginine (mM)**			**Melatonin (mM)**		**L-Arginine (mM)**	
Before HS	**Weeks after HS**	**CONT**	**0.5**	**1**	**0.5**	**1**	**CONT**	**0.5**	**1**	**0.5**	**1**
SOD (U/ml) **WB:** 3.1 ± 0.8 **SN:** 2.5 ± 0.9	**1**	3.5^{a} ± 0.3	4.1^{a} ± 0.3	5.3^{b} ± 0.3	4.5^{a} ± 0.3	5.4^{b} ± 0.2	3.1^{a} ± 0.2	4.2^{b} ± 0.2	5.5^{c} ± 0.2	4.3^{b} ± 0.2	5.6^{c} ± 0.2
	2	4.0 ± 0.3	4.5 ± 0.3	4.9 ± 0.3	4.5 ± 0.3	5.0 ± 0.3	3.8^{a} ± 0.2	4.5^{a} ± 0.2	5.3^{b} ± 0.2	4.5^{a} ± 0.3	5.3^{b} ± 0.2
	3	4.6^{a} ± 0.3	5.2^{a} ± 0.3	5.8^{b} ± 0.2	5.3^{a} ± 0.2	5.8^{b} ± 0.3	4.5^{a} ± 0.1	5.3^{a} ± 0.1	5.9^{b} ± 0.2	5.3^{a} ± 0.4	5.9^{b} ± 0.2
	4	4.9^{a} ± 0.2	5.5^{a} ± 0.2	7.1^{b} ± 0.2	5.6^{a} ± 0.2	8.2^{b} ± 0.2	5.1^{a} ± 0.1	5.9^{a} ± 0.1	6.7^{b} ± 0.1	5.9^{a} ± 0.2	7.6^{b} ± 0.1
	5	4.5^{a} ± 0.2	5.0^{a} ± 0.2	8.6^{b} ± 0.1	5.9^{c} ± 0.1	7.6^{b} ± 0.1	4.6^{a} ± 0.2	5.0^{a} ± 0.2	7.7^{b} ± 0.2	6.1^{c} ± 0.2	5.6^{b} ± 0.2
CAT (U/L)	**1**	80.6^{a} ± 2.3	82.7^{a} ± 2.0	88.8^{b} ± 3.9	82.7^{a} ± 2.1	86.7^{b} ± 3.0	80.9^{a} ± 2.4	83.9^{a} ± 2.4	88.7^{b} ± 2.4	84.7^{a} ± 2.2	88.8^{b} ± 2.6
	2	83.7^{a} ± 2.0	85.0^{a} ± 1.4	92.7^{b} ± 3.0	85.0^{a} ± 2.2	91.5^{b} ± 2.4	84.4^{a} ± 2.2	85.3^{a} ± 2.2	92.2^{b} ± 2.1	85.5^{a} ± 1.5	92.1^{b} ± 3.1

WB: 76.6 ± 0.6 **SN:** 74.7 ± 0.8	**3**	88.3^{a} ± 2.1	87.4^{a} ± 2.3	94.6^{b} ± 3.3	86.9^{a} ± 2.6	94.3^{b} ± 1.4	89.4^{a} ± 2.0	89.7^{a} ± 2.1	94.2^{b} ± 2.3	90.0^{a} ± 2.8	93.8^{b} ± 1.3
	4	89.1^{a} ± 2.2	88.4^{a} ± 2.9	96.3^{b} ± 1.0	87.6^{a} ± 2.7	95.1^{b} ± 2.7	93.9^{a} ± 1.7	92.7^{a} ± 3.1	97.7^{b} ± 1.7	93.3^{a} ± 2.0	97.7^{b} ± 2.0
	5	97.1^{a} ± 1.3	96.8^{a} ± 1.0	106.7^{b} ± 1.7	96.8^{a} ± 3.7	104.6^{b} ± 1.1	98.9^{a} ± 1.0	99.2^{a} ± 1.4	105.0^{b} ± 1.4	98.6^{a} ± 2.1	103.6^{b} ± 1.7
DFI (%) **WB:** 9.2 ±0.8 **SN:** 7.5 ± 0.6	**1**	11.9 ± 1.5	10.1 ± 1.4	10.2 ± 2.0	9.2 ± 1.3	10.1 ± 1.1	10.0 ± 0.9	8.2 ± 0.7	8.2 ± 0.8	7.7 ± 0.7	8.3 ± 0.7
	2	17.7^{a} ± 1.3	12.8^{b} ± 1.1	13.7^{b} ± 1.3	12.0^{b} ± 1.21	13.6^{b} ± 1.2	13.4 ± 0.7	11.9 ± 0.6	12.6 ± 0.6	12.2 ± 0.5	12.0 ± 0.6
	3	20.3^{a} ± 1.0	13.8^{b} ± 1.1	16.2^{b} ± 1.1	15.9^{b} ± 0.9	15.5^{b} ± 1.0	16.8^{a} ± 0.9	12.7^{b} ± 1.0	15.2^{a} ± 0.9	13.7^{b} ± 1.0	15.8^{a} ± 0.6
	4	24.5^{a} ± 0.9	17.2^{b} ± 0.9	19.5^{b} ± 0.9	17.0^{b} ± 0.8	18.9^{b} ± 0.8	21.8^{a} ± 0.5	17.3^{b} ± 0.3	18.2^{b} ± 0.4	17.7^{b} ± 0.5	17.9^{b} ± 0.3
	5	26.1^{a} ± 1.0	19.6^{b} ± 1.0	22.4^{b} ± 1.0	19.9^{b} ± 0.9	21.7^{b} ± 1.0	24.3^{a} ± 0.5	20.7^{b} ± 0.5	21.3^{b} ± 0.3	20.8^{b} ± 0.3	21.0^{b} ± 0.8
Total ROS (%) **WB:** 37.7 ± 1.0	**1**	79.4^{a} ± 1.4	68.0^{b} ± 1.4	62.1^{c} ± 1.5	70.7^{b} ± 1.4	63.28^{c} ± 1.5	77.0^{a} ± 1.0	65.0^{b} ± 0.6	67.9^{b} ± 0.8	66.6^{b} ± 0.7	64.4^{b} ± 0.7
	2	81.3^{a} ± 1.9	75.3^{b} ± 1.0	72.0^{b} ± 1.2	77.0^{b} ± 1.2	70.4^{b} ± 1.2	80.5^{a} ± 0.9	73.0^{b} ± 0.6	70.2^{b} ± 0.5	73.8^{b} ± 0.5	74.6^{b} ± 0.6
	3	78.1^{a} ± 1.2	71.4^{b} ± 1.7	68.3^{b} ± 1.6	72.6^{b} ± 0.9	69.6^{b} ± 1.8	81.6^{a} ± 0.8	74.2^{b} ± 1.6	70.5^{b} ± 0.8	78.5^{a} ± 0.8	74.7^{b} ± 0.6

SN: 35.9 ± 0.7	**4**	74.5[a] ± 1.2	67.9[b] ± 0.8	65.6[b] ± 1.4	70.1[b] ± 0.5	66.0[b] ± 1.9	74.0[a] ± 0.4	65.2[b] ± 0.3	61.4[c] ± 0.4	68.0[b] ± 0.5	60.8[c] ± 0.3
	5	65.5[a] ± 1.5	59.1[b] ± 1.4	56.5[b] ± 1.2	57.4[b] ± 1.3	54.4[b] ± 1.9	64.0[a] ± 0.5	57.9[b] ± 0.5	54.7[b] ± 0.3	55.5[b] ± 0.3	50.5[c] ± 0.6

[a-c]Within each heat model and row, means without a common letter differed ($p \leq 0.05$). For superoxide dismutase (SOD) and catalase (CAT), there were effects of week ($p = 0.01$ and $p = 0.045$, respectively); for DNA fragmentation index (DFI%), there was an effect of week ($p = 0.01$) and a group*week interaction ($p = 0.05$); and for total ROS%, there were week ($p = 0.044$) and group*week interaction ($p = 0.037$) effects.

4.5. Discussion

Our objective was to elucidate effects of melatonin or L-arginine on quality of frozen-thawed sperm from heat-stressed (HS) rams. In this experiment, we used two models of HS, namely whole-body heating and scrotal neck insulation and we added two concentrations (0.5 and 1 mM) of melatonin or L-arginine to the semen extender. Furthermore, semen collection, evaluation and treatment with melatonin and L-arginine were only done for 5 wk after HS, enabling assessment of heat influence on epididymal maturation and on much of spermatogenesis, but unfortunately, not on spermatocytogenesis. We purposefully chose a relatively mild HS insult to cause a modest depression in sperm quality. Consequently, we were unlikely to affect the very early stages of spermatogenesis as those are more resistant to HS and sperm quality would only be affected for a few weeks and then recover. All sperm quality parameters studied started to return toward normal levels at the end of this study except for DNA fragmentation index (DFI) that was still increasing.

Exogenous melatonin or L-arginine in semen extender improved quality of frozen-thawed sperm from HS rams. For nearly all end points, either 1 mM of melatonin or L-arginine was more protective than either 0.5 mM or the no additives control. All four treatments improved total and progressive motility and minimized DFI, whereas improvements in total abnormalities, acrosomal integrity, sperm SOD and CAT and total ROS were dose-dependent, i.e., 1 mM melatonin or L-arginine gave the best results. The hypothesis that melatonin or L-arginine improve the quality of frozen-thawed sperm from HS rams was supported. Bowed midpiece, ruffled acrosome, detached normal heads, and distal midpiece reflex were highest in the no additives control group. There were some abnormalities considered primary sperm abnormalities such as ruffled acrosomes and others considered secondary abnormalities such as distal midpiece

defects. There were other abnormalities that only occur after spermiation, such as distal midpiece reflex, detached normal heads and bowed midpiece defects and those abnormalities were reduced with our treatments over the 5 wk of the study.

The decrease in total sperm abnormalities and the increase in acrosome integrity were dose-dependent (greatest improvement with 1 mM of either melatonin or L-arginine), with nearly the same pattern in both HS models. This was consistent with previous studies in which 1 or 2 mM melatonin reduced total sperm morphological abnormalities in frozen-thawed rabbit [143] or bull [135] semen and ameliorated cryopreservation-induced stress. Furthermore, supplementing the extender with 0.5 or 1 mM L-arginine improved post-thaw sperm motility in rams [175] and buffaloes [183]. Doses used in this study were based on those previous reports. We inferred that melatonin or L-arginine reduced total sperm morphological abnormalities by conferring protection against deleterious effects of both HS and cryopreservation.

Improvements in SOD, CAT and total ROS% were dose-dependent, with 1 mM melatonin or L-arginine yielding the best results compared to 0.5 mM melatonin or L-arginine and no additives control. However, for the DNA fragmentation index (DFI%), all treatments reduced DFI% over the 5 wk. This was consistent with a study in humans [173] in which 1 mM melatonin alleviated oxidative damage to sperm caused by heat-induced oxidative stress by improving sperm motility, reducing mitochondrial ROS content, stabilizing mitochondrial membrane potential, reducing lipid peroxidation products, maintaining DNA integrity, and reducing apoptosis. Melatonin is expected to reduce oxidative damage both in vitro and in vivo, due to its capacity to scavenge ROS. Its antioxidant capabilities make it an excellent choice for use in cryopreservation media to protect sperm from excessive ROS, with effects on sperm quality attributed to its broad-spectrum antioxidant activity [124,184]. Moreover, based on recent

studies in several species [136,143], adding melatonin to semen extender significantly increased sperm motility, cell membrane integrity, antioxidant capacity and anti-apoptosis gene expression and decreased DNA fragmentation. Conversely, in another study [185] although melatonin improved sperm motility, DNA integrity and reduced mitochondrial superoxide production, it had no significant effect on sperm survival, lipid peroxidation, or intracellular ROS. In contrast, in our study, there was lower intracellular total ROS% and improved sperm SOD and CAT, especially with 1 mM melatonin.

To the best of our knowledge, there are no previous reports regarding adding L-arginine to the semen extender of HS males to protect sperm. Regardless, in several studies, L-arginine has been added to semen extender to alleviate oxidative stress induced by sperm cryopreservation. For example, 0.5 or 1 mM L-arginine in ram semen extender protected sperm from cryopreservation-induced damage [175]; this was accompanied by inhibition of lipid peroxidation and supporting antioxidant defence mechanisms by inducing NO and decreasing ROS. In our study, 1 mM of L-arginine was more effective than 0.5 mM to mitigate cryopreservation-induced reductions of sperm quality in semen from HS ram. Increased activities of sperm antioxidant enzymes (SOD and CAT) in this study after addition of melatonin or L-arginine to the semen extender likely contributed to reductions in total ROS% and improved sperm quality in HS ram sperm. Over the 5 wk, both total and progressive motility were significantly higher in the 1 mM of melatonin or L-arginine groups than in the no additives control group, following HS induced by either scrotal neck insulation or whole-body heating. Similarly, 1 or 2 mM improved total and progressive motility in post-thawed rabbit [186] and bull [135] sperm.

This study had some limitations. We only had 10 rams; they were all of the same breed, but not the same age. There were two methods of HS (continuous localized testicular and intermittent whole-body). Semen was collected only once weekly for 5 wk, with two methods of semen collection used; however, there was no significant difference between the two methods. Despite those limitations, the two methods of HS yielded very similar effects on sperm. Furthermore, there were many significant differences between control and treated sperm, which were generally consistent with previous reports utilizing melatonin or L-arginine. Therefore, although this study should be replicated (with care to overcome the stated limitations), the results were clear and arguably compelling.

4.6 Conclusion

In conclusion, exogenous melatonin or L-arginine in semen extender mitigated decreases in quality of frozen-thawed sperm from HS rams. Furthermore, 1 mM melatonin or L-arginine generally yielded better results in comparison to 0.5 mM or no additive controls. Future studies are needed, including fertility trials, to determine the extent that adding melatonin and L-arginine to the semen extender of HS males can improve fertility**.**

Chapter 5: Angus bulls voluntarily access shade during hot weather, reducing scrotal subcutaneous temperatures and improving sperm quality; modified from: Shahat, A. M., Juan Castillo, Thundathil, J. C., & Kastelic, J. P. (2023). Angus bulls voluntarily access shade during hot weather, reducing scrotal subcutaneous temperatures and improving sperm quality. *Canadian Journal of Veterinary Research*, 87:17-22.

5.1 Abstract

Our objectives were to establish relationships among bull location (shaded versus nonshaded), scrotal subcutaneous and ambient temperatures, and sperm quality. Six Angus bulls (4 to 5 y) were randomly allocated into two groups of three, housed in two outdoor pens, with one containing shade (shed ~3.5 x 6 m and 2.5 m high, one open side). Semen was collected by electroejaculation once weekly for 9 wk. Percentage of time a bull voluntarily accessed shade for ≥ 15 min (assessed with a game camera) increased with ambient temperature and ranged from 7.6 to 86.7% for ambient temperatures < 25 and > 33 ^{0}C, respectively. Over the 10 hottest days, scrotal subcutaneous temperature (measured hourly with an implanted data logger) in the control group had a direct association with ambient temperature. Conversely, bulls with access to shade had lower (p = 0.001) scrotal subcutaneous temperatures during high ambient temperatures, particularly when they accessed shade. During the four hottest days, bulls voluntarily accessed shade most of the time from 12.00 to 17.00 (peak ambient temperatures). For total sperm morphological abnormalities and acrosome integrity, there were group effects (p = 0.001 for each), plus a time effect for acrosome integrity (p = 0.009). For total and progressive forward sperm motility, there were group effects (p = 0.001 and 0.023, respectively). Furthermore, for sperm motility kinetics measured with CASA [average path velocity (VAP), curvilinear velocity

(VCL), straight line velocity (VSL), straightness of track (STR), and linearity of track (LIN)] there were group effects (p = 0.005, 0.011,p0.010, 0.020, and 0.046, respectively). In summary, during hot weather, bulls voluntarily accessed shade, significantly lowering scrotal subcutaneous temperatures and improving sperm quality.

5.2 Introduction

An increasing focus on managing heat stress in cattle is being driven by various factors, including increases in extreme weather events and climate variability, more cattle in livestock facilities, increased demands for livestock growth/efficiency, changing cattle demographics, and increased societal concerns regarding animal welfare [187]. Moreover, animal productivity is adversely affected by high temperatures and sun radiation. Exposure to hot environments increases testicular temperature and reduces reproductive efficiency [51]. Testicular function is extremely temperature sensitive; bull testes must be 2 to 5 °C below body temperature to produce viable and fertile sperm [31]. Heat stress and the need for heat mitigation are becoming increasingly important cattle welfare issues as global temperatures continue to rise [188]. Furthermore, it is well established that *Bos taurus* cattle are less heat-tolerant than *Bos indicus* cattle under thermal stress, due to inferior heat regulatory capacity [116,189].

When body temperature rises above a threshold, mammals have evolved a series of thermoregulatory behaviours to return to homeostasis, thereby increasing their chances of survival [190]. In response to an increase in body temperature, the preoptic region of the hypothalamus initiates behavioural and physiological cooling processes [191,192]. Concurrently, heat load alleviation solutions aim to reduce impacts of the thermal environment and to restore normal body temperature [193]. Cooling is advocated, as it lessens impacts of environmental conditions on productivity [194]. Conduction, convection, and radiation all contribute to heat

loss, but all are reliant on a thermal gradient. As ambient temperature rises, cooling shifts from non-evaporative cooling to evaporative heat loss [195].

Restricted or managed feeding programmes, reducing water temperature, increasing water access to compensate for increased water consumption, sprinkling systems, and shade are all strategies to mitigate heat stress [196]. In a hot environment, reducing incoming heat radiation by providing shade is an efficient way to assist animals in maintaining temperature regulation [116]. Given a choice, cows prefer natural shade from trees to man-made structures, as trees provide both sun protection and a radiation sink effect due to moisture evaporation from the leaves [38]. Regardless, when direct sun radiation is severe (350 W/m^2) and air temperatures exceed 28 °C, most European cattle breeds prefer shade to reduce discomfort [118]. In several studies to assess effects of artificial shade on the performance and reproductive characteristics of bulls in pastures, there were improvements in semen quality in bulls with access to shade comparison to those without shade access [119,120,197]. However, none of those studies concurrently measured scrotal subcutaneous temperature and its relationship with ambient temperature, frequency of shade access, and sperm quality. Objectives of this study were to determine associations among bull location (shade versus no shade), intrascrotal and ambient temperatures, and sperm quality. We tested the hypothesis that bulls access shade during increased ambient temperatures and have lower intrascrotal temperatures and better sperm quality than those without access to shade.

5.3 Materials and methods

This study was conducted at the University of Calgary W.A. Ranches near Madden, AB, Canada (Elev 3652 ft, 51.39 °N, 114.24 °W) from June to August 2021. During the experiment, ambient temperature data were retrieved from the *weather underground* (WU) website

containing the information from the weather station (Madden - IROCKYVI16), approximately 5 km from the study location. These data were used as the official ambient temperatures for our study. Relative humidity from the same weather station during the study never exceeded 30 to 40%.

5.3.1 Bulls and chemicals

These experiments were approved by the University of Calgary Veterinary Sciences Animal Care Committee (AC19-0106) and conducted in accordance with Canadian Council on Animal Care guidelines. Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA).

Temperature data loggers (DL; DST Micro-T, Star-Oddi, Gardabaer, Iceland), 8.3 × 25.4 mm and 3.3 g and designed to be implanted, were used to monitor intrascrotal temperatures. The DLs were activated (using the manufacturer's communication box and Mercury® software), set to record temperature every 10 min, and placed in a waterbath (28.0-40.5 °C) to verify function. Thereafter, they were set to record at a 1-h intervals, disinfected (2% glutaraldehyde for 10 h), rinsed with sterile water and surgically implanted.

5.3.2 Experimental design

Six adult Angus bulls (4 to 5 y), approximately 600 to 750 kg, were randomly allocated into two groups (three bulls/group); one group was housed in a small outdoor pen containing artificial shade (a metal structure ~3.5 x 6 m and 2.5 m high with a roof and three walls) to be accessed as desired (shaded group) and the second group were housed in an adjacent small outdoor pen without shade (non-shaded group). Both groups had *ad libitum* access to hay, water and salt.

For monitoring the shaded group, a game camera (Digital Trail Camera®, CamPark, China) was placed inside the shade structure. The field of view that covered all the shade area and captured an image whenever a bull moved into or exited the shade, with capacity to record images irrespective of light/darkness. This camera recorded the date and exact time of image captured and ambient temperature (-20 to 60 ℃) with 0.3 s trigger speed, with all data stored on removable storage media. We considered a bull to have accessed shade when he spent at least 15 consecutive minutes with the majority of his body in the shade, based on our report [26] that scrotal subcutaneous temperature could change within 15 min. We determined the percentage of time that bulls voluntarily accessed the shade at the following ambient temperature ranges (℃): < 25; > 25 & < 27; > 27 & < 29; > 29 & < 31; > 31 & < 33; and > 33.

Immediately prior to surgical implantation of DLs, bulls were given: 1 ml/10 kg oxytetracyline SQ (Bio-Mycin 200 LA, Boehringer Ingelheim, Burlington, Ontario); 0.5 mg/kg meloxicam SQ (Metacam, Boehringer Ingelheim); caudal epidural anesthesia (0.07 mg/kg xylazine in ~5 mL saline; Rompun, Bayer, Mississauga, Ontario) in the 1st intercoccygeal intervertebral space; and local subcutaneous blocks with bupivacaine (Bupivacaine, 2.5 mg/ml, Hospira Inc., Lake Forest, Illinois, USA) at incision sites. A skin incision (~ 2 cm) was made on the posterior scrotum, at the middle of the scrotum, lateral to the midline, with blunt dissection used to create a subcutaneous ‘pocket’ ventral to the surgery site, a DL placed in each site, and the incision closed with skin staples.

Starting 10 d after DLs were implanted, semen was collected weekly using an electro-ejaculator for 2 wk as control samples in April. Then during the summer (21 June to 19 August 2021), semen was collected once weekly for 9 wk, transported to the laboratory undiluted in a thermos at 33-35℃.

Once semen collection was completed at the end of the study, small incisions were made in scrotal skin using identical procedures as those described above, to enable retrieval of the DLs. Data were retrieved using Mercury software (Star-Oddi, Gardabaer, Iceland). The DLs were recovered from five bulls (but was missing in one bull in the no shade group).

5.3.3 Sperm evaluation

Immediately after samples arrived at the laboratory, semen was diluted in 1 ml 1X-TALPH, then motility (total and progressive forward) was evaluated using CASA (Sperm Vision®;[179]), morphology using eosin-nigrosin (200 sperm were evaluated at 1000x; 1 experienced operator did the evaluation for all samples and was blinded to the experimental group to avoid bias), and acrosome integrity using FITC-PSA [180], with 200 sperm evaluated. The CASA parameters studied included: average path velocity (VAP; velocity of progression along a smoothed trajectory); curvilinear velocity (VCL; velocity of progression along the entire trajectory); straight line velocity (VSL; velocity of progression from first to last coordinates); amplitude of lateral head displacement (ALH; mean lateral sperm head displacement along a smoothed trajectory); straightness of track (STR; VSL/VAP×100); and linearity of track (LIN; VSL/VCL×100) [198].

5.3.4 Statistical analyses

A General Linear Model (repeated measures) was done to compare sperm end points and scrotal subcutaneous temperatures between shaded and non-shaded groups, with a *post-hoc* LSD test used to locate differences between groups within each week. Data variance was uniform (based on Levene's tests). Results are reported as mean ± standard error of the mean (SEM). For

all analyses, $p < 0.05$ was considered significant. All statistical analyses were conducted with the IBM SPSS 28.0 Software Package (IBM Corp., New York, New York, USA).

5.4 Results

The camera captured thousands of images of bulls accessing the shade. The percentage of time that a bull accessed shade increased with ambient temperature, and it was 7.6, 21.0, 24.2, 38.0, 61.9, and 86.7% at the following ambient temperature ranges (℃): < 25; > 25 & < 27; > 27 & < 29; > 29 & < 31; > 31 & < 33; and > 33, respectively.

Over the 10 hottest days of this study, scrotal subcutaneous temperature in the no shade group had a direct relation with ambient temperature, with the highest scrotal subcutaneous temperature (36.5 ℃) when the ambient temperature was ~ 33 ℃ (Figure 5.1). Conversely, bulls with access to shade had lower ($p = 0.001$) scrotal subcutaneous temperatures during high ambient temperatures, particularly if they voluntarily accessed shade.

During the four hottest days of this study, peak ambient temperatures were 32.0 to 33.5℃ (Figure 5.2A;). For each individual bull in the shade group, from 10.00 to 19.00, we displayed when he was in the shade, ambient temperature, and scrotal subcutaneous temperatures (Figure 5.2B-D). Bulls voluntarily chose shade most of the time from 12.00 to 17.00, coinciding with peak ambient temperatures.

For all sperm quality parameters examined (sperm total and progressive motility, sperm kinematics, abnormalities, and acrosome integrity), there were no significant differences between shade and no shade groups at Week 0 (baseline). For total and progressive forward motility (Figure 5.3), there were group effects ($p = 0.001$ and 0.023, respectively). Both total and progressive forward motility differed ($p < 0.05$ for each) between shade and no shade groups

from Weeks 2 to 4, then at Week 5 for total motility and Weeks 5 and 6 for progressive motility there were no significant difference between groups. From Week 6 for total motility and Week 7 for progressive forward motility to the end of the experiment (Week 9), the two groups differed ($p < 0.05$).

For total sperm morphological abnormalities and acrosome integrity (Figure 5.3), there were group effects ($p = 0.001$ for each), plus there was a time effect for acrosome integrity ($p = 0.009$). Both end points had differences ($p < 0.05$) between shade and no shade groups from Week 3 to the end of experiment (Week 9) for acrosome integrity, whereas for total abnormalities, there was no significant differences between groups at Weeks 5 and 7, otherwise for all other weeks, there was a difference ($p < 0.05$). The most common abnormalities that were significantly higher in the no shade group were detached heads, pyriform heads, coiled tails, distal cytoplasmic droplets and distal midpiece reflexes.

Figure 5.1: Ambient temperatures during the 10 hottest days in our study and mean ±SEM of scrotal subcutaneous temperatures (°C) in bulls in shade and no shade groups. Treatment × Time effect (p = 0.001).

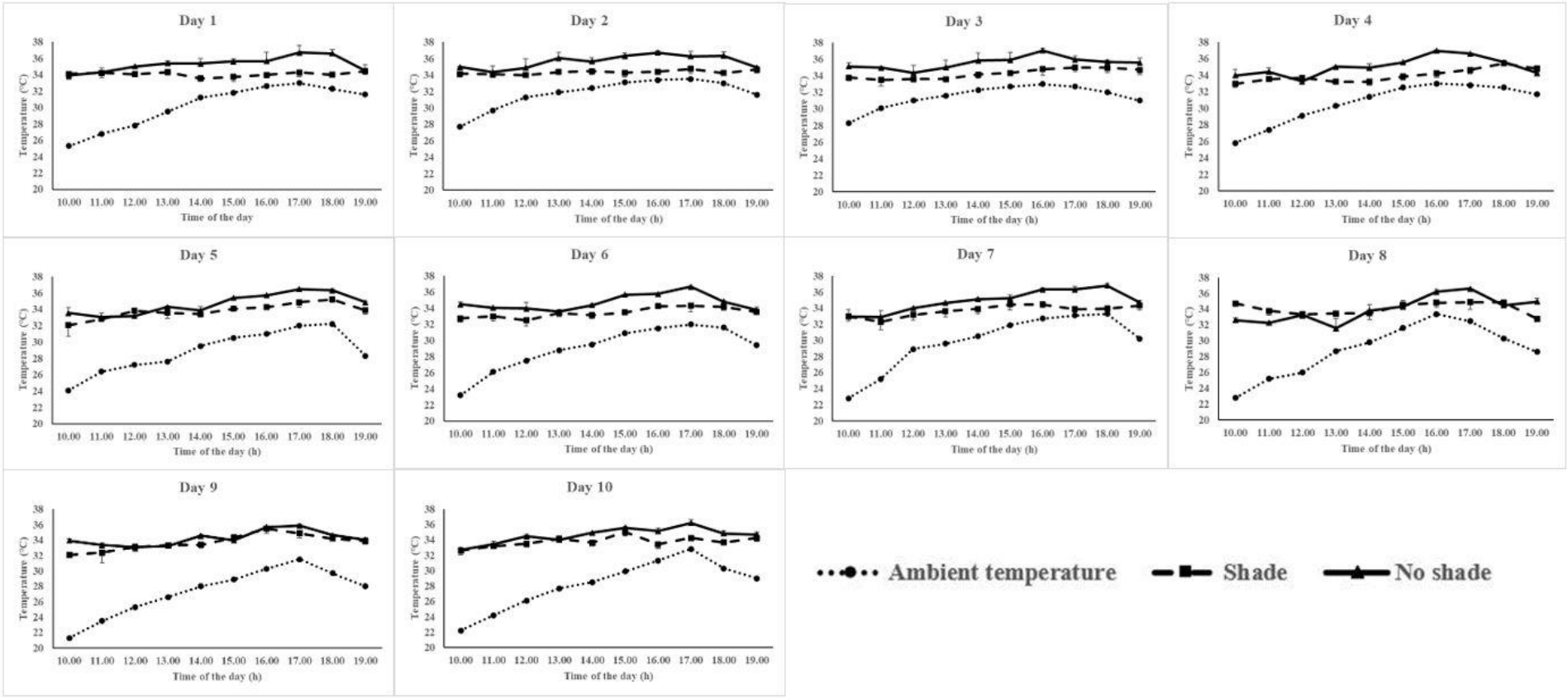
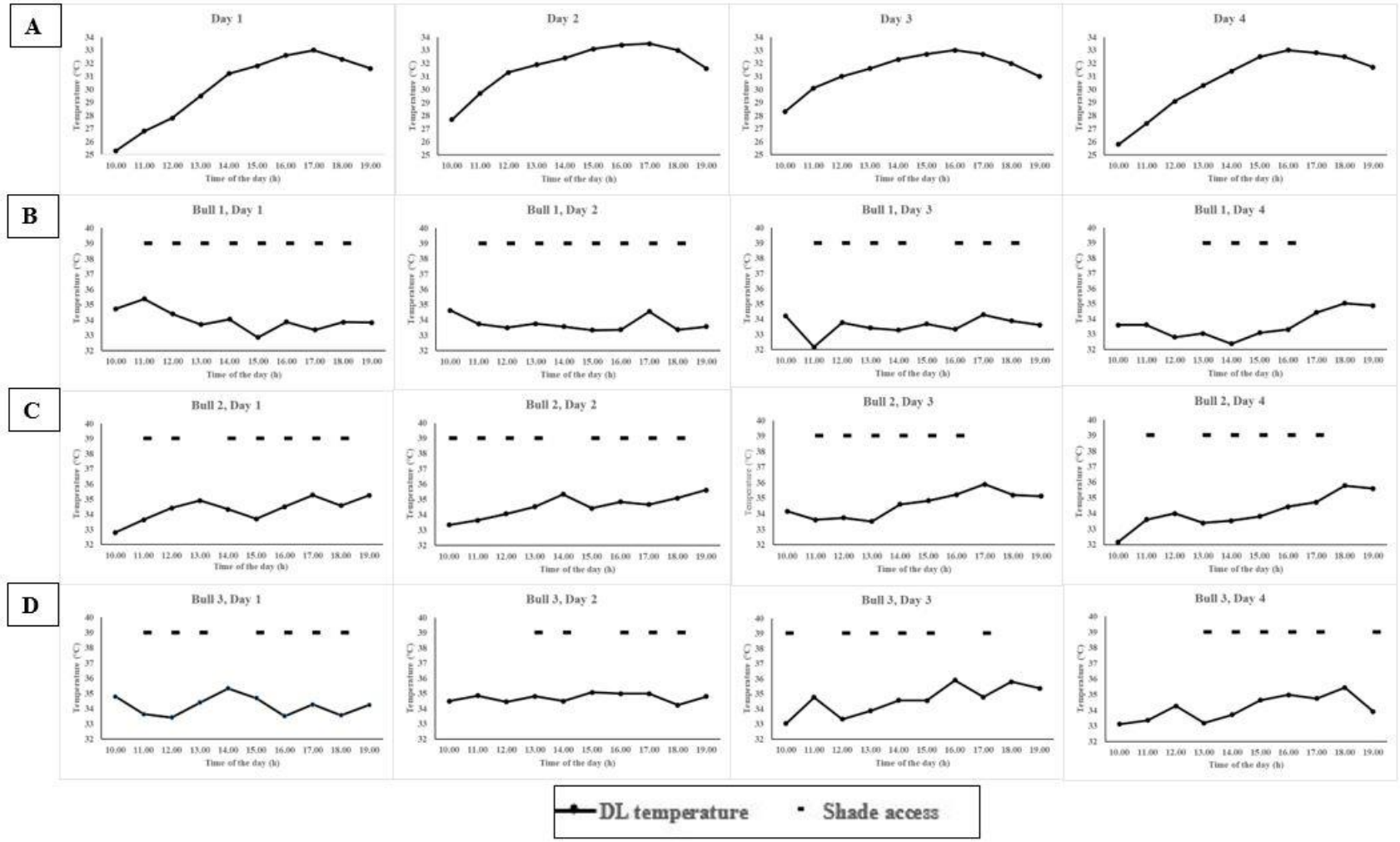


Figure 5.2: Ambient temperature (°C) during the 4 hottest days of our study. Rows B, C and D are for Bulls 1 to 3, respectively, with intrascrotal temperature (°C) and bull choosing to access the shade (indicated by the line at the top of the figure).

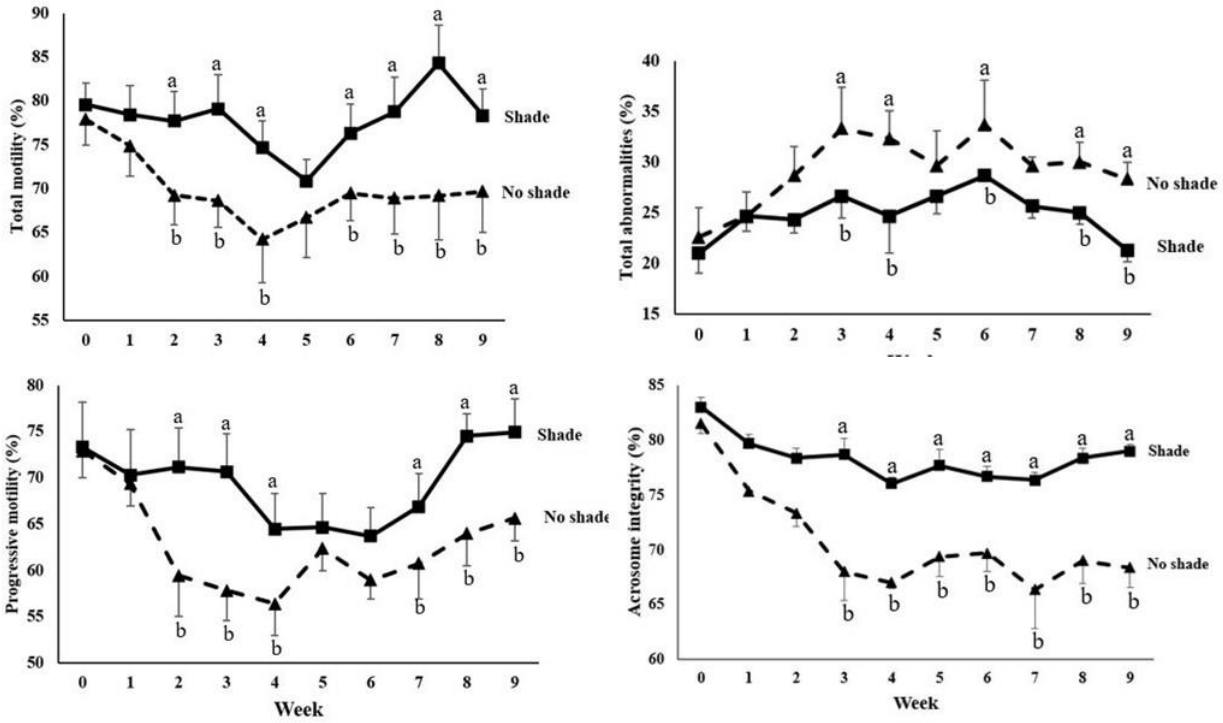


5.5 Discussion

In this study, we investigated whether bulls with access to shade during high ambient temperatures had lower intrascrotal temperatures and better sperm quality than bulls without shade. Bulls most commonly accessed shade from 12.00 to 17.00, when ambient temperatures were at their highest. The proportion of time a bull seeking shade increased with ambient temperature, ranging from 7.6 to 86.7% for temperatures < 25 and > 33 °C, respectively. Similarly, in another study [120] semi-confined young Brangus bulls spent more time in the shade from 11.00 to 14.00. To the best of our knowledge, this was the first study to use DL monitor scrotal subcutaneous temperature and determine its association with ambient temperature for bulls with or without access to shade in warm ambient temperatures. In this study, bulls with access to shade had lower scrotal subcutaneous temperature when they accessed the shade during the high ambient temperatures. In previous studies, scrotal subcutaneous temperature was studied and both whole-scrotum insulation in bulls [34] and whole-body heating in rams [26,34] increased scrotal subcutaneous temperatures.

For sperm kinematics (VAP, VCL, VSL, STR and LIN) there were group effects (p = 0.005, 0.011, 0.010, 0.020, 0.046, respectively; Table 5.1), whereas ALH had only a time effect ($p < 0.05$). There were no significant differences between the two groups for any sperm kinetic end point on Week 0. However, for Weeks 1 to 9: VAP and VCL were higher ($p < 0.05$) in shade versus no shade bulls on 6 of 9 weeks; VSL was higher ($p < 0.05$) in shade bulls on 8 of 9 weeks; and STR and LIN were higher in shade bulls on 5 of 9 weeks.

Figure 5.3: Sperm total and progressive motility, total sperm morphological abnormalities, and acrosome integrity in sperm from bulls in shade and no shade groups.



[a,b]Within a week, means without a common letter differed ($p \leq 0.05$). Effect of group for sperm total and progressive motility and total abnormalities (p = 0.001, 0.023 and 0.001, respectively), whereas there were effects of group and time effects (p = 0.001 and 0.009, respectively) for acrosome integrity.

There were no significant differences between groups for any sperm quality end point studied at baseline, as expected, as all bulls were kept together in the same paddock for 30 d prior to the start of the trial and had no access to shade. Regarding sperm total abnormalities, there was a significant difference between groups for Weeks 3, 4, 6, 8 and 9, whereas acrosome integrity was different between groups from Week 3 to the end of the experiment.

Table 5.1: Mean ± SEM of sperm kinematics of bulls with and without access to the shade.

Week	Group	VAP (µm/s)	VCL (µm/s)	VSL (µm/s)	ALH (µm)	STR (%)	LIN (%)
0	**Shade**	83.2±3.4	131.2±2.3	75.8±2.3	4.2±0.1	90.1±1.0	56.7±1.0
	No shade	80.7±3.2	128.7±2.1	74.0±2.5	3.9±0.1	90.0±1.0	55.3±1.1
1	**Shade**	79.9^{a}±4.8	126.9^{a}±3.2	71.1^{a}±3.7	4.1±0.5	88.3±1.8	55.0^{a}±1.5
	No shade	71.4^{b}±4.5	116.3^{b}±3.5	62.4^{b}±3.9	3.7±0.1	86.3±1.1	52.6^{b}±2.3
2	**Shade**	81.4^{a}±3.2	128.2^{a}±3.3	72.3^{a}±3.4	3.9±0.2	88.6^{a}±1.1	56.0^{a}±2.0
	No shade	70.6^{b}±4.3	115.0^{b}±3.4	60.8^{b}±4.0	3.8±0.1	85.0^{b}±2.5	51.6^{b}±2.7
3	**Shade**	75.6^{a}±4.7	118.2^{a}±3.9	64.2^{a}±3.8	4.9±0.4	84.3±1.2	53.6±1.2
	No shade	65.8^{b}±3.7	105.5^{b}±3.4	56.2^{b}±3.6	4.3±0.3	84.0±1.6	52.0±3.5
4	**Shade**	72.8±2.6	117.7±1.1	64.9a±1.3	3.2±0.1	88.6^{a}±1.6	55.3^{a}±1.3
	No shade	70.8±4.1	118.0±1.0	59.6^{b}±2.4	4.2±0.3	83.8^{b}±2.2	48.6^{b}±3.0
5	**Shade**	76.2^{a}±1.8	127.1^{a}±1.3	66.2^{a}±1.5	3.9±0.4	86.3±2.1	51.6±1.7
	No shade	69.9^{b}±2.2	119.6^{b}±2.0	59.0^{b}±1.9	4.0±0.2	84.0±1.1	49.0±1.1
6	**Shade**	75.8^{a}±1.4	122.0±3.1	66.0±2.0	4.5±0.6	86.5±1.2	54.3±1.0
	No shade	68.8^{b}±2.8	118.8±3.1	64.0±2.0	4.4±0.4	86.4±1.3	55.0±0.5
7	**Shade**	74.2^{a}±1.6	124.4^{a}±1.6	63.7^{a}±2.2	4.2±0.1	85.3^{a}±1.2	50.6^{a}±0.6
	No shade	67.5^{b}±2.7	118.8^{b}±2.1	55.4^{b}±2.6	4.1±0.1	81.4^{b}±1.7	46.0^{b}±1.7
8	**Shade**	75.8^{a}±3.2	126.9^{a}±2.4	63.2^{a}±2.6	4.6±0.1	83.0^{a}±1.5	49.3^{a}±0.8
	No shade	66.6^{b}±3.4	108.7^{b}±2.3	54.2^{b}±2.2	4.1±0.2	80.2^{b}±1.8	46.6^{b}±1.7
9	**Shaded**	75.1±3.2	119.5±3.6	65.5^{a}±2.7	3.8±0.2	87.0^{a}±1.1	54.6±1.8
	No shade	72.0±3.3	115.3±2.6	59.3^{b}±2.0	3.8±0.3	83.67^{b}±2.18	55.3±1.4

[a,b]Within each endpoint and column, means without a common letter differed ($p \leq 0.05$). VAP, VCL, VSL, STR and LIN had group effects (0.005, 0.011, 0.010, 0.020, and 0.046, respectively), whereas ALH had a time effect ($p < 0.05$).

Similarly, in a previous study, bulls without shade had a trend for more aged acrosomes and abnormal sperm [119]. Conversely, in several studies [120,197,199,200] total sperm morphological abnormalities were not different between bulls with versus without shade. Apparent differences between the current results and other studies may have been due to differences in breed, as *Bos taurus* breeds and crossbreds are more susceptible to heat stress in warm climates, whereas environmental strategies for management of heat stress are not necessarily needed in *Bos indicus* cattle such as Nelore [10,156]. In addition, shade type (natural or artificial) or even shade design [188], may have affected outcomes.

There was no significant difference between groups for total or progressive motility on Week 0. Almost immediately thereafter, both total and progressive motility began to decrease markedly in the no shade group, with no decrease or a relatively smaller decrease in the shade group, resulting in significant differences between the two groups on Weeks 2 to 4 and on Weeks 7 to 9, consistent with reports [119,120,197] that access to shade for bulls on pasture enhanced sperm motility. Conversely, other studies [199,200] reported conflicting results regarding effects of shade on sperm motility.

To the best of our knowledge, no previous studies reported effects of shade availability on bull sperm kinematics. Overall, differences between the two groups in the detailed kinetics of sperm motion were generally similar, with significantly higher VAP, VCL and VSL in shade

versus no shade bulls on Weeks 1 to 3 and Weeks 7 and 8, whereas STR and LIN were significantly higher in shade versus no shade bulls on Weeks 2, 4, 7, and 8. However, ALH was not significantly affected by access to shade.

This study had some limitations. We only had six bulls in total, with scrotal temperature measurements only available for five bulls and semen collected only once weekly. Regardless, we detected many significant and biologically relevant differences between the two groups, providing clear evidence that given an opportunity, bulls will seek shade when ambient temperatures are high and that this reduces intrascrotal temperature and improves sperm quality. Therefore, although this study should be replicated (with care to overcome the stated limitations), the results were clear and arguably compelling. Ideally, future work should be done using larger number of bulls and determining effects of shade availability on breeding efficiency in pasture.

In conclusion, bulls in pasture voluntarily accessed shade during hot weather, reducing scrotal subcutaneous temperatures and improving sperm quality. Future research should include a greater number of bulls and assess the influence of shade availability on pasture breeding efficiency.

Chapter 6: Melatonin improves testicular hemodynamics and sperm quality in rams subjected to mild testicular heat stress; modified from: Shahat, A. M., Thundathil, J. C., & Kastelic, J. P. (2022). Melatonin improves testicular hemodynamics and sperm quality in rams subjected to mild testicular heat stress. *Theriogenology*, 188:163-169.

6.1 Abstract

Melatonin is a potent free-radical scavenger, with anti-inflammatory, anti-oxidative, and anti-apoptotic effects. The objective was to determine whether melatonin promoted testicular blood flow and protected sperm quality in rams after mild HS (scrotal neck insulation). Twelve yearling Dorset rams with good semen quality were housed indoors (~18-20 ℃). Once weekly for 2 wk, Doppler indices (resistive index [RI] and pulsatility index [PI]) were measured in the supratesticular artery and semen collected by electroejaculation. Then, rams were randomly allocated into two equal groups, and given either 36 mg melatonin in 1 ml corn oil SQ under the ear (MEL), or only corn oil (CONT). At 15 d after treatment, all rams were subjected to mild HS for 96 h, with blood flow measurements and semen collection done once weekly for 7 wk. There were group, week and group*week interaction effects ($p < 0.005$) for total and progressive sperm motility (CASA); total sperm abnormalities and acrosome integrity had effects of group, week and group*week interaction effects ($p < 0.001$); and there were group and week effects for RI and PI ($p < 0.005$), with no significant differences before treatment. Changes in total and progressive motility and sperm abnormalities were evident at Week 1 post-HS in CONT rams, but MEL mitigated ($p < 0.05$) these effects from Weeks 2 to 7. Furthermore, both PI and RI were reduced ($p < 0.05$; i.e., significant increase in blood flow) in MEL versus CONT rams most weeks after HS. In MEL rams, sperm motility and total abnormalities had recovered at Weeks 5

and 6, respectively, whereas CONT rams had not completely recovered by Week 7. There was no difference ($p < 0.05$) between MEL and CONT groups in scrotal subcutaneous temperatures in the 4-d intervals before, during and after HS. In conclusion, melatonin significantly improved testicular blood flow and protected sperm motility and morphology in rams exposed to testicular HS. Therefore, melatonin has potential for mitigating effects of testicular HS under field conditions.

6.2 Introduction

The continued rise in global temperatures has been a source of concern for decades [201]. When temperatures rise above a physiological threshold, compensatory mechanisms are overwhelmed, resulting in heat stress (HS). Most mammalian testes are 4-5 ℃ cooler than the rest of the body [156], with increased testicular temperature adversely affecting sperm motility, morphology, and fertility. All types of testicular cells are affected by increased testicular temperature caused by scrotal insulation or other HS models; however, germ cells are more sensitive than Sertoli or Leydig cells [54], with apoptosis-induced death of germ cells being the primary effect of HS on the testis [36]. The role of ROS in apoptosis initiation, as well as death of germ cells and DNA damage, appears crucial [77]. Heme oxygenase 1 (*HMOX1*) and antioxidant enzymes such as glutathione peroxidase 1 (*GPX1*), glutathione S-transferase alpha, and superoxide dismutase 1 (*SOD1*) are upregulated in response to heat stress, indicating a powerful oxidative stress response [79].

Testicular blood flow is critical to supply the testes with oxygen and nutrients and to remove metabolic wastes. Temperature changes in the testes and/or the environment may alter testicular blood flow [202]; it increased rapidly after testicular warming of anesthetized bulls [115]. Doppler ultrasonography is a non-invasive imaging technique that evaluates blood supply

dynamics [144]. In a pulsed-wave Doppler examination, the most common blood flow measurements are the resistive index (RI), and pulsatility index (PI) [203], which are reliable indicators for identifying infertile dyspermic males in clinical practise [204]. Blood perfusion of the tissue downstream is negatively correlated with RI and PI values of an artery [205]; therefore, when RI and PI rise, vascular resistance of blood flow rises, decreasing blood perfusion, and vice versa [206]. Furthermore, testicular ultrasonic echotexture has been linked to seminiferous tubule area, sperm production, and sperm quality in bulls [207].

Melatonin (N-acetyl-5-methoxytryptamine) is a small neurohormone produced primarily by the pineal gland [208], but also by the testis [209]. It is a powerful free-radical scavenger, protecting the testis from inflammation and ROS generation, with anti-inflammatory, anti-oxidative, and anti-apoptotic roles in the testis [209]. In that regard, melatonin protected the murine testis from heat-induced damage [130] and it enhanced testicular blood flow in Ossimi rams [210] and in Shiba goats [144]. The objective was to determine whether melatonin promoted testicular blood flow and protected sperm quality in rams after mild HS (scrotal neck insulation).

6.3 Materials and methods

6.3.1 Animals and chemicals

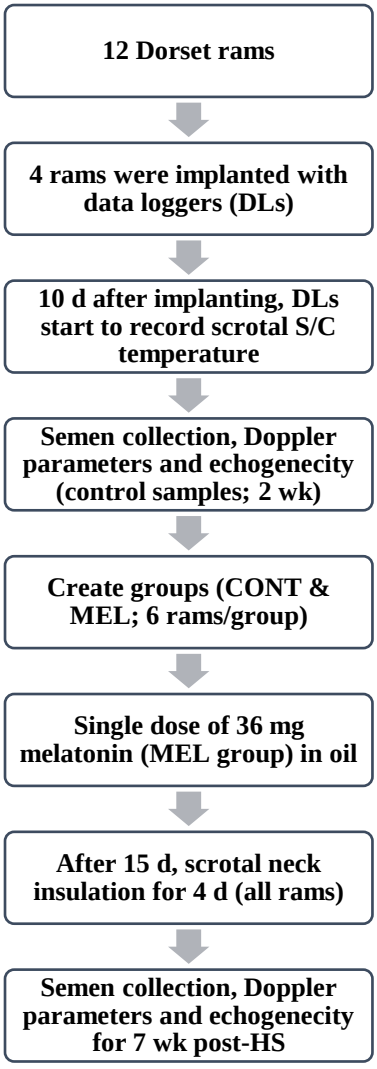
Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). This experiment was approved by the University of Calgary Veterinary Sciences Animal Care Committee (AC19-0106) and conducted in accordance with Canadian Council on Animal Care guidelines at the Spyhill Campus, University of Calgary, Canada (51.1595° N, 114.2148° W). Twelve Dorset yearling rams (65 to 80 kg) were housed indoors (two rams per

pen) at ~18-20 °C, with lights on from 07:00 to 16:30 to mimic natural breeding season conditions. At approximately 08:00 and 15:00, they were fed pellets and grass hay, with *ad libitum* access to water and salt. The experiment was done from November 2021 to February 2022.

6.3.2 Experimental design

An overview of the experimental design is given in Figure 6.1. Four rams were given 8 μg/kg dexmedetomidine IM (Dexdomitor, Zoetis, Parsippany-Troy Hills, NJ, USA), lumbo-sacral epidural anesthesia (0.07 mg/kg xylazine in ~4 mL saline; Rompun, Bayer, Mississauga, ON, Canada), and local blocks with bupivacaine (Bupivacaine, 2.5 mg/ml, Hospira Inc.) at incision sites. A skin incision (~2 cm) was made on the anterior scrotum, lateral to the midline and ~6 cm from the bottom of the scrotum, blunt dissection used to create a subcutaneous 'pocket' ventral to the surgery site, a data logger (DL; DST Micro-T, Star-Oddi, Gardabaer, Iceland; 8.3 × 25.4 mm and 3.3 g) placed, and the incision closed with staples. Implanting DLs on the anterior scrotum put the scrotal incision in apposition with the caudal abdomen during recumbency, to promote cleanliness [26]. Rams were given 1 ml/10 kg oxytetracyline SQ (Bio-Mycin 200 LA), plus 0.5 mg/kg meloxicam SQ (Metacam) before surgery. The DLs were programmed to record temperature hourly, starting 10 d post-surgery.

Figure 6.1: Schematic illustration of the experimental design.

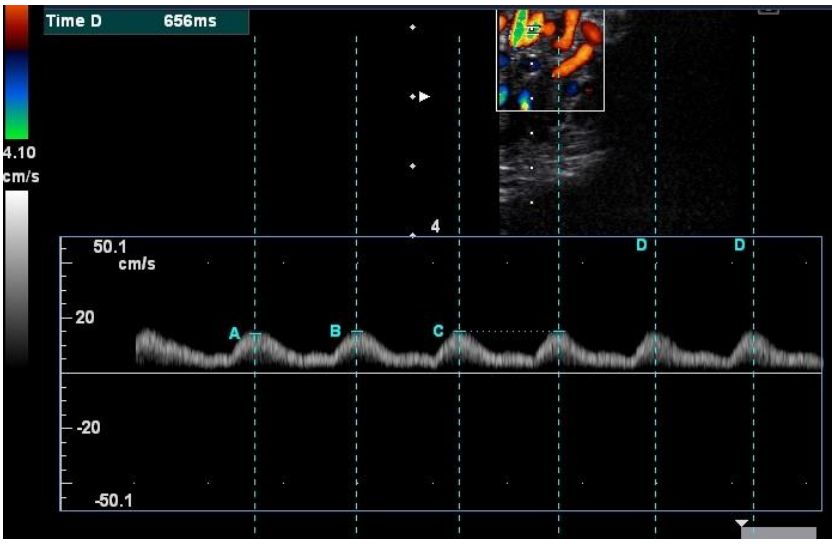


CONT, control; MEL, melatonin.

Once weekly, semen samples were collected using an electroejaculator [177] and ultrasonographic examinations were done. The same operator consistently performed all ultrasonographic examinations. To minimize effects on testicular blood flow (TBF), ultrasonography was done before any other procedures and rams were gently physically

restrained without sedation. All ultrasonographic examinations were done with a 7.5 MHz linear-array transducer (Toshiba Viamo, Tokyo, Japan). Copious ultrasonic gel was applied to the transducer, and fleece on the posterior aspect of the scrotum was shaved as needed. All ultrasound machine settings (frequency, brightness, depth, and contrast) were standardised and fixed uniformly for all assessments. The angle between the Doppler beam and the long axis of the supratesticular artery never exceeded 60^0, with a high-pass filter set at 50 Hz in the pulsed wave Doppler settings. Furthermore, the Doppler gate was held constant at 0.5 mm. The transducer was placed longitudinally on the sidewall (lateral surface) of the scrotum and the position adjusted until the supratesticular artery was visible within the vascular network at the proximal pole of the testis (testicular vascular cone). Following presentation of the supratesticular artery's spectral layout (Figure 6.2), end points assessed were Doppler indices (resistive index [RI] and pulsatility index [PI]). In addition, B-scale images of the testes were frozen and recorded and echogenicity of testicular parenchyma analyzed using ImageJ® software (NIH, Bethesda, MD, USA).

Figure 6.2: Spectral layout of the supratesticular artery in rams, using pulsed wave Doppler mode.



At 10 d after implanting DLs, semen was collected and evaluated from the 12 rams for 2 wk as a pre-treatment control (semen from each ram was kept separate, with no pooling of ejaculates). Then, rams were allocated randomly into two groups, each with six rams; one group (MEL), was given a single dose of 36 mg melatonin in 1 ml corn oil SQ under the ear, as reported [144,211], whereas the second group (CONT) was given only 1 ml corn oil. At 15 d after treatment, all rams were subjected to mild HS (insulation of the scrotal neck with a wool-blend sock; as described [34]) for 96 h, with blood flow measurements and semen collection done once weekly for the next 7 wk after HS (9 wk post-melatonin injection). In vitro sperm valuations included motility using CASA (Sperm Vision®; [179]), morphology using eosin-nigrosin and acrosome integrity using FITC-PSA [180].

Once all examinations and semen collections were completed, small incisions were made in scrotal skin using identical procedures as those described above to enable retrieval of the DLs. Data were subsequently retrieved using the manufacturer's communication box and Mercury® software. All DLs were working, there were no missing data, and no signs of inflammation or reactions where DLs were located.

6.3.3. Statistical analyses

Normality of data distributions was evaluated, and repeated measures used to analyse all data that were collected repeatedly, including main effects (group and week) and their interactions. A Bonferroni *post-hoc* test was used to compare between groups within each week. Data variance was uniform (based on Levene's tests). Results are reported as mean ± standard error of the mean (SEM). For all analyses, $p < 0.05$ was considered significant. The IBM SPSS 27.0 Software Package was used to conduct statistical analyses (IBM Corp., New York, NY, USA).

6.4 Results

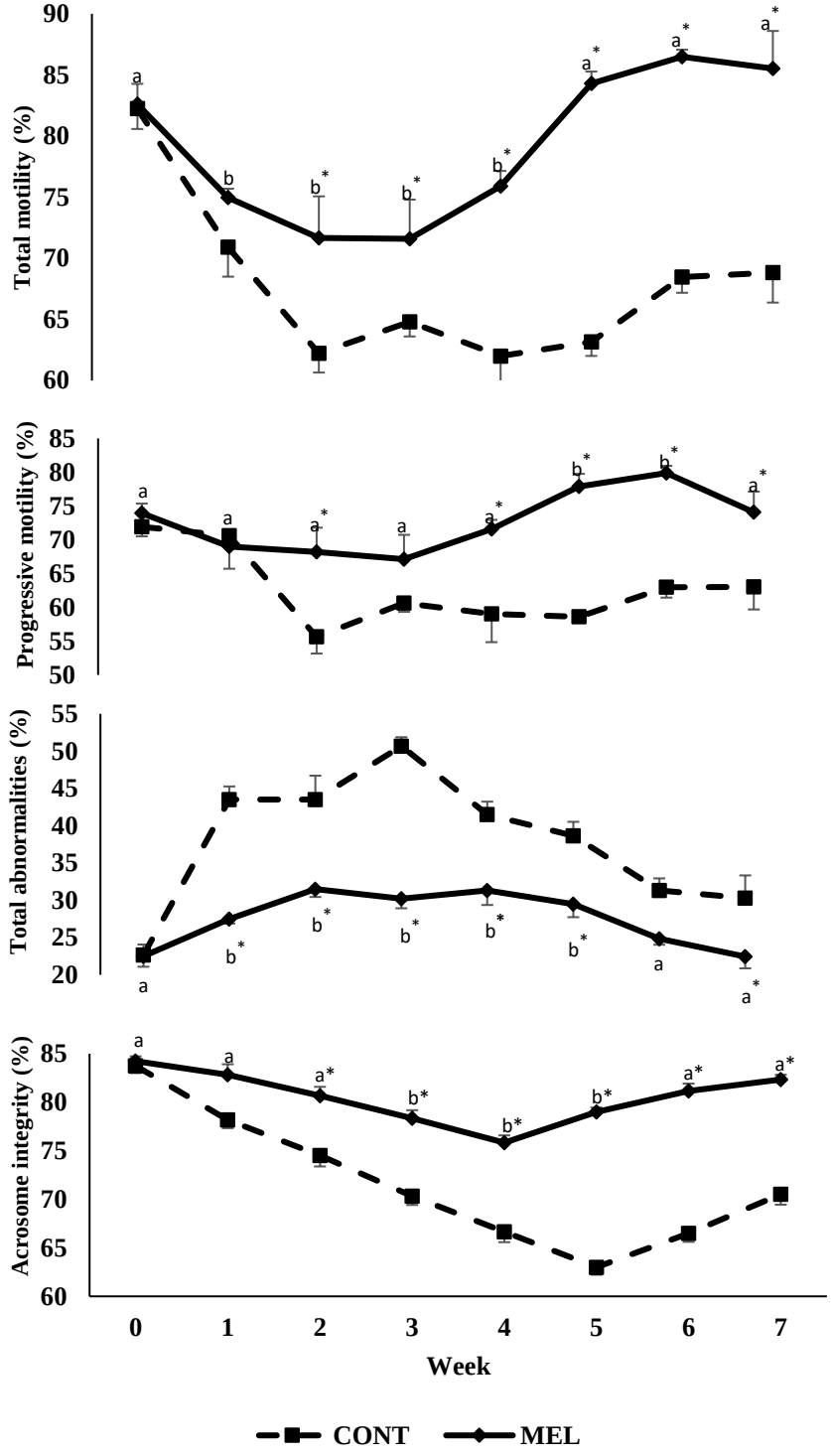
6.4.1 Total and progressive motility and sperm kinematics

For total and progressive motility, there were effects of group, week and group*week interactions ($p < 0.005$). For total motility (Figure 6.3), there was a reduction ($p < 0.05$) in sperm quality from 1 wk after HS in both groups, persisting to 4 wk after HS in the MEL group and 7 wk after HS in the CONT group, with higher total motility ($p < 0.05$) from 2 to 7 wk in MEL versus CONT rams. There was a reduction ($p < 0.05$) in progressive motility from 2 wk after HS in CONT rams, whereas rams in the MEL group had higher ($p < 0.05$) progressive motility than in the CONT group from 2 to 7 wk after HS (Figure 6.3). Regarding sperm kinematics, average path velocity (VAP) and curvilinear velocity (VCL) had significant group, week and group*week effects, linearity (LIN) had a significant week effect only, and wobble coefficient (WOB) had significant week and group* week interaction effects, with generally better kinetics in MEL versus CONT rams (Table 6.1).

6.4.2 Total sperm abnormalities and acrosome integrity

For total sperm abnormalities and acrosome integrity there were effects of group, week and group*week interaction effects ($p < 0.001$).

Figure 6.3: Mean ±SEM sperm motility and morphological abnormalities, in melatonin-treated (MEL) and control (CONT) rams subjected to testicular heat stress (HS; Week 0).



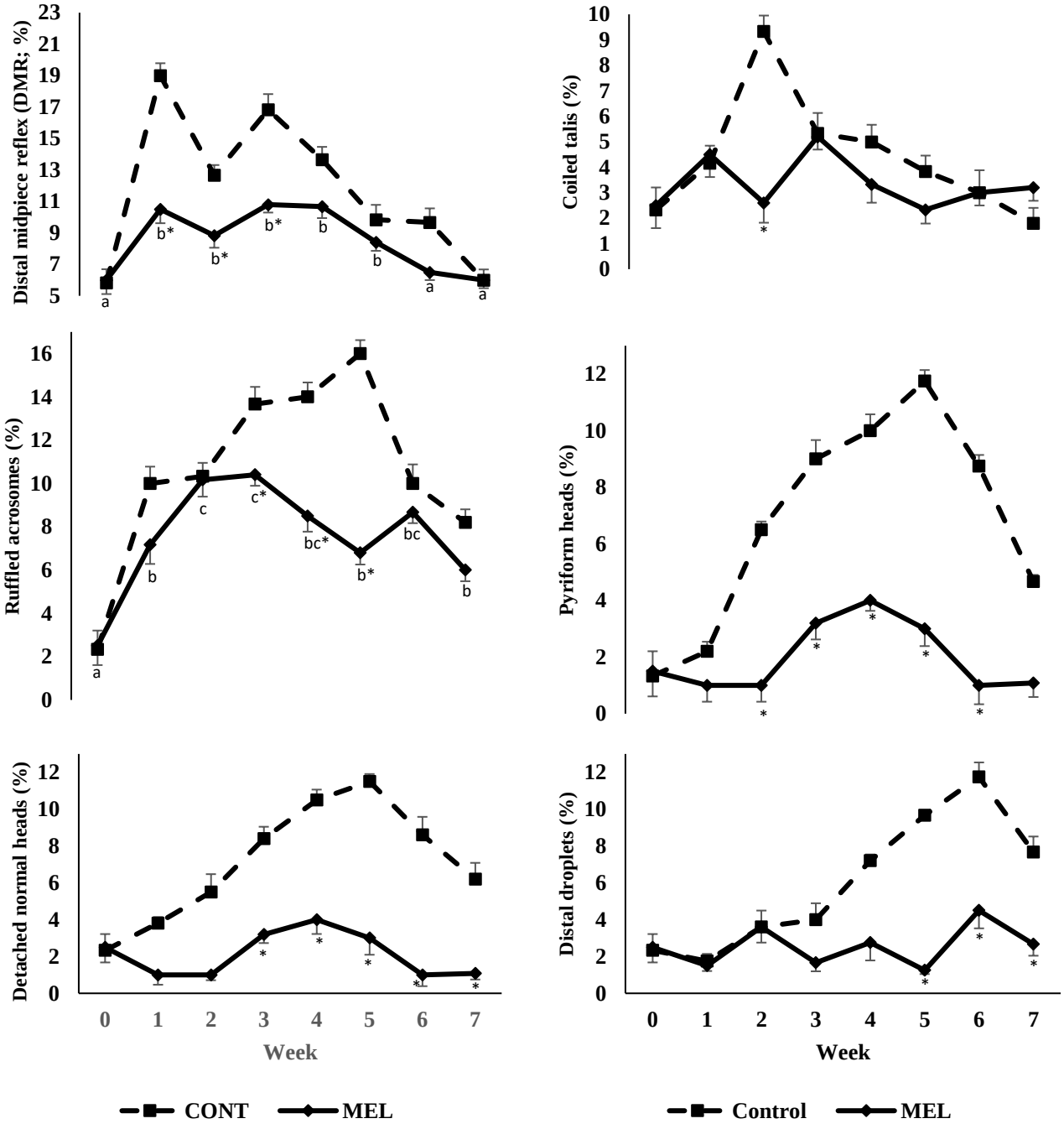
*Difference ($p < 0.05$) between groups on that week. [a,b]Within the melatonin group, means without a common superscript differed ($p < 0.05$). There were group, week and group*week interaction effects ($p < 0.005$) for total and progressive motility and for total sperm morphological abnormalities.

Although there was an increase ($p < 0.05$) in total abnormalities (Figure 6.3) in the MEL group compared to baseline values from 1 to 5 wk after HS, the MEL group had significantly fewer total abnormalities compared to the CONT group for most weeks after HS. Distal midpiece reflex (DMR), coiled tails, ruffled acrosomes, pyriform heads, detached normal heads and distal cytoplasmic droplets (Figure 6.4) were the most common sperm defects in both groups after HS. However, in most weeks after HS, the MEL group had fewer ($p < 0.05$) abnormalities than CONT. In the MEL group, acrosome integrity was lower ($p < 0.05$) at 3-5 wk after HS compared to baseline (Figure 6.3). Furthermore, the MEL group had higher ($p < 0.05$) acrosome integrity compared to the CONT group from 2 to 7 wk post HS.

6.4.3 Doppler indices (PI and RI) of supratesticular artery and testicular echogenicity

For PI, RI, and testicular echogenicity, there were group and week effects ($p < 0.001$). In the MEL group, there were reductions ($p < 0.05$) in PI and RI starting from 1 and 2 wk post HS, respectively, and persisting until the end of the study (Figure 6.5). However, both groups had reductions ($p < 0.05$) in PI and RI on the day of HS removal. Testicular echogenicity was higher ($p < 0.05$) in the MEL group compared to the CONT group from 1 to 7 wk after HS.

Figure 6.4: Mean ±SEM sperm morphological abnormalities, in melatonin-treated (MEL) and control (CONT) rams subjected to testicular heat stress (HS; Week 0).



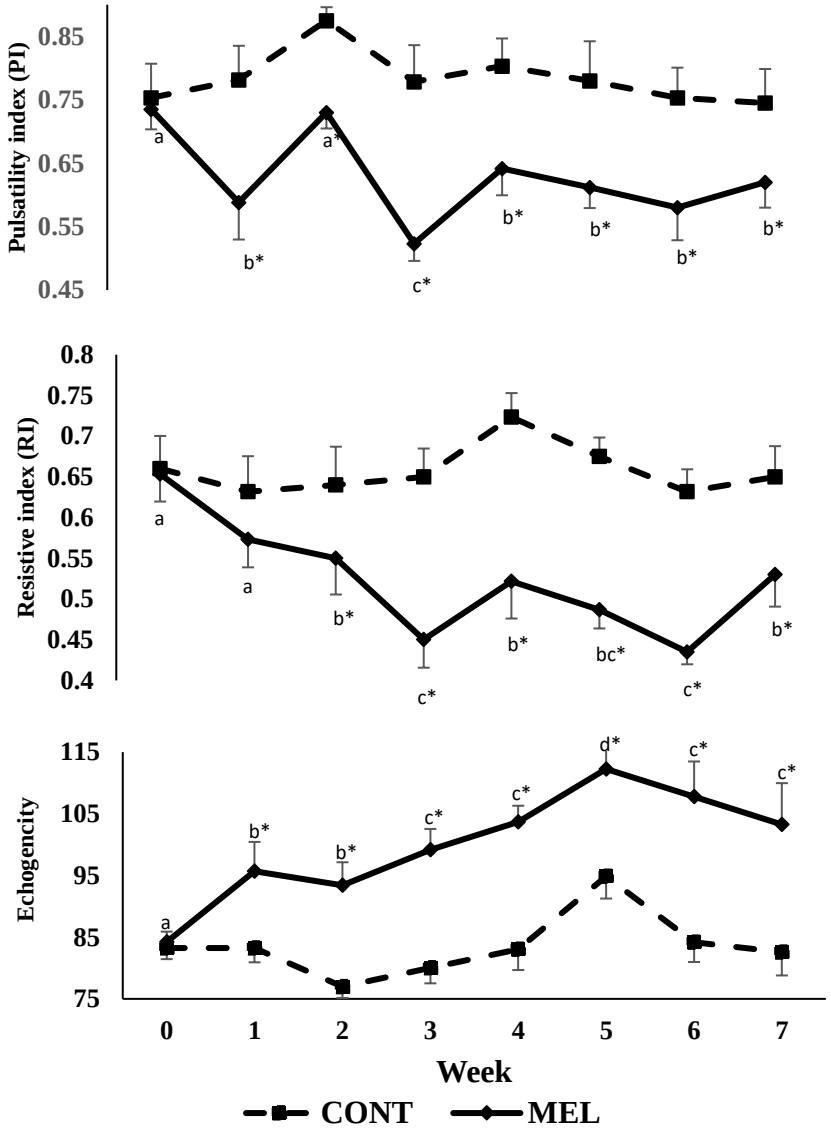
*Difference ($p < 0.05$) between groups on that week. [a-c]Within the melatonin group, means without a common superscript differed ($p < 0.05$).

Table 6.1: Mean ± SEM sperm kinematics (CASA) in rams treated with melatonin in oil (MEL) or only oil (CONT) and subjected to testicular heat stress (HS).

Interval after HS	VAP		VSL		VCL		STR		LIN		WOB	
	Control	MEL	Control	MEL	Control	MEL	Control	MEL	Control	MEL	Control	MEL
W0	79.42 2.18	77.91[a] 2.19	116.67 2.95	117.0[a] 3.44	72.48 2.87	70.45[a] 1.95	0.91 0.01	0.90 0.01	0.62 0.01	0.62[a] 0.03	0.68 0.02	0.67[a] 0.03
W1	72.84 2.51	2.88[b*] 1.58	104.21 2.70	126.19[b*] 2.50	66.75 2.99	71.29[a*] 1.58	0.91 0.01	0.89 0.01	0.63 0.01	0.58[b*] 0.02	0.69 0.01	0.64[a*] 0.03
W2	75.95 2.00	90.47[c*] 2.60	111.39 3.34	115.96[a] 2.75	68.38 3.82	77.11[b*] 1.54	0.89 0.01	0.93 0.01	0.61 0.01	0.66[c*] 0.01	0.67 0.01	0.71[b*] 0.03
W3	74.12 1.87	89.45[c*] 2.20	104.78 1.84	122.62[b*] 2.20	67.41 1.84	84.43[c*] 1.35	0.90 0.01	0.93 0.01	0.64 0.01	0.68[c*] 0.01	0.70 0.01	0.73[b] 0.01
W4	78.12 1.82	105.61[d*] 2.09	110.17 2.71	123.82[b*] 1.35	71.41 1.99	82.03[c*] 1.71	0.91 0.01	0.91 0.01	0.64 0.02	0.66[c*] 0.01	0.71 0.01	0.72[b] 0.01
W5	70.53 1.80	82.05[b*] 1.49	103.84 2.19	150.00[c*] 2.41	63.78 2.08	94.83[d*] 1.66	0.89 0.01	0.89 0.01	0.61 0.02	0.63[a] 0.01	0.68 0.01	0.71[b] 0.01
W6	76.70 1.95	79.37[a] 1.00	112.75 3.34	131.85[d*] 3.19	69.06 1.95	74.73[ab] 1.03	0.89 0.01	0.91 0.01	0.61 0.03	0.56[b*] 0.01	0.67 0.02	0.62[a*] 0.02
W7	78.18 1.67	87.54[c*] 1.59	138.01 2.45	134.22[d] 2.14	59.86 2.35	80.63[c*] 1.35	0.76 0.01	0.92 0.01	0.43 0.03	0.61[a*] 0.02	0.56 0.01	0.65[a*] 0.02

Difference ($p < 0.05$) between groups on that week. [a-c]Within the melatonin group, means without a common superscript differed ($p < 0.05$). VAP and VSL had group, week and group*week effects (0.001, 0.016 and 0.001; 0.001, 0.028 and 0.001, respectively). For VCL, there were group and group* week interaction effects (0.001 and 0.04, respectively), LIN had week effect only (0.02), and WOB had week and group* week interaction effects (0.003 and 0.04, respectively).

Figure 6.5: Mean ±SEM of Doppler indices (Pulsatility index; PI and Resistive index; RI) of supratesticular artery and testicular echogenicity from rams in control and melatonin groups.



*Difference ($p < 0.05$) between groups on that week. [a-d]Within the melatonin group, means without a common superscript differed ($p < 0.05$). There were group and week effects ($p < 0.001$) for PI, RI and testicular echogenicity. On the day of HS removal PI, RI and echogenicity were 0.56, 0.48 and 86.90, respectively, in the control group and were 0.54, 0.45 and 88.02 in the melatonin group.

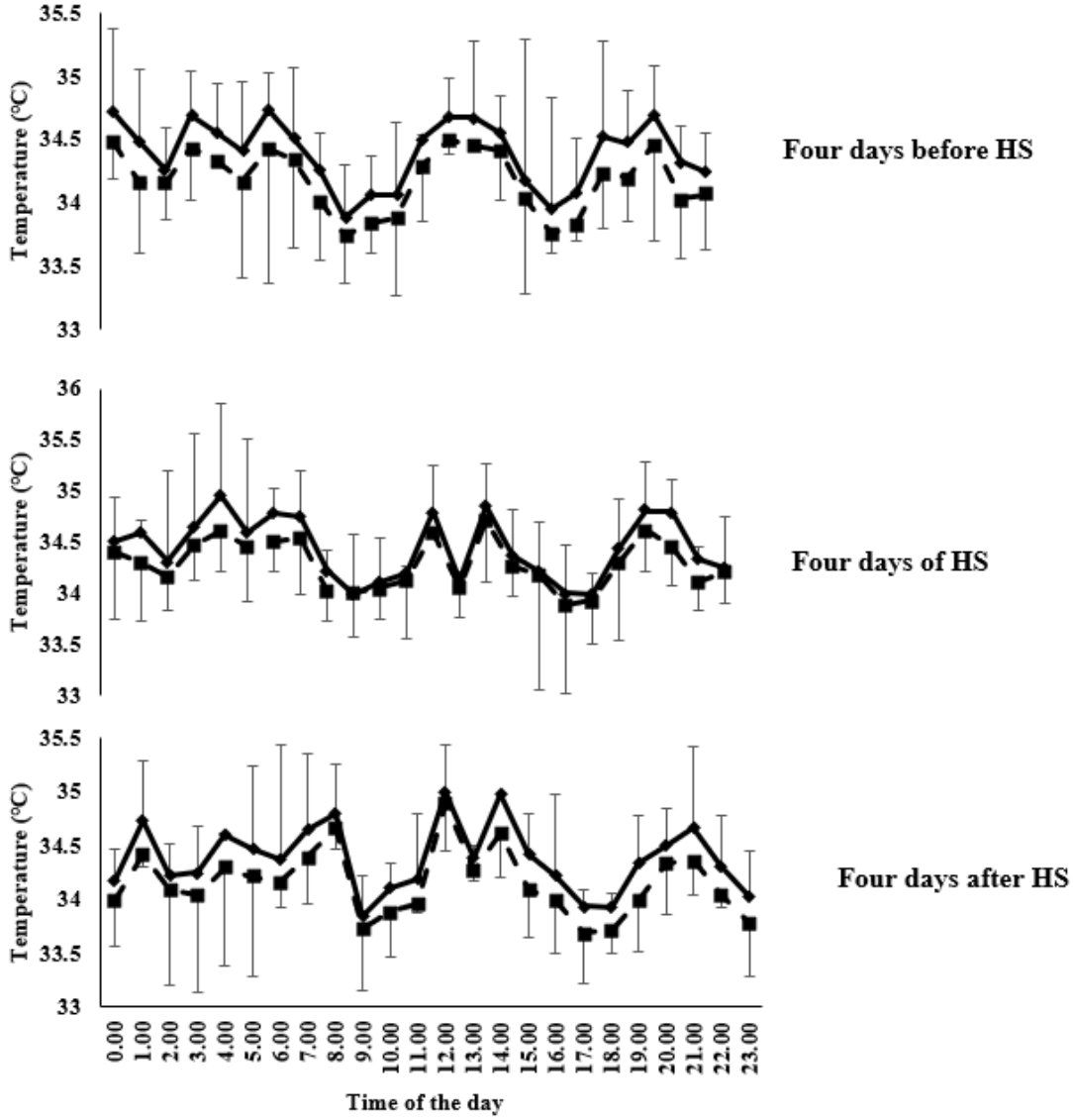
6.4.4 Scrotal subcutaneous temperature

There was no difference ($p < 0.05$) between MEL and CONT groups in scrotal subcutaneous temperatures over the 4-d intervals before, during, or after HS (Figure 6.6).

6.5 Discussion

Our objective was to elucidate if melatonin affected testicular blood flow and sperm quality in rams following mild HS (scrotal neck insulation). To the best of our knowledge, this is the first report on the effects of melatonin to protect the testes against HS in farm animals. In this experiment, the MEL group had higher progressive and total motility from 2 to 7 wk after HS with generally better sperm kinetics. Furthermore, the MEL group had significantly fewer total abnormalities compared to the CONT group for most weeks after HS and higher acrosome integrity compared to the CONT group from 2 to 7 wk post HS. These findings supported our hypothesis that melatonin significantly improves testicular blood flow and protects sperm motility and morphology in rams exposed to testicular HS. Furthermore, this was consistent with previous studies in which melatonin injections improved sperm quality in the non-breeding season in rams [211,212] and goats [144]. These improvements were attributed to melatonin's systemic or local roles in the testis, as well as increases in testicular blood flow, which could be a potential trigger for the testis' endocrine function [156]. Furthermore, this could be also be due to its antioxidant and anti-apoptotic properties in testes and sperm [130].

Figure 6.6: Mean ± SEM of data logger (DL) temperatures for 4 d before heat stress (HS), 4 d of HS and 4 d after HS in rams in control and melatonin groups. There was no difference ($p < 0.05$) between MEL and CONT groups in scrotal subcutaneous temperatures over the 4-d intervals before, during, or after HS.



In the MEL group, there were reductions in PI and RI starting from 1 and 2 wk post HS, respectively, and persisting until the end of the study (except at Week 2 for PI). However, both groups had reductions in PI and RI at HS removal. Furthermore, testicular echogenicity was higher in the MEL group compared to the CONT group from 1 to 7 wk after HS, and overall, changes in echogenicity were inversely proportional to changes in the PI and the RI, with the higher flow increasing the echogenicity. Similarly, in previous studies [10,115], HS increased testicular blood flow, ensuring ample O_2 to support increased testicular metabolic needs and avoid hypoxia. Regarding melatonin administration, results were consistent with previous studies where melatonin injections in rams in either breeding or non-breeding seasons [210,211] reduced PI and RI, thereby increasing testicular blood flow after melatonin injection, with similar effects in goats [144].

There was no difference between MEL and CONT groups in scrotal subcutaneous temperatures in the 4-d intervals before, during and after HS. This was consistent with our previous report [34] of no differences in scrotal subcutaneous temperature before, during, or after HS when using the scrotal neck insulation model of HS. Perhaps sheep have a higher thermoregulatory capacity than cattle are therefore are less sensitive to HS [158,159]. It could also be due to differences in how the scrotal neck was insulated, thereby affecting the extent of the HS and body responses. Another possibility was that the testicular thermoregulatory ability was not impaired by the scrotal neck insulation model used in our study. Despite the increased blood flow in the MEL group, there was no increase in scrotal subcutaneous temperature, although blood flow is the main source of heat in the testes [112]. As melatonin has been reported to affect thermoregulatory function [213], despite the increased blood flow in MEL, perhaps these rams were able to thermoregulate and maintain testicular temperature within the same range as the control.

There were significant effects of Group, Week, and Group x Week interactions for total and progressive motility and for total sperm morphological abnormalities. For all of these end points, as would be expected, at the outset, there were very similar values in the two groups. However, soon thereafter, the values substantially differed, with a much more pronounced deterioration in the CONT group compared to the much milder deterioration in the MEL group, and a more rapid return to baseline values, thereby accounting for the significant main effects and interactions. Furthermore, individual sperm morphological abnormalities (Fig. 6.4) were very consistent with total sperm morphological abnormalities (Fig. 6.3).

6.6 Conclusion

In conclusion, melatonin administration before HS heat stress significantly improved testicular blood flow and protected sperm motility and morphology, consistent with our hypothesis. Therefore, melatonin has potential for mitigating effects of testicular HS under field conditions.

Chapter 7: Melatonin improves post-thaw sperm quality after mild testicular heat stress in rams; modified from: Shahat, A. M., Thundathil, J. C., & Kastelic, J. P. (2022). Melatonin improves post-thaw sperm quality after mild testicular heat stress in rams. Accepted (December 2022) in *Reproduction in Domestic Animals* journal.

7.1 Abstract

The objective was to determine effects of slow-release melatonin on post-thaw sperm quality in rams exposed to mild testicular heat stress (HS; scrotal neck insulation). Twelve yearling Dorset rams were randomly and equally allocated to receive either 36 mg melatonin in 1 ml corn oil or 1 ml corn oil injected subcutaneously (SQ); 15 d later, all rams had HS for 96 h (start of HS = start of Week 0). Semen was collected before HS and once weekly from Weeks 1 to 7, extended in Steridyl CSS One Step, held at 5 ℃ for ~3 h, loaded into 0.5 ml straws, held 5 cm above liquid nitrogen for 10 min, and then plunged. Computer assisted semen analysis (CASA) was conducted on frozen-thawed sperm. There were group and week effects for total and progressive motility ($p = 0.001$), plus group and week effects and group*week interactions ($p = 0.001$) for post-thaw total abnormalities, acrosome integrity, post-thaw sperm DNA fragmentation index (DFI), and high mitochondrial membrane potential (HMMP). Post-thaw sperm total and progressive motility, acrosome integrity and HMMP were higher ($p < 0.05$) in melatonin versus control groups from Weeks 1 to 7, and for the melatonin group reached baseline level (pre-heat stress) at Week 7 (75.79 ± 0.96, 65.48 ± 1.51, 75.00 ± 0.89, and 67.00 ± 1.06, respectively; mean ± SEM). Conversely, post-thaw sperm total abnormalities and DFI were lower ($p < 0.05$) in melatonin versus control and both reached baseline at Week 7 in the melatonin group (26.00 ± 0.57 and 5.66 ± 0.17, respectively). Coiled tails, distal midpiece reflexes, distal cytoplasmic droplets, ruffled acrosomes, bowed midpieces, pyriform heads, and knobbed acrosomes were the most common abnormalities in both groups, with lower percentages in melatonin-treated rams. Results supported our

hypothesis that HS reduces post-thaw sperm quality, and that melatonin lessens those reductions, manifested by significantly better total and progressive motility, acrosome integrity and HMMP, and fewer sperm total abnormalities and DFI.

7.2 Introduction

Heat stress (HS) is detrimental to animal health and fertility, often caused by extreme heat, and exacerbated by climate change [155,214]. Most mammalian testes need to be 4 to 5 °C below body core temperature to produce morphologically normal, motile, and viable sperm [24]. Temperatures exceeding physiological tolerance and ability to compensate cause HS; it often affects the entire body, but can be localised [35].

Effects of higher testicular temperatures depend on their duration and intensity; a slight increase may momentarily reduce sperm quality, whereas a constant and/or severe increase is likely to cause infertility [10,156]. All types of testicular cells are affected, with germ cells being most sensitive [54]. Testicular HS reduces sperm production and quality, with morphologically aberrant sperm appearing after a few days [95]. Mild HS (scrotal insulation for 8 h) reduced quality of frozen-thawed bull sperm [215].

Sperm cryopreservation is valuable for managing and preserving male fertility in humans and animals. Despite "optimal" cooling/thawing protocols, roughly half of sperm die [216]. There are also deleterious effects on sperm kinematics, DNA integrity, membrane integrity, and composition, attributed to increased reactive oxygen species (ROS) [217]. Moreover, cryopreserved sperm have decreased antioxidant activity [218], especially those from heat-stressed males [97].

Melatonin (N-acetyl-5-methoxytryptamine, MW= 232), synthesized from tryptophan via serotonin and secreted by the pineal gland [219], regulates the circadian rhythm [213] and seasonal

reproduction in some mammals. It also has powerful antioxidant properties as a hydroxyl radical (•OH) scavenger [165], detoxifying various reactive oxygen and nitrogen species, including singlet oxygen (O2), NO, and the ONOO anion, as well as peroxynitrous acid and H_2O_2. Melatonin has been widely used, both *in vitro* to improve post-thaw sperm quality in various species [143,172,220] and *in vivo* in rams (slow-release injection) to improve testicular blood flow and sperm quality [210,211,221]. Slow-release melatonin reached peak plasma concentrations (1.3-1.5 ng/ml) at Weeks 3 and 4 after injection then decreased, reaching basal concentrations (0.1-0.2 ng/ml) at Week 8 [144]. Furthermore, melatonin improved post-thaw sperm quality [222,223].

We reported that mild HS in rams induced deleterious effects on fresh sperm (total and progressive motility, total abnormalities and acrosome integrity), but these were mitigated by slow-release melatonin injected before HS [221]. Our objective was to determine effects of slow-release melatonin on post-thaw sperm quality in rams exposed to mild testicular HS. We hypothesized that melatonin lessens reductions in post-thaw sperm quality following mild testicular HS.

7.3 Materials and methods

7.3.1 Animals and chemicals

Unless otherwise specified, all chemicals were from Sigma-Aldrich (St. Louis, MO, USA). The University of Calgary Veterinary Sciences Animal Care Committee approved this experiment (AC19-0106), conducted in accordance with Canadian Council on Animal Care guidelines at the University of Calgary, Canada (51.1595° N, 114.2148° W) from November 2021 to February 2022. Rams and collection and evaluation of semen collection were reported (Shahat et al., 2022a). Yearling Dorset rams (n=12, 65 to 80 kg) were housed indoors at 18-20 °C, with lights on from 07:00 to 16:30, and fed pellets and grass hay, with *ad libitum* access to water and salt.

7.3.2 Experimental design

As a pre-treatment control, semen was collected and evaluated from each ram once weekly for 2 weeks (semen from each ram was kept separate). Then, rams were allocated randomly into two equal groups, with one group (MEL) given 36 mg melatonin in 1 ml corn oil subcutaneously (SQ) under the ear, whereas the second group (CONT) received only 1 ml corn oil. Fifteen days later, all rams were subjected to mild HS (scrotal neck insulation with a wool-blend sock) for 96 h (start of HS = start of Week 0). Semen collection was done once weekly for Weeks 1-7, starting 7 d after HS was initiated.

All ejaculates were cryopreserved. Semen was extended (Steridyl CSS One Step ®; Minitube Canada Ltd., Ingersoll, ON, Canada) and kept in a refrigerator for ~3 h until it reached + 5 °C. Thereafter, it was loaded into 0.5 ml straws, held horizontally 5 cm above liquid nitrogen in a styrofoam box (42×42×27 cm) for 10 min, then plunged into liquid nitrogen (-196 °C) and subsequently stored in liquid nitrogen tanks [178].

7.3.3 Evaluation of frozen-thawed sperm

Straws were thawed at 37 °C for 35 s (two straws/week/ram/group) and immediate post-thaw motility (total and progressive forward) and kinematics evaluated using Computer Assisted Semen Analysis (CASA; Sperm Vision®;[179]). For CASA, 5 µl of post-thawed semen was put into a pre-heated 20 µm Leja slide (IMV®), examined with a ZEISS microscope Axioskop 40 and AccuPiXEL camera, and analyzed with Sperm Vision software (Minitube®). For morphology, a drop of semen and of eosin-nigrosin were placed on a pre-heated slide, mixed, and a smear was made and subsequently evaluated (200 sperm per slide at 1000 X). We assessed the following morphological defects: proximal and distal cytoplasmic droplets, distal midpiece reflexes, bowed midpieces, pyriform and tapered heads,

detached normal and abnormal heads, microcephalic heads, coiled and double tails, knobbed, and ruffled acrosomes. Acrosome integrity was evaluated using FITC-PSA ([180]; 200 sperm per slide at 1000 X).

7.3.3.1 Assessment of sperm characteristics using flow cytometry

To assess DNA integrity, a sperm chromatin structure assay (SCSA) was done with flow cytometry (BDTM LSR II; BD Biosciences, East Rutherford, NJ, USA), with 200 events/s and 10,000 events/sample (done in duplicate). To exclude debris and aggregates, sperm were gated using 90° and forward-angle light scatter. The flow cytometer laser was 488 nm excitation wavelength and 10 – 30 mW power, with FL1 and FL3 detectors used to detect green (515-530 nm band pass) and red fluorescence (>630 nm long pass), respectively. For each sample, two straws were thawed (37 °C, 35 s), pooled and extended to 2 $\times 10^6$ sperm/mL using TNE buffer (0.01 M TRIS-HCl, 0.15 M NaCl, and 1 mM EDTA; pH 7.4) immediately after thawing [181]. Sperm DNA was denatured by mixing 200 µL of extended semen and 400 µL of acid-detergent solution (0.1% v:v Triton X-100, 0.15 M NaCl, and 0.08 N HCl; pH 1.2). After 30 s, sperm were stained with 300 µL of acridine orange (AO; 10 mg chromatographically purified AO/mL). The AO stain buffer contained 370 ml 0.10 M citric acid buffer, 630 ml 0.20 M Na2PO4 buffer, 372 mg EDTA (disodium, FW = 372.24; 1 mM), and 8.77 g NaCl (0.15 M). Stained sperm were incubated on ice for 3 min before flow cytometry. In each sample, individual DNA fragmentation index for each spermatozoon (DNA fragmentation index; DFI), calculated by the ratio red/(red + green) fluorescence multiplied by 1000, was determined as described [182]. Each sample had two controls, negative (control semen) and positive (heat-treated; semen from HS males was heated to 95 °C then placed on ice).

Mitochondrial membrane potential (MMP) was assessed using JC-1 (Invitrogen, Eugene, OR, USA). In cells with low (LMMP) or medium (MMMP) mitochondrial membrane potential, this probe emits green fluorescent light, whereas cells with high mitochondrial potential (HMMP) emit red

fluorescent light [224]. For each sample, 1×10^6 sperm were suspended in 1 ml of warm PBS, 2 µM of JC-1 solution added, and semen incubated at room temperature for 30 min. Using a flow cytometer (BDTM LSR II flow cytometer (BD Biosciences, East Rutherford, NJ, USA), 20×10^3 events from each sample were examined (flow rate of ~200 sperm/s). Green (FL1) and orange (FL2) fluorescence, representing low and high mitochondrial membrane potential, respectively, were measured using emission filters of 530 and 585 nm bandpass, and proportion of cells with HMMP calculated [225]. The flow cytometer laser was 488 nm excitation wavelength and 10 – 30 mW power.

7.3.4 Statistical analyses

Normality of data distributions were evaluated, and repeated measures ANOVA used to determine main effects (treatment, group, and week) and their interactions. A Bonferroni *post-hoc* test was used to compare between groups within each week. Data variance (Levene's tests) was uniform. Results are reported as mean ± standard error of the mean (SEM). For all analyses, $p < 0.05$ was considered significant. The IBM SPSS 27.0 Software Package (IBM Corp., New York, NY, USA) was used.

7.4 Results

7.4.1 Motility and kinematics

There were group and week effects for both total and progressive motility ($p = 0.001$). At Week 0 (pre-HS baseline), there was no significant difference between CONT and MEL for total or progressive motility (Figure 7.1). Conversely, MEL had higher ($p < 0.05$) post-thaw total and progressive motility than CONT for Weeks 1-7. In MEL, post-thaw total and progressive motility were lower ($p < 0.05$) than baseline for Weeks 1 to 3 and 1 to 5, respectively, then back to baseline from Weeks 4 and 6 to Week 7.

Post-thaw VAP, VSL, VCL and STR had significant week and group*week effects, whereas LIN and WOB had significant week and group*week interactions (Figure 7.2). For all post-thaw sperm kinematics, there was no significant difference between CONT and MEL at Week 0 (Figure 7.2), but MEL had significantly higher VAP, VSL, STR and LIN than CONT on several weeks.

7.4.2 Total abnormalities and acrosome integrity

There were group, week and group*week interactions for post-thaw sperm total abnormalities and acrosome integrity ($p = 0.001$). There was no significant difference between groups at Week 0 for total abnormalities or acrosome integrity (Figure 7.1). However, MEL had fewer ($p < 0.05$) abnormalities compared to CONT from Weeks 1 to 7, with total abnormalities exceeding baseline on Weeks 2-5. Conversely, acrosome integrity in MEL was higher ($p < 0.05$) than in CONT from Weeks 1 to 7, with lower ($p < 0.05$) acrosome integrity from Weeks 2 to 5. For most morphological abnormalities, there were significant differences between groups, with significantly lower percentages in MEL versus CONT during various weeks (Figures 7.3 and 7.4).

7.4.3 DNA Fragmentation Index (DFI%) and High Mitochondrial Membrane Potential (HMMP%)

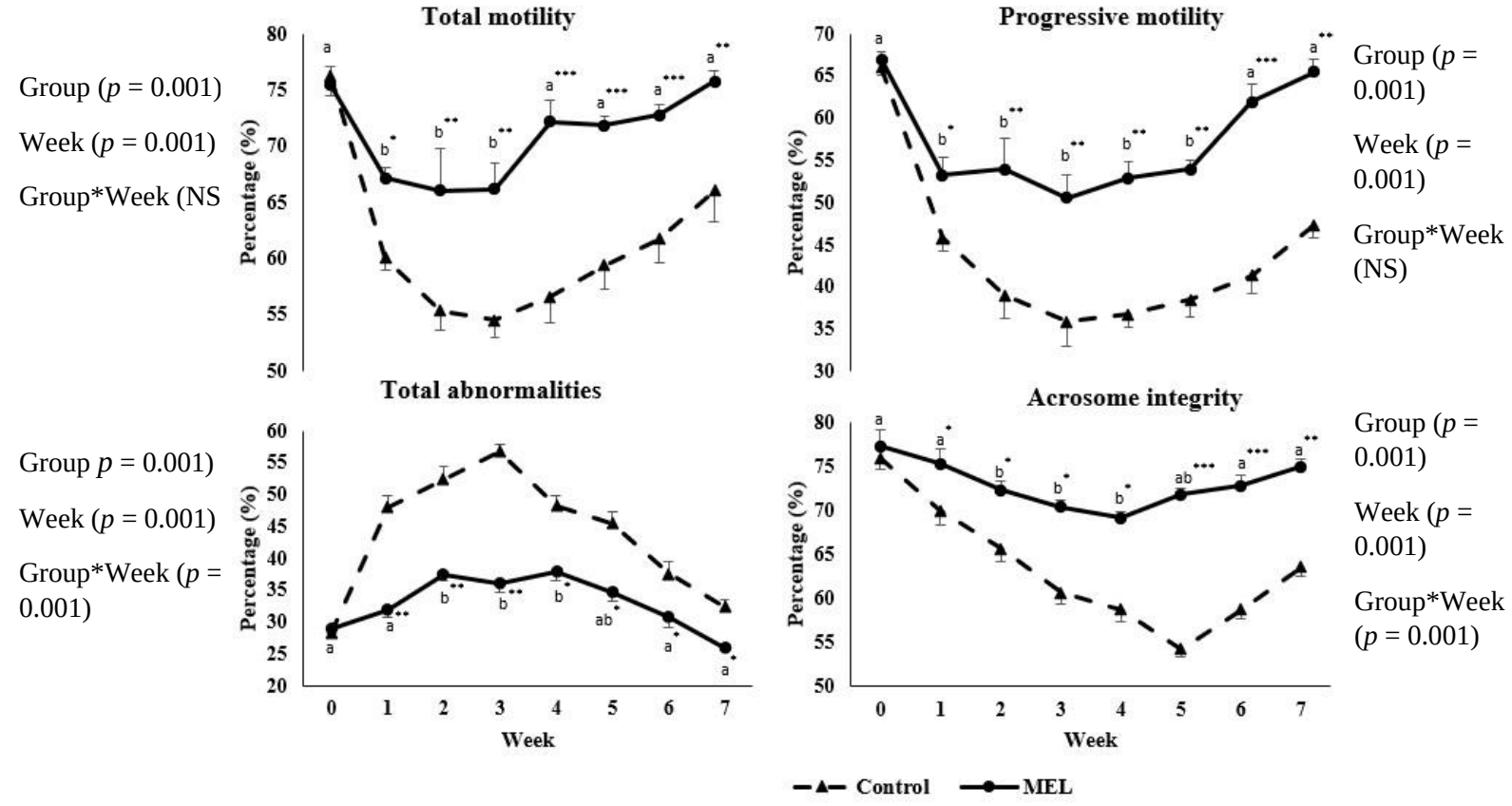
Both post-thaw sperm DFI% and HMMP% had group, week, and group*week interaction effects ($p = 0.001$). There was no significant difference between groups at Week 0 (baseline; prior to heat stress) for DFI% or HMMP% (Figure 7.5). For DFI%, MEL had a lower ($p < 0.05$) percentage than CONT starting from Week 4. However, HMMP% was significantly higher in MEL than CONT starting from Week 2.

7.5 Discussion

This is apparently the first report regarding effects of melatonin injection on post-thaw sperm quality of rams exposed to mild testicular HS, with melatonin conferring post-thaw sperm with

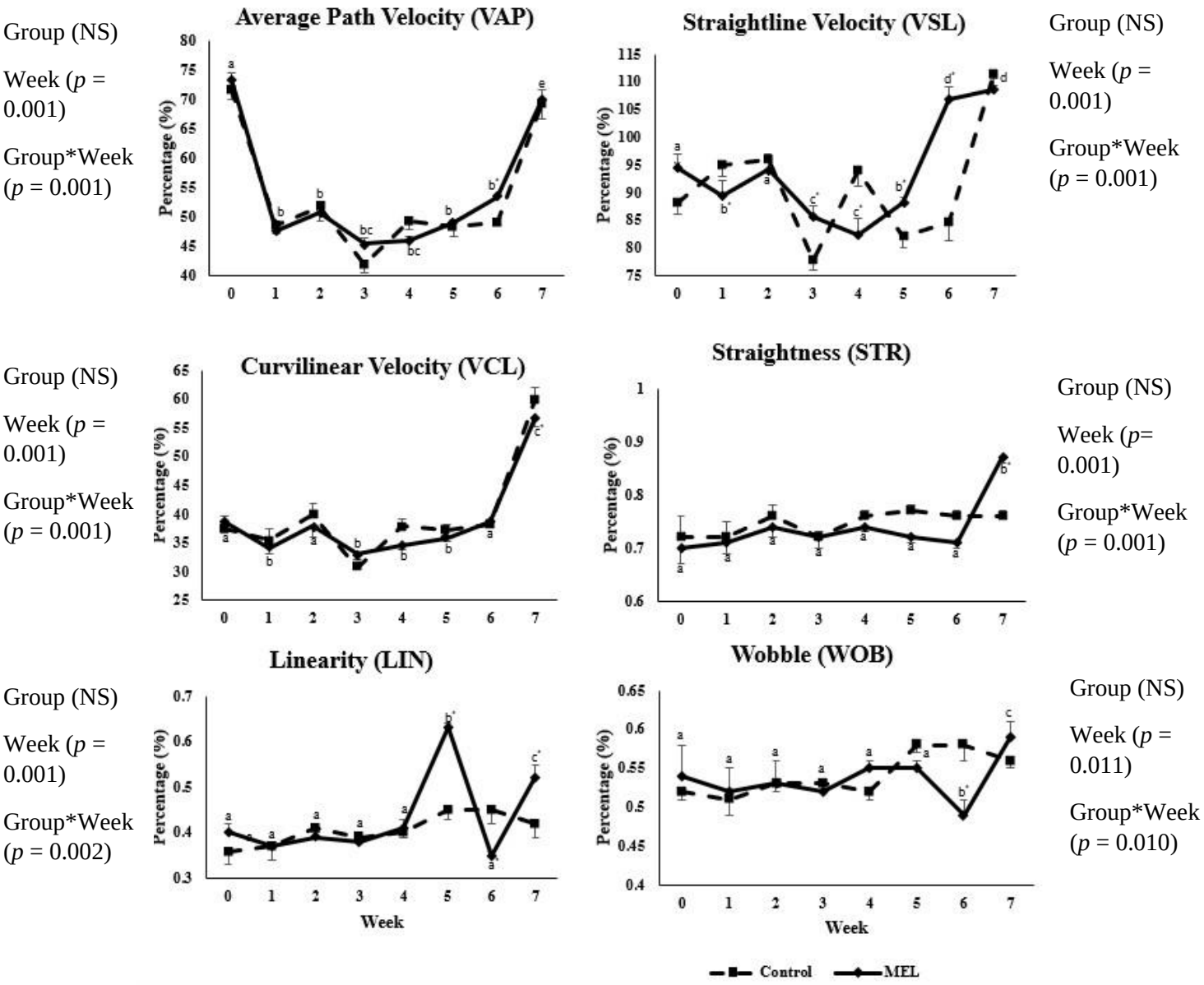
significantly better total and progressive motility, acrosome integrity and mitochondrial membrane potential, plus lower sperm abnormalities and DFI%.

Figure 7.1 Mean ± SEM post-thaw sperm characteristics in melatonin-treated (MEL) and control (CONT) rams (six per group) subjected to testicular heat stress (HS; Week 0).



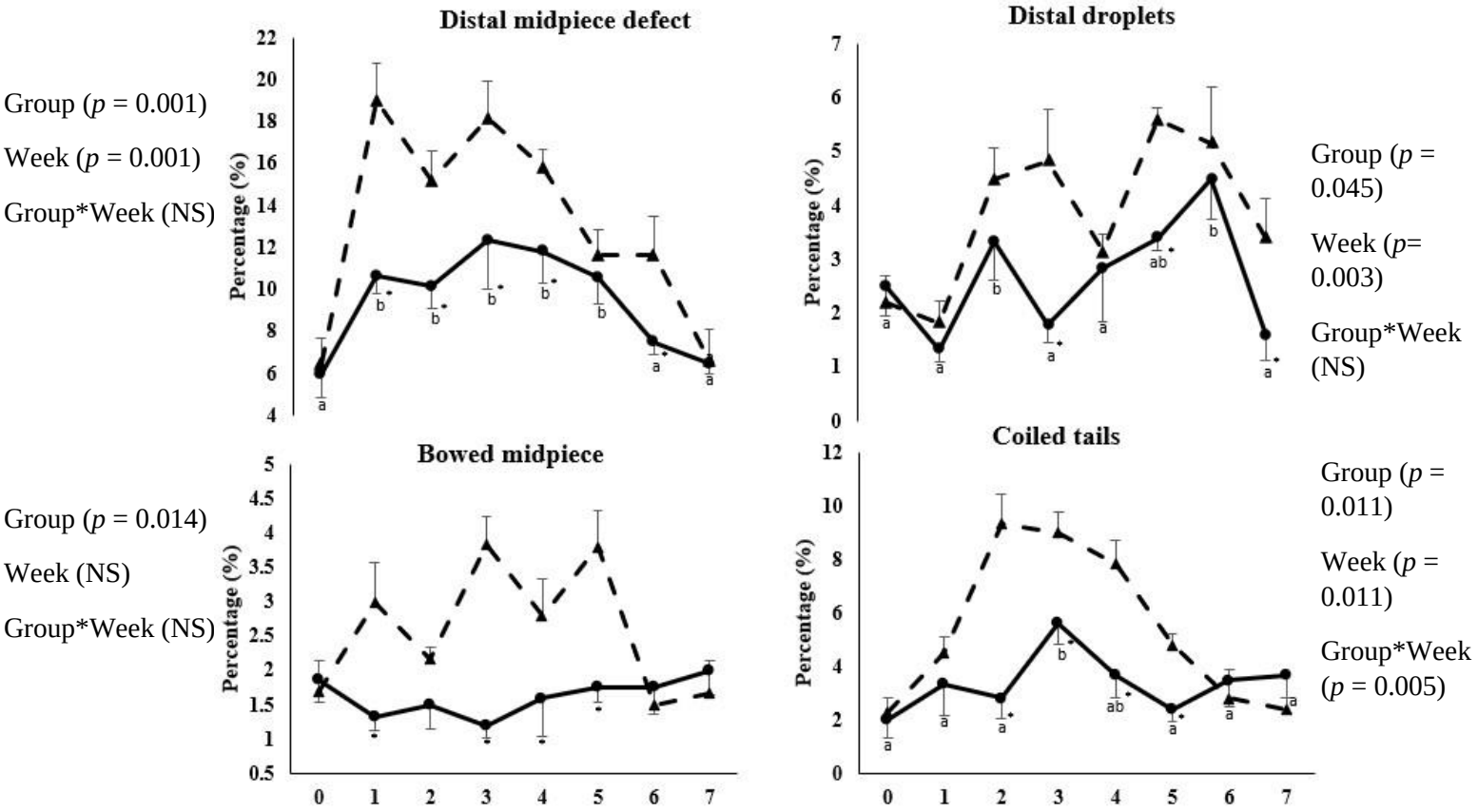
* ** and *** Difference ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) between groups on that week. [a,b]Within the melatonin group, means without a common superscript differed ($p < 0.05$). NS means non-significant.

Figure 7.2 Mean ± SEM of post-thaw sperm kinematics (CASA) in rams treated with melatonin in oil (MEL) or only oil (CONT) and subjected to testicular heat stress (HS).



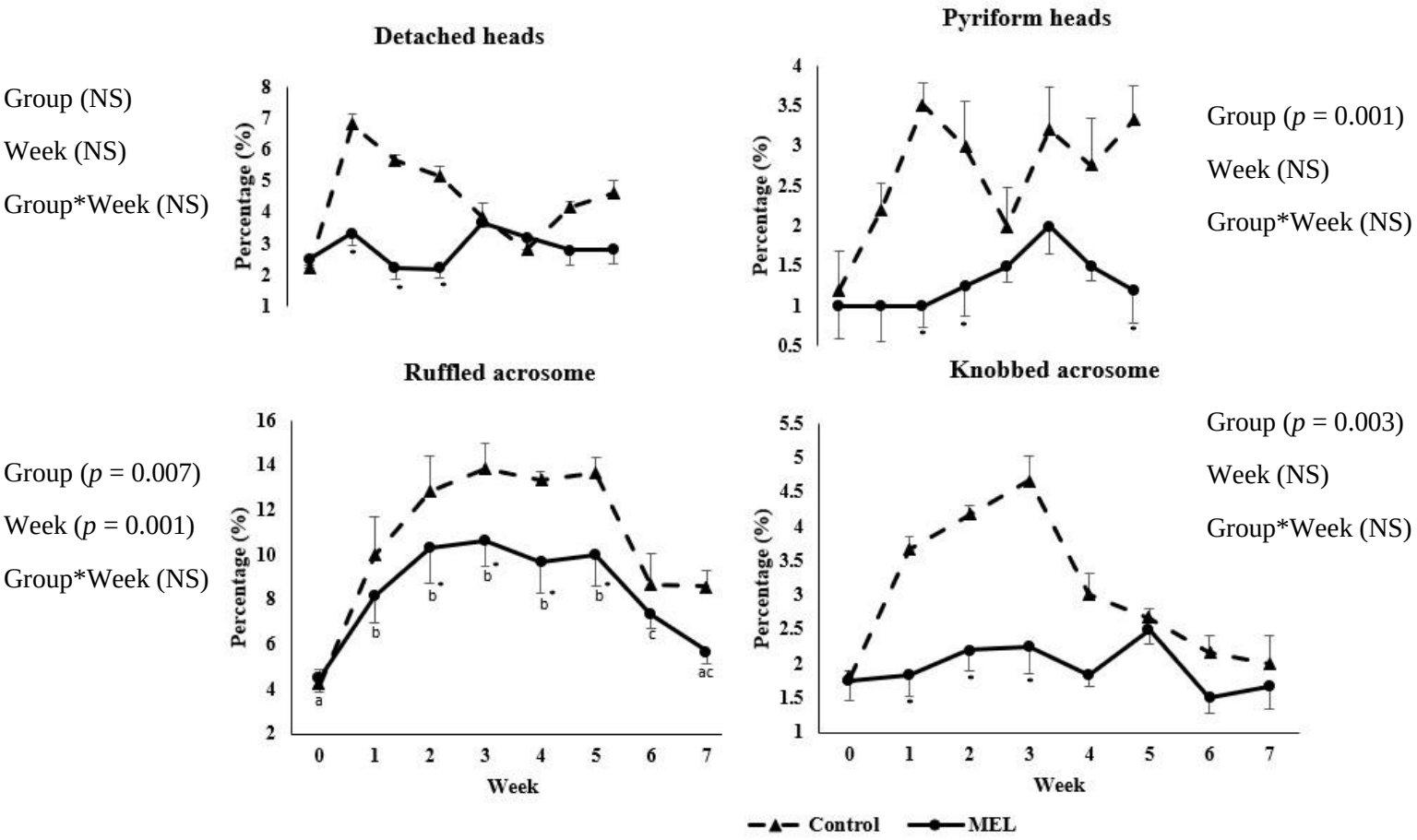
* Difference ($p < .05$) between groups on that week. [a-e]Within the melatonin group, means without a common superscript differed ($p < .05$). NS means non-significant.

Figure 7.3 Mean ±SEM post-thaw sperm morphological abnormalities (in melatonin-treated (MEL) and control (CONT) rams (six per group) subjected to testicular heat stress (HS; Week 0).



*Difference ($p < 0.05$) between groups on that week. [a-c]Within the melatonin group, means without a common superscript differed ($p < 0.05$). NS means non-significant.

Figure 7.4 Mean ±SEM post-thaw sperm morphological abnormalities in melatonin-treated (MEL) and control (CONT) rams (six per group) subjected to testicular heat stress (HS; Week 0).



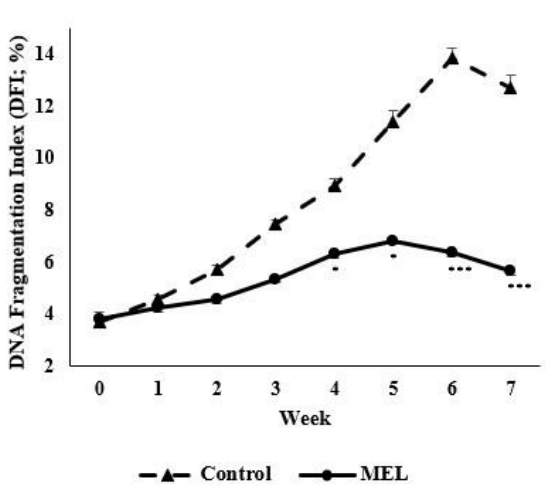
*Difference ($p < 0.05$) between groups on that week. [a-c]Within the melatonin group, means without a common superscript differed ($p < 0.05$). NS means non-significant.

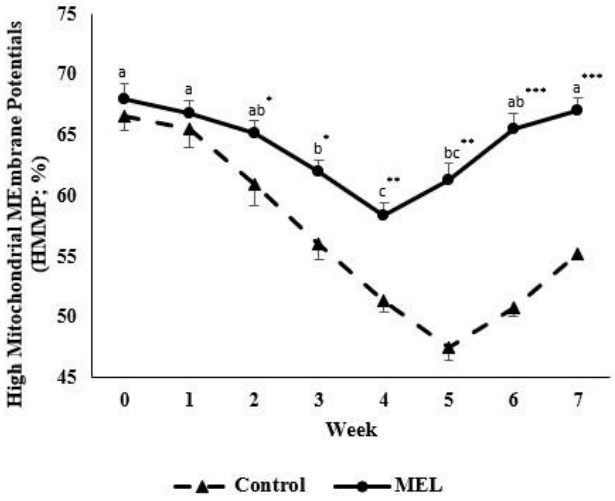
Figure 7.5 Mean ± SEM of post-thaw sperm DFI and HMMP (%) in melatonin-treated (MEL) and control (CONT) rams (six per group) subjected to testicular heat stress (HS; Week 0).

Group (p = 0.001)

Week (p = 0.001)

Group*Week (p = 0.001)





Group (p = 0.001)

Week (p = 0.001)

Group*Week (p = 0.001)

* ** and *** Difference ($p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively) between groups on that week. [a-c]Within the melatonin group, means without a common superscript differed ($p < 0.05$).

We injected melatonin 2 wk before HS, as it needs 2-4 wk to take effect [144,210,211]. These findings were consistent with reported benefits of melatonin implantation or injection during breeding and non-breeding seasons in rams [210,211,226,227], indicating that slow-release melatonin enhanced functionality of frozen-thawed sperm in rams after mild testicular HS.

Mammalian testes must be 2 to 5 °C below body temperature for optimal spermatogenesis [157]. Decreased sperm concentration, motility, viability, normal morphology, acrosome integrity, and chromatin stability due to elevated testicular temperature, acute or long-term, cause infertility [51,156]. Scrotal neck insulation, a model to induce testicular HS, reduced ram sperm quality without altering scrotal subcutaneous tempertaure [34]. Impacts of HS on sperm quality were mitigated by feeding l-arginine to HS boars [176] or adding melatonin or l-arginine to semen extender to post-thawed sperm

from HS rams [220]. Moreover, melatonin injections in mice reduced impacts of HS on testes and sperm quality [130]. As we reported for these rams [221], melatonin significantly protected sperm motility and morphology of fresh semen and also improved testicular blood flow, presumably contributing to effects of melatonin on post-thaw sperm quality reported herein.

In this study, melatonin significantly improved post-thaw sperm total and progressive motility, plus sperm kinematics, consistent with reports that melatonin implants improved progressive motility [222,223,228]. However, one study [223] detected no significant improvements in post-thaw total motility, perhaps due to no HS in the control group. In addition, various sperm kinematics (VAP, VSL, STR, and LIN) were significantly better in MEL at various weeks after HS, consistent with a previous study in cryopreserved sperm from *Bos frontalis* cattle [222]. Melatonin had bi-phasic effects on the male reproductive system, with short-term impacts on sperm progression and medium-term impacts on kinematic motility and velocity parameters, due to inhibition of prolactin and stimulation of gonadotrophins [226].

In this study, melatonin enhanced acrosome integrity and reduced total sperm abnormalties, with coiled tails, distal midpiece reflexes, distal cytoplasmic droplets, ruffled acrosomes, bowed midpieces, pyriform heads, and knobbed acrosomes being the most common abnormalities in both groups. Similarly, melatonin improved post-thaw acrosomal integrity and reduced sperm morphological abnormalities [222,228]. Furthermore, the MEL group had a signficant reduction in post-thaw DFI% and a significant increase in HMMP%. Similarly, melatonin decreased DFI% at 7 and 23 wk in rams in the non-breeding season [223] and significantly improved post-thaw sperm mitochondrial membrane potential in *Bos frontalis* cattle [222].

High concentrations of polyunsaturated fatty acids (PUFA) in mammalian sperm membranes make them extremely vulnerable to lipid peroxidation (LPO), oxidation of membrane lipids by partially

reduced oxygen molecules (e.g., superoxide, H_2O_2, and hydroxyl radicals; [229]). Lipid peroxidation of the sperm membrane impairs sperm function and reduces fertility, due to altered sperm motility, decreased membrane integrity, damaged sperm DNA, and formation of cytotoxic aldehydes. This oxidative stress is more pronounced following HS, which causes oxidative stress and apototic changes in sperm [10,156,230].

Melatonin protects mitochondria and plasma membrane from free radicals, lipid peroxides, and ROS, which may increase intracellular ATP concentrations, mitochondrial membrane potential, membrane fluidity, acrosome integrity, and reduce lipid peroxidation [231]. Furthermore, melatonin treatment before HS significantly protected fresh sperm motility and morphology [130,221], and increased testicular blood flow. Improvements were linked to melatonin's systemic or local functions in the testis, plus increased testicular blood flow, potentially enhancing testis' endocrine activity [156]. Antioxidant and anti-apoptotic effects on testes and sperm may also have contributed [130]. Moreover, many end points had a significant interaction, attributed to protective effects of the melatonin resulting in less reduction in sperm quality and more rapid improvement.

This study had some limitations; we only had 12 rams; semen was collected only once weekly for 7 wk, blood melatonin concentrations were not measured, and no fertility data generated. Regardless, melatonin significantly improved post-thaw sperm quality, generally consistent with reports using slow-release melatonin in rams. Therefore, this study should be replicated, and fertility assessed.

7.6 Conclusion

Melatonin treatment of rams subjected to mild testicular HS improved post-thaw sperm total and progressive motility, acrosome integrity and HMMP% and reduced total morphological

abnormalities and DFI%. Based on current and previous results, slow-release melatonin has substantial potential to protect sperm quality after testicular HS.

Chapter 8: General discussion

Global warming can cause an increase in body temperature above its physiological homeothermic point (heat stress [HS]), affecting the ability of people and animals to reproduce. Males of most mammalian species have a scrotum and systems have evolved to keep testes 3-5 ℃ below body temperature, as spermatogenesis is extremely sensitive to increases in body or environmental temperatures [8,54]. Although deleterious effects of HS on sperm quality have been widely studied and reported, the underlying mechanisms of action are not yet fully understood. Therefore, a better understanding of the pathogenesis of HS on sperm quality is needed to develop appropriate mitigation.

A recent series of studies [113,115,232] disproved the long-held belief that hypoxia is the underlying pathogenesis of reductions in sperm motility, morphology, and fertility that occur when testes are warmed [35,54,96]. In those studies, increased testicular temperature increased testicular metabolism, with corresponding increases in testicular blood flow and oxygen extraction from arterial blood and no indications of hypoxia, demonstrating that the rise in but not hypoxia caused the decline in sperm quality after HS.

This thesis research was carefully planned to address several objectives, using rams and bulls as models. Our first aim (Chapter 2) was to establish a technique for measuring intrascrotal temperatures in a continuous way without anaesthesia, restraint or altering animal behaviour, and validate it with needle thermocouples, the gold standard to measure the scrotal subcutaneous and testicular temperatures. We monitored variations in intrascrotal temperature in rams using implantable data loggers (DL; ~8 x 25 mm). The DL temperature (DLT) was compared to actual intrascrotal temperatures. DLT was sharply different in rams with scrotal insulation versus a cool environment. Therefore, we concluded that DL accurately determined intrascrotal temperatures. They are also perfectly suited for our intended use and minimally invasive. At DL removal, one of the four rams had a small accumulation of clear fluid.

Similarly, it was reported that two of the six bulls with DL implanted inside the scrotal vaginal canal had scrotal hematomas that resolved with antibacterial and anti-inflammatory treatment [233], with scar tissue but no testicular adhesions when the DL was removed. This was the first time to validate these data loggers to measure intrascrotal temperature in rams over an extended interval, without anesthesia, inflammation or hemorrhage as commonly occur with needle thermocouples. Limitations for this study included only four rams and an inability to collect semen from those rams. Regardless, we were able to validate the use of those data loggers and they worked well in subsequent experiments.

Our second objective (Chapter 3) was to determine testicular heat stress effects on sperm quality and link those changes to data logger temperatures. We postulated that testicular heat stress has negative effects on sperm quality and that these effects are related to intrascrotal temperatures recorded by data loggers. For this study, we conducted two experiments, with temperature sensors surgically implanted in scrotal subcutaneous tissues and semen collected and evaluated once weekly. In Experiment 1, we used two Angus bulls with classical whole-scrotum insulation for 96 h; they had increased scrotal subcutaneous temperatures (~2.0-2.5 ℃) with total and progressive motility, normal sperm morphology and acrosome integrity decreasing significantly and reaching nadirs 3 wk after heat stress. In Experiment 2, we used 10 Dorset rams randomly allocated into two equal groups that were either: 1) exposed to 28 ℃ ambient temperature and 30-34% RH for 8 h/d for 4 d; or 2) subjected to scrotal neck insulation that was applied and removed at the same time as the start and completion, respectively, of heat exposures in the other rams.

Scrotal subcutaneous temperatures increased (~0.8 ℃), in response to whole-body heating whereas scrotal neck insulation had no noticeable impact. Regardless, decreases in sperm quality were generally similar between treatments, reaching at nadir at 4 wk after HS, when total and progressive motility were reduced by 10% and morphologically normal sperm by 10 and 20%, respectively,

depending on whether the rams were subjected to whole-body heating or scrotal neck insulation. This was apparently the first study that described serial recording of the bull's and ram's intrascrotal temperatures during scrotal insulation, along with the simultaneous collection of semen and evaluation of sperm quality. Sperm quality was not concurrently evaluated in earlier investigations that comprised serial monitoring of intrascrotal temperatures in bulls [233] or our ram study [26]. Our findings supported earlier reports that whole-scrotum insulation reduced sperm production and semen quality in bulls [100,101]. Increased scrotal subcutaneous temperature, plus subsequent decreases in sperm quality, were expected with whole-body heating.

What was unexpected was that scrotal neck insulation failed to affect scrotal subcutaneous temperature, despite a similar decrease in sperm quality compared to whole-body heating. However, in a previous report, scrotal neck insulation in bulls did not change scrotal surface temperature but did increase scrotal subcutaneous and testicular temperatures [24]. That sheep have a better thermoregulatory capacity than cattle and are less vulnerable to HS could explain this discrepancy [158,159]. Furthermore, variations in materials used to insulate the scrotal neck could have also affected the degree of HS. In spite of no increases in scrotal subcutaneous temperature, scrotal neck insulation may have been somewhat stressful, increasing systemic cortisol concentrations and decreasing LH and testosterone concentrations and lowering sperm quality, as reported in bulls [99]. In the latter study [99], dexamethasone injection as a model for stress was compared to scrotal insulation. Changes in sperm motility and morphology were similar in the two models, similar to the responses in our study. Consequently, we continued to use the scrotal neck insulation model, as it was easily applied and had the same effects as whole-body heating on sperm quality.

Small sample size, especially in Experiment 1 (two bulls) was a substantial limitation. Therefore, these findings need to be confirmed by additional research with more animals. We inferred that whole-

body heating was a more realistic model for environmental HS, but the scrotal neck insulation model could be used as a very accessible and easy to apply model having the same deleterious effects on sperm quality as whole-body heating. Future studies, with assessment of antioxidant capacity in serum or seminal plasma and plasma cortisol concentrations would provide valuable insights to understand the mechanisms of sperm quality deterioration, especially in the scrotal neck insulation model.

Our third objective (Chapter 4) was to assess effects of melatonin or l-arginine supplementation on sperm quality following testicular HS in rams. Our hypothesis was that these substances improve sperm produced after testicular HS. Ten Dorset rams were randomly allocated to either scrotal neck insulation for 3.5 d or whole-body heating (28 °C and 30-34% RH for 8 h/d for four consecutive days). Semen was collected before HS then once weekly for 1 to 5 wk, extended and divided into five aliquots: control (no additive) or 0.5- or 1-mM of melatonin or L-arginine. All four treatments improved motility (~5 to 10 percentage points), whereas 1 mM of either compound optimized abnormalities and acrosomal integrity (~7 and 12 percentage points, respectively). For superoxide dismutase, catalase and total ROS%, 1 mM of both additives yielded best result. For DNA fragmentation index (DFI%), all four treatments reduced DFI%, with 0.5 mM yielding the best results.

As we specifically chose a moderate HS insult, we were unlikely to influence very early phases of spermatogenesis as they are more resistant to HS and the quality of the sperm would only be compromised for a short interval before returning to normal. Various concentrations of melatonin [143,171,172] and l-arginine [175,234,235] have been used as semen extender additives to ameliorate cryopreservation-induced stress in several species, with reductions in total sperm morphological abnormalities in frozen-thawed sperm and better post-thaw sperm motility. Furthermore, melatonin has been used to alleviate oxidative damage to sperm caused by heat-induced oxidative stress in human by improving sperm motility, reducing mitochondrial ROS content, stabilizing mitochondrial membrane

potential, reducing lipid peroxidation products, maintaining DNA integrity, and reducing apoptosis. Melatonin is expected to reduce oxidative damage both *in vitro* and *in vivo*, due to its capacity to scavenge ROS. Its antioxidant capabilities make it an excellent choice for use in cryopreservation media to protect sperm from excessive ROS, with effects on sperm quality attributed to its broad-spectrum antioxidant activity [124,184]. Similarly, l-arginine inhibits lipid peroxidation and supports antioxidant defence mechanisms by inducing NO and decreasing ROS [174,183,236].

There were some limitations to this study. We only had 10 rams, all of the same breed but not all of the same age. There were two methods of HS (localised testicular and intermittent whole-body). Semen was collected only once weekly for 5 wk, using two methods (albeit with no significant difference between them). Despite these limitations, the two HS methods produced very similar effects on sperm. Furthermore, there were numerous significant differences between control and treated sperm, which were generally consistent with previous studies involving melatonin or L-arginine. Therefore, although this study should be replicated (taking care to overcome the stated limitations), the findings were clear and arguably compelling.

Our fourth objective (Chapter 5) was to investigate the relationship between intrascrotal and ambient temperatures, bull location (shaded or not), and sperm quality in bulls. We hypothesised that in hot ambient temperatures, bulls seek shade, lowering intrascrotal temperature and improving sperm quality. For this study, six Angus bulls were randomly allocated into two groups of three, with only one group having access to shade. Semen was collected by electroejaculation once weekly for 9 wk and weather data were retrieved from a nearby weather station. For monitoring the shaded group, a game camera was placed inside the shade structure. The percentage of time a bull voluntarily accessed shade for more than 15 min increased with ambient temperature, ranging from 7.6 to 86.7% for ambient temperatures of 25 and > 33 °C, respectively.

During the 10 hottest days of this study, the control group's scrotal subcutaneous temperature (measured hourly with an implanted data logger) had a direct relationship with ambient temperature. Conversely, bulls with access to shade had lower scrotal subcutaneous temperatures during high ambient temperatures, especially when they accessed shade. During the four hottest days, bulls voluntarily sought shade from 12.00 to 17.00 (peak ambient temperatures). Moreover, bulls in the shade group had better sperm quality (total and progressive motility, sperm kinematics, sperm morphology and acrosome integrity) consistent with differences between groups in intrascrotal temperature.

To the best of our knowledge, this was the first study to use DL to monitor scrotal subcutaneous temperature and determine its relationship with ambient temperature in bulls with or without access to shade in hot weather. Sperm morphological abnormalities were not consistent with several previous studies [120,197,199,200] in which total sperm morphological abnormalities did not significantly differ between bulls with versus without shade. Perhaps these inconsistencies were due to breed differences, as *Bos taurus* breeds and crossbreds are more susceptible to heat stress, whereas environmental strategies for heat stress management are not always required for *Bos indicus* cattle, e.g., Nelore [10,156]. Furthermore, shade type (natural or artificial) and even shade design [188], may have influenced outcomes. Our findings on sperm motility were consistent with previous research [119,120,197] that access to shade for bulls on pasture enhanced sperm motility. In contrast, other studies [199,200] reported contradictory findings regarding effects of shade on sperm motility.

A main limitation of this study was the sample size, with six bulls in total, but only five bulls having scrotal temperature measurements and semen being collected only once a week. Regardless, there were many significant and biologically relevant differences between groups, providing clear evidence that when given the opportunity, bulls will seek shade when ambient temperatures are high, lowering intrascrotal temperature and improving sperm quality. We recommend using GPS collars for

future replications, to address bull location and bull-bull interaction and dominancy issues. Another recommendation is a fertility trial to assess bull natural breeding behaviour and outcomes in the presence or absence of shade. These refinements would be a better way to access a wide range of factors that could be affected by bulls having access to shade while on pasture or even factors that may affect a bull's decision to access the shade.

Our fifth goal (Chapter 6) was to investigate effects of melatonin on testes function, testicular blood flow, and sperm quality after testicular HS in rams. We speculated that heat stress reduces sperm quality, whereas melatonin supplementation protects testes function, improves testicular blood flow, and reduces sperm quality reductions after testicular HS. Twelve yearling Dorset rams with good sperm quality were housed indoors (at temperatures ranging from 18 to 20 °C). Doppler indices (RI and PI) were measured in the supratesticular artery once a week for 2 wk, and semen was collected via electroejaculation. The rams were then allocated into two equal groups and given either 36 mg melatonin in 1 ml corn oil SQ under the ear (MEL) or corn oil alone (CONT). All rams were subjected to mild HS for 96 h, 15 d after treatment, with blood flow measurements and semen collection performed once weekly for 7 wk. Changes in total and progressive motility, as well as sperm abnormalities, were observed in CONT rams at Week 1 post-HS, but MEL mitigated these effects from Weeks 2 to 7. Furthermore, most weeks after HS, both PI and RI were reduced (i.e., a significant increase in blood flow) in MEL versus CONT rams. Sperm motility and total abnormalities had recovered in MEL rams by Weeks 5 and 6, respectively, whereas CONT rams had not recovered completely by Week 7. There was no difference in scrotal subcutaneous temperatures between the MEL and CONT groups in the 4-d intervals before, during, and after HS.

To our knowledge, this was the first study on the use of melatonin to protect farm animal testes from HS. Our findings related to the sperm quality was consistent with previous studies in which

melatonin injections improved sperm quality in the non-breeding season in rams [211,212] and goats [144]. These improvements were attributed to melatonin's systemic or local roles in the testis, as well as increases in testicular blood flow, which could be a potential trigger for testis' endocrine function [156]. Furthermore, this could be also be due to its antioxidant and anti-apoptotic properties in testes and sperm [130]. Moreover, our blood flow data were consistent with the findings from previous studies in previous studies [10,115], in which HS increased testicular blood flow, ensuring ample O_2 to support increased testicular metabolic needs and avoid hypoxia. Regarding melatonin administration, results were consistent with previous studies where melatonin injections in rams in either breeding or non-breeding seasons [210,211] reduced PI and RI, thereby increasing testicular blood flow after melatonin injection, with similar effects in goats [144].

One of the unexpected findings that we had in this study that there was no difference between MEL and CONT groups in scrotal subcutaneous temperatures in the 4-d intervals before, during and after HS. Regardless, this was consistent with our previous report [34] of no differences in scrotal subcutaneous temperature before, during, or after HS when using the scrotal neck insulation model of HS. It might also have been due to variations in the insulation of the scrotal neck, which might have an impact on how strongly the body and HS react. Another possibility was that the scrotal neck insulation model employed in our investigation did not impair the testicular ability to regulate its own body temperature. Although blood flow is the primary source of heat in the testes, there was no rise in scrotal subcutaneous temperature in the MEL group [112]. Melatonin has been linked to a decrease in thermoregulatory efficiency [213]; although there was higher blood flow in MEL, perhaps these rams were still able to thermoregulate and keep their testes in the same range as the control.

This study had some limitations, one of them that we did not measure melatonin concentrations, as this would have documented increased melatonin concentrations in treated rams and relate that to

changes in sperm quality and blood flow. A future recommendation is to replicate this study, measuring melatonin concentrations in blood and seminal plasma throughout the experimental period. Furthermore, include a fertility trial to see how changes in sperm quality and blood flow are related to fertility, an important consideration in heat-stressed animals.

Our sixth goal (Chapter 7) was to ascertain effects of melatonin injection on post-thaw sperm quality in rams following mild HS. We hypothesized that whereas melatonin supplementation protects testicular function, it minimises losses in post-thaw sperm quality after mild HS. This study was a continuation of the study in Chapter 6, with semen collected before HS and once weekly from Weeks 1 to 7 and then extended, cryopreserved and subsequently thawed and assessed. For most assessments between Weeks 1 and 7, melatonin groups outperformed control groups in terms of post-thaw sperm total and progressive motility, acrosome integrity, and HMMP. Conversely, melatonin had a lesser difference than control in post-thaw sperm total abnormalities and DFI. The most frequent sperm morphological abnormalities were coiled tails, distal midpiece reflexes, distal cytoplasmic droplets, ruffled acrosomes, bowed midpieces, pyriform heads, and knobbed acrosomes in both groups, with lower percentages in rams treated with melatonin. This was apparently the first report of melatonin injection on rams with mild testicular HS and consequences for post-thaw sperm quality.

Melatonin was injected 2 wk before HS, based on reports that slow-release melatonin needs 2-4 wk to start to exert effects [144,210,211]. Furthermore, our findings were consistent with reported benefits of melatonin-incubated sperm [135,172,186,220]. Improvements in sperm motility and kinematics were consistent with results from reports that melatonin implants improved progressive motility [222,223,228]. However, one study [223], detected no significant improvements in post-thaw total motility, perhaps due to the absence of HS in the control group. Furthermore, improvements in

post-thaw sperm acrosome integrity, DNA integrity and mitochindrial membrane potential were in line with previous studies [222,223,228].

High concentrations of polyunsaturated fatty acids (PUFA) in mammalian sperm membranes make them extremely vulnerable to lipid peroxidation (LPO), oxidation of membrane lipids by partially reduced oxygen molecules (e.g., superoxide, hydrogen peroxide, and hydroxyl radicals [229]) as a result of cryopreservation stress. This oxidative stress is more pronounced with sperm from HS males, as HS causes oxidative stress and apototic changes in sperm [10,156,230]. Melatonin defends against free radicals, lipid peroxides, and reactive oxygen species in the mitochondria and plasma membrane, which may boost intracellular ATP concentrations, mitochondrial membrane potential, membrane fluidity, acrosome integrity, and lipid peroxidation [231]. Furthermore, melatonin given before HS significantly protected fresh sperm motility and morphology [130,221], and also increased testicular blood flow.

This study had some weaknesses, including only 12 rams, semen was only collected once weekly for 7 wk, and there were no assessments of blood melatonin concentrations or fertility. Regardless, melatonin considerably increased the quality of post-thaw sperm, which is generally consistent with previous studies. Ideally, this investigation should be repeated, and fertility should be evaluated utilising either natural breeding or in vitro embryo production.

One of the main limitations of this thesis research was the absence of the fertility data for all experiments. Therefore, we recommended to replicate these experiments with more focus to overcome limitations, particularly those related to fertility data. Regardless, the current studies provided promising results to build on, as sperm quality parameters we measured have some association with fertility, as explained below.

Fertility is a complex process that is dependent on sperm quality, female fertility, proper herd management, and precise timing when using artificial insemination (AI). Furthermore, bull subfertility has been linked to a substantial percentage of reproductive failures in dairy cattle [1], and subfertile bulls can result in considerable financial losses [237]. Furthermore, sperm quality parameters are correlated and could be used as a predictor for *in vivo* and *in vitro* fertility. Sperm motility, as assessed by CASA, is widely regarded as one of the most important characteristics associated with fertilizing potential. CASA gives objective and relevant information on numerous sperm motility parameters, which aid in predicting the reproductive status of sperm samples [238,239].

In addition, the presence of an intact acrosome on a spermatozoon is required for oocyte penetration and enhanced chances of fertilization. Furthermore, viable spermatozoa with an intact acrosome were strongly correlated with *in vivo* fertility [225,238]. Sperm mitochondrial transmembrane potential is regarded as one of the most sensitive tests for assessing sperm quality and determining field fertility. *In vivo* fertility has been linked to viable sperm with increased mitochondrial transmembrane potential [240]. Furthermore, after combining sperm features, there was a high association between pregnancy rates with live normal sperm and stable deoxyribonucleic acid complex measured with flow cytometry [241]. In contrast, morphologically aberrant sperm have a negative association with fertility [242].

In summary, we validated data loggers as a reliable, less invasive method to serially measure intrascrotal temperature. Furthermore, in two animal models, we related intrascrotal temperature measured with data loggers to sperm quality and how changes in intrascrotal temperatures affected sperm quality. In addition, we aimed to mitigate HS impacts on sperm quality. *In vitro,* we added two concentrations of melatonin and l-arginine to semen extender for extending semen collected from HS rams. Overall, 1 mM of either additive generally gave the best results. *In vivo,* we injected rams with

slow-release melatonin and then exposed them to heat stress to determine the protective effect of melatonin on testicular blood flow, intrascrotal temperature, and both fresh and post-thaw sperm quality. Melatonin-treated rams had better testicular blood flow and fewer effects of HS on sperm quality (both fresh and post-thaw).

Using a bull model, we tested whether providing access to a shade structure during hot days affected scrotal subcutaneous temperature and sperm quality. Bulls voluntarily accessed shade under hot conditions, and they had lower scrotal subcutaneous temperature and better sperm quality.

These studies validated a new method to monitor scrotal subcutaneous temperature. Furthermore, in response to testicular HS, we demonstrated benefits of melatonin and l-arginine *in vitro* to improve sperm quality, as well as benefits of either melatonin *in vivo* or access to shade. Therefore, this work yielded new knowledge regarding scrotal/testicular thermoregulation and indicated methods to mitigate the deleterious effects of testicular HS on sperm quality either *in vitro* or *in vivo* with a future recommendation to include direct assessments of fertility.

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Appendix A: Table 1.1: Various substances used to ameliorate effects of HS (Modified from: AM Shahat, G Rizzoto, JP Kastelic. (2020). Amelioration of heat stress-induced damage to testes and sperm quality. Theriogenology 158:84-96.)

Ref.	Substance	Insult	Dose & route	Action	Species
I.	**Amino Acids**				
[176]	**1. L-Arginine**	Hot ambient temperatures	0.6, 0.8 or 1.0% of basal diet (oral)	* ↑ Semen quality and libido during hot weather. * 1.0% group had great semen quality and antioxidant capacity. * 0.8% group had better libido.	Pig
[243]		Experimental cryptorchidism	15 mg/100 kg BW (oral)	* ↓ Testicular oxidative stress but did not improve outcomes with cryptorchidism	Rat

II. Antibiotics

[244]	**1. Minocycline** (semisynthetic, 2nd generation tetracycline)	Immersion in hot water (42 ℃ for 15 min)	a. Spermatogenic cells cultured *in vitro* for 12 h b. 45 mg/kg BW (i/p)	* Suppressed release of cytochrome c which triggers HS-induced apoptosis from mitochondria (*in vivo* and *in vitro)*. * Significantly ↓ number of TUNEL-positive cells (Terminal deoxynucleotidyl transferase-mediated deoxy-UTP end labelling).	Rat
[245]		7 mg/kg BW dexamethasone i/p	100 mg/kg BW for 7 d (oral)	* Inhibited germ cell apoptosis induced by dexamethasone. * ↑ Johnsen score and sperm/testis.	Mouse
[246]	**2. Pentoxifylline**	Surround scrotum with thermal bag with air at 50 ℃, for 1 h, 2x/d, 2 d	17 mg/kg BW, 2x/d for 30 d (oral)	* ↑ Total motility and rapid sperm within 14 d * ↓ Lipid peroxidation. * Prevented apoptosis and ↓ total sperm defects.	Horse

III.	**Antioxidant cocktail**				
[247]	**1. Mixed multi-antioxidant supplement**	Tropical summer temperatures	100 g/d for 42 or 84 d (oral)	* Antioxidant supplementation for 42 or 84 d in tropical summer tended to ↓ harmful effects of HS on DNA integrity, but not on sperm concentration or motion end points	Pig
[248]		High-dose ionizing irradiation	One capsule in 10 ml saline; 1 mL i/p (once)	* ↓ Testicular damage caused by oxidative stress, apoptosis in germ cells and ↑ spermatogenesis; protected fertility after irradiation.	Rat

IV.	**Enzyme inhibitors**				
[249]	**1. Xanthine oxidase inhibitors (Allopurinol and BOF-4272)**	Surgically induced cryptorchidism	**Allopurinol:** 0.1-100 mg/kg BW (i/p) daily for 7 d after surgery **BOF-4272:** 300 µg/kg	* Both compounds attenuated weight reductions of cryptorchid testis. * ↓ Apoptosis in germ cells (*in-situ* staining of fragmented DNA). * Potential treatment of male infertility due to HS.	Rat
V.	**Hormones**				
[250]	**1. GnRH agonist and anti-androgen**	Heating testes to 43 ℃ for 30 min in water bath	Implant GnRH agonist + daily injections of anti-androgen for 14 d	* Did not prevent initial decline in testicular mass or % abnormal sperm after heating (35 d post-heating), but lessened subsequent decreases in testicular mass and sperm count (182 d post-heating).	Rat

[251]	**2. Melatonin**	Hot summer temperatures	Melatonin (3 mg/kg BW) for 20 d (oral)	* ↑ % normal live sperm and ↓ abnormal and dead sperm. * Following AI, melatonin had positive impact on hatch weight and relative spleen weight of chicks. * % fertile eggs not affected.	Chicken
[252]		Experimental cryptorchidism	0.7 mg/kg BW for 56 d (i/p)	* Epididymis of ipsilateral testes and bilateral cryptorchid with melatonin were oligospermic compared to azoospermic control. * No change in sperm concentration or motility, or testosterone concentrations.	Rat
[253]		Sublethal irradiation (8 Gy) to total body or abdomino-pelvic region	Given 24 h before (10 mg/kg), immediately before (20 mg/kg) and 24 h after	* ↓ Apoptosis (big ↓ immunoreactivity of caspase-3 (apoptosis marker). * Based on electron microscopy, inhibited degenerative changes in spermatogenic	Rat

		irradiation (10 mg/kg; all i/p)	cells after irradiation, especially 1^0 spermatocytes.	
[130]	Single HS treatment in 39 or 42 ℃ in water bath for 20 min	* 20 mg/kg BW at 2 h before 42 ℃ treatment (i/p)	**<u>Melatonin pre-treatment</u>** * ↓ Vacuolization of seminiferous tubules; ↑ tubules with regular seminiferous epithelium and multiple layers of germ cells post-HS * Significantly ↓ malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) generation (markers of ROS), but ↑ SOD and catalase (CAT) activity; implied alleviation of heat-induced oxidative stress * Suppressed activation of JNK and p38 mitogen-activated protein kinase (MAPK) (apoptosis-related signaling pathway) and ↑ HSPA2 and anti-apoptotic B-cell	Mouse

				lymphoma 2 (BCL-2) in testes (mitigated heat-induced damage).	
[130]			* 20 mg/kg BW daily (immediately after 42 ℃ treatment) for 14 d (i/p)	**<u>Melatonin post-treatment</u>** * Faster recovery of spermatogenesis post-heat treatment apparent at 7 versus 14 d without melatonin. * Preserved integrity of Sertoli cell tight junction (may be essential for faster recovery from testicular HS).	Mouse
VI.	**Minerals**				
[254]	**1. Selenium (Se)**	Scrotal hyperthermia (42 °C for 30 min) after 8 wk Se supplementation	0.5 ppm/kg for 8 wk (oral)	* ↓ Apoptotic index of spermatocytes, round and elongated spermatids. * ↑ % motile sperm and ↓ ROS generation. * ↓ Caspases 3, 8 and 9 and ↑ BCl2, ↓ apoptosis.	Mouse

[255]	**2. Selenium and Vit E**	Scrotal insulation (SI) for 18 d	Selenium (0.1 mg/kg BW) and vitamin E (0.3 IU/ kg BW) for 120 d: 60 d prior to SI, 18 d during SI and 42 d post SI	* Maintained scrotal circumference, but failed to protect testes against degenerative changes 18 d after insulation. *Improved tubular diameter and seminiferous epithelium height at 42 d after SI; supplementation hastened spermatogenesis recovery after HS.	Goat
[256,257]	**3. Zinc**	Heat testes to 43 ℃ for 15 min in water bath	10 mg/kg of zinc sulphate (i/p) every 2 d for 60 d	* Promoted restoration of normal testicular structure 15 d after HS. * Sperm motility, concentration and hypo-osmotic sperm test (HOST) positive sperm maintained without further deterioration at 30 d post-HS. * Maintained fetus weight obtained from paternal heat treatment.	Mouse

[258]		Whole-body HS (40 ℃ ambient temperature).	300 mg/kg BW zinc sulphate orally for 1 mo before HS	* Maintained relatively intact testicular structure with slight degeneration. * ↑ Cu-Zn SOD, ↓ MDA, maintained Nrf2 protein (key regulator against oxidative stress) expression.	Mouse
[259]		After 30 d zinc supplementation, mice kept at 40 °C for 5 h/d for 8 d	Zinc sulphate 1500 mg/kg BW for 30 d (oral)	* ↓ MDA and glutathione (GSH) in epididymis (alleviated oxidative stress). * Restored integrity of caput epididymis epithelium and ↓ stress response.	Pig
VII.	**Natural substances**				
[260]	**1. Betaine** (methylamine naturally occurring in plant and animal tissues; used as feed additive)	Summer HS	0.63 or 1.26% for 10 wk (oral)	* ↑ Total sperm in ejaculate by 6 or 13% (0.63 and 1.26%, respectively). * 1.26% betaine ↑ % sperm with distal midpiece reflex (DMR), but 0.63% did not affect sperm.	Boar

[261]	Testicular immersion in water at 42 ℃	250 mg/kg BW/d for 14 d, before or after testicular HS	* Treatment before and after HS ↑ antioxidant defense (↑ activity of CAT and GPX enzymes). * Accelerated germinal epithelium regeneration, no change in epididymal sperm. * Improved epididymal sperm in intact mice (no HS).	Mouse
[262]	Ambient temperature 30.2 ℃	* **BET**; 0.63% of 96% betaine + 250 phytase units * **BET**; 0.63% of 96% betaine + super-dosed Phytase (2,500 phytase units)	* Partially mitigated effects of HS on sperm morphology; minimal effects on total or progressive motility. * No improvement in semen production.	Boar

		* For 16 wk (4 wk before HS, 4 wk during HS and 8 wk after HS)		
[263]	Chronic HS at 38 ℃; 55-65% RH for 4 h for 3 successive days/wk	* **BET only:** 1000 mg/kg BW * **BET (**1000 mg/kg BW) + **Vit C** (200 mg/kg BW * **BET (**1000 mg/kg BW) + **Vit E** (150 mg/kg BW) * **BET (**1000 mg/kg BW) + **Vit C** (200 mg/kg BW + **Vit E** (150 mg/kg BW)	* Antioxidants, either individually or combined, induced complete recovery in sperm concentration, % live, pH, and fertility. * All treatments restored total protein, globulin, AST, ALT, TAC, and MDA. * Enhanced semen quality of roosters in hot regions.	Chicken

[264]	**2. Coenzyme Q10**	Temperature humidity index (THI) of 29 (optimum = 23).	10 or 20 mg/kg BW for 8 wk (oral)	* Both doses improved semen quality and male fertility, but induced sperm DNA damage and altered testes histology. * ↓ Oxidative stress by ↑ main antioxidant testicular enzyme (GSH) and ↓ MDA.	Rabbit
[265]	**3. Gherlin** (endogenous antioxidant)	Testes in 43 ℃ water for 15 min	At onset of heating, 2 nmol s/c, every 2 d, up to d 60	* Partial recovery in miotic index, spermatogenesis rate, presence of spermatocytes. * Hastened testicular regeneration by 30 d.	Rat
[266]	**4. Hydrogen sulphide (H_2S)** (Endogenous gaseous transmitter)	HS 42 °C, 30 min/d for 3 d	Before HS, NaHS 5.6 mg/kg BW (i/p)	* Attenuated apoptosis in 1^0 cultures of testicular germ cells (inhibited effects of H_2S on release of cytochrome C and Bax/Bcl-2 ratio). * Enhanced mitochondrial function by ↑ O_2 intake and ↑ ATP. * ↑ SOD activity and ↓ ROS.	Mouse

VIII. Phenolic compounds					
[267,268]	**1. Tert-butylhydroquinone** (tBHQ, synthetic phenolic antioxidant)	A single scrotal heat exposure of 42 °C for 25 min.	* 10 mg/g tBHQ **diet for 1 wk** and then treated with scrotal heat. ***Single i/p** injection of 100 mg/kg BW tBHQ, scrotal heat 3 h later. ***Single intra-testis** injection of 12.5 mg/kg BW tBHQ and were exposed to heat before injection.	* Testes from oral and i/p tBHQ-treated mice had ↑ compact interstitial cells and ↓ germ cell loss. * ↓ testosterone and ↓ expression of cytochrome P450 17α-hydroxylase/17,20-lyase (CYP 17), a microsomal enzyme used to assess androgen output. * ↓ MDA * Induced mild oxidative stress and further enhanced ability of cellular antioxidants to protect testicular cells from HS via the Nrf2 antioxidant system.	Mouse

			* 1% tBHQ (w/w) for 1 wk in diet		
[269]	**2. Quercetin**	Summer HS	Quercetin hydrate in diet (30 mg/kg) for 8 wk	* Maintained semen quality, ↓ oxidative stress.	Rabbit
IX.	**Vitamins**				
[270]	**1. Ascorbic acid**	* 34.5, 37, 39.5 °C. * Same temperatures + H_2O_2 (200 μmol/L)	Ascorbic acid (400 and 600 μmol/L) in **sperm suspension**	* 600 μmol/L had more pronounced ↓ in static oxidation reduction potential (sORP) compared to 400 μmol/L. * ↓ heat-induced oxidative stress (*in vitro*).	Human

[271]	**2. Vit E (α-tocopherol)**	Experimental cryptorchidism	30 or 100 mg/kg BW (I/P)	* Long-term ↑ in seminiferous epithelium region and maturation, ↓ apoptosis and histological alterations. * α-tocopherol before orchidopexia, particularly 30 mg/kg, partially protected undescended testis from ROS damage.	Rat
[254]	**2. Vit E (Alpha-tocopherol)**	Scrotal hyperthermia (42 °C, 30 min), after Vit E for 8 wk	200 mg/kg diet for 8 wk (oral)	* ↓ apoptotic index of spermatocytes, round spermatids and elongated spermatids. * ↑ % motile sperm. * ↓ ROS generation, ↓ caspases 3, 8 and 9 and apoptosis, ↑ BCl2.	Mouse
[272]		Twice/wk in well-maintained (50 to 55% humidity and 42 ˚C) incubator for 10 min for 75 d.	20 IU/kg BW/d for 75 d (oral)	* ↓ Free radicals. * ↑ Sperm velocity straight line (**SVSL**), sperm velocity curved line. (**SVCL)** and sperm velocity average path (**SVAP).** ***** Normalized seminiferous epithelium.	Mouse

[273]	**3. Vit E + organic Se**	High ambient temperature (33-36 ℃).	* 200 mg α-tocopherol/kg diet * 0.3 mg organic Se/kg diet * 200 mg α-tocopherol/kg diet + 0.3 mg Se/kg diet	* All treatments ↑ semen quality. * The combination ↓ seminal plasma thiobarbituric acid reactive substances (TBARS) to ~28% of controls and doubled activity of seminal plasma glutathione peroxidase.	Chicken
[274]	**4. Retinol**	* Refrigerator temp (4 ℃). * Environmental temp (22 ℃). * Scrotal temp (32 ℃) * Hot (41.5 ℃)	6 µM retinol as an antioxidant added to storage extender	* ↑ % live, active and progressive sperm with ↑ % membrane integrity at 41.5 ℃ supplemented with retinol compared to control.	Cattle

X. Traditional herbs

[275]	**1. *Lycium barbarum*** (LBP, Chinese medicinal herb)	Testes in 43 °C water bath for 15 min	10, 50, 100, and 200 mg/kg/d for 14 consecutive days	* ↑ Testis and epididymis weights (first three doses). * ↑ SOD activity and ↓ MDA (all doses). * Most seminiferous tubules had intact structure (especially 10 mg/kg dose).	Rat
[276]	**2. Radix *Puerariae*** **(**Chinese herbal medicine)	Bovine Sertoli cell culture exposed to HS (42 ℃ for 1 h)	15 μM	* Supressed ROS production and ↓ oxidative damage. * Prevented initiation of mitochondria-dependent apoptotic pathway. * ↑ expression of HSP 72.	Bovine Sertoli cell culture
[277]	**3. Baicalin** (extracted from the dried root of *Scutellaria baicalensis Georgi)*	Bovine Sertoli cell culture exposed to HS (43 ℃ for 1 h)	0.1, 1, 10, 20 μg/mL of baicalin	* Mitigated HS-induced cell apoptosis by modulating cell survival rate by activating the Fas / FasL pathway. * ↑ HSP 72 expression in Sertoli cells.	Bovine Sertoli cell culture

[278]		Testes in 41 °C water with 80% RH for 2 h on the Day 8 of baicalin	50 mg/kg BW daily for 7 d	* ↑ Activities of SOD, CAT and GSH-Px enzymes. * ↓ MDA content and cellular apoptosis by blocking Fas/FasL pathway.	Mouse
[279,280]	**4. Panax ginseng** (Korean red ginseng, KRG)	32 ± 2°C, 2 h/day/7 wk (intermittent sub-chronic high temp.)	* 100 or 200 mg/kg/d orally for 8 wk (starting from 1 wk before HS) or 100 or 200 mg/kg/day orally for 25 wk	* Protected antioxidant related enzymes, proteins associated with spermatogenesis, sex hormone receptors and sperm quality in heat-stressed rats.	Rat
[281]	**5. Kyung-Ok-Ko (KOK),** also known in China as Qiong-yu-gao	Testes in 43 °C water for 10 min	0.25, 0.50 or 2.0 g/kg/d for 5 wk	* ↑ Testes weights, sperm concentration and motility. * Morphological appearance of seminiferous tubules was restored.	Mouse

	(traditional Korean medicinal formula)			* ↑ Antioxidant enzyme activity, ↓ protein expressions of testicular apoptosis.	
[282]	**6. *Platycodon grandiflorum* (PGS) saponins** (named Jiegeng in China, Kilkyong in Korea and Kikyo in Japan)	Testes in 43 °C for 18 min on the 7th d of PGS treatment	15 or 30 mg/kg) as a gavage for 14 d	* Restored testicular structure to normal standards, with well-preserved tubular morphology. * Prevented activation of MAPK signaling pathway, which contributes to oxidative stress and apoptosis. * Lower dose (15 mg/kg) more protective than higher dose (30 mg/kg).	Mouse
[283]	**7. *Angelica keiskei*** (Ashitaba, contains two chalcones, xanthoangelol [XA] and 4-hydroxyder ricin [4HD])	Testes in 41°C water for 15 min and 42 °C for 20 min	* *Angelica keiskei* (Ashitaba) powder (57.5 mg/kg) * Xanthoangelol (functional	* Prevented impairment in sperm densities, progressive motility and lateral head displacement amplitude. * Prevented reduction of the expression of *Hspa1l* and *Hspa2*.	Mouse

			component, 3 mg/kg)		
[284]	**8. Decursin** (*Angelica gigas* Nakai, Apiaceae)	Surgically induced unilateral cryptorchidism	400 mg/kg orally for 4 wk after unilateral cryptorchidism.	* ↑ mean weight of cryptorchid testis. * Maintained sperm counts, motility, and spermatogenic cell density. * ↑ SOD, ↑ Nrf2 and HMOX1 and ↓ apoptosis.	Rat
[285]	**9. *Nigella sativa* Linn (**Black Seed)	Testes in 43 °C water for 15 min	10 and 20% in diet	* Enhanced spermatogenesis and ↑ testosterone. * ↓ MDA and ↑ antioxidant enzyme activity.	Mouse
[286]	**10. *Mallotus roxburghianus* Muell**	Testes in 43 °C water for 30 min	Methanol extract (400 mg/kg)	* Suppressed lipid peroxidation. * Restored antioxidant enzymes and testosterone, restored spermatogenesis.	Rat

[287]	**11. Apigenin** (4, 5, 7-trihydroxyflavone)	Testes in 43 °C water for 30 min/d for 6 d	10, 20 or 50 mg/kg once daily for 35 d	* ↑ Testosterone and inhibin B. * ↑ SOD and GSH-Px activity, ↓ MDA. * Preserved seminiferous tubule diameter	Mouse
[288]		Summer HS	0.3, 0.6 and 0.9 ml Parsley oil/kg diet (Parsley oil contains Apigenin)	* Improved semen end points and ↓ negative alterations in seminiferous tubules. * Positive effects of Parsley oil attributed to antioxidant activity.	Japanese quail
[289]	**12. Green tea** (*Camellia sinensis*)	Testes in 42 °C water for 20 min	500 or 750 mg/kg orally for 49 consecutive days	* Recovered adverse effects HS on sperm concentration, total and progressive motility and hypo-osmotic swelling test (HOST) within 28 d after HS, compared to 42 d in control.	Mouse
[290]	**13. Cinnamon** (Cinnamon bark oil, CBO)	Ambient temperature of 34 °C for 8 h/d	250 or 500 ppm	* ↓ Testicular lipid peroxidation, MDA. * Prevented ↓ spermatids, sperm. * Improved testicular histology.	Japanese quail

[291]	**14. *β*-carotene and curcumin**	Testes in 43 ℃ for 15 min on d 7 of supplementation	*β*-carotene (10 mg/kg) and curcumin (20 mg/kg) orally once a day for 14 d	* Restored normal testes weight and structure. * ↓ MDA and ↑ SOD. * ↓ mRNA for BCL2-associated X protein and caspase-3 (antiapoptotic).	Mouse
[292]	**15. Guava leaves essential oil (GLEO)**	Ambient temperature 45 °C 7 h/d for 60 d	100 μL GLEO/kg BW	* ↓ Rate of free radical formation and thus lipid peroxidation.	Guinea pig
[293]	**16. Red grape (*Vitis vinifera*)**	Testes in 43 ℃ for 30 min/d for 15 d	0.8 mL/rat/d of red grape juice (RGJ)	* Restored antioxidant status of testis and intact testicular structure. * Maintained normal serum testosterone, testicular SOD, catalase, glutathione and lipid peroxidase and the apoptotic enzyme caspase-3 of testis.	Rat
[294]	**17. Royal Jelly (RJ)**	Summer HS	200, 400, or 800 mg/kg BW once a week	* ↑ Testosterone, ejaculate volume, motility, sperm total output * ↓ abnormal and dead sperm. * ↑ Seminal plasma fructose.	Rabbit

[295]	Testes in 43, 39 or 37°C water for 20 min/d	100 mg/kg/d	* Enhanced sperm characteristics. * ↓ MDA and % of sperm with chromatin abnormality and DNA damage. * ↑ Numbers of zygote and 2-cell, blastocyst stage and hatched embryos, ↓ % arrested embryos after IVF.	Rat

APPENDIX B: PERMISSION TO PUBLISH

Chapters 1 to 7 are published (as noted below). As indicated on the websites of each of the six journals, these papers can be included in a graduate student thesis.

Chapter 1: AM Shahat, G Rizzoto, JP Kastelic. (2020). Amelioration of heat stress-induced damage to testes and sperm quality. *Theriogenology* 158:84-96.

Chapter 2: Abdallah M Shahat, Jacob C Thundathil, John P Kastelic (2020). Data loggers in scrotal subcutaneous tissues reliably assess intrascrotal temperatures in rams. *Small Ruminant Research* 193:106247

Chapter 3: Shahat AM, Thundathil JC, Kastelic JP (2021). Scrotal subcutaneous temperature is increased by scrotal insulation or whole-body heating, but not by scrotal neck insulation; however, all three heat-stress models decrease sperm quality in bulls and rams. *Journal of Thermal Biology 100*:103064.

Chapter 4: Shahat AM, Thundathil JC, Kastelic JP (2022). Melatonin or L-arginine in semen extender mitigate reductions in quality of frozen-thawed sperm from heat-stressed rams. *Animal Reproduction Science* 238:106934.

Chapter 5:

Shahat, A. M., Juan Castillo, Thundathil, J. C., & Kastelic, J. P. (2023). Angus bulls voluntarily access shade during hot weather, reducing scrotal subcutaneous temperatures and improving sperm quality. *Canadian Journal of Veterinary Research*, 87:17-22.

Chapter 6: Shahat AM, Thundathil JC, Kastelic JP (2022). Melatonin improves testicular hemodynamics and sperm quality in rams subjected to mild testicular heat stress. *Theriogenology* 188:163-9.

Chapter 7: Shahat AM, Thundathil JC, Kastelic JP (2022). Melatonin improves post-thaw sperm quality after mild testicular heat stress in rams. Accepted in *Reproduction in Domestic Animals* Journal.