2020-09-04

Pathogenesis of Heat-Induced Infertility in Male Mammals

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Pathogenesis of Heat-Induced Infertility in Male Mammals

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN VETERINARY MEDICAL SCIENCES

CALGARY, ALBERTA

SEPTEMBER, 2020

Abstract

Testicular temperature must be 3-5 ºC below body temperature for physiological spermatogenesis and testicular function. Therefore, increased testicular temperatures, either the entire body or just the testes, reduce sperm quality and fertility. Our understanding regarding the pathophysiology of testicular heat stress is unclear. There is a long-standing dogma that as testicular temperature increases, there is no change in blood flow, and the testes, which are regarded as physiologically functioning on the brink of hypoxia, undergo frank hypoxia. However, recent data challenged this dogma, indicating that temperature itself was the major pathological agent. Therefore, this thesis was developed to further investigate the subject. In a series of five studies, the overall aim was to investigate changes in testicular blood flow in response to testicular heat stress and its pathophysiology on testes and testicular function. In the first two studies, we investigated how heat stress and hypoxia affect testicular blood flow and metabolism in rams; both treatments increased testicular blood flow which supported metabolic needs, with no indications of hypoxia. The third study was a comparison of responses between *Bos indicus* and *Bos taurus* bulls to increased testicular temperature. Once again, testicular blood flow significantly increased, supporting metabolic needs, with no indications of hypoxia. These three studies provided new knowledge to debunk the previous dogma and to support the new understanding that temperature itself was the main pathological factor of testicular heat stress. In the last two studies, we investigated how heat stress modulates gene expression in bull and mouse testes. Heat stress caused modulation of gene *P53* and components of the *P53*-dependent apoptotic pathway, also upregulation of genes associated with the antioxidant (*GPX1*) and chaperone systems (Hsp70) and downregulation of the *StAR* gene and reduced testosterone concentrations (impaired steroidogenesis). Collectively, these studies provided novel information

regarding testicular vascular physiology under local heat stress and described several factors associated with its pathophysiology in the testes. Lastly, it is expected that these findings will serve as a strong base for new studies in this area, to elucidate in more detail, how heat stress affects reproduction in male mammals.

Keywords: Heat stress, testes, sperm, gene expression

Acknowledgements

Throughout the 4.5 years of my graduate studies at University of Calgary I received much assistance and support from faculty and colleagues; therefore, the completion of this thesis work, although led by my effort, also reflects the input of many others.

First and foremost, I acknowledge the support, advice and friendship of my supervisor. Dr. Kastelic offered numerous opportunities for personal and professional development. Working under his supervision has been a true gift and molded me in several ways, as he taught me to strive for my goals and dreams, but always to support other persons around me. Dr. Kastelic teaches through his examples, and I feel honored and truly blessed to have had the opportunity to count him as a mentor and as a friend.

I also acknowledge the committee that supported me in the development of this thesis and supported our ideas. Drs. Claudia Klein, Nigel Caulkett and Jacob Thundathil always provided very constructive input and ideas that contributed to the elaboration of each chapter of this document and the overall unity of the findings herein described. Furthermore, I also acknowledge my-workers and colleagues that were involved in the projects herein described and who provided assistance whenever needed.

A special mention is directed to Dr. John Tyberg and Cheryl Hall; their support and expertise were key to the studies that constituted the basis of this thesis work, and they were gracious to provide continuing support as new challenges and possibilities were identified throughout the program.

It is important to indicate the crucial assistance of Dr. João Carlos Pinheiro Ferreira as a major collaborator, as well as all the faculty, workers and fellow students at São Paulo State

University (UNESP) that were fundamental for the development of the research projects conducted there and who provided attention to every detail and need.

Dedication

To my family, 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 Jenn, for her love, support and patience, for helping me every single day to reach our goals and dreams, for the little family we are and the big family we will be.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
ADP	Adenosine diphosphate
A-V	Arterial-Venous
Ach	Acetylcholine
BAX	BCL-2-associated X protein
BCL2	B-cell lymphoma 2
BE	Base excess
Bi	*Bos indicus*
Bt	*Bos taurus*
CVO2	Venous O_2 content
CYP17	Cytochrome P450 17A1
DAZ	Deleted in azoospermia
ECG	Eletrocardiogram
FADD	FAS cell surface death domain
FAS	FAS cell surface
FASL	FAS ligand
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase
GSI	Gonadosomatic index
GPX1	Glutathione peroxidase 1
GSTA	Glutathione S-transferase
HCO3-	Bicarbonate
HIF1	Hypoxia induced factor 1
HMOX1	Heme oxygenase 1
HRE	Hypoxia-response element
HSF1	Heat shock factor 1
H2AFZ	H2A Z variant histone 1
MAP	Mean arterial pressure
NO	Nitric oxide
O_2	Oxygen
PCO_2	CO_2 pressure
pH	Potential of hydrogen
PO_2	Pressure of O_2
PPIA	Peptidylprolyl isomerase A
P53	Protein 53
Q(t)	Testicular blood flow
ROS	Reactive species of O_2
SEM	Standard error of the mean
StAR	Steroidogenic acute regulatory protein
TBARS	Thiobarbituric acid reactive substances
TDO2	Testicular O_2 delivery rate
TVO2	Testicular O_2 metabolic rate

TUNEL	Terminal deoxynucleotidyl transferase-mediated deoxy-UTP end labelling
TVC	Testicular vascular cone
V-A	Venous-Arterial

Epigraph

"The key difference between the investigations of scientists and those of painters and poets is an insistence on measurement to check the validity of scientific arguments, especially the governing the physical laws"

From: Is the Temperature Rising? The Uncertain Science of Global Warming, by S. George Philander

Chapter 1: A new paradigm regarding testicular thermoregulation in ruminants?

1.1 Introduction

Global warming and frequent episodes of high ambient temperature peaks in several countries (Weinberg et al., 2017; Ho et al., 2017; Smoyer-Tomic et al., 2003) are likely to adversely impact food animal productivity, as heat stress has deleterious effects on animal health and reproduction (Kastelic et al., 2017,2018; Rojas-Downing et al., 2017. Heat stress occurs when an animal is exposed to a temperature exceeding the animal's ability to compensate. It usually involves the entire body, although it may be restricted to a specific organ/anatomical region, including testis (Setchell et al., 1966), with duration of exposure ranging from peracute to chronic.

Effects of environmental and physiological conditions on mammalian reproduction have been studied for many years (Miztal et al., 2017; Setchell, 1998; Waites and Moule, 1961). Heat stress can adversely affect mammalian reproduction in at least two ways. First is whole-body effects; for example, a heat-stressed animal has reduced feed intake that can impair energy balance. Second, increased temperatures can have direct or indirect deleterious effects on reproductive cell function (Hansen, 2009; Sailer et al., 1997), depending on extent and duration of exposure. Testicular temperature is critically important for male reproduction. For many species, testes must 4 to 5 ºC below body core temperature to sustain production of morphologically normal, motile and fertile sperm (Saab et al., 2011; Kastelic et al., 1995; Skinner et al., 1966).

1.2 Mechanisms of heat transfer

Heat exchange between mammals and the environment, a fundamental component of thermoregulation, occurs primarily in four ways: conduction, convection, radiation and evaporation (Finch, 1986). According to the heat balance equation, heat stored is the sum of heat produced and heat absorbed, minus heat lost to the environment. To increase body temperature, the sum of heat produced and absorbed must exceed heat loss, whereas to maintain body temperature, these values must be equal. Several factors affect heat transfer in animals, including color, type of coat and environmental conditions (Finch, 1986).

During the day, heat gain is a combination of solar radiation plus body metabolism, whereas at night, heat flow is generally reversed, with heat dissipating from the body to the environment. The balance between heat gain and heat loss drives thermoregulation. Furthermore, heat exchange resistance in an animal is affected by several factors, including coat density and color, air resistance and evaporative resistance. Regarding effect of coat, in *Bos indicus* (*Bi*) bulls (Finch, 1986), inward heat flow in bulls with a black coat was 16 or 56% higher than those with brown or white coats, respectively. Furthermore, hair coats on *Bi* cattle support heat loss through convection and conduction (Hansen, 2004) and their dense flat hair coats have greater potential to reflect solar radiation (Finch, 1986).

Conduction is heat transfer between two bodies in physical contact; water and blood flow are important agents of heat transfer via conduction in animals. As air and fur have lower conductive capability and if contact of the animal with the ground is also limited, conduction may have a more important function within a body compared to exchanges between the body and the environment. Conversely, convection is heat transfer by bulk movement of particles,

including blood and air within an organism. All surfaces have a boundary layer, that is a transition zone for temperature exchange; a thicker layer increases insulation and reduces the convection coefficient. Regardless, wind can cause forced convection, reducing the thickness of the boundary layer. For an animal, the boundary layer is proportional to body's diameter, therefore smaller/skinnier animals have a thinner boundary layer and are more predisposed to heat loss to the environment through convection (Finch, 1985,1986).

Transfer of magnetic energy, radiation, is another important mechanism of heat exchange. Animals receive and transmit radiation, with surface temperature and emissivity regulating intensity of radiation. Once more, heat flows from the warmer to the colder object. Therefore, animals can receive heat through solar radiation and concurrently emit radiation to a cooler animal or object. Furthermore, animals absorb ~60-80% of incident radiation; the amount is a function of skin color, as darker coats tend to have higher absorptivity, although emissivity is not affected by coat/skin color, but by temperature. Lastly, as evaporation of water from skin and lungs is a major mechanism of heat loss, sweating and panting have substantial thermoregulatory potential (Hansen, 2004; Finch, 1985,1986; Turner, 1964).

1.3 Testicular thermoregulatory mechanisms

Testicular thermoregulation in animals can be divided in two components: non-vascular and vascular. Non-vascular thermoregulation includes physiological responses, e.g. sweating, changes in testis location relative to the abdomen and other behavioral approaches, e.g. seeking shade. In contrast, vascular responses include countercurrent heat exchange and peripheral vasodilation.

1.3.1 Non-vascular thermoregulatory mechanisms

One of the first descriptions of non-vascular thermoregulatory mechanisms was a report that exposure to cold temperatures causes scrotal contraction and wrinkling, whereas at warmer temperatures, the scrotum is relaxed and extended (Cooper, 1830). In animals with a scrotum, the tunica dartos, the cremaster muscle and sweat glands all contribute to testicular cooling (Senger, 2003). The tunica dartos, smooth muscle in the scrotal wall that contracts and relaxes to raise or lower testes, is an important regulatory factor in ambient temperatures up to 30 ºC (Beakley and Findlay, 1955). Furthermore, the scrotal ligament has an important function, attaching the tunica dartos to the ventral pole of the testis and connecting the tunica dartos and the cremaster muscle, thereby synchronizing their actions. Furthermore, the striated cremaster muscle, attached to the internal abdominal oblique muscle, contracts to draw testes closer to the body in cold weather, but relaxes to allow testes to move away from the body when exposed to higher temperatures (Senger, 2003).

Scrotal sweat glands respond to heat stress by releasing sweat in a pulsatile fashion, with evaporation of sweat promoting testicular cooling (Waites and Voglmayr, 1963; Robertshaw and Vercoe, 1980). In bulls and rams, scrotal sweat glands are controlled by sympathetic adrenergic innervation and influenced by adrenal hormones (Waites and Voglmayr, 1963; Robertshaw and Vercoe, 1980). In rams, scrotal skin temperatures >35 ºC activate synchronous discharges (intervals of 2 to 14 min) of apocrine sweat glands. Furthermore, *Bi* start to sweat at a lower temperature than *Bos taurus* (*Bt*) and unlike the latter, they usually do not reach a plateau of sweat production but rather they are able to keep increasing sweating production (Amakiri,

1974). Interestingly, scrotal sweat glands in bulls are more densely distributed, larger, and produce more sweat than those elsewhere in the body (Robertshaw and Vercoe, 1980; Amakiri, 1974; Waites and Woglmayr, 1963; Nay and Hayman, 1956). There are also breed differences, with *Bi* bulls having a higher sweat gland perimeter and more functional sweat glands compared to *Bt* and cross-bred bulls, indicating important adaptations (Carvalho et al., 1995).

Scrotal hair coat or wool coverage can affect thermoregulatory responses. When rams are exposed to local testicular heat stress, thermoregulatory responses are distinctly different between fully fleeced versus shorn rams (Hales and Hutchinson, 1971), since the wool serves as an important thermo isolation component, once removed, the thermoregulatory capability will rely on other mechanisms such as sweat gland activity and evaporation, for example. In response to heat stress, fully fleeced rams have a faster respiratory rate (panting) compared to shorn rams and more intense peripheral (skin) vasodilation. However, this panting response is lost if fully fleeced rams are shorn. In the same study, shorn rams had lower body temperatures, but they compensated by increased oxygen (O_2) consumption. When these rams were exposed to local heat stress (testicular insulation), there was a reduction in metabolic activity, but no vasomotor or respiratory responses.

In another study (Waites and Woglmayr, 1980), rams with a wool-covered scrotum versus short scrotal wool were compared regarding scrotal insulation. In the former, there was an initial increase in scrotal temperature (due to an exothermic response between wool keratin and skin), followed by a reduction in scrotal temperature. Interestingly, rams with short scrotal wool had acute decreases in skin temperature, due to rapid evaporation of sweat. These responses were attributed to the presence of warm receptors in the scrotum and a neuronal-based thermoregulatory response. Another indication of neurological control of thermoregulation in

rams is that scrotal sweat discharges and panting are abolished by blocking superior perineal nerves (Waites and Woglmayr, 1980). Rats have warm and cold receptors in their scrotum, with stimulation initiated at ambient temperatures of 35 to 45 and at 13 to 33 ºC, respectively (Hensel, 1983).

1.3.2. Vascular thermoregulatory mechanisms

There are several theories regarding evolution of the scrotum in mammals and its role in temperature homeostasis, as not all mammals have a scrotum (Freeman, 1990). Perhaps mammals evolved from a scrotal to an ascrotal phenotype, due to challenges inherent in having a pendular testes (Werdelin and Nilsone, 1999). Regardless, for both configurations, counter-current heat exchange in the testicular vascular cone (TVC) seems to be key for reducing testicular temperature (Freeman, 1990). The TVC is located dorsal to the testis and comprised of the highly coiled testicular artery, surrounded by the venous pampinform plexus, with blood flow in these vessels going in opposite directions. Consequently, the TVC acts as a counter-current heat exchanger, cooling arterial blood before it enters the testis (Hees et al., 1984; Cook et al., 1994).

Based on comparative anatomical studies of the pampiniform plexus, there is much similarity among several mammals. However, for the testicular artery, there are notable distributional differences among species, although closely related species are more likely to have anatomic similarities (Harrison, 1948). In the bovine testis, the testicular artery exits the bottom of the TVC and courses ventrally, under the corpus epididymis. At the ventral pole of the testis, it divides into three or four branches that remain superficial (caudal aspect of testis) and meander vertically and laterally before entering the testicular parenchyma and dividing into numerous

branches to perfuse all testicular lobes, emphasizing the importance of the artery for delivery of O_2 and nutrients to the testis and for maintaining temperature homeostasis (Harrison, 1949).

In an *in vitro* model, heat transfer from the testicular artery to the pampiniform plexus was highly efficient (91%), emphasizing the importance of countercurrent heat exchange in the TVC for testicular cooling (Glad et al., 1991). It was also stated that this exchange is not a regulatory mechanism *per* se due to its passivity, although its function is highly beneficial for testicular temperature control (Waites and Moule, 1961). The most important location for heat transfer is the base of the TVC, where the testicular artery has a larger luminal area and slower blood flow and it is surrounded by a network of small veins, facilitating exchange (Waites and Moule, 1961).

In bulls, development of the testicular artery peaks between 6 and 12 mo of age and its length, diameter and distance between the vein and artery in the spermatic cord all increase with age, whereas artery wall thickness decreases (Cook et al., 1994). In monthly ultrasonographic examinations in bulls, TVC diameter increased until 13.5 mo of age (Brito et al., 2004). All these characteristics promoted increased heat exchange in the TVC after puberty, consistent with the need to keep testes cooler than core body temperature.

1.4 Scrotal and testicular temperatures

Temperatures of the bovine scrotum and testes have been well described (Kastelic et al., 1995). Scrotal surface temperatures decrease from top to bottom, resulting in a positive temperature gradient (top temperature minus bottom temperature) inversely proportional to ambient temperature (range from ~4 to 1 ºC when ambient temperatures were 5 and 35 ºC,

respectively). In contrast, the intratesticular temperature gradient is negative, with testes being ~1 ºC cooler at the top versus the bottom. Blood in the testicular artery is cooled in the TVC, but arterial blood temperatures at the bottom of the TVC and the bottom of the testis are not significantly different. However, arterial blood temperature decreased significantly between the bottom of the testis compared to the point of insertion into testicular parenchyma (Kastelic et al., 1996). Therefore, the testis is warmest at the origin of its blood supply (bottom of testis), but cooler distal to the blood supply (more dorsal).

Furthermore, based on the assumption that the scrotum is vascularized from top to bottom, it is also warmer at the origin of the blood supply (top of scrotum) and cooler distally (bottom of scrotum). It was concluded that the testes and scrotum have opposing, albeit complementary, temperature gradients that work together to keep testes cooler than body temperature (Kastelic et al., 2018). Epidydimal temperatures also have to be taken into consideration, as the cauda epididymis was warmer than the testis, but the tail was cooler than both (Kastelic et al., 1995). Epididymal sperm can be impacted by heat stress (Cameron and Blackshaw, 1980) and sperm production was positively associated with lower epidydimal temperature (Brito et al., 2002). Therefore, epididymal temperature and the capability to maintain temperature gradients are critical for maintenance of sperm quality.

Another important aspect that must be considered is position of testis relative to the body and characteristics of the scrotum and testicular vascular cone. When comparing *Bi* and *Bt* bulls, scrotal temperature was lower in *Bi* than *Bt*; furthermore, *Bi* bulls had thicker scrotal necks, associated with shorter scrotal necks and less pendular scrotums (Brito et al., 2004). In the same study, despite correlations between testicular and scrotal morphological characteristics and sperm quality, semen quality was not significantly different between *Bi* versus *Bt*.

Fat deposition in scrotum and scrotal neck impairs heat loss through radiation (Cook et al., 1994) increasing testicular temperature and reducing sperm quality. Increased deposition of fat in scrotal neck occurs as bulls age (Cook et al., 1994), with potential to reduce sperm quality. In particular, high-energy diets increase fat deposition in the scrotum (Barth et al., 2008). Bulls fed a high-energy diet had lower sperm quality, higher percentage of nonfunctional seminiferous tubules and more scrotal fat than those fed low- or medium-energy diets (Swanepoel et al., 2008). Similarly, high nutritional plane in Holstein bulls increased both fat deposition in scrotum and scrotal surface temperature (Vogler et al., 1991).

Scrotal insulation, an experimental model to increase testis temperature, mostly impairs scrotal heat loss through radiation and evaporation. This approach has been widely used in various species, particularly cattle (Vogler et al., 1991; Barth and Bowman, 1994). Under physiologic conditions, testicular and scrotal temperatures are carefully controlled (Kastelic et al., 2018). Therefore, addition of any material, either experimental scrotal insulation or fat deposition as described above, impairs heat exchange with the environment and results in increased testicular temperatures.

1.5 Heat stress impacts vary among cattle breeds

There are breed differences in ability of bulls to withstand heat stress. For example, *Bi* bulls usually have relatively good productivity in tropical climates, whereas *Bt* bulls are under heat stress in the same conditions. The disparate response under similar conditions generates questions regarding breed differences at morphological, physiological, or perhaps even genetic levels. There are also important differences for dairy versus beef breeds (Rahman et al., 2018;

Wildeus and Hammond, 1993), indicating a possible influence of morpho-physiological traits in heat resistance.

There are several morpho-physiological differences and potential genetic and molecular mechanisms underlying higher resistance of *Bi* cattle to heat stress. Higher environmental temperatures are more likely to affect *Bt*-based production systems, with reductions in production and animal welfare, as these cattle are not well adapted to warm climates (Rojas-Downing, 2017). Although *Bi* cattle are better adapted to heat stress than *Bt*, underlying mechanisms are not fully understood (Entwistle, 1992). Heat stress (~27 ºC for 10 d) reduced feed intake in *Bt* bulls, whereas intake in *Bi* bulls was not altered (Valente et al., 2015).

Factors such as morphological traits and skin colour can have important roles in thermoregulation. There are morphological differences between *Bi* and *Bt* cattle; obvious traits in the former are loose skin, shorter hair and a hump (Cardoso et al., 2015). Furthermore, radiation absorption is lesser in *Bi* breeds and they have less resistance to transfer of metabolic heat to the skin compared to *Bt*, indicating greater cooling capability (Finch, 1986). Furthermore, *Bi* bulls have greater resistance to heat stress and morphological adaptations to improve heat loss and decrease arterial blood temperature in the testicular vascular cone (TVC) than *Bt* bulls (Hansen, 2004; Brito et al., 2004; Tuner, 1964). For example, *Bi* bulls had the thinnest testicular artery wall and shortest arterial-venous distance in the TVC, features expected to facilitate heat exchange between arterial and venous blood (Brito et al., 2012).

Following whole-body exposure to high temperatures, *Bi* and *Bt* had several differences (Skinner and Louw, 1966). Specifically, *Bi* bulls had greater sperm motility and viability, better sperm morphology and their sperm quality recovered more quickly after heat stress when compared to *Bt* bulls. Furthermore, *Bi* bulls had a lower rectal temperature (Skinner and Louw,

1966). Therefore, it was concluded that *Bi* bulls are more resistant to the influence of heat. In addition, *Bi* cattle had ~5 ºC rectal-testicular temperature gradient in either direct sun or shade, indicating greater testicular thermoregulatory capability (Godinho and Cardoso, 2015). Capability of maintaining significantly lower temperatures under relatively high field temperatures may contribute to reduced impacts on testicular and germ cells and faster recovery.

There were also seasonal differences between *Bt* and *Bi* bulls in semen quality. Despite more sperm abnormalities in summer for both breeds, there were greater lipid peroxidation and proportion of sperm abnormalities in *Bt* versus *Bi* bulls (Nichi et al., 2006). Similarly, sperm quality was maintained in *Bi* but reduced in *Bt* during warmer seasons (Koivisto et al., 2009).

The ability to adapt to a new environment must be considered. In a comparison of effects of a tropical climate on native *Bi*, adapted *Bt* and non-adapted *Bt* cattle (Carvalho et al., 1995), although adapted *Bt* bulls were accustomed to heat, they had higher rectal temperatures and respiratory rates compared to *Bi* bulls ($P<0.01$). Furthermore, non-adapted *Bt* bulls had the greatest increases in rectal temperature and respiratory rate compared to the other two groups ($P<0.01$), indicating importance of adaptation to an environment and breed differences (independent of adaptation). In a comparison of bulls adapted versus non-adopted to the climate conditions (higher temperature), on average, the former had a lower rectal temperature, larger scrotal circumference and fewer morphologically abnormal sperm (Wildeus and Hammond, 1994).

In addition to important differences between *Bt* and *Bi* cattle, there were breed-specific differences among *Bt* breeds (Pereiera et al., 2014), indicating that adaptation to climate was an important aspect of thermoregulatory capability. In a comparison of Sahiwal (*Bi*) and (Frieswal) crossbred cattle, higher expression of Hsp90 under heat stress conferred protection against cell

death and regulated body temperature, as crossbred cattle had a higher rectal temperature than *Bi* cattle exposed to ambient temperatures of 37-45 ºC (Deb et al., 2014).

Another study using a microarray approach studied impacts of heat stress on mRNA expression patterns in blood of *Bi* cattle (Mehla et al., 2014). After exposure to 42 ºC for 4 h, and assessment at three time points (4, 24 and 48 h), there were changes in expression of several genes, including up-regulation of 140 transcripts including *HSP1* and Heat Shock Proteins (*HSPs*), and also down-regulation of 77 transcripts. Most upregulated genes were associated with protein folding, synthesis and transport, metabolism of carbohydrates, apoptosis induction and production of transcriptional factors, whereas downregulated genes were associated with oxidative phosphorylation, lipid metabolism and cell division. Another study compared responses of dermofibroblast of *Bi* and crossbred cattle (Singh et al., 2014). Exposure to 40 or 44 ºC for 3 h increased expression of *HSP70* genes, with significantly higher expression in crossbred cattle compared to *Bi*. Overall, dermal fibroblasts of *Bi* had greater thermal tolerance than those of crossbred animals, indicating a genetic basis of heat resistance.

1.6 Consequences of increased testicular temperature

Testicular temperature can be considered an assessment of energy state, namely sum of metabolism and blood flow, with blood flow having the largest contribution (Barros-Adwell et al., 2018), minus heat loss via venous blood, skin convection, sweat evaporation and to a lesser extent, radiation (Waites, 1991; Finch, 1986). Effects of increased testicular temperature depend on extent and duration of testicular heating. Whereas a brief, mild increase in testicular temperature may cause only temporary reduction in sperm quality, prolonged and/or substantial

heating are likely to cause infertility. Furthermore, a severe thermal insult may cause permanent cessation of spermatogenesis.

Several pathological states have a profound influence on sperm quality, fertility and can impair reproductive soundness. Cryptorchids have impaired reproductive capability (Bedford et al., 1982). Using rodent models of induced cryptorchidism (moving testes to the abdominal cavity), testis temperature was increased 1.5 ºC, testicular weight decreased (Bedford et al., 1982) and although spermatogenesis continued, rate of sperm production was reduced and there were more morphologically abnormal sperm (Bedford et al., 1982). In another study in rodents (Bedford et al., 1978), following relocation of epididymides to the abdominal cavity (without altering location of testes), after a few months, sperm quality was highly impaired, although fertility was maintained, presumably due to compensable sperm abnormalities and the huge number of sperm in a rodent ejaculate.

Sperm maturation in the epididymis is supported by a highly specialized epithelium (Senger, 2003) However, when epididymal temperatures approach body core temperature, there are changes in fluid secretion and content. The cauda epididymis rapidly loses its capability to maintain viable sperm; furthermore, in rodents, there were changes in secretion of water and ions into caudal epididymal fluid following exposure to increased temperatures. However, if testes are relocated to the scrotum, sperm quality can be restored (Wong et al., 1983).

Impacts of heat stress on spermatogenesis may not be readily apparent in ejaculates collected soon after testes are warmed. For example, morphologically abnormal sperm were first observed in ejaculates starting ~2 wk after testicular hyperthermia in bulls (Hansen, 2004). In another study, bull semen collected a few days after increased testicular temperature had apparently normal sperm; however, changes in sperm were evident after routine cryopreservation

and thawing (Vogler et al., 1991). In addition, short-term testicular hyperthermia in rats induced cellular apoptosis, with spermatocytes and early spermatids most affected (Lue et al., 1999). In that study, treatment with a GnRH antagonist caused intense apoptosis, mostly impacting spermatocytes at pachytene and diplotene stages, dividing spermatocytes and early spermatids, indicating testosterone may protect testes against heat stress. Furthermore, in bulls exposed to high ambient temperatures (35 ºC), blood testosterone concentrations decreased, but after 2 wk, returned to pre-treatment values, although sperm quality remained impaired (Rhynes and Ewing, 1973). In rams, prolonged (28 d) scrotal insulation reduced blood testosterone concentrations and severely affected sperm morphology and testicular architecture (Byers and Glovers, 1984).

After testicular insulation in Holstein bulls, morphological abnormalities generally followed a consistent sequence up to 30 d after insulation (Vogler et al., 1993). Although specific defects appeared at predictable times, there were differences among bulls in percentage of sperm affected. In beef bulls, dexamethasone (20 mg/d for 7 d) or scrotal insulation (4 d), as models of stress and high temperature, respectively, had similar impacts on the spermiogram (Barth and Bowman, 1994). Chronic exposure to heat stress in Angus bulls impaired sperm quality and ejaculate volume (semen collected by artificial vagina) but did not alter sperm concentration in the ejaculate. However, as expected, there was an increase in percentage of morphologically abnormal sperm (Meyerhoffer et al., 1985).

Heat stress in rats reduced testicular size and sperm concentration in rete testes fluid, although concentrations of major components in rete testes fluid were unaffected (Setchell and Waites, 1972). Interestingly, boars exposed to heat stress in a climate chamber had increases in percentage of morphologically abnormal sperm, but no changes in total volume or sperm concentration (Paul et al., 2008). Furthermore, there were indications that effects on semen

quality were more influenced by an acute temperature increase than duration of exposure [39]. Reduced sperm concentrations and more morphologically abnormal sperm in rams exposed to local heating of the testes when compared to animals with whole-body exposure to high temperature (climatic chamber) indicated that physiological regulation aspects may be differentially activated, based on the nature of the exposure (Saab et al., 2011). Furthermore, in the same study, there were indications that a primary heat stress episode may confer some degree of resistance through development of adaptative mechanisms mitigating negative effects in subsequent exposures (Saab et al., 2011).

Regarding fertility and embryo development, heat stress damaged DNA in rat epididymal sperm and increased embryo resorption and reduced litter size (Pérez-Crespo, 2008), with indications that heat may differentially affect germ cell lines. There were major impacts on spermatocytes and round spermatids (Yaeram et al., 2006) and a similar impact on epididymal sperm (Sailer et al., 1997), based on chromatin structure and sperm motility and morphology, although fertility was not directly assessed.

Male fertility rates, both *in vitro* and *in vivo,* were negatively affected by increased temperatures (Paul et al., 2008; Pérez-Crespo et al., 2008; Yaeram et al., 2006). Exposure of mice to 42 ºC for 30 min reduced pregnancy rate, placental weight and litter size; furthermore, they also impaired embryo development, as described above (Paul et al., 2008). Exposure of mice to 36 ºC for two 12-h episodes reduced sperm concentrations, fertility and litter size (Yaeram et al., 2006). Furthermore, there were changes in male/female ratios of progeny (Pérez-Crespo et al., 2008), and even transient delays in embryo development (Setchell, 1998). In cattle, *Bi* embryos are more resistant to heat shock compared to those from *Bt* cattle or crossbreds, indicating that heat tolerance ranged from the whole-animal to the early embryo (Silva et al.,

2013; Eberhardt et al., 2009). In boars, genetic selection of a line more resistant to heat stress appeared to improve male fertility (Flowers, 2018).

Heat stress differentially affects various stages of the spermatogenic cycle, with greater impact on pachytene spermatocytes, whereas spermatogonia (Celino et al., 2011) and elongated spermatids (Cataldo et al., 1997) were more resistant. Furthermore, high temperatures alter testicular biochemistry, including rate of incorporation of amino acids and modifications in enzyme concentrations. Impacts of increased temperature affect all testicular cells, including Leydig (Kanter and Aktas, 2009) and Sertoli (Danno et al., 2000). In cryptorchid or heat-stressed animals, Sertoli cells had variations in protein expression patterns and impaired translation initiation (Cataldo et al., 1997; Guo et al., 2007; Kanter and Aktas, 2009).

Steroidogenesis by Leydig cells was impaired after heat stress, mostly attributed to repression of *StAR* (Bozkaya et al., 2017; Oka et al., 2017; Li et al., 2016). Furthermore, DNA damage can be present in sperm collected from epididymis 6 h after heat stress exposure, with decreased DNA quality in sperm cells undergoing development at moment of heat exposure (Pérez-Crespo et al., 2008). In addition, there are indications heat stress causes epigenetic changes that may be transferred to progeny (Rodgers et al. 2013; Chao et al., 2012).

Metabolic parameters within testes can also be modified by heat stress. Regarding sources of heat for testes, blood flow had a much greater contribution than testicular metabolism (Barros-Adwell et al., 2018). Several studies reported metabolic changes in testicular cells after heat stress. For example, there were several changes in Sertoli cells (Xu et al., 2015), including increased inflammatory cytokines and impaired production of several factors associated with the blood-testis barrier. Furthermore, in rats, heat stress affected energy metabolism pathways and the hypothalamic pituitary adrenal axis (Hou et al., 2015). Heat stress also modified sperm

metabolic parameters, in both classical (Voglmayr et al., 1971) and recent studies (Sabés-Alsina et a., 2016), altering sperm metabolic rate and composition of fluids secreted in the rete testes.

1.7 O_2 delivery, metabolic rate and extraction

To further understand impacts of changes in blood flow, several metabolic concepts need to be understood. To maintain organ function, cardiovascular and respiratory systems must interact to ensure appropriate amounts of O_2 are delivered to a tissue, with adequate O_2 extraction to support metabolic needs (Burstcher, 2013). Interestingly, O_2 utilization, delivery and extraction can be influenced by temperature, including both hyperthermia (Kerner et al. 1999) and hypothermia (Roe et al., 1966), emphasizing the importance of these functions.

1.7.1 O_2 delivery

O_2 delivery, i.e. the amount of O_2 reaching an organ, can be either a measurement of blood flow in a vessel (if O_2 content remains consistent), or a function of O_2 saturation, cardiac output and hemoglobin concentration and hemoglobin-O_2 binding coefficient (Burstcher, 2013). Total blood O_2 content is affected by the hemoglobin-O_2 bond and O_2 dissolution in plasma (Habler and Messmer, 1997). Maintenance of appropriate tissue oxygenation to support metabolic needs under various conditions is achieved through modulation of O_2 delivery. Therefore, if metabolism changes, O_2 delivery is adjusted to address tissue needs (Mortensen et al., 2005). One of the biggest challenges regarding heat stress is loss of the ability to modulate

blood flow to match O_2 requirements, as changes in flow are primarily modulated by the need to dissipate heat; therefore, there is no assurance that a particular tissue or organ receives appropriate blood flow to meet O_2 demands.

One of the most important systems involved in tissue oxygenation is the cardiovascular system, which under heat stress is challenged between supplying O_2 to the organs and increasing the blood flow to the peripheral regions of the body and skin to increase heat loss (Cramer et al., 2017). Under heat stress conditions, there is both reduced blood flow to internal organs and a reduction in metabolic rate; consequently, increased blood flow to the skin can occur without adverse effects on the function of the internal organs. Under normothermic conditions, the majority of the blood flow in the body of mammals is directed towards internal organs. It is noteworthy that vascular beds irrigating the liver, pancreas, spleen and gastrointestinal system often receive up to 50% of the cardiac output (Crandall et al., 2015; Rowell et al., 1965).

Furthermore, at normothermic temperatures, there is substantial vasoconstriction in the skin. Consequently, this limits blood flow to peripheral areas, thereby reducing heat loss and maintaining core-body temperature. However, there are profound changes in response to heat stress. Peripheral vasodilation is a key mechanism that promotes heat loss, as a major portion of the cardiac output is directed towards the skin, where heat is dissipated, both directly, and in association with evaporation of sweat (Kellog, 2006). One of the most important consequences is the concurrent reduction of blood flow to several organs, including liver and spleen, however, based recent data, it indicates that an increase in testicular blood flow takes place (Barros-Adwell et al., 2018), but more studies are required to confirm the data.

An important consequence of hypoxia is the pathological cascade associated with production of reactive O_2 species (ROS). These compounds are mostly generated through

oxidative phosphorylation in mitochondria. Under hypoxic conditions, there is increased ROS production due to the metabolic strain induced by inadequate O_2 (Hamilton et al., 2016). In appropriate concentrations, ROS molecules are integral components of physiologic reactions. Regardless, they are probably best known for their negative impacts when present in excessive concentrations, overcoming antioxidant defenses and damaging cells and tissues and impairing organ function.

Increased testicular temperature in mature rats did not influence testicular blood flow, but substantially modified vasomotion (rhythmic tone variation in vessels; Setchell et al., 1995). The importance of vasomotion is debatable, but there are indications that its pattern is related to nutrient exchange within tissues. However, there's important indications that vasomotion is an androgen-regulated rhythmical contraction mechanism present in smooth muscle, and specifically in the testes, have the ability to regulate interstitial fluid dynamics (Welsh et al., 2010), including factors such as redox mechanisms and supporting the maintenance of nutritive perfusion to muscle and neighboring tissues under perfusion failure (Rucker et al., 2000). For rats, normal vasomotion was described for physiological temperature, but between 36 and 42 ºC, the vasomotion pattern disappeared (Setchell et al., 1995). In another study, vasomotion in testes was dependent on testosterone and Sertoli cells (Collin et al., 1993). Vasomotion in testicular tissue has been described in a few mammalian species and may have importance in testicular function (Collin et al., 2000), although further studies are needed. It is important to indicate, however, that more studies are required to understand the function of vasomotion in the questions investigated in this thesis.

1.7.2 O_2 utilization (metabolic rate)

In addition to modulations in respiratory rate, the body has two metabolic defense mechanisms to compensate for hypoxia, namely regulation of O_2 metabolic rate and increased O_2 extraction rate.

The O_2 metabolic rate for a structure for a specific time point is obtained by multiplying blood flow by arterial-venous O_2 content difference (Caulkett et al., 1996). Furthermore, metabolic need is a major factor affecting O_2 extraction. In the case of hypoxia, despite reduced metabolism, reduced blood flow increases O_2 extraction to support metabolic needs (Samsel and Schumaker, 1991). Consumption of O_2 is related to organ activity. For example, exercise increases O_2 consumption by exercised muscle (Gonzáles-Alonso et al., 1998). In contrast, reduced activity in internal organs during heat stress may reduce their metabolic rate (Neufer et al., 1989).

Temperature is an important regulator of metabolic rate in animals (Heldmaier and Ruf, 1992) and humans (Saxton, 1981). Reductions in organ function and metabolic rate to preserve tissue integrity and reduce strain on the cardiovascular system are well established. For example, reduced activity in the stomach and intestine under heat stress (Neufer et al., 1989) are likely due to a lower metabolic rate.

1.7.3 O_2 extraction

O_2 extraction is defined as the metabolic rate of O_2 divided by O_2 delivery,

reported on a percentage basis, to indicate the amount of O_2 used by the organ at a certain time point. Extraction reflects tissue conditions and activity and is a very important end point regarding organ function (Shoemaker et al., 1988).

As previously described, reduced blood flow to internal organs also decreases O_2 delivery, stimulating increased O_2 extraction (Gonzáles-Alonso et al., 2003), an important defense mechanism in response to either heat stress or hypoxia (Samsel and Schumaker, 1991; Trangmar et al., 2014). Furthermore, increased extraction is also a consequence of an increased metabolic rate. For example, increased O_2 extraction contributes to maintenance of O_2 delivery, even under non-physiological conditions (Fan et al., 1985).

Reductions in blood flow to an organ can cause hypoxia. A hypoxic condition can reduce O_2 delivery and often results in increased O_2 extraction (Samsel and Schumaker, 1991). For example, a reduction in blood flow to the brain under exercise in heat stress is compensated by increased O_2 extraction to sustain O_2 metabolic rate (Trangmar et al., 2014). Furthermore, classical studies described increased O_2 extraction in the liver during exercise and heat stress, regarded as compensation for reduced hepatic perfusion (Rowell et al., 1965).

1.7.4 Potential regulators of testicular blood flow

Several local factors were recently described, providing new insights into vasoconstriction/vasodilation stimuli. ATP is an important vasodilatory agent. Based on *in vivo* and *in vitro* studies, increased ATP release by erythrocytes and not endothelial cells was responsible for increased blood flow to the human arm under heat stress (Kalsi et al., 2017). Interestingly, prostaglandins and nitric oxide (NO) are described as downstream effectors of the

ATP in the vasodilation process (Mortensen et al., 2009). Furthermore, NO is a well described agent in blood flow modulation (Fitzgerald et al., 2007). Release of NO in the skin occurs during vasodilation by endothelial cells (Burtscher, 2013), through the influence of acetylcholine (ACh) and activation of other receptors (Kellog et al., 1985).

Prostaglandins are also important vasodilators in skin (Kellog et al., 1985). Recent studies described inhibition of prostaglandins and NO in the exercised leg, with reductions in blood flow to skeletal muscles (Heinonen et al., 2011; Kellog et al., 1995). Studies regarding mechanisms of skin vasodilation described that co-release of ACh and other neurotransmitters are necessary to elicit a full response (Mortensen et al., 2005). Importantly, NO is also fundamental for vasodilation to achieve maximum potential, acting synergistically with neurotransmitters (Kellog et al., 1995). Vasodilation starts with temperature stimuli that elicit a response from the nervous system, with NO following previous stimuli and sustaining vasodilation. However, mechanisms discussed above have apparently not yet been reported for testicular vasculature.

1.8 Pathophysiology of heat stress – Molecular mechanisms

Male germ cells are differentially affected by heat stress (Lue et al., 1999; Paul et al., 2008). Rat spermatogonia are considered more resistant to heat stress than other testicular cell types (Mclean et al., 2002), consistent with decreased sperm quality in bulls after testicular hyperthermia, followed by subsequent restoration, with a new generation of germ cells derived from spermatogonia (Barth and Bowman, 1994). Testicular heating may have the most profound effects on spermatids and primary spermatocytes (Vogler et al., 1991).

There are several reports regarding molecular pathways that protect or damage testicular cells and sperm after heat stress (Durairajanayagam et al., 2015; Kim et al., 2013). One of the most important factors is the *HSF1* gene and its product *HSF1* (Heat Shock Factor 1) that regulates production of Heat Shock Proteins (*HSPs*), conferring protection after heat stress (Zhang et al., 2002). The most common *HSP*s in various tissues are *HSP70* and *90* (Vendrell et al., 2016; Zhao et al., 2010), although other *HSP*s are described (Pei et al., 2012). In a heat stress episode, *HSF1* and *HSP* genes are up-regulated, with increased protein expression for their products (Vendrell et al., 2016; Zhang et al., 2002), with reduced protein denaturation. The *DAZ* (Deleted in Azoospermia) gene and its products, including *DAZL* protein, are involved in cellular protection after heat stress, including removal of signaling molecules and impeding the apoptosis pathway (Kim et al., 2013). Various other factors are also involved in protection against heat stress, including increased expression of anti-oxidant factors such as *HMOX1* (Heme oxygenase 1), *GPX1* (Glutathione Peroxidase 1) and *GSTA* (Glutathione S-Transferase Alpha) (Paul et al., 2009) to protect against reactive O_2 species (ROS) and *BCL-2* gene and protein (Apoptotic inhibitor) (Chen et al., 2008).

Several other factors are related to cellular destruction and functional impairment after heat stress. For example, up-regulation of gene *P53* and expression of its product are apoptotic inducers (Gu et al., 2015). There is also increased expression of *CASP 8* and *10* (Chao et al., 2012). Other important genes and their products also seem to be involved, such as up-regulation of *BAX* (accelerator of apoptosis) (Mete et al., 2012) and downregulation of Bag1 (chaperone protein - protective) (Zhao et al., 2010).

Furthermore, hormonal imbalances seem to occur under heat stress, including down-regulation of *StAR* (Steroidogenic Acute Regulatory Protein) gene and protein (Brugnon et al.,

2012). Reduced activity of this enzyme, a critical step in steroidogenesis, would decrease production of testosterone, a hormone with critical roles supporting spermatogenesis and testicular integrity (Bozkaya et al., 2017). Furthermore, reports described the reduction in *StAR* and testosterone (serum) soon after either chronic or intense heat stress exposure (Bergh et al., 1984a, 1984b; Au et al., 1983). Enzymes involved in testosterone synthesis such as *CYP17* and Cytochrome P450) had reduced activity after heat exposure (43 ºC/30 min) in rats (Li et al., 2009).

Expression of *HSP70* genes in skin, as a marker of heat resistance, was measured throughout the year in *Bi* and crossbred cattle (Maibam et al., 2017); although both breeds had increased expression of *HSP* genes in summer, *Bi* had higher expression, better heat tolerance and adaptability to higher temperatures. Furthermore, another important heat shock protein (*HSP90*) had higher mRNA and protein concentrations in peripheral blood mononuclear cells in *Bi* versus crossbred animals after *in vitro* heat shock (42 ºC for 1 h; Koivisto et al., 2009).

Under low O_2, the increase in expression of Hypoxia induced factor 1 alpha (*HIF-1*) is a pathway that is associated with oxidative stress adaptation (Reyes et al., 2012). One of the most important consequences of *HIF-1* is increased expression of Vascular Endothelial Growth Factor (*VEGF*), with receptors in testicular cells (Kilinç et al., 2004; Ergün et al., 1997). It is noteworthy that *VEGF* stimulates angiogenesis, a defense mechanism in response to hypoxia, as an hypoxic organ uses angiogenesis to increase its blood delivery. However, this mechanism is a longer-term versus immediate response to hypoxia.

Maintaining homeostasis is fundamental for proper organ function, with O_2 availability a major driver of cellular function. In addition to testes, another system that operates with low O_2 concentrations is the renal system. Although the kidneys receive one of the highest portions of

cardiac output, the renal medulla receives much less flow than the renal cortex (Layton et al., 2016). Although physiological, a risk of imminent hypoxia is present (Chen et al., 2009), similar to that reported for testes. Furthermore, both organs have a countercurrent arteriovenous heat exchange (Kastelic et al., 2018; Röed and Aukland, 1969).

One of the most common pathological conditions associated with testicular hypoxia and oxidative stress is varicocele, which is defined as an increased tortuosity and higher diameter of vessels in the pampiniform plexus in an abnormal proportion, impairing proper blood drainage and oxygenation (Razi et al., 2015). Interestingly, expression of *HIF-1* and *VEGF* in animals with varicocele have characterized in several studies (Goren et al., 2017; Madrid et al., 2013; Kilinç et al., 2004). Reduced O_2 availability leads to oxidative damage, as release of radical O_2 species (ROS) can be very detrimental for the seminiferous epithelium in laboratory animals and ruminants (Paul et al., 2009; Hamilton et al., 2006). Furthermore, in several species, antioxidant treatments reduce impact of ROS on cells (Martin-Hidalgo., 2019; Peña et al., 2019). Other fundamental markers associated with hypoxia are alterations in oxidation reduction potential (Samanta et al., 2018; Agarwal et al., 2016) and observation of seminal oxidative stress (Saleh et al., 2002), which are common in the varicocele literature. Therefore, parameters such as those should be included when investigating testicular hypoxia.

Release of ROS is not due exclusively to hypoxia, as described in other models (Haddad et al., 2001); perhaps, ROS are associated with the apoptotic pathway triggered by exposure to increased temperatures (Durairajanayagam et al., 2015), as both conditions are associated with cell death due to expression of P53 and its pathway, leading to apoptosis (Durairajanayagam et al., 2015; Liang et al., 2015).

Based on the above descriptions, the *P53*-dependent apoptotic pathway is a logical candidate, since it was described in other organs and systems as a result of heat stress (Hikin et al., 2003). Although a review paper suggested that this pathway was involved in testicular heat stress pathophysiology, no data were cited to support that statement (Durairajanayagam et al., 2015).

The *P53*-dependent apoptotic pathway has two main pathways, namely, intrinsic and extrinsic, with the *P53* gene being a central factor and the initiator of both response cascades. Also known as "guardian of the genome", the *P53* acts as a cell screener and therefore a determinant of cell fate, as described for various systems (Durairajanayagam et al., 2015). In the extrinsic pathway, *P53* binds to *FAS-FADD* complex, which then activates the initiator *CASPases 8* and *10* and at the last step, effector *CASPases 3, 6* and *7*. For the intrinsic pathway, *P53* alters the balance of the *BAX-BCL2* complex, downregulation of *BAX*, or upregulation of *BCL2* results in cell death, through release of cytochrome c, that binds to Apaf1, leading to recruitment and activation of initiator *CASP*ase 9 and the effector *CASP*ases 3, 6 and 7, similar to the sequence indicated for the external pathway. Interestingly, another fundamental component of the heat stress response is the chaperone system, *Hsp70* gene and protein, that can also upregulate the *P53* gene, leading to activation of either the intrinsic and/or extrinsic pathways.

There are examples in the literature of activation of the intrinsic pathway after heat stress exposure, e.g. murine liver cells (Pagliari et al., 2005) and surgically induced cryptorchidism in monkeys (Zhang et al, 2003). Furthermore, in men, downregulation *of BCL2* is associated with a reduction in germ cell count and infertility, however, the *BAX:BCL2* ratio at a normal level is correlated with overall sperm quality (motility, morphology and concentration) (Mostafa et al., 2014). When mice were exposed to 42 ºC for 15 min, there was increased cytochrome c release.

In addition, when minocycline (*CYTB* inhibitor) was administered, a reduction in sperm TUNEL+ cells and apoptosis level was observed (Matsuki et al., 2003), indicating potential involvement of the *P53*-dependent intrinsic apoptotic pathway in heat stress pathophysiology. Regarding the extrinsic pathway, the activity of *FAS/FASL* was responsible to mediate apoptosis in testes under oxidative stress conditions (Yang et al., 2015). Furthermore, it is clear that *FAS/FASL* is an upstream factor to *CASP8* (Ding et al., 2003), which is upregulated after exposure of rat testicular tissues to 43 ºC for 15 min (Hikin et al., 2003).

1.9 Does testicular hyperthermia cause hypoxia?

There is a longstanding paradigm that testes operate on the brink of hypoxia, with barely enough O_2 to fulfill physiological needs and that blood flow to the testes does not increase in response to increased testicular temperature. However, increased temperature increases metabolism and cellular demand for O_2. Therefore, according to this paradigm, hypoxia is the underlying cause of heat-induced changes in sperm morphology and function (Setchell et al., 1966). Despite being a well-accepted paradigm, remarkably, there are limited experimental data to support it.

Effects of hyperthermia on testicular function and fertility have been studied in laboratory and farm animals, whereas effects of hypoxia have been studied within contexts of disrupted blood flow and hypobaric hypoxia, as models of testicular torsion and living at high altitudes, respectively (Reyes et al., 2012; Bustos-Obregón et al., 2006). Male mice exposed to 36 ºC for two 12-h intervals (on successive days) had lower pregnancy rates and litter sizes when mated to females 10 or 14 d after heat exposure (Pérez-Crespo et al., 2008). In another study, mice

exposed to 36 ºC had an increase of ~1 ºC in body temperature and ~5 ºC in testicular temperature when measured 12 h after the onset of exposure (Zhu et al., 2004). In that study, blood flow to the testis remained practically unaltered during heat exposure, but O_2 consumption had a two-fold increase, supporting the hypoxic testis theory (Main and Waites, 1977).

Regarding O_2 concentrations, atmospheric air is approximately 21% O_2, whereas 10.8 and 16.0% O_2 in inspired air constitute hypoxia and mild hypoxia, respectively, in rats (Chen et al., 2007). Mice breathing air with 12.5, 15.0, 21.0 and 100% O_2 had testicular O_2 concentrations of 16, 24, 36, and 102 μmole/L (Baker and Lindop, 1970). Furthermore, breathing 100% O_2 doubled O_2 saturation in rat testes (Kram et al., 1989). Based on that, there is apparently an association between O_2 content of inspired air and O_2 content of testes, enabling testicular O_2 content to be varied from approximately 50 to >200% of physiologic concentrations. Ability to independently alter testicular temperature and testicular O_2 content provided a novel opportunity to critically test effects of hyperthermia and hypoxia on spermatogenesis, as discussed below.

1.10 Challenging the long-standing paradigm

Two recent collaborative studies were performed to study effects of hypoxia and hyperthermia on testes of mice (Kastelic et al., 2019) and rams (Kastelic et al., 2017). The first study used 48 mice in a 2x3 factorial design involving two temperatures (20 and 36 ºC) and three concentrations of O_2 in inspired air (13, 21 and 95%). Mice were exposed to two intervals of experimental conditions, each lasting 12 h, separated by a 12-h interval (at standard ambient conditions). At two time points (14 and 20 d after initial exposure), testes were recovered and sperm morphology, production and reserves assessed. Outcomes were mostly due to temperature,

with reductions in daily sperm production, testis weight and seminiferous tubule diameter ($P<0.06$, $P<0.03$ and $P<0.005$, respectively) in mice exposed to 36 ºC when compared to those exposed to 20 ºC.

Interestingly, sperm morphology and specific stages of sperm cell development were altered in mice exposed to 36 ºC, including increases in percentage of sperm with defective heads ($P<0.0001$) or tails ($P<0.001$) and percentage of altered elongated spermatids ($P<0.001$). Regarding effects due to O_2 variations, seminiferous tubule diameter and epididymal sperm reserves were reduced in the 13% O_2 group, but sperm quality and production were not consistently disrupted by hypoxia. In addition, no hyperthermia-induced disruptions were prevented by hyperoxia, indicating a major role of increased temperature, but not hypoxia.

The second experiment involved 18 rams, in a 2x3 factorial essentially replicating the mouse study, with testicular insulation (yes/no) and three O_2 concentrations (14, 21 and 85%) in inspired air (rams were placed in chambers for 30 h). Semen was collected twice weekly for 4 wk. then once weekly for 2 wk. Outcomes were similar to the mouse study, with primarily deleterious effects of increased testicular temperature on sperm motility and morphology and failures of hyperoxia to prevent these changes or hypoxia to replicate them (Kastelic et al., 2017). Similarly, in another collaborative study in our laboratory (Barros-Adwell et al., 2018), as ambient temperature increased from 5 to 35ºC, testicular blood flow increased (4.9 ± 0.7 versus 8.2 ± 0.9 ml/100 g testis/min, respectively; $P<0.05$).

Results summarized immediately above supported the hypothesis that the pathophysiology of heat stress is due to increased temperature itself, rather than hypoxia, as stated in the dogma that under heat stress, there is no increase in blood flow to the testes (Waites and Setchell, 1964), leading to an hypoxic environment in the testes, and as consequence,

impaired sperm quality and infertility. **Therefore, a critical question to be addressed in this thesis is as follows: Is the long-standing dogma that increased testicular temperature does not cause increased blood flow and the resulting hypoxia is responsible for decreases in sperm motility, morphology and fertility incorrect? In addition to refuting the role of blood flow and hypoxia in the pathogenesis of heat-induced changes in sperm, involvement of the *P53*-dependent intrinsic and extrinsic pathways on the pathophysiology of heat stress on testes and sperm quality were also characterized.** To address questions described above and to further characterize the heat stress pathophysiology, we developed various objectives and aims of this thesis work herein presented.

1.11 Aims, hypotheses and objectives

1.11.1 Overall aim, hypotheses and objectives of thesis

The overall aim of this thesis work was to investigate modulations in testicular blood flow in response to testicular heat stress and pathophysiology of heat stress on testes and testicular function. The hypothesis established for each chapter in association with objectives and key results obtained are described below:

1.11.2 Chapters 2 and 3

The aim of the first two chapters was to investigate the response of testicular blood flow to three O_2 concentrations (hyperoxia, normoxia and hypoxia) and three testicular temperatures

(34, 37 and 40 ºC), respectively. The hypotheses were that there is an increase in testicular blood flow, without signs of hypoxia, as O_2 decreased (Chapter 2) or as testicular temperature increased (Chapter 3), in the two independent studies.

Chapter 2 – Published in *Scientific Reports*:

Rizzoto G, Hall C, Tyberg J V, Thundathil JC, Caulkett NA, Kastelic JP. Increased testicular blood flow maintains O_2 delivery and avoids testicular hypoxia in response to reduced O_2 content in inspired air. Scientific Reports 2018;8:10905.

Chapter 3 – Published in *Reproduction, Fertility and Development*:

Rizzoto G, Hall C, Tyberg J V, Thundathil JC, Caulkett NA, Kastelic JP. Testicular hyperthermia increases blood flow that maintains aerobic metabolism in rams. Reproduction Fertility and Development 2019;31:683-8.

Key findings:

In the two studies, as testicular temperature increased or O_2 decreased, there was a significant increase in testicular blood flow and increases in metabolic rate and O_2 extraction, without changes in lactate production or pH.

1.12.3 Chapter 4

The aims of this study were to: 1) investigate the impact of mild heat stress on testicular blood flow in cattle; and 2) compare responses between *Bi* and *Bt* bulls. The hypothesis was that testicular blood flow increases in response to the elevation of testicular temperature and *Bi* bulls

have better ability to modulate testicular blood flow and O_2 delivery in response to heat stress than *Bt* bulls.

Chapter 4 – Published in *Theriognology*:

Rizzoto G, Ferreira JCP, Mogollón Garcia HD, Teixeira-Neto FJ, Bardella LC, Martins CL, Silva JRB, Thundathil JC, Kastelic JP. Short-term testicular warming under anesthesia causes similar increases in testicular blood flow in *Bos taurus* versus *Bos indicus* bulls, but no apparent hypoxia. Theriogenology 2020;145:94-9.

Key findings: An increase in testicular temperature elicited an increase in testicular blood flow that matched metabolic needs, without producing any signs of testicular hypoxia.

1.12.4 Chapter 5

The objective of the study was to characterize effects of short-term testicular hyperthermia (scrotal insulation) on testosterone concentrations and gene expression in *B. indicus* testes. The hypotheses were that: 1) testicular hyperthermia reduces testosterone concentrations; and 2) alters expression of candidate genes in testes.

Chapter 5 – Published in *Theriogenology*:

Rizzoto G, Ferreira JCP, Codognoto VM, Oliveira KC, Mogollón Garcia HD, Pupulim AGR, Teixeira-Neto FJ, Castilho A, Nunes SG, Thundathil JC, Kastelic JP. Testicular hyperthermia

reduces testosterone concentrations and alters gene expression in testes of Nelore bulls. Theriogenology 2020;152:64-8

Key findings: Testicular heat stress (due to scrotal insulation) profoundly reduced testicular testosterone concentrations and enabled us to elucidate some of the mechanisms involved in the pathway through which: 1) *Bi* bulls responded to heat stress, i.e. increased expression of antioxidant molecules (*GPX1*) and upregulation of chaperones (*HSP*70); and 2) how heat stress damaged testicular cells, including down regulation of *StAR* and BCL-2, fundamental for spermatogenesis and sperm quality and anti-apoptotic activity, respectively.

1.12.5 Chapter 6

The objectives were to investigate: 1) Impacts of mild acute heat stress on sperm and testes; and 2) Involvement of *StAR* and P53 genes and P53-dependent intrinsic and extrinsic pathways. We tested the hypotheses that mild acute heat stress: 1) upregulates chaperone (Hsp70) and antioxidant systems (*GPX1*); 2) causes early downregulation of *StAR*; and 3) activates *P53*-dependent apoptosis through both intrinsic (*BCL2*) and extrinsic (*CASP* 8) genes pathways.

Rizzoto G, Boe-Hansen G, Klein C, Thundathil JC, Kastelic JP. Acute mild heat stress strategically alters gene expression in testes and reduces sperm quality in mice. Under review at *Theriogenology*, 2020.

Key findings: Acute mild heat stress: 1) Profoundly impaired sperm morphology, motility and acrosome integrity and reduced seminiferous tubule diameter and testicular weight; 2) chaperone (*HSP*70) and antioxidant systems (*GPX1*) were activated soon after heat exposure; and 3) *P53*-dependent intrinsic (*BCL2*) and extrinsic (*CASP* 8) apoptotic pathways were activated.

Chapter 2: Increased testicular blood flow maintains oxygen delivery and avoids testicular hypoxia in response to reduced oxygen content in inspired air

2.1 Abstract

Despite a long-standing assertion that mammalian testes operate near hypoxia and increased testicular temperature causes frank hypoxia, we have preliminary evidence that changes are due to hyperthermia *per se*. The objective was to determine how variations in inspired O_2 concentration affected testicular blood flow, O_2 delivery and extraction, testicular temperature and lactate production. Eight rams were maintained under general anesthesia, with successive decreases in O_2 concentration in inspired air (100, 21 and 13%). As O_2 concentration decreased (100 to 13%), testicular blood flow increased (9.6 ± 1.7 vs 12.9 ± 1.9 ml/min/100 g of testis, $P<0.05$; mean ± SEM). Conductance (normalized flow) increased from 0.46 ± 0.07 to 1.28 ± 0.19 ml/min/mm Hg/100 g testis ($P<0.05$). Increased testicular blood flow maintained O_2 delivery and increased testicular temperature by ~1 ^{0}C; this increase was correlated to increased testicular blood flow ($r = 0.35$, $P<0.0001$). Furthermore, O_2 utilization increased concomitantly and there were no significant differences among O_2 concentrations in blood pH, HCO_3^- or base excess, and no effects of venous-arterial differences in lactate production. We concluded that, under acute hypoxic conditions, testes maintained O_2 delivery and uptake by increasing blood flow and O_2 extraction, with no indication of anaerobic metabolism.

2.2 Introduction

Maintenance of testicular temperature 3-4 ºC lower than the body core temperature is essential for production of morphologically normal and motile sperm in most mammals (Freeman, 1990; Kastelic et al., 1995). There is a long-standing paradigm that the testicular microenvironment functions on the brink of hypoxia (Kastelic et al., 2018) and that with increasing testicular temperature, there is increased testicular metabolism and increased O_2 demands, but no change in testicular blood flow. Thus, decreases in percentage of morphologically normal and motile sperm that follow testicular hyperthermia are usually attributed to secondary effects of hypoxia and not directly to hyperthermia (Bergh et al., 2001; Hamilton et al., 2016).

With ischemic conditions due to blood flow compromised by obstruction of testicular vessels (e.g., varicocele and testicular torsion) or with hypobaric hypoxia (e.g., reduced O_2 pressure at high altitudes) spermatogenesis and fertility are impaired, similar to changes after testicular warming (Damsgard et al., 2016; Ates et al., 2015; Reyes et al., 2012; Paul et al., 2009). Notwithstanding, these observations were not clear evidence that the pathogenesis of increased testicular temperature was due to hypoxia.

Markers of hypoxia have been detected after exposure to hyperthermia (Hamilton et al., 2016; Verrati et al., 2008a), supporting the assertion that effects of testicular hyperthermia are due to hypoxia. However, in those studies, neither testicular blood flow nor O_2 delivery/utilization were measured. Although hyperemia was reported when testes not covered by the scrotum were exposed to increased temperatures (Verrati et al., 2008b), this was not regarded as sufficient evidence to challenge the classical view that hypoxia mediates the damage

caused by testicular hyperthermia. In a previous preliminary study (Mieusset et al., 1992), conscious rams breathed inspired air containing 85, 21 or 14% O_2 for 30 h. The scrota were insulated in half of the rams (a well-established model to increase testicular temperature); in those rams, the percentages of morphologically normal sperm and motile sperm were significantly decreased from ~2 to 5 wk after exposure. Furthermore, in that study, hyperoxia did not mitigate effects of scrotal insulation and hypoxia did not cause subsequent decreases in morphologically normal or motile sperm. Therefore, based on that preliminary study, we inferred that hyperthermia *per se*, and not hypoxia, was the underlying cause of reductions in morphologically normal and motile sperm following testicular hyperthermia. However, neither testicular blood flow nor O_2 delivery were measured. Therefore, objectives of the current study were to determine how variations in O_2 concentrations in inspired air affect testicular blood flow, O_2 delivery and extraction, lactate production and testicular temperature.

2.3 Materials and methods

2.3.1 Animals and anesthetic procedure

Eight crossbred rams (12-15 mo, 40-56 kg) were used. Rams were pre-medicated with 8 μg/kg dexmedetomidine (Dexdomitor (0.5 mg/ml, Zoetis, Parsippany-Troy Hills, NJ, USA) and 2 mg/kg of alfaloxone (Alfaxan 10 mg/ml, Jurox Pty Ltd, Rutherford, NSW Australia) administered IM. After approximately 15 min, anesthesia was induced with 2 mg/kg of alfaloxone administered IV. Thereafter, rams were intubated, anesthesia was maintained by inhalation of isoflurane (1.0-2.0%; Fresenius Kabi Animal Health, Richmond Hill, ON, Canada)

and a constant volume ventilator (Harvard Apparatus – 12 breaths/min – 10 ml/kg stroke volume) was used throughout the study. To reduce the depth of anesthesia needed, epidural analgesia (0.07 mg/kg of xylazine (Rompum, 20 mg/kg, Bayer, Mississauga, ON, Canada) in ~4 mL of saline) and local anesthetic blocks (bupivacaine (Bupivacaine, 2.5 mg/ml, Hospira Inc., Lake Forest, IL, USA), ~2 ml/site SC) were performed at incision sites. All rams were maintained under general anesthesia throughout the procedure and euthanized (saturated potassium chloride given IV under deep anesthesia) at the end of the study. This study was reviewed and approved by the University of Calgary Health Sciences Animal Care Committee (AC16-0010) and all methods were conducted in accordance with the guidelines.

Each ram was exposed to three O_2 concentrations (100, 21 and 13% sequentially) in inspired air, by combining O_2 and nitrogen. Following reductions in oxygen concentration, rams were allowed ~20 min to adapt, and thereafter exposure was maintained for another 45 min, with measurements recorded at 30 min (temperature measured at 0, 15, 30 and 45 min). Oxygen concentrations were determined with an O_2 analyzer (MySign®O, Wilmar, MV, Germany).

2.3.3 Animal instrumentation pre-assessment

All invasive procedures were performed under anesthesia. Testicular temperatures were measured by inserting a needle thermocouple (20-gauge x 2.5 cm (21)), through the scrotal skin (anterior aspect of testis) and into the testis. This thermocouple was inserted at the beginning of the intervention and remained *in situ* throughout the study. Standard ECG leads were attached (for cardiac monitoring). The right carotid artery was isolated and a 14-gauge polyvinyl catheter was placed for monitoring arterial pressure and determining arterial blood gases. The right

jugular was isolated for intravenous administration of saline (5 ml/kg/h) and drug administration. An incision (~12 cm) was made between the right external inguinal ring and the attachment of the scrotum to the body wall. The spermatic cord was identified and the testicular artery and vein isolated. A 20-gauge catheter was inserted in the left testicular vein (distal to the testis) for blood gas and lactate measurement.

2.3.4 Blood sampling and blood flow measurement

Blood samples were collected from the carotid artery and testicular vein to measure blood gases and lactate (Nova Biomedical, Stat Profile® pHOx Ultra®, Waltham, MA, USA). An ultrasonic flow probe (2SB1551; Transonic® Flowprobe, Ithaca, NY, USA) was placed around testicular artery to measure blood flow. Measurements were performed and recorded for arterial and venous blood samples collected at 30 min after the start of the monitored interval. Blood flow was obtained with specific software (Sonometrics Corp. System, London, ON, Canada) and data were further converted using custom software (CV Works, AccuDAQ Inc, Calgary, AB, Canada). Testicular perfusion and oxygenation were calculated as described (Table 1). In addition, for testicular vasculature, arterial-venous differences were calculated for O_2 content, $P_{O2,}$ $P_{CO2,}$ pH, HCO_{3-} or base excess and venous-arterial differences were calculated for lactate.

2.3.5 Statistical analyses

One-way analysis of variance for repeated measures, followed by a Dunnet's *t*-test, was used to compare, among the three groups, data recorded at 30 min. Pearson's correlation analysis

was used to determine linear correlations. All statistical analyses were performed with GraphPad Prism Version 6.0 (GraphPad Software Inc, La Jolla, CA, USA) and $P<0.05$ was considered significant.

Table 2.1. Formulas for testicular perfusion and oxygenation.

Testicular O_2 delivery (ml/min)	$TDO_2 = Q(t) * CaO_2/100$
Testicular metabolic rate (ml/min)	$TVO_2 = Q(t)*(CaO_2 - CvO_2)/100$
Testicular O_2 extraction (%)	O_2 extraction = TVO_2/ TDO_2
Testicular conductance ($mL/min^{-1}/g^{-1}/mmHq^{-1}$)	Testicular conductance = (Q(t)/testicular weight)/aortic blood pressure

*Q(t) = testicular blood flow (ml/min); CaO_2 = arterial O_2 content (ml/dl); CvO_2 = venous O_2 content (ml/dl). (Caulkett et al., 1996; Semeniuk et al., 1998).

2.4 Results

As O_2 content in inspired air decreased, there were significant increases in testicular blood flow (Fig. 2.1A) and conductance (Fig 2.1B), accompanied by concurrent increases in intra-testicular temperature (Fig. 2.1C), although body temperature remained constant. Furthermore, testicular blood flow and intra-testicular temperature were correlated ($r = 0.35$, $P<0.0001$; Fig 2.1D).

Testicular O_2 delivery was maintained throughout the entire experiment; therefore, O_2 delivery to the testes was not affected by reductions in O_2 concentration in inspired air (Fig. 2.2A). Furthermore, as O_2 concentrations decreased, there were increases in testicular metabolic rate (Fig. 2.2B) and O_2 extraction (Fig. 2.2C).

Figure 2.1 A) Testicular blood flow and B) testicular conductance (mean ± SEM) in eight rams exposed to three concentrations of oxygen in inspired air. C) Testicular and body temperatures over time (mean ± SEM). D) Correlations between testicular temperature and testicular blood flow (r = 0.35, P<0.0001). *P≤0.05; **P≤0.01.

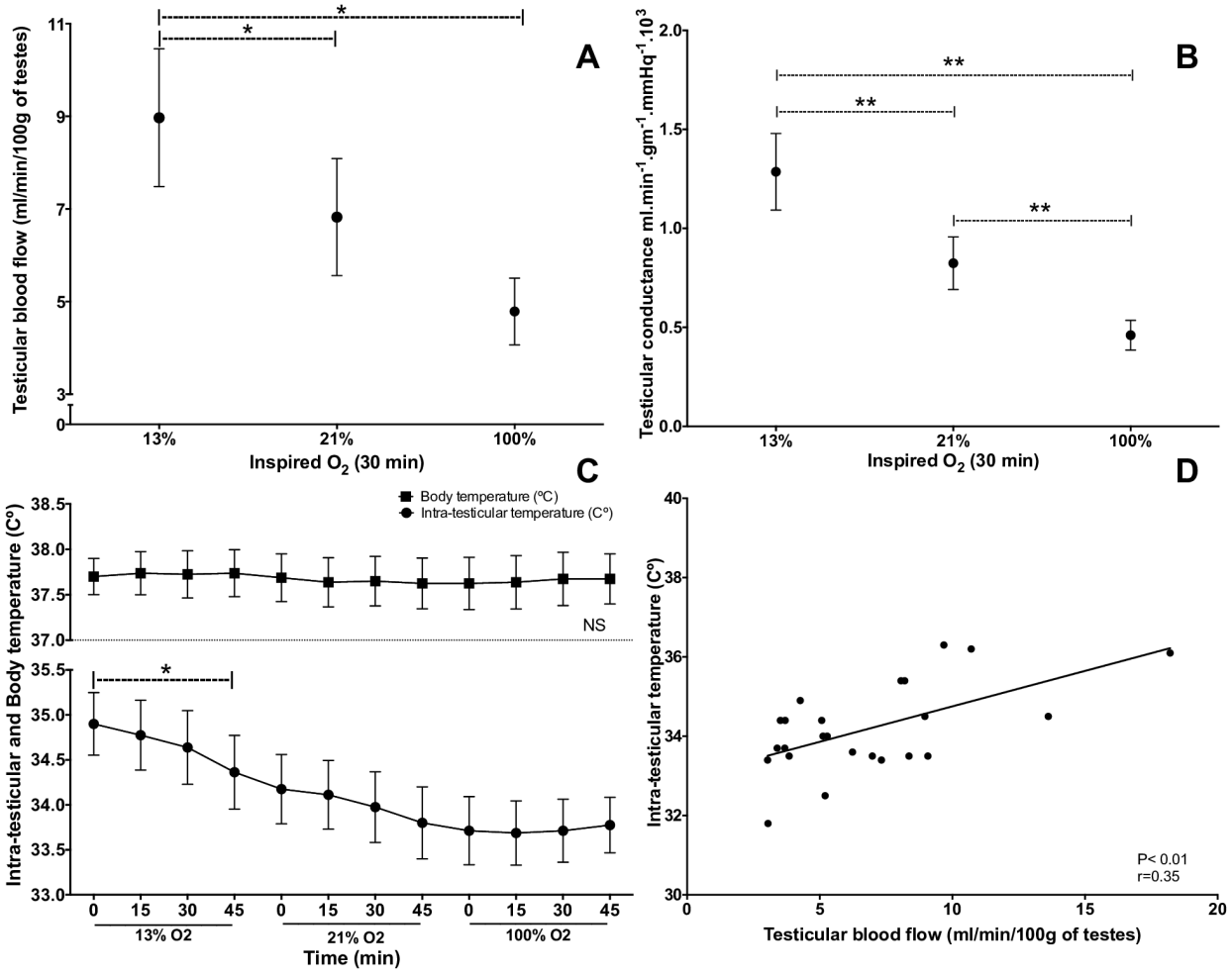


Figure 2.2 A) Testicular oxygen delivery, B) testicular metabolic rate and C) testicular oxygen extraction (mean ± SEM) in eight rams exposed to three concentrations of oxygen in inspired air. One-way analysis of variance for repeated measures, followed by a Dunnet's *t*-test, was used to compare, among groups, data recorded at 30 min. *P≤0.05; **P≤0.01.

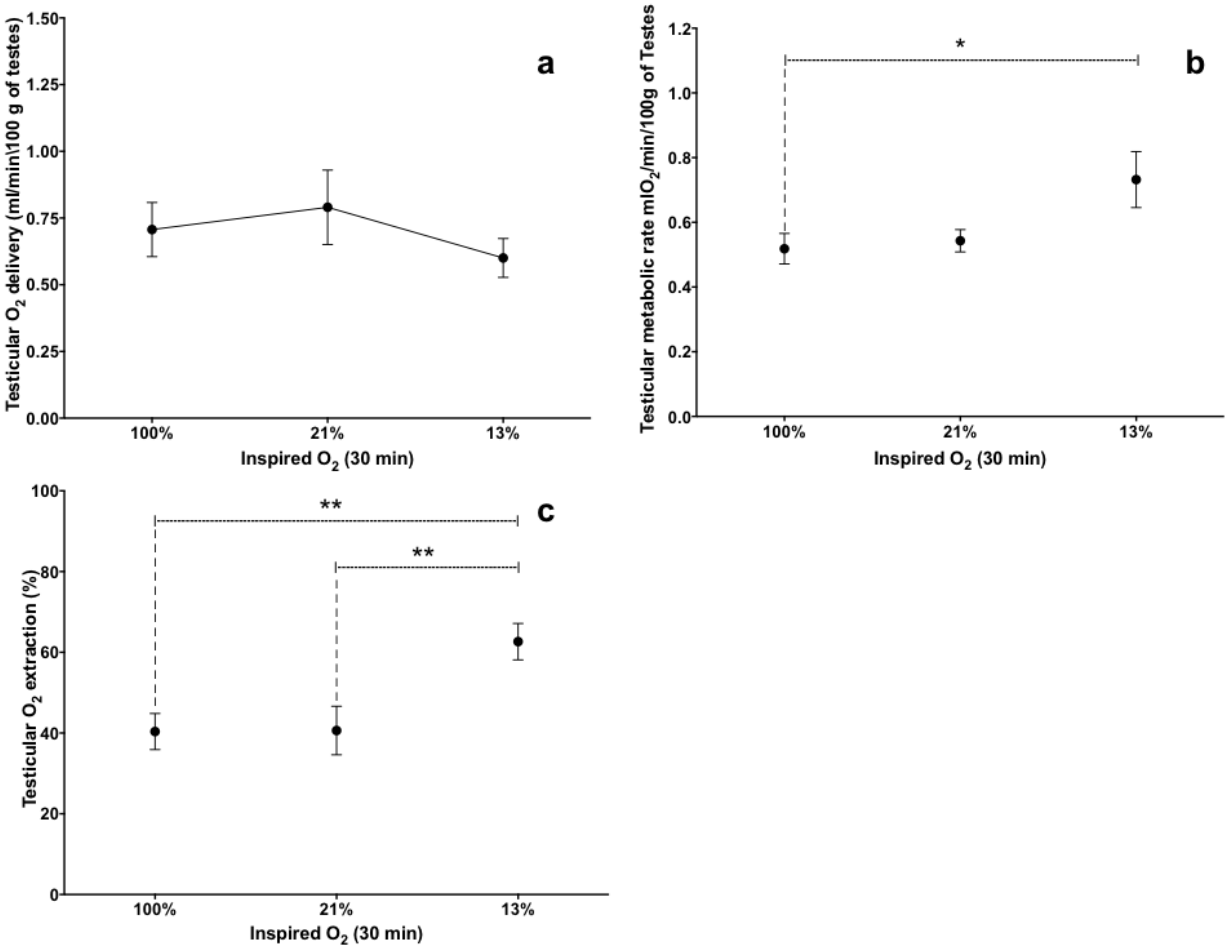
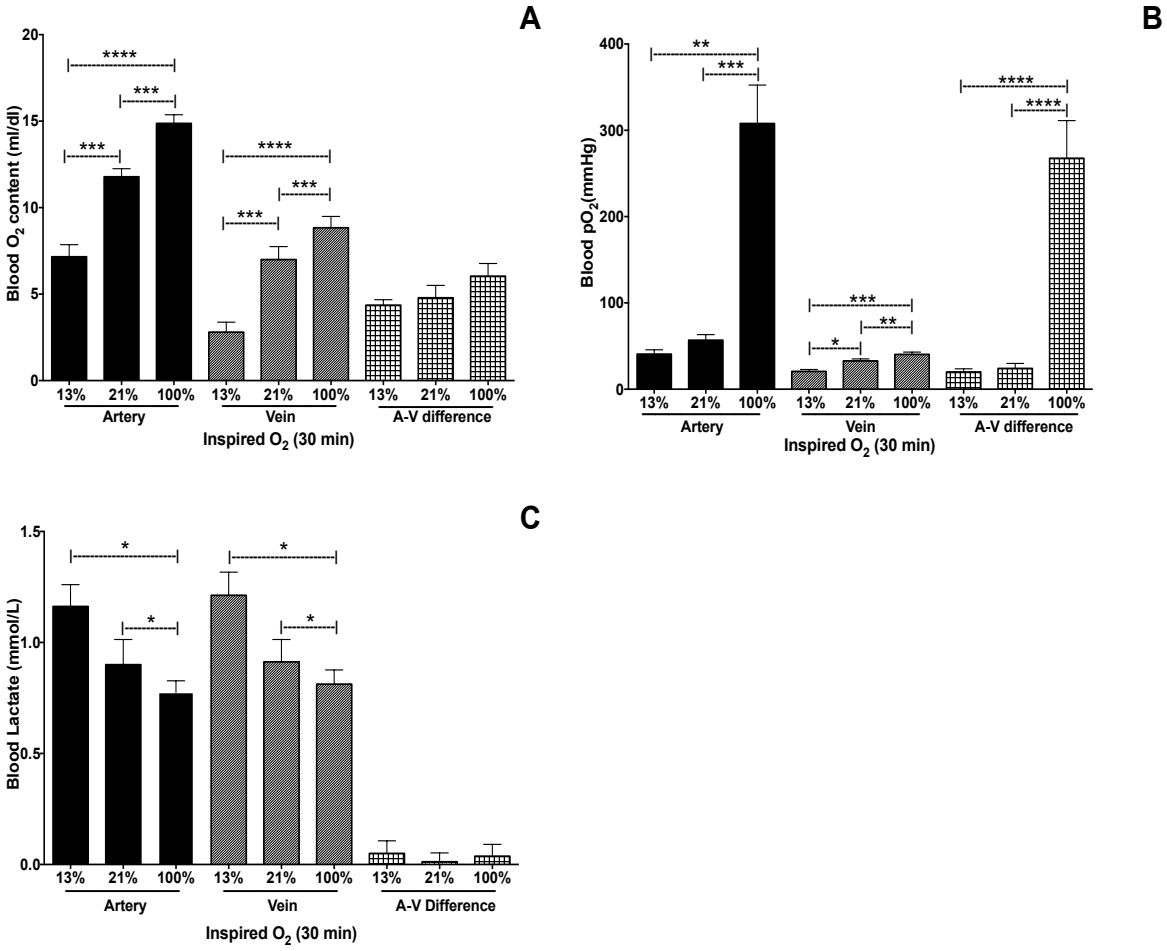


Figure 2.3 Testicular arterial, venous, and A-V difference in A) venous blood oxygen content, B) PO2; and C) lactate concentrations and net production (mean ± SEM) in eight rams exposed to three concentrations of oxygen in inspired air. One-way analysis of variance for repeated measures, followed by a Dunnet's *t*-test, was used to compare, among the three groups, data recorded at 30 min.*P≤0.05; **P≤0.01; *** P≤0.001; ****P≤0.0001.



Consistent with our experimental design, there were large and significant differences among groups for testicular arterial and venous O_2 content (Fig. 2.3A) and arterial and venous P_{O2} (Fig. 2.3B). There were no significant differences among groups for P_{CO2} in either the testicular artery or vein.

Lactate concentrations in the testicular artery and the testicular vein were higher ($P<0.05$) at 13% O_2 versus at 21 or 100%, but there was no significant difference among groups for V-A lactate concentrations (Fig. 2.3C). Furthermore, there were no significant differences among groups for arterial or venous pH, HCO_{3-} or base excess, and no significant A-V differences for pH, HCO_{3-} or base excess.

2.5 Discussion

Although regulation of blood flow in response to varying O_2 concentrations in inspired air has been extensively studied in several organs (Kastelic et al., 2017; Hoiland et al., 2016; Pitmann, 2011; Segal and Kurijaka, 1995), this was apparently the first study to characterize effects of varying inspired-air O_2 concentrations on testicular blood flow, O_2 delivery, O_2 uptake, and temperature in rams. In this study, as O_2 concentrations in inspired air decreased from 100 to 13%, blood flow increased concurrently and sustained O_2 delivery to the testis. This increased blood flow increased testicular temperature by ~1^0 C and concomitantly increased metabolism and O_2 utilization. There was a small (albeit significant) increase in venous lactate concentrations but no significant increase in the V-A difference. Therefore, under the acute conditions of this study, we concluded that there were no indications of a shift to anaerobic metabolism over a broad range of inspired-air O_2 concentrations.

In the present study, hypoxia (i.e., 13% O_2) significantly increased the supply of blood to the testes, either measured as blood flow or as conductance (flow normalized by arterial pressure). Although testicular hypoxia is considered to cause male infertility (Steinback and Poulin, 2016; Gat et al., 2010; Okumura et al., 2003), repeated complete interruptions of blood flow to the testes (occlusion of testicular artery for 1-h intervals) did not cause long-term impairment of sperm production in rams (Wang et al., 2017). Regardless, chronic exposure to severe intermittent hypoxia in male rats (5% O_2 in inspired air) reduced sperm motility and fertility (Van Vliet et al., 1987). Perhaps the degree and duration of testicular hypoxia affects the ability to compensate.

Testicular blood flow and testicular temperature were significantly correlated ($r = 0.35$) in the current study. Clearly, hypoxia-induced increases in blood flow increased testicular temperature and metabolism. Furthermore, rectal temperature remained constant, suggesting that increased blood flow to the testis was not a systemic response, consistent with a previous report (Torres et al., 2014). Acute or chronic hypoxia decreased O_2 content in rat muscle, although blood flow was not altered (Barros-Adwell et al., 2018). In the human brain, hypoxia (14% for 18 min) increased O_2 metabolic rate and blood flow (compared to 21% O_2 (Maloney and Mitchell, 1996)) consistent with the present study. In some organs, a reduced O_2 supply increases blood flow (Xu et al., 2012; Marshall et al., 1999) due to release of vasodilators (decreased O_2 impairs re-phosphorylation of adenosine diphosphate (ADP), which is subsequently degraded to adenosine, and that compound causes vasodilation (Pitmann et al., 2011).

Another consequence of induced hypoxia was increased O_2 extraction from arterial blood, from ~40% at 21% O_2 to ~60% at 13% O_2 ($P<0.01$), thereby reducing O_2 content and P_{O2} in the testicular vein, although P_{CO2} remained unchanged. Observed effects were similar to those

reported by Pittman (2011) and Hoffman (1992), indicating that under hypoxic conditions, the first physiological reflex was to open capillaries to increase available area for O_2 exchange, accounting for the observed increase in O_2 extraction. Interestingly, prolonged exposure to reduced O_2 concentrations (e.g., at high altitudes) may result in adaptation with no increase in extraction after long-term exposure, suggesting a more important role for increased extraction under acute exposures (Zerbini et al., 2013).

Both hyperoxia and hypoxia can cause oxidative damage and lipid peroxidation (11 Verrati et al., 2008A). Chronic exposure to hyperoxia reduced P_{O2}, pH and increased P_{CO2} in old versus young rats, indicating that age affects tolerance to this condition (Andrade et al., 2014; Aljerian and Haffor, 2015). Furthermore, hyperoxia increased the activity of radical O_2 species and might impair diaphragm contractility, thereby potentially affecting body oxygenation (Torres et al., 2014). Exposure to ~100% O_2 in inspired air decreased blood flow to the rat brain (Xu et al., 2012; Kuwahira et al., 1993), reduced (~8-30%) cardiac blood flow in humans (Farquhar et al., 2009), and caused a 7% reduction in renal blood flow (Jain et al., 2017), all of which were consistent with our findings (lowest testicular blood flow at 100% O_2).

Lactate is a well-defined marker of hypoxia (Lee et al., 2015; Adeva-Andany et al., 2014). In bulls, lactate concentrations in the rete testis were similar to those in the blood (Voglmayr et al., 1970), although for humans they can be slightly different (Lee et al., 2015). Lactate is produced in testes as a product of glucose metabolism by Sertoli cells (Bodansky, 1975); increased concentrations were reported in pathological conditions in rats, including cryptorchidism (Harkonen and Kornano, 1971) and hypoxia (Shevantaeva et al., 2006). Furthermore, a severe reduction in lactate was also associated with infertility (Alves et al., 2016). In the present study, lactate concentrations in the testicular vein were increased ($P<0.05$) when

rams were exposed to 13% O_2. However, V-A differences were not significant among groups, nor were there significant A-V differences in pH, $HCO3^-$ or base excess. Therefore, there was no evidence of reduced O_2 concentrations in inspired air causing anaerobic metabolism.

In the present study, only blood samples were collected and analyzed. Although retrieval of testicular biopsies was considered, it was expected that this would cause hemorrhage and inflammation that could invalidate the study. Furthermore, we did not sample tissues to detect angiogenesis, as these changes were not detected at 1 d after the onset of hypoxia in mice (Madrid et al., 2005), although they were detected at 5 d after the onset of hypoxia in rats (Farías et al., 2005). There are alternative approaches to detect a change from aerobic to anaerobic metabolism. Important examples are genes that contain the Hypoxia-Response Element (HRE) in their promoter region; hypoxia is associated with increased gene expression of those specific genes, including Hypoxia Induced Factor (HIF) I and II (Loboda et al., 2010; Lin et al., 2010). Furthermore, identification of thiobarbituric acid reactive substances (TBARS) and Reactive Oxygen Species (ROS) are important markers of tissue hypoxia and oxidative damage in the testes (Cvetkovi et al., 2012; Zepeda et al., 2012). Unfortunately, in the present work, due to the experimental design and the potential for damage, testicular biopsies were not collected. Therefore, in future studies, recovery of testicular tissue and assessment of cellular and molecular evidence of tissue hypoxia should be done. In addition, more prolonged exposure to hypoxia in rams to determine long-term effects on testicular tissue, sperm and blood testosterone concentrations, are indicated.

Our experiment was designed to minimize the impact of anesthesia. Each ram was initially subjected to 100% O_2, followed by two successive reductions in O_2 content in inspired air; therefore, each ram served as its own control. In addition, anesthetic depth was maintained at

as constant a plane as possible throughout the experiment. Furthermore, adult animals were used, since the testicular vascular cone (TVC) undergoes development until puberty (Brito et al., 2012; Cook et al., 2004) and is fundamental for thermoregulatory capabilities of the testes (Kastelic et al., 2018). To our knowledge, there are no studies in pre-pubertal animals regarding testicular blood flow response to heat stress.

2.6 Conclusion

In conclusion, under acute hypoxic conditions (13% O_2 in inspired air), the testis was able to maintain O_2 delivery and uptake by increasing blood flow and O_2 extraction without indication of a shift to anaerobic metabolism. Similarly, in our previous study in conscious rams, exposure of control rams (no scrotal insulation) to 85, 21 and 14% O_2 for 30 h had no significant effect on semen quality (Kastelic et al., 2017). Therefore, the testis was able to compensate for decreased O_2 concentrations in inspired air.

2.7 Acknowledgments

We appreciate the efforts of the animal care staff for maintaining and transporting the rams.

Chapter 3: Testicular hyperthermia increases blood flow that maintains aerobic metabolism in rams

3.1 Abstract

There is a paradigm that testicular hyperthermia fails to increase testicular blood flow and that an ensuing hypoxia impairs spermatogenesis. However, in our previous studies, decreases in normal and motile sperm after testicular warming were neither prevented by concurrent hyperoxia nor replicated by hypoxia. The objective of the current study was to determine effects of increasing testicular temperature on testicular blood flow, O_2 delivery and uptake, and to detect evidence of hypoxia. Under general anesthesia, testicular temperatures of nine crossbred rams were sequentially maintained at approximately 33, 37 and 40 ºC (± 0.5 ºC; 45 min/temperature). As testicular temperature increased from 33 to 40 ºC, there were increases in testicular blood flow (13.2 ± 2.7 vs 17.7 ± 3.2 ml/min/100 g of testes; mean ± SEM; $P<0.05$), O_2 extraction (31.2 ± 5.0 vs 47.3 ± 3.1%; $P<0.0001$) and O_2 consumption (0.35 ± 0.04 vs 0.64 ± 0.06 mL/min/100 g of testes; $P<0.0001$). There was no evidence of anaerobic metabolism, based on a lack of change in lactate, pH, HCO_3-, and base excess. In conclusion, these data challenged the paradigm regarding scrotal/testicular thermoregulation, as acute testicular hyperthermia increased blood flow and tended to increase O_2 delivery and uptake, with no indication of hypoxia.

3.2 Introduction

In most mammals, testes must remain ~3-4 ºC cooler than body temperature for production of morphologically normal and motile sperm (Kastelic et al., 1995; Freeman et al., 1990). Increased testicular temperature reduces percentages of morphologically normal and motile sperm and their fertility (Kastelic et al., 2017; Brito et al., 2012). The paradigm regarding scrotal/testicular thermoregulation is that testes are almost hypoxic; increases in testicular temperature increase cellular metabolism but blood flow fails to increase and therefore testes become hypoxic (Waites and Setchell, 1964). This assertion has apparently never been critically tested.

In a preliminary study (Kastelic et al., 2017), a classic scrotal insulation model was used to increase testicular temperature in rams. In this model, percentages of morphologically normal and fertile sperm decreased as expected. However, it was noteworthy that these changes were neither prevented by hyperoxia nor were they replicated by hypoxia. Furthermore, in a study in bulls (Barros-Adwell et al., 2018), increasing ambient temperature from 5 to 35 ºC resulted in increases in both testicular temperature (31.8 vs 34.9 ºC; $P<0.001$) and blood flow (2.45 vs 4.22 ml/100 g testis/min; $P<0.05$). Thus, these previous studies suggested that testicular hyperthermia might not cause testicular hypoxia.

In the current study, our objective was to determine effects of increasing testicular temperature on testicular blood flow, O_2 delivery (TDO2), metabolic rate (TVO2) and O_2 extraction and to detect evidence of hypoxia.

3.3 Materials and methods

3.3.1 Animals and modulation of testicular temperature

This project was reviewed and approved by the University of Calgary Health Sciences Animal Care Committee (AC16-0010). Nine crossbred rams (~1 y of age and 49.4 ± 5.4; mean ± SD) kg) were used.

Each ram was exposed to three testicular temperatures (33, 37 and 40 ºC, in that order). To modulate temperature, ice packs or heating pads were placed next to the scrotum, an infrared heat lamp was placed 30 cm away, or the scrotum was placed in a custom-made, double-walled glass container that was heated by circulating warm water from a water-bath. Once the target testicular temperature had been reached, it was maintained for 45 min and then temperature was increased. It took ~20 min to reach each temperature; once the target was achieved, temperature was carefully monitored and maintained.

3.3.2 Anesthetic protocol

Dexmedetomidine (8 μg/kg; Dexdomitor; Zoetis, Parsippany-Troy Hills, NJ, USA) and alfaloxone (2 mg/kg; Alfaxan; Jurox Pty Ltd, Rutherford, NSW Australia) were administered IM for premedication. Approximately 15 min later, anesthesia was induced with IV alfaloxone (2 mg/kg) and maintained with 1-2% isofluorane (Fresenius Kabi Animal Health, Richmond Hill, ON, Canada) and constant-rate IV infusion of dexmedetomidine (2 ug/kg/h). Rams were mechanically ventilated (Harvard Apparatus; 12 breaths/min; stroke volume, 10 ml/kg) and were placed on a pad heated with warm water to maintain body temperature. The FiO_2 was adjusted (26.14 ± 1.1%) to maintain a physiological PaO_2 (~90 mmHg) throughout the study. To

minimize depth of anesthesia, a xylazine epidural was administered (0.07 mg/kg; Rompun, Bayer, Mississauga, ON, Canada) and local anesthetic blocks were used (bupivacaine; Bupivacaine, 2.5 mg/ml, Hospira Inc, Lake Forest, IL, USA; ~ 2 ml/site SC) at blood-vessel access sites. At the end of the study, rams were euthanized by IV injection of saturated potassium chloride (~15 ml), administered under deep anesthesia. Tissues were made available for secondary use in other research.

3.3.3 Instrumentation pre-assessment and sampling methodology

Testicular temperatures were determined with a needle thermocouple (20-gauge x 1 inch; Kastelic et al. 2017), inserted via the anterior aspect of the scrotum (through the scrotal skin and into the testis) and kept *in situ* throughout the study. Body temperature was monitored with a hand-held monitor and probe (8402-00; Cole-Palmer® Thermistor thermometer, Vernon Hills, IL, USA) inserted into the rectum. For cardiac monitoring, standard ECG leads were used.

Vascular access for blood pressure monitoring and collection of arterial samples was obtained by isolation and catheterization (14-gauge polyvinyl catheter) of the right carotid artery. Furthermore, the right jugular vein was similarly isolated and catheterized to provide a means to administer saline (maintenance dose, 5 ml/kg/h) and dexmedetomidine.

An incision was made approximately half-way between the external inguinal ring and the top of the testis on the right side. The testicular artery and vein were identified and isolated. The right testicular artery was used for ultrasonic measurement of blood flow (2SB1551; Transonic® Flowprobe, Ithaca, NY, USA). Blood flow data were captured (Sonometrics Corp. System, London, ON, Canada) and converted (CV Works, AccuDAQ Inc, Calgary, AB, Canada). Blood

samples were collected from the right testicular vein to determine carotid artery and blood gases, blood pH and lactate concentrations (Nova Biomedical, Stat Profile® pHOx Ultra®, Waltham, MA, USA).

At 30 min after each targeted testicular temperature (33, 37 and 40 ºC) was achieved, blood flow data and body temperature were recorded and arterial and venous blood samples collected. Formulas to calculate testicular perfusion and oxygenation are shown in Table 3.1. In addition, testicular venous-arterial differences in P_{O2}, pH, P_{CO2} and HCO_{3-} and lactate were calculated.

Table 3.1 Formulas for testicular perfusion and oxygenation

Testicular O_2 delivery (ml/min)	$TDO_2 = Q(t) * CaO_2/100$
Testicular metabolic rate (ml/min)	$TVO_2 = Q(t)*(CaO_2 - CvO_2)/100$
Testicular O_2 extraction (%)	O_2 extraction = TVO_2/ TDO_2
Testicular conductance ($mL/min^{-1}/g^{-1}/mmHq^{-1}$)	Testicular conductance = (Q(t)/testicular weight)/aortic blood pressure

*Q(t) = testicular blood flow (ml/min); CaO_2 = arterial O_2 content (ml/dl);

CvO2 = venous O2 content (ml/dl). (Semeniuk et al., 1998; Caulkett et al., 1996).

3.3.4 Statistical analyses

One-way analysis of variance for repeated measures, followed by a Dunnet's *t*-test, were performed on 30-min data for the following end points: testicular arterial blood flow, testicular

conductance, testicular and body temperatures, TDO_2, TVO_2, O_2 extraction, testicular arterial and venous O_2 content, P_{O2} and P_{CO2}, lactate, pH, HCO_{3-}, base excess and hemoglobin. All statistical analyses were performed with GraphPad Prism Version 6.0 (GraphPad Software Inc, La Jolla, CA, USA) and $P<0.05$ was considered significant.

3.4 Results

Testicular blood flow ($P<0.05$) and conductance ($P<0.01$) increased when comparing 33 and 37 to 40 ºC (13.2 ± 2.7, 14.8 ± 2.8 and 17.7 ± 3.2 ml/min; and 1.1 ± 0.2, 1.2 ± 0.2 and 1.6 ± 0.3, respectively); both increased in association with increasing testicular temperature, peaking at 40 ºC (Fig. 3.1A and B). Effects of heating were restricted to the scrotum and testes, with no significant change in body temperature throughout the experiment (Fig. 3.1C). TDO_2 did not significantly change (1.29 ± 0.19; 1.40 ± 0.17; 1.55 ± 0.17 ml/min/100 g of testis, respectively; Fig. 2a). Oxygen saturation was not significantly different during the experiment (Fig. 3.2B), but total hemoglobin was reduced ($P<0.05$) at 40 versus 37 ºC (9.15 ± 0.46 vs 9.81 ± 0.46 g/dL; Fig. 2c). Arterial O_2 content was not significantly different among the three target temperatures.

However, venous O_2 content and the venous-arterial difference differed ($P<0.05$ and $P<0.01$, respectively) among temperatures (Fig. 3.3A). For P_{O2}, there was no arterial-venous difference (Fig. 3.3B), but venous P_{O2}, was lower ($P<0.05$) at 40º vs 33 ºC (36.63 ± 1.00; 44.33 ± 1.55, respectively). The TVO_2 differed ($P<0.01$-<0.0001) among all temperatures (0.35 ± 0.04; 0.55 ± 0.07; 0.69 ± 0.05, respectively; Fig. 3.3C) and O_2 extraction was higher ($P<0.0001$) at 40 versus 33 ºC (31.2 ± 4.7 and 47.3 ± 3.6, respectively; Fig. 3.3C). There were no significant

differences among temperatures for lactate concentration, pH, HCO_3- or base excess (0.72 ± 0.02, 7.42 ± 0.01, 28.40 ± 0.47 and 4.06 ± 0.40, respectively).

Figure 3.1 (A) Testicular blood flow, (B) testicular conductance and (C) testicular and body temperatures in 9 rams exposed to increasing testicular temperatures. Mean ± SEM; *P≤0.05; **P≤0.01

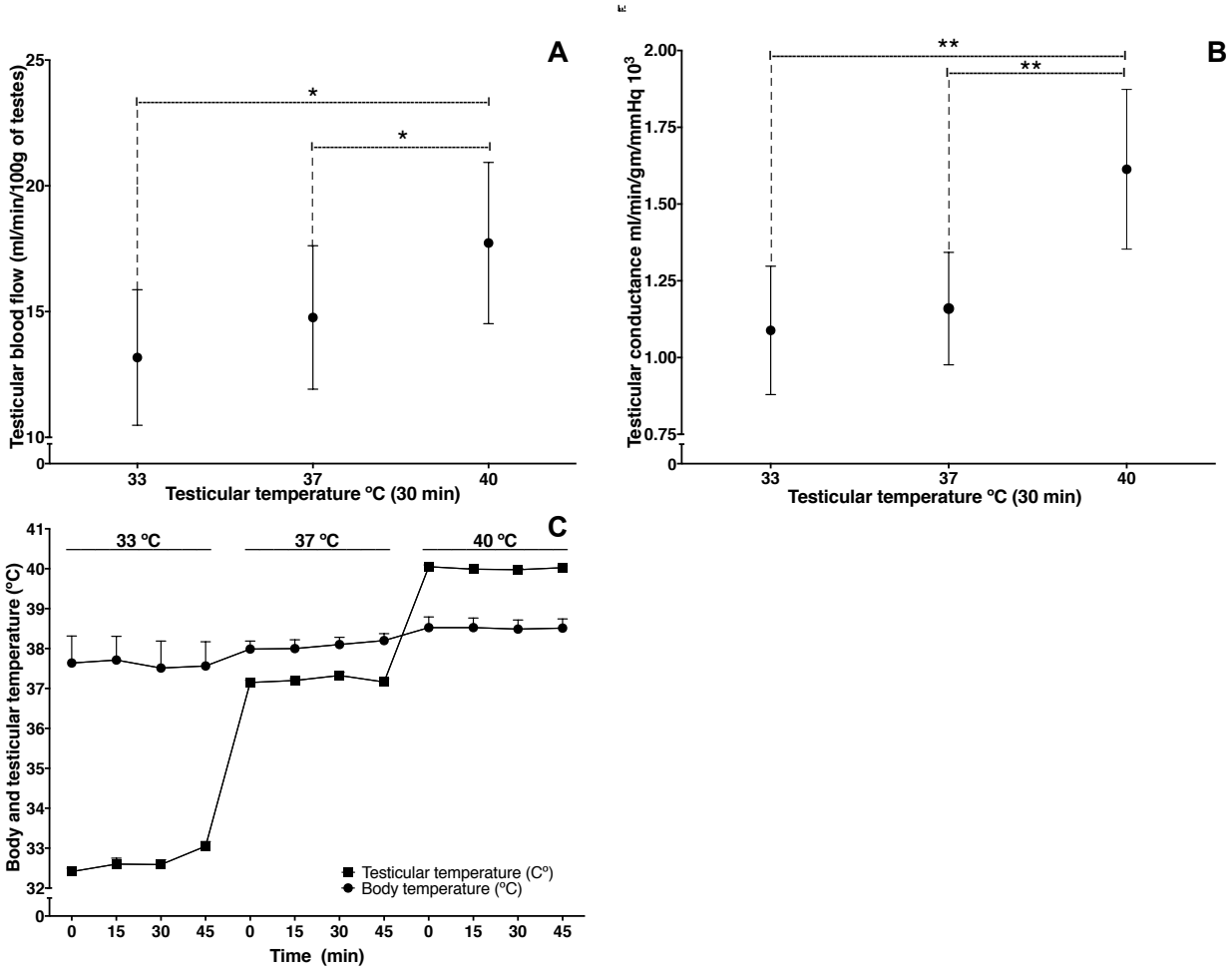


Figure 3.2 (A) Testicular O_2 delivery, (B) arterial O_2 saturation and (C) total hemoglobin in nine rams exposed to increasing testicular temperatures. Mean + SEM; *P≤0.05

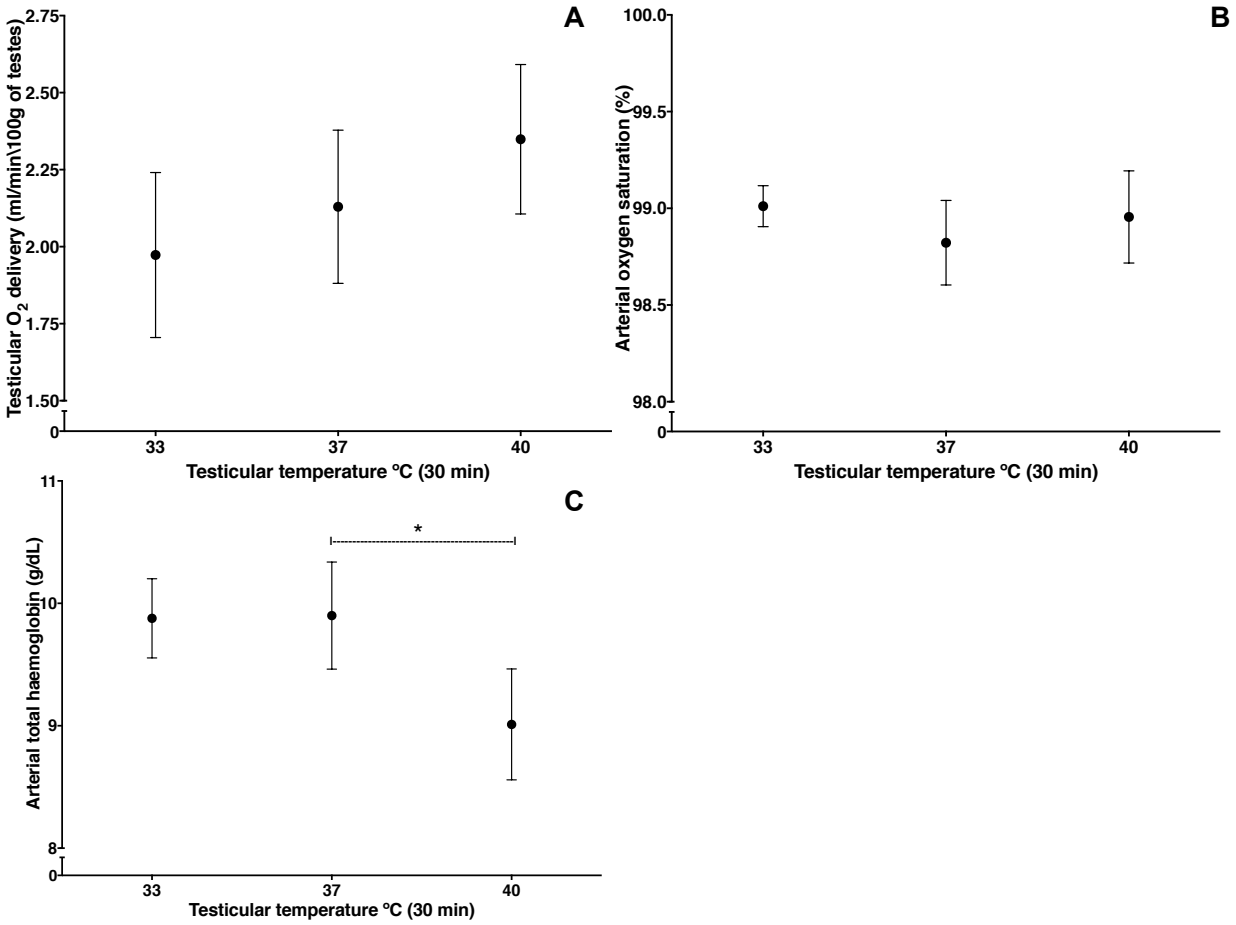
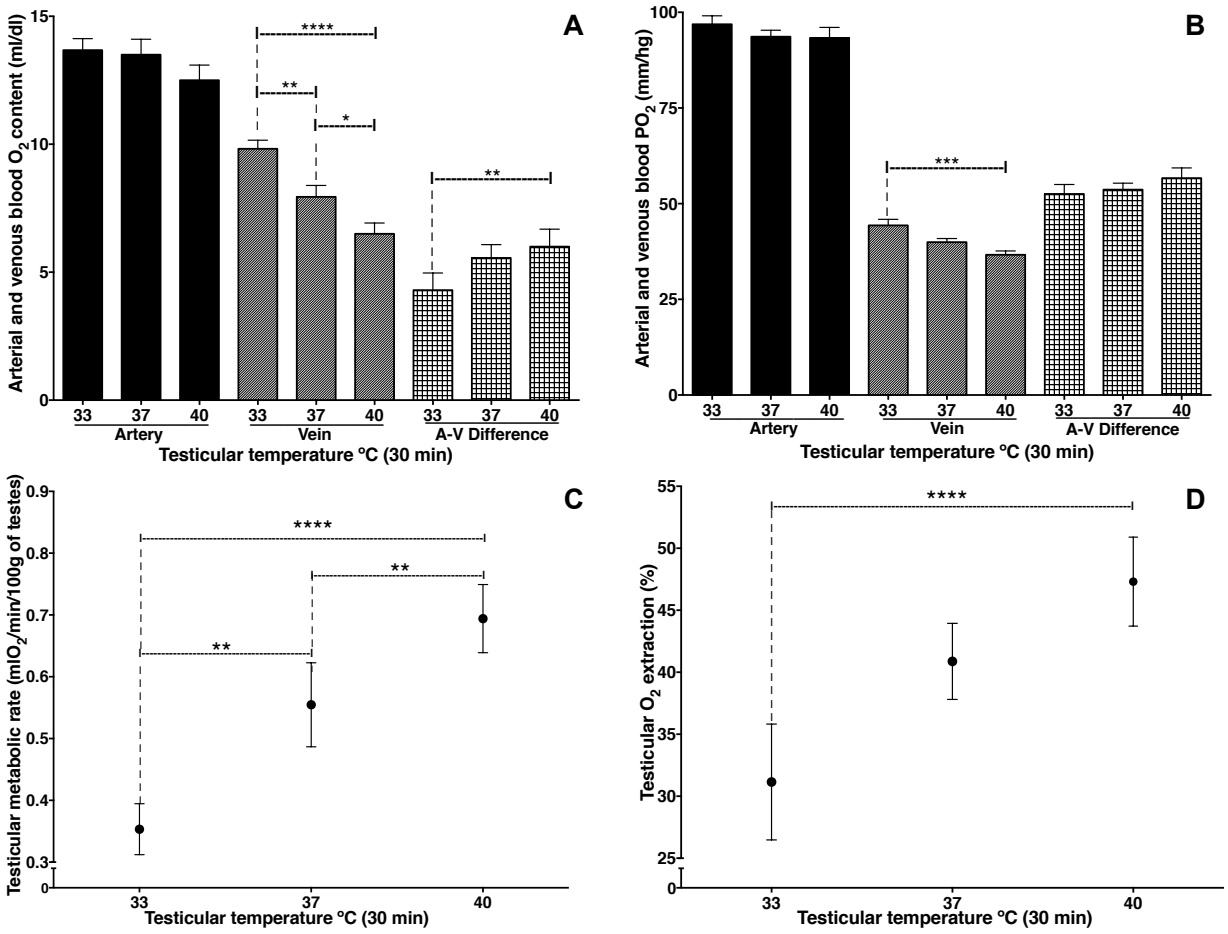


Figure 3.3 Testicular arterial, venous, and A-V difference for (A) blood O2 content, (B) PO_2, (C) O_2 metabolic rate and (D) O_2 extraction in nine rams exposed to increasing testicular temperatures. Mean ± SEM; *P≤0.05; **, P≤0.01; ***, P≤0.001; ****, P≤0.0001.



3.5 Discussion

This is apparently the first report that heating the testes increased testicular blood flow in rams with an intact scrotum. In a classic study (Waites and Setchell, 1964), heating ram testes to

39 ºC for 2.0 or 2.5 h did not alter testicular blood flow. That study involved only four treated and two control rams, whereas the current study used nine rams, each serving as its own control. In another study in rams (Mieusset et al., 1992), one testis was retained in the scrotum and maintained at 33 ºC, whereas the other was removed from the scrotum and testicular temperature sequentially increased from 33 (control) to 36 and ultimately to 39 ºC (for 60, 60 and 30 min, respectively), with an average 78% greater blood plasma flow to the testis at 39 ºC compared to the control. However, as this testis was outside the scrotum, it was concluded that these findings did not disprove the classic assertion that blood flow does not increase when testes are warmed. Furthermore, in another study (Barros-Adwell et al., 2018) in bulls at ambient temperatures of 5, 15 and 35 ºC, at the highest versus lowest temperatures, there were increases in testicular temperature (34.9 vs 31.8 ºC, $P<0.001$) and blood flow (4.22 vs 2.45 ml/100 g testis/min, $P<0.05$). It is noteworthy that the present data directly challenged the long-standing assertion that testicular hyperthermia does not increase testicular blood flow in ruminants.

Perhaps an absence of increased blood flow in response to increased testicular temperatures in previous reports was due to a different method of measurement. Previous studies used tritiated water (Waites and Setchel, 1964) and *p*-aminohippurate infusion (Mieusset et al., 1992). In the present study, we used an ultrasonic flow probe, similar to the equipment used in Barros-Adwell et al., (2018).

Testicular blood flow at 33 ºC was consistent with a previous report of testicular blood flow in rams (9.6 ml/100 g testis/min; Waites and Setchell, 1964). The importance of using conductance (i.e., flow at a given pressure gradient) was demonstrated by the greater significance compared to blood flow at 37 and 40 ºC ($P<0.01$ and $P<0.05$, respectively). Furthermore, body temperature was not significantly affected by modulation of testicular temperature, indicating

that increases in testicular temperature were responsible for the local increase in blood flow, similar to a previous report (Maloney and Mitchell, 1996).

In the present study, testicular metabolic rate nearly doubled (96% increase) between the lowest and highest temperatures. Concurrently, there was greater blood flow (25%) and conductance (48%), as reported in other organs (Gonzáles-Alonso et al., 2015; Carlsson et al., 1976). Although there was no difference in O_2 delivery, extraction increased by 52%, similar to responses to hyperthermia (41 ºC) in dogs (Schumacker et al., 1987). In the present study, despite no significant change in arterial O_2 content, there was a 34% decrease in venous O_2 content, similar to a previous study in rams with increased testicular temperature (Waites and Setchell, 1964), and a significant reduction in venous P_{O2} at 40 vs 33 ºC.

There was a significant decrease in total hemoglobin at 40 ^{0}C. The decreased hemoglobin was attributed in part to collection of multiple blood samples. In addition, decreased hemoglobin during anesthesia in rams has been reported (Ceylam et al., 2007), perhaps due to sequestration of hemoglobin in the spleen or non-splenic sites during anesthesia (Wilson et al., 2004).

Increased testicular metabolism apparently triggered increased testicular blood flow in the present study. Similar results were observed in human limbs during exercise and at rest; increased blood temperature was associated with substantial increases in blood flow and VO_2 during activity (Gonzáles-Alonso, 2015). Furthermore, it was suggested that thermoregulatory mechanisms have key roles in blood flow and metabolism (Gonzáles-Alonso et al., 2015). At 37 ºC in the present study, there were numerical increases in blood flow, conductance, TDO_2 and extraction; however, all of these end points (except TDO_2) were significantly increased at 40 ºC. Perhaps there is a threshold of testicular hyperthermia needed to increase blood flow. In addition, duration of exposure also may have an important role in other parameters, e.g. O_2 extraction

(Drescher et al., 2017).

In the present study, there were no significant changes in lactate concentrations (a hypoxia marker; Chang et al., 2015; Lee et al., 2015; Adeva-Andany et al., 2014), pH, HCO_3-, or base excess. Therefore, increases in blood flow and in particular, blood O_2 extraction, supported the nearly doubled O_2 metabolic rate, with no indications of hypoxia or a change from aerobic to anaerobic metabolism. In a previous study (Kastelic et al., 2017), a classic scrotal insulation model was used to increase testicular temperature in rams. As expected, this reduced percentages of morphologically normal and motile sperm; however, these changes were neither prevented by hyperoxia (85% O_2 in inspired air) nor were they replicated by hypoxia (14% O_2). Therefore, it was concluded that disruptions in spermatogenesis (reductions from 55 to 35% and from 80 to 30%, for morphologically normal and motile sperm, respectively) were due to hyperthermia *per se* and not hypoxia. Although blood flow and O_2 delivery were not assessed in the previous study, the current work supported previous interpretations.

In the present study, to minimize deterioration of ram condition and responses due to prolonged anesthesia, each testicular temperature was maintained for only 45 min. It is noteworthy that in a previous study (Kastelic et al., 2017), rams were exposed to testicular insulation for 30 h and no impact of hypoxia was observed, implying that the adequate oxygenation was maintained for that interval. Furthermore, although the anesthetic drugs used in this study do have some impact on cardiac output, vascular resistance and blood flow (Raekallio et al., 2010; Schlunzen et al., 2006), each ram underwent the three treatments in a successive manner and therefore each acted as its own control. In addition, specific efforts were made to maintain a consistent depth of anesthesia. Responses to testicular warming occurred rapidly and a stable equilibrium was soon reached. Therefore, we compared data recorded after 30 min.

3.6 Conclusion

In conclusion, successive increases in testicular temperature nearly doubled the testicular metabolic rate. Remarkably, there were no indications of testicular hypoxia or anaerobic metabolism, due to increases in blood flow (25%), conductance (48%) and in particular, O_2 extraction (52%). Therefore, these data, in combination with other reports, challenged the paradigm that testicular hyperthermia fails to increase testicular blood flow and the ensuing hypoxia disrupts spermatogenesis.

Chapter 4: Testicular warming causes similar increases in testicular blood flow in *Bos taurus* versus *Bos indicus* bulls, but no apparent hypoxia

4.1 Abstract

Bull testes must be 4-5 ºC below body temperature, with testicular warming more likely to cause poor-quality sperm in *Bos taurus* (*Bt*; European/British) versus *Bos indicus* (*Bi*; Indian/zebu) bulls. Despite a long-standing dogma that testicular hyperthermia causes hypoxia, we reported that increasing testicular temperature in bulls and rams enhanced testicular blood flow and O_2 delivery/uptake, without hypoxia. Our objective was to determine effects of short-term testicular hyperthermia on testicular blood flow, O_2 delivery and uptake and evidence of testicular hypoxia in pubertal Angus (*Bt*) and Nelore (*Bi*) bulls (nine per breed) under isoflurane anesthesia. As testes were warmed from 34 to 40 ºC, there were increases ($P<0.0001$, but no breed effects) in testicular blood flow (mean $\pm$ SEM, 9.59 ± 0.10 vs 17.67 ± 0.29 mL/min/100 g, respectively), O_2 delivery (1.79 ± 0.06 vs 3.44 ± 0.11 mL O_2/min/100 g) and O_2 consumption (0.69 ± 0.07 vs 1.25 ± 0.54 mL O_2/min/100 g), but no indications of testicular hypoxia. Hypotheses that: 1) both breeds increase testicular blood flow in response to testicular warming; and 2) neither breed has testicular hypoxia, were supported; however, the hypothesis that the relative increase in blood flow is greater in Angus versus Nelore was not supported. Although these were short-term increases in testicular temperature in anesthetized bulls, results did not support the long-standing dogma that increased testicular temperature does not increase testicular blood flow and an ensuing hypoxia is responsible for decreases in motile, morphologically normal and fertile sperm.

4.2 Introduction

Bull testes must be 4-5 ºC below body temperature for production of motile, morphologically normal and fertile sperm (Kastelic et al., 2013; Saab et al., 2011; Skinner et al., 1966). Continental or British breeds (*Bt*) are generally very susceptible to deleterious effects of increased testicular temperature on sperm quality (Nichi et al., 2006), whereas *Bi* (Indian or zebu) bulls are better adapted to warm environments. *Bi* bulls have a significantly thinner testicular artery wall and shorter distance between venous and arterial blood in the testicular vascular cone, facilitating heat exchange (Brito et al., 2004). Furthermore, they also have a more pendulous scrotum (Brito et al., 2004), larger sweat gland perimeter and more functional sweat glands (Carvalho et al., 1995).

There is a long-standing paradigm that: mammalian testes operate near hypoxia (i.e. O_2 delivery to the testis is just above an anaerobic threshold); when testes are warmed, blood flow/O_2 delivery does not increase to address increased metabolic demands; and consequently, testicular hypoxia reduces percentages of morphologically normal and motile sperm (Waites and Setchell, 1964). However, we have substantial evidence challenging this paradigm. In rams (Kastelic et al., 2017) and mice (Kastelic et al., 2019), whereas hyperthermia (testicular or systemic, respectively) decreased percentages of morphologically normal and motile sperm, breathing hyperoxic air did not prevent effects of hyperthermia, nor did breathing hypoxic air replicate these effects.

In Angus bulls, as ambient temperatures increased from 5 to 35 ºC, there were increases in testicular temperature (mean $\pm$ SEM, 31.8 vs 34.9 ºC; $P<0.01$) and blood flow (2.45 vs 4.23 mL/min/100 g testis, $P<0.05$; (Barros-Adwell et al., 2018). In anesthetized rams (Rizzoto et al.,

2019), warming testes from 33 to 40 ºC increased testicular blood flow (13.2 ± 2.7 vs 17.7 ± 3.2 mL/min/100 g of testis, $P<0.05$), O_2 extraction (31.2 ± 5.0 vs 47.3 ± 3.1%, $P<0.0001$) and O_2 consumption (0.35 ± 0.04 vs 0.64 ± 0.06 mL/min/100 g of testis, $P<0.0001$). Remarkably, based on no significant changes in neither lactate nor pH, we concluded that there was no evidence of anaerobic metabolism nor testicular hypoxia. Furthermore, in another recent study in anesthetized rams (Rizzoto et al., 2018), when we decreased O_2 in inspired air from 100 to 13%, testicular blood flow increased (9.6 ± 1.7 vs 12.9 ± 1.9 mL/min/100 g of testis, $P<0.05$) and sustained O_2 delivery, with no evidence of testicular hypoxia or anaerobic metabolism. Collectively, based on these recent studies, we inferred that testicular hyperthermia may not cause testicular hypoxia, in direct contrast with long-standing dogma (Waites and Setchell, 1964).

Based on increased testicular blood flow compensating for testicular hyperthermia and systemic hypoxia and profound differences between *Bt* and *Bi* bulls in their susceptibility to deleterious effects of increased testicular temperature on sperm quality, our objective was to determine local physiological and vascular responses in Angus and Nelore bulls as testicular temperature was increased. We tested the following hypotheses: In response to testicular warming: 1) both breeds increase testicular blood flow, O_2 delivery and uptake; 2) relative increase in blood flow is greater in Angus bulls; and 3) neither breed has testicular hypoxia.

4.3 Materials and methods

4.3.1 Animals and experimental design

The experimental protocol was reviewed and approved by Institutional Animal Care and Use Committees at the University of Calgary (Protocol 18-0141) as well as Unesp-Botucatu, (Protocol 029/2018). Furthermore, this experiment was performed in accordance with all relevant guidelines and regulations regarding use of animals in research. There were two groups of bulls (nine per group), Angus (*Bt)* and Nelore (*Bi*), ~19 and 24 mo old, respectively, with average body weights of ~250 and 450 kg (no difference in body condition score was observed). Immediately prior to the study, all bulls were subjected to a standard breeding soundness evaluation (CBRA, 2013) and deemed acceptable (total sperm motility >70% and normal sperm morphology >70% for both breeds; scrotal circumference >32 and >31 cm for Angus and Nelore, respectively). Bulls were group-housed in outdoor pens, fed hay and grain, with *ad libitum* access to water.

As an overview of the experimental design, under general anesthesia, all bulls were exposed to three consecutive testicular temperatures (34, 37 and 40 ºC) for short intervals. Testicular temperature was measured with a needle thermocouple and modulated with heat packs or bags of ice placed in contact with scrotal skin. Once a target temperature was reached, it was maintained for 45 min to enable assessments of blood flow and blood gases. Thereafter, ~15-20 min was needed to warm testes to reach the next target temperature.

4.3.2 Anesthetic protocol

Bulls were subjected to general anesthesia to facilitate placement of instruments and catheters and facilitate collection of data and blood samples. The anesthetic protocol was designed to minimize blunting effects of anesthetics on the autonomic nervous system and regulation of blood flow. After being fasted according to recommendations for preventing/minimizing regurgitation of gastric contents during general anesthesia (48 h off feed and 18-24 h off water), a 14-gauge Teflon catheter was placed in a jugular vein for intravenous administration of xylazine (0.05 mg/kg, Xilazin®, Syntec do Brasil Ltda, Santana do Parnaíba SP, Brazil), followed 5 min later by induction of anesthesia with intravenous ketamine (2.5 mg/kg, Dopalen, Ceva Saúde Animal Ltda, Paulínea, SP, Brasil) and midazolam (0.1 mg/kg, Dormire, Cristália Prod. Quim. Farm. Ltda, Itapira, SP, Brasil). An endotracheal tube was placed, the bull positioned in lateral recumbency on a padded table and anesthesia maintained with isoflurane (Isoforine, Cristália Prod. Quim. Farm. Ltda, Itapira, SP, Brasil) in O_2 under mechanical ventilation (2800C, Mallard Medical/AB Medical Technologies Inc, Redding CA, USA). Intravenous administration of physiological saline (3 mL/kg/h) was done throughout anesthesia.

Because inhalational anesthetics supress autonomic control of blood flow and cause cardiovascular depression in a dose-related fashion (Seagard et al., 1984), end-expired isoflurane concentrations were monitored (Cardiocap 5, Datex-Ohmeda Ltd, Helsinki, Finland) and adjusted to produce a superficial depth of anesthesia (mean arterial pressure >100 mm Hg). Mechanical ventilation was adjusted to maintain arterial CO_2 tension close to physiological (40-45 mm Hg) with a FiO_2 of 98 ± 1.12%. End-expired isoflurane concentrations, corrected to sea level that produced the above-mentioned criteria (range of 0.9 to 1.07 and 0.8 to 1.16% Vol% in Angus and Nelore bulls, respectively) were maintained for each bull.

Epidural xylazine (0.05 mg/kg diluted to a volume of 20 mL with physiological saline), was administered into the sacrococcygeal joint, and intravenous nonsteroidal anti-inflammatory (flunixin meglumine, 1.1 mg/kg, Banimine, MSD Saúde Animal, Cruzeiro, SP, Brazil) aided analgesia. Prophylactic antibiotic therapy (ceftiofur 1.1 mg/kg im, Cef_{50}, União Química Farmacêutica Nacional S/A, Embu-Guaçu, SP, Brazil) was given before surgical interventions.

4.3.3 Instrumentation pre-assessment

An incision was made over the scrotal neck to isolate the testicular artery and testicular vein. An ultrasonic flowprobe (2SB1551; Transonic® Flowprobe, Ithaca, NY, USA) was placed around the left testicular artery, as described (Rizzoto et al., 2018;2019). Blood flow data were captured with a USB acquisition system (DATAQ® DL-1100) and evaluated using WinDaq software (DATAQ® Instruments Inc., Akron, OH, USA). A 20-gauge Teflon catheter was percutaneously inserted into an auricular artery and connected to a fluid-filled pressure transducer (TruWave™ PX260, Edwards Lifesciences, Irvine, CA, USA) for measuring mean arterial pressure (Cardiocap 5) and for collection of samples for arterial blood gas analysis/arterial blood lactate concentrations (ABL80 Flex Basic, Radiometer Medical ApS, Bronshoj, Denmark). An identical catheter was placed in the left testicular vein for collecting samples for venous blood gas analysis/venous blood lactate concentrations (ABL80 Flex Basic). Arterial and venous blood gases were corrected according to body (rectal) temperature. Blood flow in the testicular artery, mean arterial pressure and arterial/venous blood gases were used to calculate conductance plus O_2 delivery, extraction and consumption (Table 1).

Table 4.1: Formulas for testicular circulatory and metabolic parameters

Testicular O_2 delivery (ml/min)	$TDO_2 = Q(t) * CaO_2/100$
Testicular metabolic rate (ml/min)	$TVO_2 = Q(t)*(CaO_2 - CvO_2)/100$
Testicular O_2 extraction (%)	O_2 extraction = TVO_2/ TDO_2
Testicular conductance ($mL/min^{-1}/g^{-1}/mmHq^{-1}$)	Testicular conductance = (Q(t)/testicular weight)/aortic blood pressure

*Q(t) = testicular blood flow (ml/min); CaO_2 = arterial O_2 content (ml/dl);

*Caulkett et al. (1996) and Semeniuk et al. (1998).

4.3.4 Measurements, sample collection and recovery

Testicular temperature was measured with a needle thermocouple (20-gauge x 2.5 cm) [11,12], placed through the scrotal skin and kept *in situ* throughout the study. All bulls were exposed to three consecutive testicular temperatures (34, 37 and 40 ºC). To modulate temperature, heat packs warmed to ≤45 ºC or bags of ice were placed in contact with scrotal skin. Once a target temperature was reached, it was maintained for 45 min to enable assessments. Thereafter, ~15-20 min was needed to warm testes to reach the next target temperature. Rectal temperature was measured with a clinical thermometer.

For all end points studied, blood sample collection and data recording were done every 15 min (0, 15, 30 and 45 min) for each testicular temperature (34, 37 and 40 ºC). After completion of sampling, a bilateral scrotal incision was made for castration, with ligation of testicular vessels to provide hemostasis, followed by recovery from anesthesia. Post-operatively,

the animals were given antimicrobials and non-steroidal anti-inflammatories and closely monitored.

4.3.5 Statistical analyses

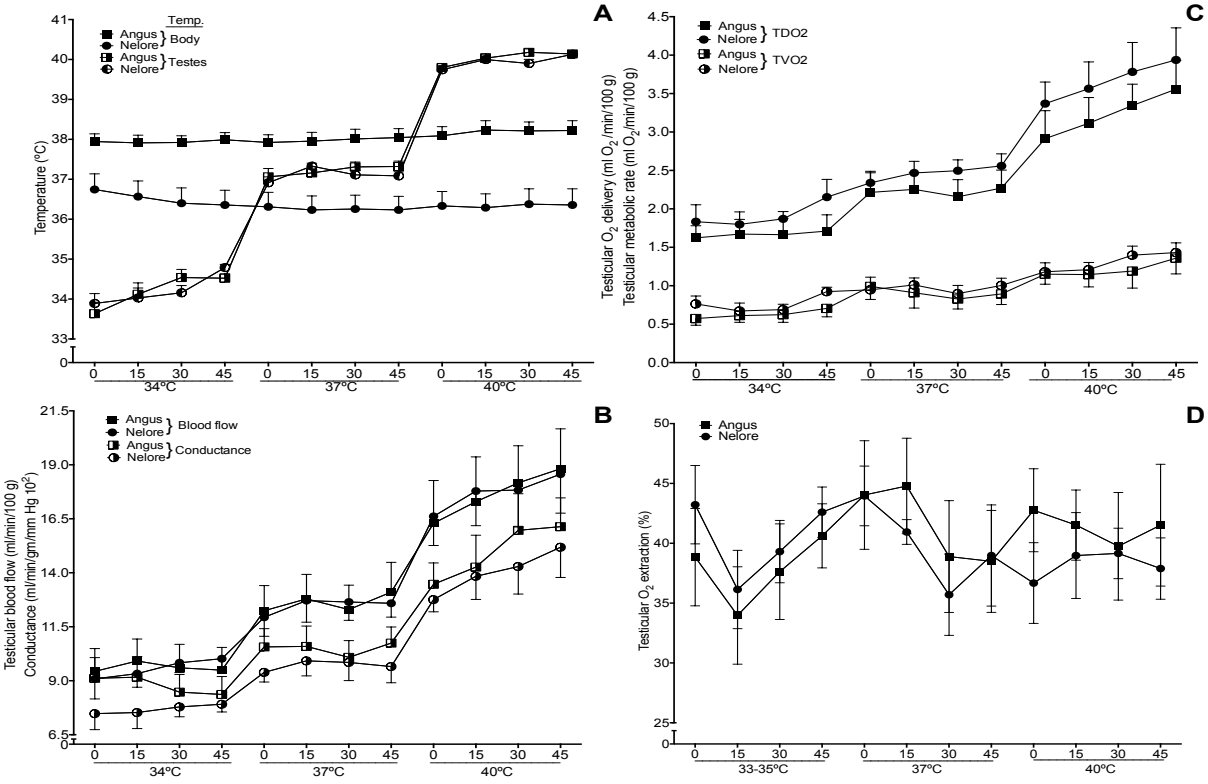
Student's *t*-tests were used to compare between breeds, testicular weight and testicular weight/body weight. Pearson's correlation analysis was used to determine linear correlations between testicular temperature and testicular blood flow, using averages of the four time points (0, 15, 30 and 45 min) for each target temperature, with a separate analysis for each breed. These statistical analyses were done with GraphPad Prism Version 6.0 (GraphPad Software Inc, La Jolla, CA, USA). For all end points measured four times at each temperature, a linear mixed-effects model was used, accounting for breed, temperature, time and the three two-way and one three-way interactions. These analyses were performed using functions nlme and ssmeans from the software R (R Foundation for Statistical Computing, Vienna, Austria). For all statistical analyses, $P<0.05$ was considered significant.

4.4 Results

Testicular temperature was consistently maintained within approximately ± 0.5 ºC of target temperatures (Fig. 4.1A). Angus bulls had a higher ($P<0.05$) rectal temperature than Nelore (average 38.04 ± 0.3 ºC vs 36.36 ± 0.04 ºC, respectively; Fig. 4.1A). Testicular warming increased ($P<0.0001$) testicular blood flow, conductance (Fig. 4.1B), O_2 delivery and O_2 metabolic rate (Fig. 4.1C); all approximately doubled between 34 and 40 ºC. However, there was no significant difference for testicular O_2 extraction (Fig. 4.1D). Increases in testicular

temperature and blood flow were highly correlated (P<0.001) in both Angus (r=0.61) and Nelore (r=0.69) bulls (data not shown).

Figure 4.1 Comparisons between Angus and Nelore bulls (nine of each breed) subjected to increasing testicular target temperatures (34, 37 and 40 ºC), with data recording and sample collection at 15-min intervals for 45 min at each target temperature. Testicular and rectal temperature (A); Angus bulls had a higher (P<0.05) rectal temperature than Nelore. T. Testicular blood flow and conductance (B), O2 delivery (TDO2) and metabolic rate (TVO2) (C), and O2 extraction (D). Testicular warming increased (P<0.0001) testicular blood flow, conductance, O2 delivery and O2 metabolic rate, but there was no significant change in testicular O2 extraction



Nelore bulls had higher (P<0.01) arterial and venous hemoglobin concentrations than Angus (Fig. 4.2A). There was a breed x temperature interaction (P<0.01) for venous lactate concentrations; at 34 ºC, Nelore bulls had higher venous lactate (P<0.001) concentrations than Angus (Fig. 4.2B). There were no significant effects for pH, base excess (BE) or H_2CO_3 in either arterial or venous samples (data not shown).

Figure 4.2 Comparisons between Angus and Nelore bulls (nine of each breed) subjected to increasing testicular target temperatures (34, 37 and 40 ºC), with data recording and sample collection at 15-min intervals for 45 min at each target temperature. Arterial and venous hemoglobin concentrations (A) and arterial and venous lactate concentrations (B).

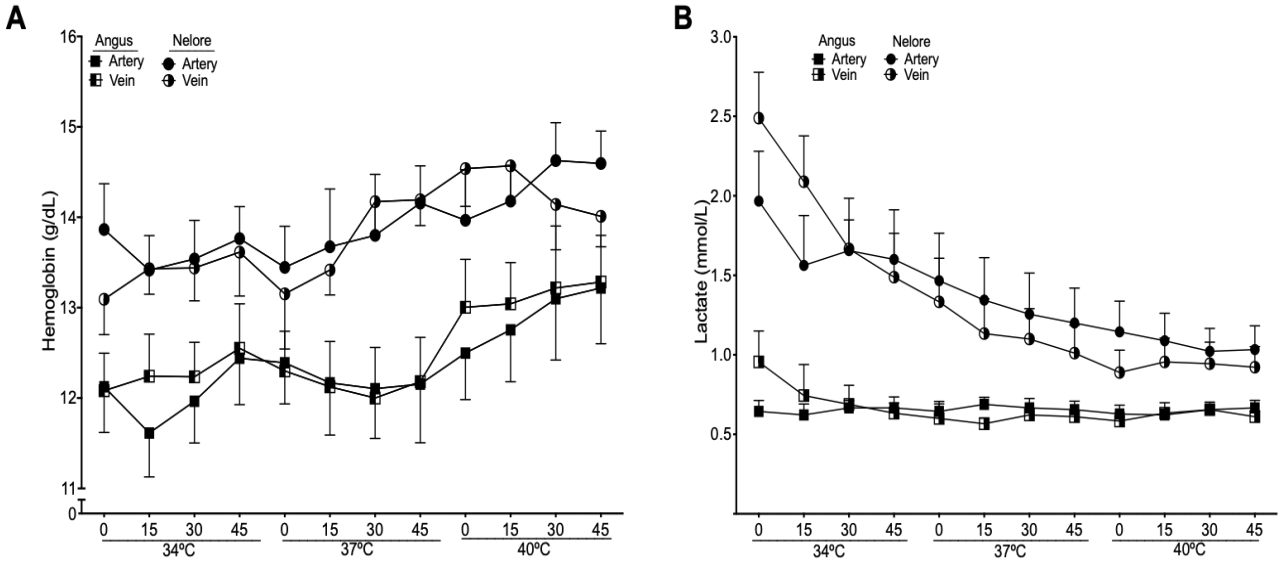
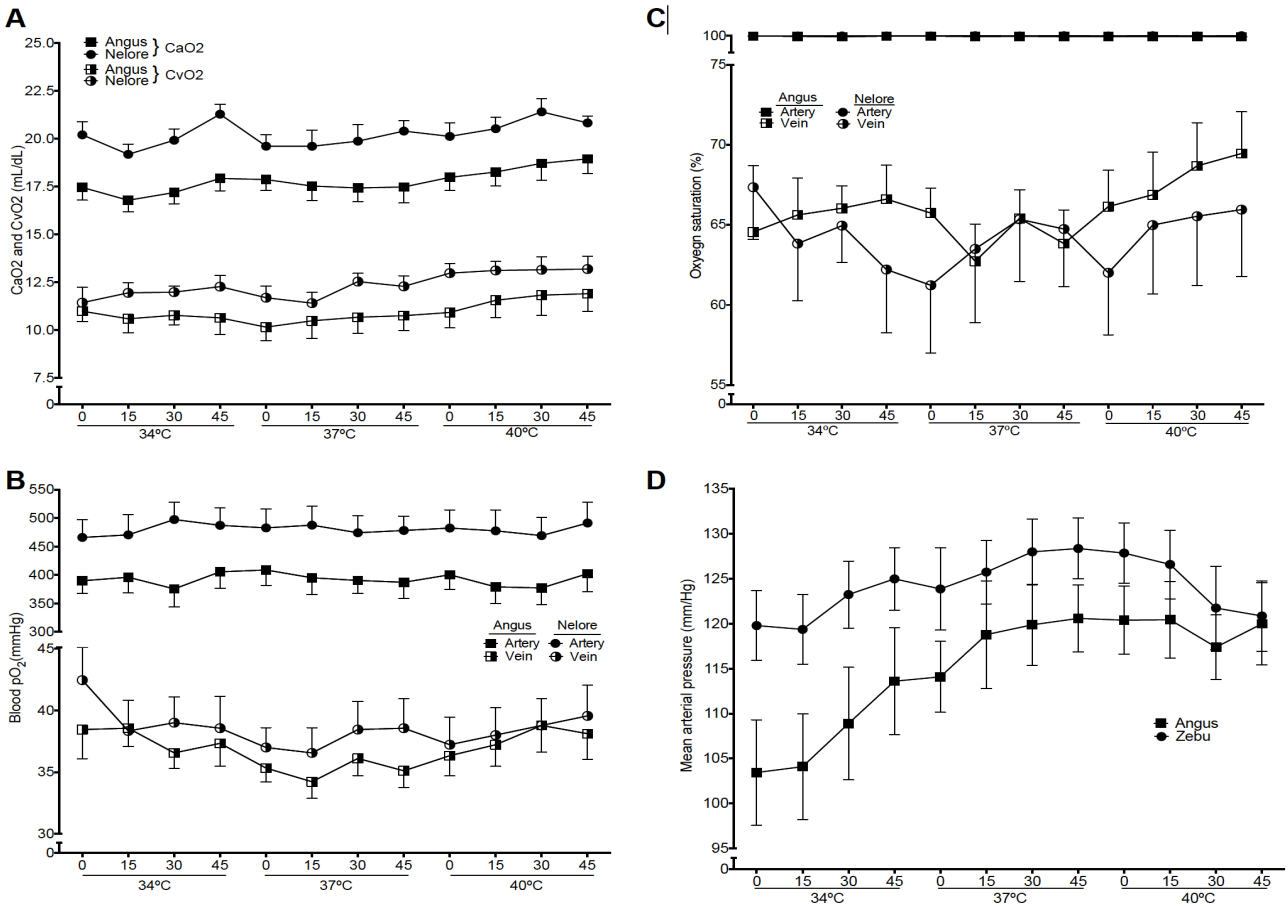


Figure 4.3 Comparisons between Angus and Nelore bulls (nine of each breed) subjected to increasing testicular target temperatures (34, 37 and 40 ºC), with data recording and sample collection at 15-min intervals for 45 min at each target temperature. Systemic CaO_2 and CvO_2 (A). Nelore bulls had higher (P<0.05) arterial O_2 content (CaO_2) compared to Angus, whereas for both breeds, venous O_2 content (CvO_2) was higher (P<0.05) at 40 ºC compared to other temperatures. Systemic pO_2 (B); Nelore bulls had higher (P<0.05) arterial PO_2 than Nelore bulls throughout the study, with no difference between breeds for venous PO_2. Systemic O_2 saturation (C) and mean arterial pressure (D). There were significant breed x temperature interactions for venous O_2 saturation and mean arterial pressure.



Nelore bulls had higher ($P<0.005$) arterial O_2 content (CaO_2) compared to Angus. For both breeds, venous O_2 content (CvO_2) was higher ($P<0.05$) at 40 ºC compared to other temperatures (Fig. 4.3A). Nelore bulls had higher ($P<0.05$) arterial PO_2 than Nelore bulls throughout the study (Fig. 4.3B), with no difference between breeds for venous PO_2.

There were breed x temperature interactions ($P<0.05$) for venous O_2 saturation (Fig. 4.3C) and for mean arterial pressure (Fig. 4.3D). Testes weight was not different between breeds (402.7 ± 14.95 and 411.7 ± 16.03 g for Angus and Nelore, respectively), but testes weight as a percentage of body weight was greater ($P<0.001$) for Angus versus Nelore bulls (0.16 ± 0.005 and 0.08 ± 0.003%).

4.5 Discussion

For both breeds, as testicular temperature increased from 34 to 40 ºC, there were highly significant increases (~100%) in testicular blood flow, conductance, O_2 delivery and consumption. In the absence of increases in testicular O_2 extraction with testicular warming, testicular blood flow and O_2 delivery matched increased metabolic demands in both breeds. Based on this, coupled with no increases in testicular venous lactate concentrations, we inferred that testes of both *Bt* and *Bi* cattle breeds were not subjected to hypoxia under short-duration heat stress. However, our hypothesis that the relative increase in blood flow is greater in Angus versus Nelore bulls was not supported. It is important to emphasize that these bulls were under general anesthesia and only exposed to short-term testicular hyperthermia.

As testes were warmed in the present study, there was a corresponding increase in testicular metabolic rate/O_2 consumption. Consequently, blood flow increased to provide adequate O_2 to support increased metabolic demands. There was a high correlation between testicular temperature and blood flow in both breeds, due to approximate doubling of both blood flow and conductance as testes were warmed from 34 to 40 ^oC. These findings were in direct contrast to the long-standing dogma that testicular blood flow does not change in response to increased testicular temperature (Waites and Setchell, 1964). In our previous study in rams (Rizzoto et al., 2019), using the same target testicular temperatures, blood flow increased only ~30% between 34 and 40 ^oC, although that increase was able to meet heat-induced increases in metabolic demands, with no indications of testicular hypoxia. It is important to indicate however, that the studies differed regarding the inspired O_2; in the ram model, the FiO_2 was adjusted (26.14 ± 1.1%) to maintain physiological PaO_2 (~90 mmHg), whereas in this study, an FiO_2 of FiO2 of 98 ± 1.12% was used, leading to a higher saturation of the blood and therefore no activation of the higher extraction state, explaining the lack of significant difference with regards to the extraction rate (Fig. 4.1D).

Increased O_2 extraction is a physiologic mechanism to maintain O_2 concentrations in the micro-environment and support aerobic metabolism (Maginiss et al., 1994). Although O_2 extraction was not significantly altered in the present study, it was significantly increased when we used the same model of testicular hyperthermia as in our recent ram study (Rizzoto et al., 2017), presumably due to the much smaller magnitude of increased blood flow/O_2 delivery. For unknown reasons, the relative increase in blood flow/O_2 delivery in bulls was more than three-

fold greater than the increase in rams; further studies are needed to verify and elucidate this apparent difference in these two ruminant species.

There was a significant breed x temperature interaction for venous lactate concentrations, due to lactate being ~0.5 mmol/L higher in the vein than the artery of Nelore bulls at the start of the study (Time 0 at 34 ºC). Perhaps this was stress related to pre-surgical handling, as it is well known that *Bi* are more easily stressed than *Bt cattle* (Burrow, 1997) and blood lactate concentrations can be increased as part of a stress response (Mitchell, 1988). Regardless, it declined relatively rapidly thereafter. Furthermore, as testicular temperature increased, venous lactate concentrations were similar to or slightly lower than arterial venous lactate concentrations. This was critical, as lactate is one of the most important markers of hypoxia (Chang et al., 2015; Lee et al., 2015).

Rectal temperature was significantly lower (~1 to 1.5 ºC) in Nelore than in Angus bulls during anesthesia. This difference can be attributed to different ability to maintain body temperature or better thermoregulatory capability of tropical *versus* European cattle breeds *(*Hammond et al., 1999). The *Bt* cattle, previously adapted to hot environmental conditions had higher rectal temperatures and respiratory rates compared to *Bi* ($P<0.01$), whereas non-adapted *Bt* had the largest increases in rectal temperatures and respiratory rates compared to other groups ($P<0.01$), indicating the importance of previous adaptation to an environment and breed differences in thermoregulatory capabilities (Carvalho et al., 1995).

Testes should be cooler than body temperature to allow adequate spermatogenesis. In Nelore bulls (Godinho et al., 1980), the rectal-testicular temperature gradient remained ~5 ºC,

irrespective of whether they were exposed to sun or shade, consistent with their ability to tolerate high ambient temperatures. Regulation of testicular blood flow under heat stress may represent an important mechanism to keep testes cooler than body temperature, as we reported that testicular blood flow was the main source of testicular heat (Barros-Adwell et al., 2018); consequently, we hypothesized Nelore bulls would have lower increases in testicular blood flow in response to heat stress than Angus bulls. However, there was no significant breed effect on testicular blood flow in response to increasing testicular temperature. Therefore, we inferred that superior thermal resistance in *Bi. indicus* bulls may be associated with physiological thermoregulatory components other than a better ability to modulate testicular blood flow.

Nelore bulls had higher CaO_2 and arterial pO_2 compared to Angus bulls; however, there was no difference between breeds for CvO_2. In a previous study, CvO_2 was higher for *Bi* versus *Bt* bulls under long-term (15 d) heat stress (Beatty et al., 2006), indicating better adaptability to warm environments. Furthermore, Nelore had consistently higher arterial hemoglobin than Angus. Hemoglobin concentrations vary among cattle breeds (Bachmann et al., 1978; Penny et al., 1966). At 34 ºC, Nelore bulls had higher hemoglobin concentrations, accounting for their higher CaO_2 value, based on the formula for calculating CaO_2 (Table 1).

There were no significant differences between breeds in testes weight. However, testes weight as percentage of body weight (gonadosomatic index, GSI) was higher for Angus than Nelore, consistent with a previous report (Lunstra et al., 1993).

Evaluation of effects of testicular hyperthermia on local blood flow/metabolism under general anesthesia was the main limitation of the present study. Because isoflurane anesthesia

decreases sympathetic efferent activity and causes cardiovascular depression in a dose-dependent fashion (Seagard et al., 1984), end-tidal isoflurane concentrations were reduced to maintain MAP > 100 mm Hg. Inhalant anesthetic concentrations used the present study were equal or less than the minimum alveolar concentration of isoflurane reported in Holstein cows (1.14% at sea level) and likely represented a light plane of anesthesia (Cantalapiedra et al., 2000).

The analgesic effects of epidural xylazine contributed to reduce isoflurane requirements for maintaining anesthesia and probably assisted to minimize blunting effects of inhalant anesthesia on autonomic regulation of testicular blood flow. Although alpha-2 agonists such as xylazine, also have central sympatholytic effects and inhibit sympathetic ganglionic transmission *in vitro* (McCallum et al., 1988), epidural xylazine was given to provide intra- and postoperative analgesia due to the invasive nature of the procedures. Although this model facilitated evaluation of local control of circulation to the testis, it could not fully mimic effects of neurohormonal changes of bulls exposed to environmental heat stress, which could also modify testicular blood flow and O_2 delivery and consumption.

Environmental heat stress in humans causes changes in autonomic balance and results in a hyperdynamic state (increased heart rate and cardiac output) associated with small decreases in arterial pressure due to decreased systemic vascular resistance (Crandall et al., 2015). Vasodilation increased blood flow to the skin dissipates heat to maintain thermal homeostasis. Whether European or tropical breeds of cattle differ in their hemodynamic response/autonomic nervous system changes when exposed to hot environmental conditions and how these changes alter regional vascular resistance/blood flow to the testis, remain unknown.

4.6 Conclusion

In conclusion, both *Bt* and *Bi* bulls responded to testicular warming by profoundly increasing testicular blood flow, providing ample O_2 to support increased testicular metabolic needs and avoiding hypoxia. Although it is well established that *Bt* bulls have much greater decreases in sperm quality than *Bi* bulls in response to increased ambient temperatures, there were no significant breed effects in relative increase in testicular blood flow. Our experimental design included warming testes of both breeds to 40 ^{o}C; notwithstanding, under field conditions, we expect testicular temperatures in Nelore bulls are unlikely to reach this level. Regardless, increased blood flow fully met increased metabolic demands for O_2 and there were no indications whatsoever of hypoxia. Therefore, these findings, in conjunction with our other recent reports, provided compelling evidence contradicting the long-standing paradigm that mammalian testes operate near hypoxia and that testicular hyperthermia does not provoke increased testicular blood flow, resulting in frank hypoxia.

4.7 Acknowledgements

We thank Dr. John Tyberg and Cheryl Hall for providing access to flow-monitoring equipment and software and all those who assisted with bull care and handling.

Chapter 5: Testicular hyperthermia reduces testosterone concentrations and alters gene expression in testes of Nelore bulls.

5.1 Abstract

Increased testicular temperature reduces sperm motility, morphology and fertility. Our objectives were to characterize effects of testicular hyperthermia (scrotal insulation) on acute testosterone concentrations and gene expression in *Bos indicus* (I) testes. Nelore bulls (n=20), ~27 mo of age, 375 kg, scrotal circumference >31 cm, with ≥30% motile and ≥70% morphologically normal sperm, were allocated into four groups (n=5/group): non-insulated (Control) and insulation removed after 12, 24, or 48 h. Immediately after insulation, intratesticular temperatures (needle thermocouples) were coolest in Control bulls and warmest in 48-h bulls (mean ± SEM, 35.28 ± 0.31 vs 38.62 ± 0.57 ^{0}C, P<0.05). Bulls were castrated and testes recovered. Testicular testosterone concentrations were higher in Control versus 48-h bulls (3119 ± 973.3 and 295.5 ± 122.8 ng/g of tissue, respectively, P<0.05). Total RNA was extracted, reverse transcribed and RT-qPCR done. For *StAR*, mRNA abundance decreased from Control to 48 h (1.14 ± 0.32 vs 0.32 ± 0.5, P<0.05). For *BCL2*, expression decreased from Control to 24 h (1.00 ± 0.07 vs 0.70 ± 0.12, P<0.05), but then rebounded. In addition, *GPX1* had a 70% increase (P<0.05) at 48 h, whereas *HSP*70 had a 34-fold increase (P<0.05) at 12 h and 2- and 14-fold increases (P<0.05) at 24 and 48 h, respectively. *HSF1*, *BAX*, P53 and *CASP* 8 remained unchanged. Downregulation of *StAR*, critical in androgen production, was consistent with reduced testosterone concentrations, whereas increased *GPX1* enhanced testicular antioxidative capability. Huge increases in *HSP*70 conferred protection again apoptosis and cell destruction,

whereas reduced *BCL2* promoted apoptosis. These findings provided novel insights into acute tissue responses (testosterone and gene activity) to testicular hyperthermia in *Bi* bulls.

5.2 Introduction

Heat stress is a major impediment to bull reproduction, as testicular temperature needs to be 3-5 ºC below body core temperature for production of morphologically normal, motile and fertile sperm (Kastelic et al., 1995; Freeman, 1990). Consequently, beef bulls have reduced sperm quality when testicular temperature is increased, e.g. due to elevated temperature, or experimentally, testicular insulation (Fernandes et al., 2008).

Zebu (*Bos indicus; Bi)* breeds are important in world food production, particularly in tropical environments, as they are relatively more resistant to hotter climates than British or continental breeds (*Bos taurus*; *Bt)* (Beatty et al., 2004; Hansen, 2004; Brito et al., 2002; Carvalho et al., 1995; Skinner and Louw, 1996). There are several morphological differences between these two types of bulls in reproductive anatomy. In the testicular vascular cone (Brito et al., 2004), *Bi* bulls have testicular arteries with thinner walls that are in closer proximity to the testicular vein than *Bt* bulls, thereby promoting countercurrent heat exchange and contributing to testicular cooling (Brito et al., 2004). Notwithstanding, when exposed to high ambient temperatures, *Bi* bulls have impaired sperm production and reduced sperm quality (Nichi et al., 2006; Skinner and Louw, 1966). Furthermore, although blood testosterone concentrations decreased following heat stress (Alves et al., 2016; Gomes et al., 1971), testicular testosterone concentrations have apparently not been reported. In addition, underlying mechanistic pathways through which heat stress impacts testicular function in cattle are not well characterized.

Changes in genes and proteins in response to increased testicular temperature have been reported (Maibam et al., 2017; Misztal et al., 2017; Deb et al., 20140. For example, regarding an intrinsic apoptotic pathway (Durairajanayagam et al., 2015), the P53 gene, acts upon *BAX* and BCL-2, which are pro- and anti-apoptotic, respectively, leading to apoptosis through activation of various *CASP*ases, including *CASP*ases 8, 9 and 10. Furthermore, there are other defense mechanisms, including anti-oxidative activity and activation of chaperone molecules (e.g. *HSF1* activates *HSP*s). These genes and pathways have primarily been studied in laboratory animals, but apparently not in bulls. Our objectives were to characterize effects of acute testicular hyperthermia, induced by scrotal insulation, on testosterone concentrations and gene expression in testicular tissue of *Bi* bulls.

5.3 Materials and methods

5.3.1 Bulls and experimental design

Twenty *Bi* (Nelore) bulls, 27 mo of age and ~375 kg, were used. Bulls were kept on pasture throughout the study and restrained in a livestock handling chute for procedures. All animal activities were reviewed and approved by Institutional Animal Care and Use Committees at the University of Calgary (Protocol 18-0141) as well as Unesp-Botucatu, (Protocol 029/2018). In addition, this work was done in compliance with relevant guidelines and regulations regarding use of animals in research.

Bulls were subjected to a standard bull breeding soundness examination, including assessment of internal and external reproductive structures, measurement of scrotal

circumference and collection of semen via electroejaculation. Sperm motility and morphology were assessed by visual examination with phase-contrast microscopy at 400 and 1000X, respectively. Disposable baby diapers, held in place with adhesive tape, were used to insulate the scrotum and cause acute testicular hyperthermia. Bulls were randomly allocated into four groups (n=5/group): non-insulated (control) and insulation removed after 12, 24, or 48 h. At the scheduled end of insulation, caudal epidural analgesia was performed (xylazine, 0.05 mg/kg, Xilazin®, Syntec do Brasil Ltda, Santana do Parnaíba SP, Brazil), diapers and tape were removed and a needle thermocouple used to measure intratesticular temperature, as described (Rizzoto et al., 2020).

Immediately thereafter, another sperm sample was collected via electroejaculation and sperm motility was analyzed. Then, bulls were castrated, with a scalpel used to make a lateral incision in scrotal skin and an emasculator used to crush and cut the spermatic cord. Testicular tissue was collected, cut into cubes (~400 mm^3) that were immediately placed in liquid nitrogen. To mitigate pain and inflammation, a nonsteroidal anti-inflammatory (flunixin meglumine, 1.1 mg/kg, IV, Banamine, MSD Saúde Animal, Cruzeiro, SP, Brazil) was given. In addition, ceftiofur (1.1 mg/kg, IM; Cef_{50}, União Química Farmacêutica Nacional S/A, Embu-Guaçu, SP, Brazil) was also given.

5.3.2 Testicular testosterone concentrations

Testicular tissue samples were thawed at 4 ºC, placed in a 15-mL tube, 4.5 mL of ethanol added, and tissue homogenized with a sonicator (seven, 30-s cycles; Brandson 250 Digital Sonifier, Danbury, CT, USA) and centrifuged (1000 x g for 30 min at 4 ºC; Centrifuge 5810R,

Eppendorff, Hamburg, Germany). Supernatant was placed in 15 mL tubes and concentrated at 50 ºC on speed vacuum (Speedvac Concentrator, SPD1010, Savant, Thermo Fisher Scientific, Waltham, MA, USA) for 180 min. The pellet was then dissolved in 0.5 mL of ELISA buffer solution (400060, Cayman Chemical, Ann Arbor, MI, USA) and stored at -20 ºC pending analysis. Samples were thawed at 4 ºC and a commercial assay was used (Testosterone Elisa Kit, 582701, Cayman Chemical), with a sensitivity of 3.9 pg/mL. One assay was used for all samples and the intra-assay CV was 7.4.

5.3.3. RNA extraction and expression of target genes

Testis samples (50 mg) were thawed, homogenized and total RNA extracted with Trizol® (Invitrogen, São Paulo, SP, Brazil) and stored at -80 °C. All samples were incubated with DNAse I (1 IU/µg; Invitrogen), at 65 °C for 10 min to lyse genomic DNA. Immediately after incubation, total RNA (1 µg) from each sample was submitted to reverse transcription using random primers, in accordance with manufacturer's instructions (High Capacity Kit, Applied Biosystems®, Foster City, CA, USA).

To perform RT-qPCR for each target gene (Table 1), QuantStudio ™ 7 Flex equipment using the Power Sybr Green® (Applied Biosystems®) system was used, together with specific primers for each gene of interest. The qPCR reactions were carried out in 25-µL volumes, with 1 µL of cDNA, 12.5 µL of Power Syber Green PCR Master Mix (Applied Biosystems®), 300 mM of each forward and reverse primers, and 9 µL of water. The qPCR cycling conditions were 95

°C for 10 min for initial denaturing and then 40 cycles of 95° C for 10 s, followed by primer annealing and extension at 60 °C for 1 min. Reactions were optimized to achieve maximum amplification efficiency for each gene (90-110%). To determine specificity of each PCR product, a dissociation curve was performed in association with confirmation of fragment size, using 1.5% agarose gel electrophoresis. Samples were analyzed in duplicate and as negative control, water was added to the mix in lieu of cDNA (in each plate).

NormFinder software (Department of Molecular Medicine [MOMA], Aarhus University Hospital, Aarhus, Denmark) was used to select appropriate housekeeping genes; amplification profiles of isomeryl peptidylprolyl isomerase A (*PPIA*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and histone H2A (*H2AFZ*) were analyzed, with PPIA identified as the most suitable gene. Lastly, to quantify relative expression, the ΔΔCt method (target genes / PPIA) was used, with correction of efficiency for each target gene (Livak and Schmittgen, 2001).

5.3.4 Statistical analyses

Differences among groups in testicular temperature, sperm quality end points and relative mRNA abundance of target genes were evaluated by one-way ANOVA, with a Tukey-Kramer test used to locate differences. Testicular testosterone concentrations in Control versus 48-h bulls were compared using a Student's *t*-test. All statistical analyses were done with GraphPad Prism software, Version 8.2.1 (GraphPad Software Inc, La Jolla, CA, USA) and data presented as mean ± SEM. Differences were considered significant when $P \leq 0.05$.

Table 5.1. Primers used for RT-qPCR to assess mRNA abundance of genes in testicular tissues.

Gene	Forward sequence	Reverse sequence	Conc. (mM) †	Temp. (°C) ‡
ATCB	5'GCGTGGCTACAGCTTCACC 3'	5'TTGATGTCACGGACGATTTC 3'	300	60
BAX	5'TGTTTTCTGACGGCA ACTTCA 3'	5'CGAAGGAAGTCCAATGTCCAG 3'	300	60
BCL2	5'TTCGCCGAGATGTCCAGTCAGC 3'	5'TTGACGCTCTCCACACACA 3'	300	59
CASP8	5'CAGAACAGATGGAAGCCTAT 3'	5'GGTTAGGATGGTCAGAATGT 3'	300	60
GAPDH	5'GGCGTGAACCACGAGAAGTATAA 3'	5'CCCTCCACGATGCCAAAG 3'	200	60
GPX1	5'GGGACTACACCCAGATGAATGA 3'	5'GCATAAAGTTGGGCTCGAA 3'	300	60
HSF1	5'CCTCGAGAACATCAAGAGGAAAG 3'	5'GTCGGTCAACAGCTTGGTAA 3'	300	60
HSP70	5'AACAAGATCACCATCACCAAACG 3'	5'TCCTTCTCCGCCAAGGTGTTG 3'	300	60
PPIA	5'GCCATGGAGCGCTTTGG 3'	5'CCACAGTCAGCAATGGTGATCT 3'	300	60
P53	5'GCACAAACACGCACCTCAA 3'	5'CCCATCCTCACCATCATCAC 3'	300	60
RPL15	5'CTCATCGTTGGTGCCAATGCAAGT 3'	5'TCACATCCACCCTGGGAAACAGAA 3'	300	60
RPL30	5'TGGTGTCCATCACTACAGTGGCAA 3'	5'ACCAGTCTGTTCTGGCATGCTTCT 3'	300	60
StAR	5'CCCAGCAGAAGGGTG TCATC 3'	5'TGCGAGAGGACCTGGTTGAT 3'	200	62

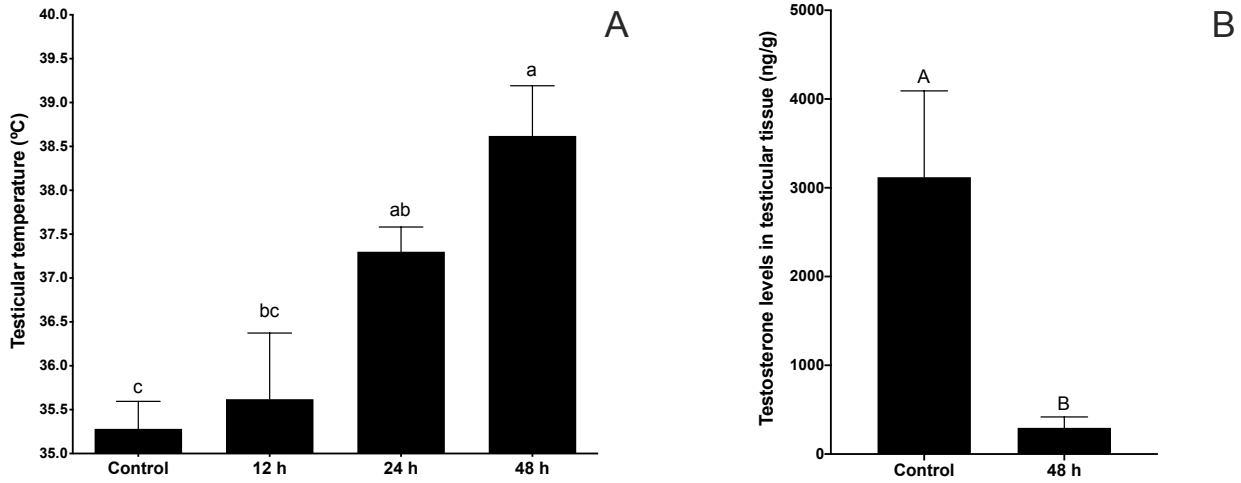
† Primer concentration per PCR reaction (mM); ‡ Temperature of annealing and extension (ºC)

5.4 Results

Testicular insulation increased intratesticular temperature from 24 h onwards, peaking at 48 h (Fig. 5.1A). There were no significant differences among groups in percentage

morphologically normal or motile sperm (data not shown). Testicular testosterone concentrations were >10-fold higher in Control versus 48-h bulls (3119 ± 973.3 and 295.5 ± 122.8 ng/g of tissue, respectively; P<0.05, Fig. 5.1B)

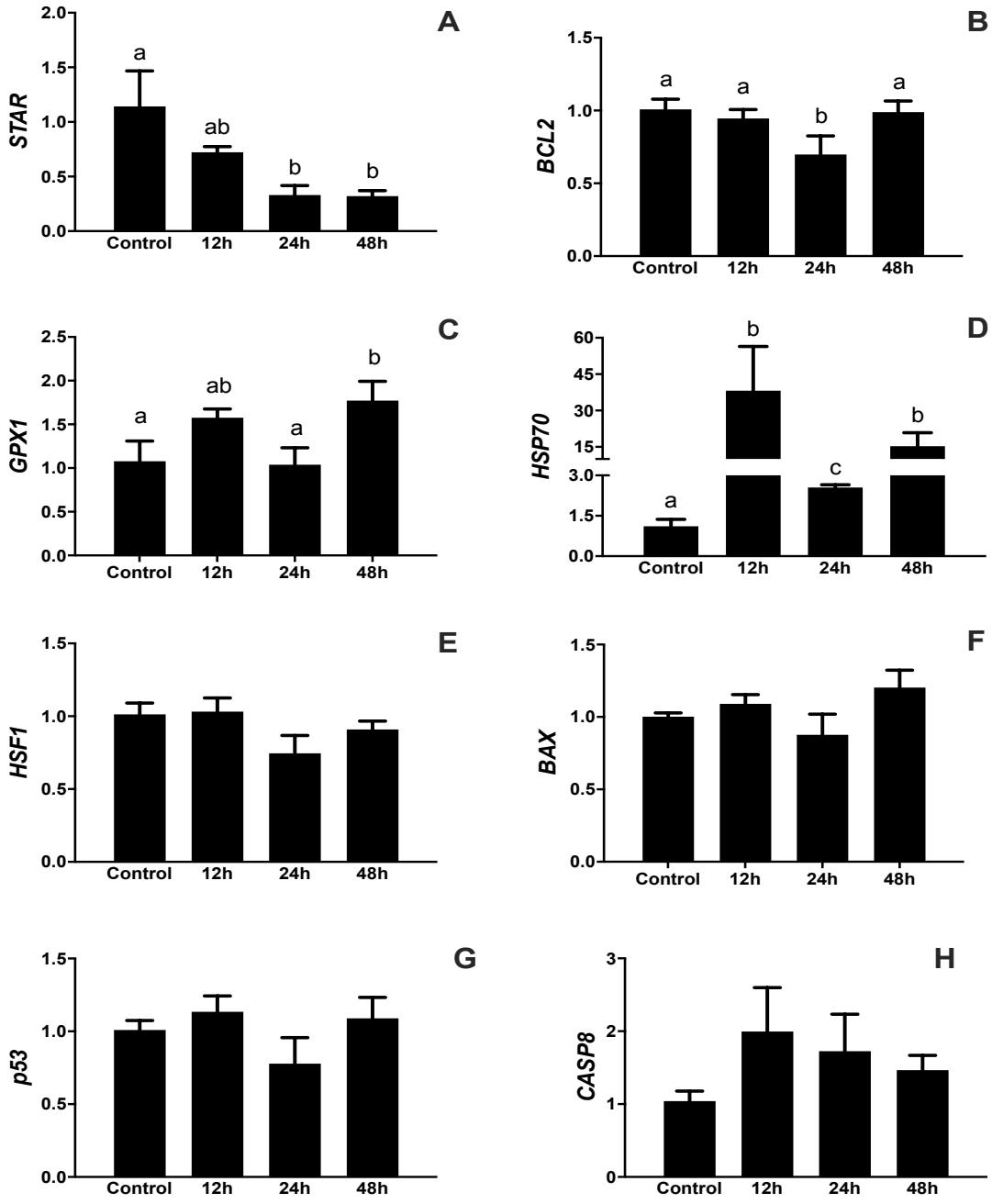
Figure 5.1 A) Mean (± SEM) intratesticular temperature (ºC) and; B) testosterone concentrations in testicular tissue (ng/g) in Nelore bulls after insulation for 12 to 48 h.



[a-c]Columns without a common superscript differed (P<0.05).

There were significant changes in mRNA transcript abundance for *StAR*, *BCL2*, *GPX1*, *HSP*70 (Fig 5.2). (A) *StAR* had marked down-regulation, starting at 12 h and becoming more evident at 24 and 48 h. (B) *BCL2* expression decreased from Control to 24 h (P<0.05), but then rebounded to Control values. (C) *GPX1* had a 70% increase (P<0.05) at 48 h, whereas (D) *HSP*70 had a 34-fold increase (P<0.05) at 12 h, followed by 2- and 14-fold increases (P<0.05) at 24 and 48 h, respectively. There were no significant changes in *HSF1* (E), *BAX* (F), P53(G) or *CASP* 8 (H).

Figure 5.2 Mean (± SEM) fold change in expression of selected genes in testes of *Bos indicus* bulls to determine effects of testicular insulation on mRNA abundance of key molecules associated with heat impact and resistance.



[a-c]Within a row, means with a different superscript differed (P<0.05).

5.5 Discussion

Testicular insulation is a common model to increase testicular temperature (Kastelic et al., 1995. In the present study, intratesticular temperature, measured immediately after insulation was removed, increased ~3 ºC after 48 h of insulation ($P<0.05$). An increase of this magnitude, even of short duration, alters spermatogenesis and reduces sperm quality in various species (Wechalekar et al., 2008; Rockett et al., 2001; Barth and Bowman, 1994).

Increased testicular temperature induced a profound (10-fold) reduction in testicular testosterone concentrations after 48 h. To our knowledge, this is the first report of change in testicular testosterone concentrations in bulls with testicular hyperthermia, although changes in serum testosterone concentration have been reported. For example, rams submitted to scrotal insulation for 72 h presented decreased serum testosterone (Alves et al., 2016) and rats exposed to 43 ºC for 30 min during 3, 5 or 10 consecutives days had a marked decrease in serum testosterone concentration (~45% of control concentrations) (Li et al., 2016).

Although all cells involved in spermatogenesis are affected by increased testicular temperatures (Cataldo et al., 1997), important damage occurs in Sertoli cells (Au et al., 1987) and in particular, Leydig cells (Bergh et al., 1984A,B; Keel and Abney, 1981); the latter produce testosterone, fundamental for spermatogenesis and sperm function. Higher temperatures alter testicular biochemistry, impairing enzymes such as hyaluronidase and DNA polymerases (Fujisawa et al., 1988). Steroidogenesis is also impaired, with reports of reduced *StAR* activity (Bozkaya et al., 2017; Li et al., 2016). *StAR* is one of the most important factors associated with the steroidogenic pathway, including testosterone production in Leydig cells. In our study, *StAR*

was down-regulated at 12 h, presumably contributing to reduced testicular testosterone concentrations at 48 h. It is noteworthy that in cryptorchids, increased testicular temperatures impaired cholesterol transport and *StAR* function in Leydig cells (Oka et al., 2017). Acute testicular heat stress in rats (43 ºC for 30 min) also reduced activity of other important enzymes for steroidogenesis, including cytochrome P450 and CYP17 (Li et al., 2016). Interestingly, heat stress reduced testosterone production by Leydig cell tumors (Kim et al., 2016), consistent with current observations and other literature discussed above.

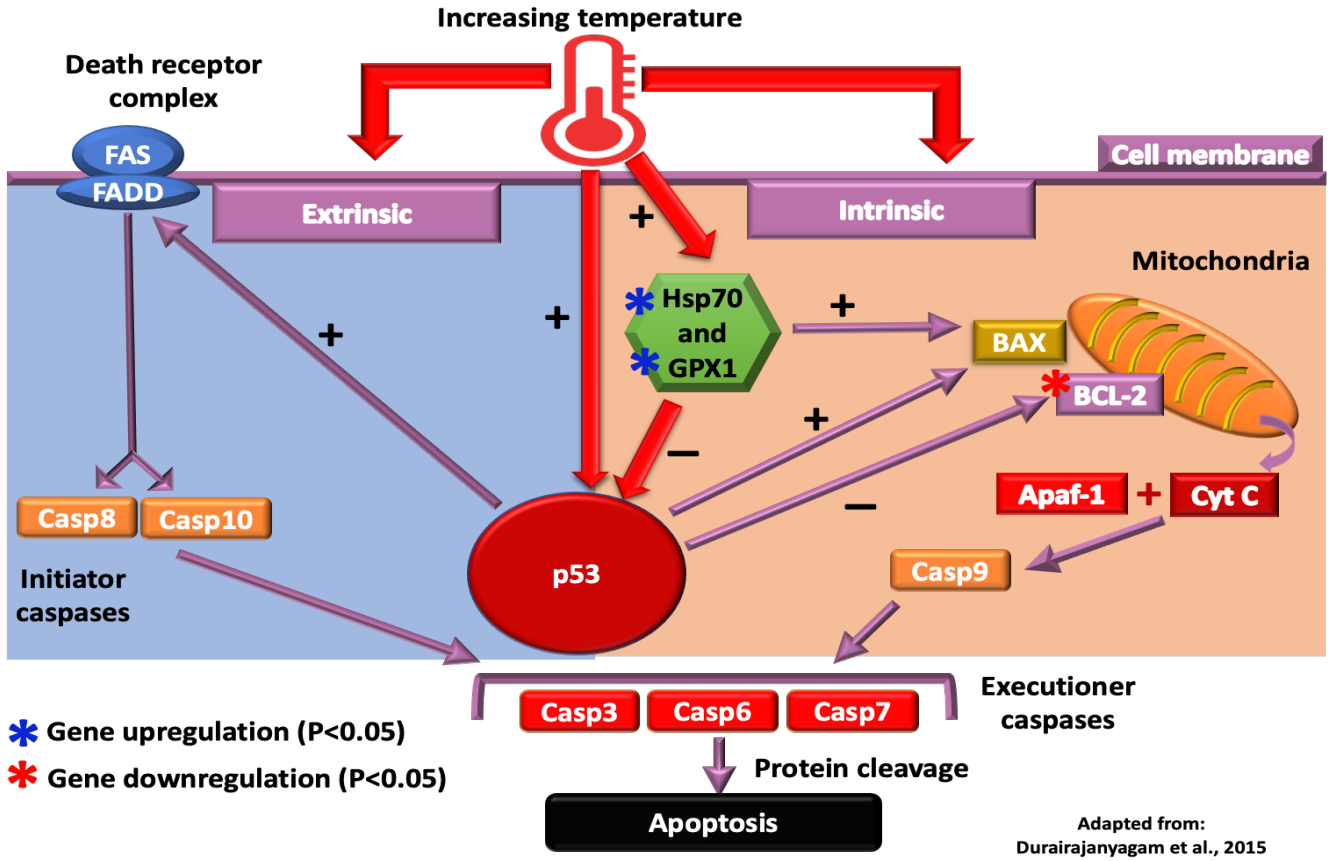
In the present study, scrotal insulation increased testicular antioxidative capability, namely *GPX1*, after 12 h of insulation, with a markedly greater increase at 48 h. Similarly, in rams, scrotal insulation for 240 h, increased testicular temperature 5 ºC and enhanced immunolocalization of *GPX1* in testes, with a broader range of cell types than other antioxidant enzymes (Hamilton et al., 2019). Testicular oxidative stress is often reported as a sequelae to heat stress, with involvement in pathogenesis of reductions in sperm quality and fertility (Hamilton et al., 2019; Pau et al., 2009). Antioxidants are abundant in seminal plasma and sperm (Kankofer et al., 2005). Glutathione peroxidase is important to prevent lipid peroxidation in sperm, similar to, for example, glutathione reductase and superoxide dismutase (Kankofer et al., 2005; Gangadhram et al., 2001). Based on present and previous results, we inferred that GPX 1 is a key antioxidant to protect testicular cells defense against heat stress.

Another fundamental defense mechanism within testes is expression of *HSF1* and heat shock proteins, the so-called heat shock response (Yu et al., 2015; Paul et al., 2009). Increase in *HSF1* expression was associated with heat stress resistance in cattle (Baena et al., 2018; Kumar et al., 2015; Li et al., 2011; Collier et al., 2008). Furthermore, increased *HSF1*gene expression

was described in *Bi* bulls in summer, consistent with heat resistance conferred by this gene and its role in heat shock (Kumar et al., 2015). When heat stress occurs, *HSF1* binds to promoters of all *HSP*s (Paul et al., 2009), thereby increasing synthesis of *HSP* mRNA. *HSP*70, one of the most important *HSP*s (Korfanty et al., 2014), acts to prevent apoptosis and cell destruction in association with other chaperone molecules. Interestingly, in our study, there was no significant difference in expression of *HSF1*, whereas profound up-regulation of *HSP*70 occurred from 12 h onwards ($P<0.05$). Presumably increased *HSF1* occurred within the first few hours of exposure, as reported in mouse testes (Korfanty et al., 2014; Kus-Liśkiewicz et al., 2013), resulting in increased *HSP*70 after 12 h of insulation.

Interactions of *BAX*, *BCL2*, P53 and *CASP*ase 8 are important responses to tissue heating. *BAX* and *BCL2* are pro and anti-apoptotic factors, respectively; the balance between these molecules is fundamental to determine cell fate. Upregulation of *BAX* and/or down regulation of BCL-2 promote cell death through apoptosis, initiating effects of apical *CASP*ases such as *CASP*ase 8, for example, and completion through other *CASP*ases, so-called effectors (et al., 2015). The key factor associated with this apoptotic pathway is P53, which can modulate both *BAX* and BCL-2 and therefore, determine cell fate. In our study, neither P53 nor *BAX* were significantly altered. As P53 can be very rapidly activated (Wittlinger et al., 2007), perhaps it initially increased and then subsequently subsided, with our first assessment done too late to document the increase. An overview of the proposed pathway is described in Fig. 5.2 with the modifications in the genes observed in this study indicated by an asterisk.

Fig.5.3 Suggested pathways of heat stress impacts and responses in testes (adapted from Durairajanayagam et al., 2015). Heat stress activates thep53 gene, leading to the activation of the extrinsic and intrinsic p53-dependent apoptotic pathways. Regarding the extrinsic pathway, heat stress-induced upregulation of pr53 upregulates the FAS gene, leading to formation of the FAS + FADD (FAS associated death domain) complex, which upregulates initiator caspases Casp8 and 10. In the intrinsic pathway, an activated p53 upregulates BAX and downregulates BCL-2, thereby increasing the BAX-BCL2 ratio, stimulating the release of Cyt C from mitochondria and having it form a complex with Apaf-1 that activates Casp9 (initiator). The initiator caspases, activated through either the extrinsic or intrinsic pathways, promote apoptosis by signaling to executioner caspases (Casp 3,6 and 7), thereby completing the apoptotic process. Furthermore, the chaperone system (Hsp70) and antioxidant system (GPX1) are physiological responses that downregulate p53 function and as consequence, suppress apoptosis. *Fold change (P<0.05) in gene (blue = upregulation and red = downregulation).



In conclusion, scrotal insulation was used to induce testicular hyperthermia, confirmed with measurement of testicular temperatures. The resulting heat stress profoundly reduced testicular testosterone concentrations and enabled us to elucidate some of the mechanisms involved in the pathway through which: 1) *Bi* bulls responded to heat stress, i.e. increased expression of antioxidant molecules (*GPX1*) and increased production of chaperones (*HSP*70); and 2) how heat stress damaged testicular cells, including down regulation of *StAR* and BCL-2, fundamental for spermatogenesis and sperm quality and anti-apoptotic activity, respectively. Further studies are required to investigate other genes and factors that may be involved with heat resistance and heat impact.

5.6 Acknowledgements

We thank all those who assisted with bull care and handling.

Chapter 6: Acute mild heat stress strategically alters gene expression in testes and reduces sperm quality in mice

6.1 Abstract

Our objective was to investigate the impacts of mild acute heat stress on sperm and testes, and based on mRNA, elucidate involvement of StAR, Trp53 and Trp53-dependent intrinsic and extrinsic apoptotic pathways in pathophysiology of testicular heat stress. Forty-eight C57BL/6 elite male mice were equally allocated into six groups, anesthetized and the distal third of their body immersed in a water-bath at 40 or 30 ºC (heat treatment and control, respectively) for 20 min. Intervals from heat exposure (Day 0) to euthanasia were: 8 and 24 h and 7, 14 and 21 d (plus a control group at 14 d). The epididymides were excised, minced and placed in Tyrode albumin lactate pyruvate hepes (TALPH) at 37 ºC for 15 min to recover sperm. Based on computer assisted sperm analysis (CASA), heat treatment reduced total and progressive motility ~40% ($P<0.05$) on Days 14 and 21. Furthermore, percentage morphologically normal sperm was significantly decreased on Day 7, with greater reductions on Days 14 and 21, mostly due to increased midpiece defects. Acrosome integrity (FITC PSA) was decreased ~35% at 8 h ($P<0.05$) and reached a nadir on Day 14. There were decreases ($P<0.05$) in seminiferous tubule diameter and testicular weight (relative to body weight) on Day 14. Testicular RNA was extracted, reverse-transcribed and cDNA used for PCR. Expression of genes Hspa1b (Hsp70) and Gpx1 had 7- and 10-fold increases ($P<0.001$ for each), at 8 h and 24 h, respectively, with Hspa1b remaining upregulated at 24 h, whereas StAR peaked at Day 14 (15-fold, $P<0.0001$) and had returned to baseline on Day 21. Both Trp53 and Casp8 were upregulated ($P<0.05$) on Day 14, whereas Bcl-2 was decreased ($P<0.05$)

on Days 7 and 14. In conclusion, acute mild heat stress severely reduced sperm quality and based on mRNA, there was upregulation of chaperone and antioxidant systems and Trp53-dependent intrinsic and extrinsic apoptotic pathways, with deleterious effects on sperm, spermatocytes and spermatids. These findings provided insights into the pathophysiology of heat stress and should contribute to development of evidence-based approaches to mitigate effects of testicular heating.

Key words: testicular thermoregulation; Trp53; apoptosis; apoptotic pathways; testes; semen; mouse

1. Introduction

In mammals, testicular temperature must be 3-5 ºC below body core temperature for production of motile and fertile sperm sperm (Lunstra et al., 1997; Kastelic et al., 1996; Cook et al., 1994). A long-standing paradigm, based on very limited data, is that increased testicular temperature is not accompanied by increased testicular blood flow; consequently, reductions in motile and morphologically normal sperm and fertility following increased testicular temperature are attributed to hypoxia (Hamilton et al., 2019; Waites and Setchell, 1964). However, in recent studies in rams and bulls, increased testicular temperature was accompanied by increased testicular blood flow, with no signs of hypoxia (Rizzoto et al., 2020;2018), implying direct effects of testicular hyperthermia on spermatogenesis and sperm.

Cellular responses to heat stress include chaperone and antioxidant systems (Durairajanayagam et al., 2015; Paul et al., 2008; Rocket et al., 2001). However, models used to study these systems usually involve intense and/or prolonged heat exposure (Kastelic et al.,

2019; Durairajanayagam et al., 2015; Wechalekar et al., 2008), which are not the most typical heat stress conditions encountered by animals, either in nature or production systems. Furthermore, few studies have been performed on the male reproductive system, the genes studied have often belonged to a specific family, or changes in gene expression were not well characterized over time.

Another important consideration is the apoptotic pathway that causes cell death following heat stress. Although hypothesized (Durairajanayagam et al., 2015; Paul et al., 2008), confirmation of the apoptotic pathway in testes has apparently not been reported. There are two pathways in the Trp53-dependent apoptotic pathway causing heat stress-related apoptosis (Gu et al., 2015); they are important markers of cellular apoptosis, as described for testes under oxidative stress (Duan et al., 2016) and in cryptorchid mice (Yin et al., 2002). In the intrinsic pathway, Trp53 downregulates the anti-apoptotic gene Bcl-2 which after some intermediate steps, activates a caspase cascade, whereas in the extrinsic pathway, Trp53 upregulates function of the FAS-FADD complex that activates Casp8, causing apoptosis (Durairajanayagam et al., 2015). One function of Hsp70 is downregulation of the TrP53 gene, thereby inhibiting the apoptotic cascade (Gu et al., 2015).

In various models of systemic heat stress or increased testicular temperature, blood testosterone concentrations were decreased (Rhynes and Ewing, 1973; Minton et al., 1981). There are indications that the steroidogenic acute regulatory protein (StAR) gene, with a critical role in steroidogenesis, was down-regulated by heat stress (Bozkaya et al., 2017). Therefore, involvement of this gene in testicular heat stress pathophysiology should be clarified.

Our objectives were to investigate impacts of mild acute heat stress on sperm and testes and to elucidate involvement of StAR and Trp53 genes and Trp53-dependent intrinsic and

extrinsic pathways in pathophysiology of testicular heat stress. We tested the hypotheses that mild acute heat stress: 1) upregulates mRNA in chaperone and antioxidant systems; 2) causes early downregulation of StAR mRNA; and 3) upregulates the mRNA of Trp53-dependent apoptosis factors in both intrinsic and extrinsic pathways.

6.3 Materials and methods

6.3.1 Animals and experimental design

Forty-eight C57BL/6 elite male mice (9 wk old) were used. These mice were housed individually, with *ad libitum* access to food and water. Mice were randomly allocated into six groups (eight mice per group) and intervals from heat exposure to euthanasia and collection of samples were: 8 and 24 h and 7, 14 and 21 d (plus a control group at 14 d). Heat exposure was designated Day 0. This study was reviewed and approved by the University of Calgary, Health Sciences Animal Care Committee (Protocol AC17-07).

6.3.2 Anesthesia and heat exposure

For heat exposure, a water-bath at constant temperature (40 ºC) was used, with water temperature monitored while the water-bath was in use. Prior to immersion in water, mice were anesthetized with a combination of 10 mg/kg of xylazine (Rompun, Bayer, Mississauga, ON, Canada) and 40 mg/kg of alfaxolone (Alfaxan, Abbott Laboratories, Ltd, Saint-Laurent, QC, Canada), diluted in distilled water, combined and injected intraperitoneal. To support the mice

during water exposure, a raft was made from a 20-mL plastic syringe case that had been cut in half lengthwise, with the interior surface and cut edges covered with tape. Mice were secured on the raft with a rubber band (~8 mm wide), placed transversely, approximately half-way between the front and rear legs and encircling the abdomen and underside of the plastic case. The raft was placed at an angle of approximately 45°, with the distal third of the body (including the scrotum) immersed for 20 min in water at either 40 or 30 ºC (heat treatment and control groups, respectively). Following water bath exposure, mice were allowed to spontaneously recover from anesthesia and then returned to their cages until they were euthanized and samples collected.

6.3.3 Sampling, testicular histology and sperm analyses

At designated sampling times, mice were euthanized by cervical dislocation and weighed. Then, the scrotum was opened and testes removed and weighed and paired testicular weight as a percentage of body weight calculated. To recover sperm, epididymides were excised, washed in PBS at 37 ºC, then immediately placed in a 1 mL drop of TALPH (Zhang et al., 2012) on a warming plate at 37 ºC and minced with small scissors. After 15 min, the fluid was mixed and an aliquot was collected to assess motility using a computerized system with a mouse-specific sperm program (CASA Spermvision®, Version 3.5.6.2, Minitube, Verona, WI, USA), with eight fields evaluated for each sample, assessing motility, progressive motility and other kinetic end points (e.g. linearity average path velocity, etc). A second aliquot was removed and used to prepare: 1) slides stained with eosin/nigrosin for sperm morphology analysis (Mortimer, 1985; Barth and Oko, 1989); and 2) unstained slides that were fixed and used for evaluation of acrosome integrity with fluorescein-conjugated *Pisum sativum agglutinin* (FITC PSA), as

described (Lybaert et al., 2009). For each of these two analyses, 200 sperm were assessed with phase-contrast microscopy (1000 X).

One testis was snap-frozen and stored at -80 ºC for subsequent RNA extraction, whereas the other testis was processed for histology and used to assess seminiferous tubule diameter. For this, pieces of testicular parenchyma (~ 0.5 cm^3) were fixed in Bouin's for 24 h, then washed and placed in 70% alcohol. Samples were embedded in blocks of paraffin and sections (5-µm thick) were prepared, placed on slides and stained with hematoxylin and eosin. Slides were examined at 400 x with a Leica RM 2500 microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA) with a camera attached. Digital images were captured and evaluated with the ImageJ® software (National Institutes of Health, Bethesda, MD, USA), with 100 tubules/slide subjected to cross-sectional evaluation (Berndtson et al., 1989).

6.3.4 Recovery of mRNA and reverse transcription to form cDNA

To assess gene expression using real time polymerase chain reaction (PCR), Trizol® reagent mediated extraction was performed by homogenizing ~80 mg of testis in 1 mL of Trizol® (Invitrogen, Burlington, ON, Canada), in accordance with manufacturer's protocol. Total RNA was diluted in 100 µL of UltraPure ™ water (Invitrogen), concentration determined with spectrophotometry (Nanodrop 2000/2000c, Thermo Fisher Scientific, Waltham, MA, USA) and the sample stored at -80 ºC. Samples were subsequently thawed on ice and 1 µg RNA was digested at 65 ºC for 10 min with DNAse I, Amplification Grade (Invitrogen) to avoid DNA contamination during synthesis of complementary DNA (cDNA). Immediately after incubation, samples were reverse-transcribed using a commercial kit, in accordance with the manufacturer's

instructions (High Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor, Applied Biosystems, Foster City, CA, USA).

6.3.5 Housekeeper gene and primers

Based on samples from all groups, among the tested housekeeping genes, the Hypoxanthine phosphoribosyltransferase 1 (Hprt1) was selected, as it was stable (P>0.05) among treatments in its amplification pattern in RTPCR. Primers (Table 1) were designed using the Primer-BLAST online system (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer efficiency was confirmed using 10-fold serial dilutions of cDNA pooled from samples; all primers used in this study had PCR efficiency from 90-110%. In addition, to confirm stability of the possible housekeeping genes to be used, PCR amplification was done with the respective primers and samples from each group, with no difference (P>0.05) in results, confirming stability of the housekeeping gene across treatments. Furthermore, specificity of each primer was confirmed with melt-curve analysis, with a single melting temperature obtained for each product.

6.3.6. cDNA purification and real time PCR

Following reverse transcription, cDNA was purified (E.Z.N.A.® Cycle Pure Kit, V-spin; Omega Bio-Tek, Norcross, GA, USA) and its concentration determined with a NanoPhotometer® NP80 system (IMPLEN, Westlake Village, CA, USA) and adjusted to 10 µg/µL. Each PCR reaction had a total volume of 10 µL, comprised of 5 µL of SYBR Green (Fast SYBR master mix, Applied Biosystems), 0.5 µL (each) 10 µM forward and reverse primers, 1 µL of cDNA and

3 µL of UltraPure ™ water. Reactions were optimized to achieve maximum amplification efficiency for each gene (90-110%). Furthermore, product specificity was based on evaluation of a dissociation curve. Samples were analyzed in duplicate and a non-template control was used as a negative control. Results were expressed as fold changes (increased or decreased expression relative to control). Furthermore, relative differences in mRNA of target genes were calculated as follows: $\Delta\Delta Cq = \Delta Cq\ (sample) - \Delta Cq\ (calibrator)$ (Schmittgen and Lyvak, 2008; Livak and Schmittgen, 2001).

Table 6.1 Primers used for RT-qPCR to assess mRNA abundance of genes in testicular tissues.

Gene	Forward sequence	Reverse sequence	Product length	NCBI accession no
HRPT1	5'CCATCACATTGTGGCCCTCT3'	5'AATGTAATCCAGCAGGTCAGCA3'	70	NM_013556.2
*HSP*70	5'GTGCACTGTACCAGGGGATTAT3'	5'CCCAGGCTACTGGAACACTG3'	95	NM_0302013
GPX1	5'CTTCCCTGTTCCTCGATGGG3'	5'CATCGCTTTCTTTCCGGCTG3'	91	NM_008160.6
StAR	5'ACTTCAGGAGTGTGCCTTCG3'	5'GTGCTTGCTGCCTACCCTAA 3'	70	NM_011485.05
BCL2	5'AGGATAACGGAGGCTGGGAT3'	5' TCACTTGTGGCCCAGGTATG 3'	152	NM_0097415
CASP 8	5' TGAGAGGCTCTACCCTCCAG 3'	5' TTCAGGCCAGCAAAAGACCC 3'	146	NM_006495634.4
P53	5'TGCACGTATCTCCTCCCTCAA3'	5'TCCGTCATGTGCTGTGACTT3'	141	NM_009812.2

6.5 Statistical analyses

For all end points, a normal distribution was verified and differences among groups determined by one-way ANOVA, with a Tukey-Kramer test used to detect differences.

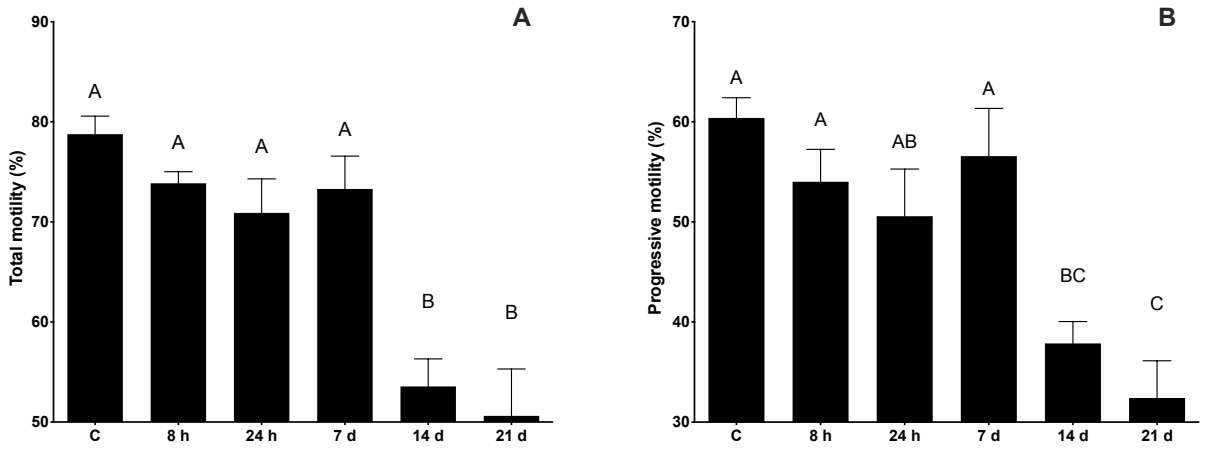
GraphPad Prism software, Version 8.2.1 (GraphPad Software Inc., La Jolla, CA, USA) was used for all analyses and data are presented as mean ± SEM. Differences were considered significant when $P \leq 0.05$.

6.4. Results

Both total and progressive motility (Fig. 6.1A and 6.1B, respectively) were markedly decreased on Days 14 and 21. There were no significant differences for any other sperm kinetic end point, as measured by the CASA system (data not shown).

At 8 and 24 h, there was a small (albeit not significant) increase in the proportion of sperm with midpiece defects. Percentage morphologically normal sperm was significantly decreased on Day 7 and remained low until Day 21 (Fig. 6.2A), with the predominant morphological abnormality being midpiece defects (Fig. 6.2B). Acrosome integrity was significantly lower at 8 h and reached a nadir at Day 14 (Fig. 6,2C).

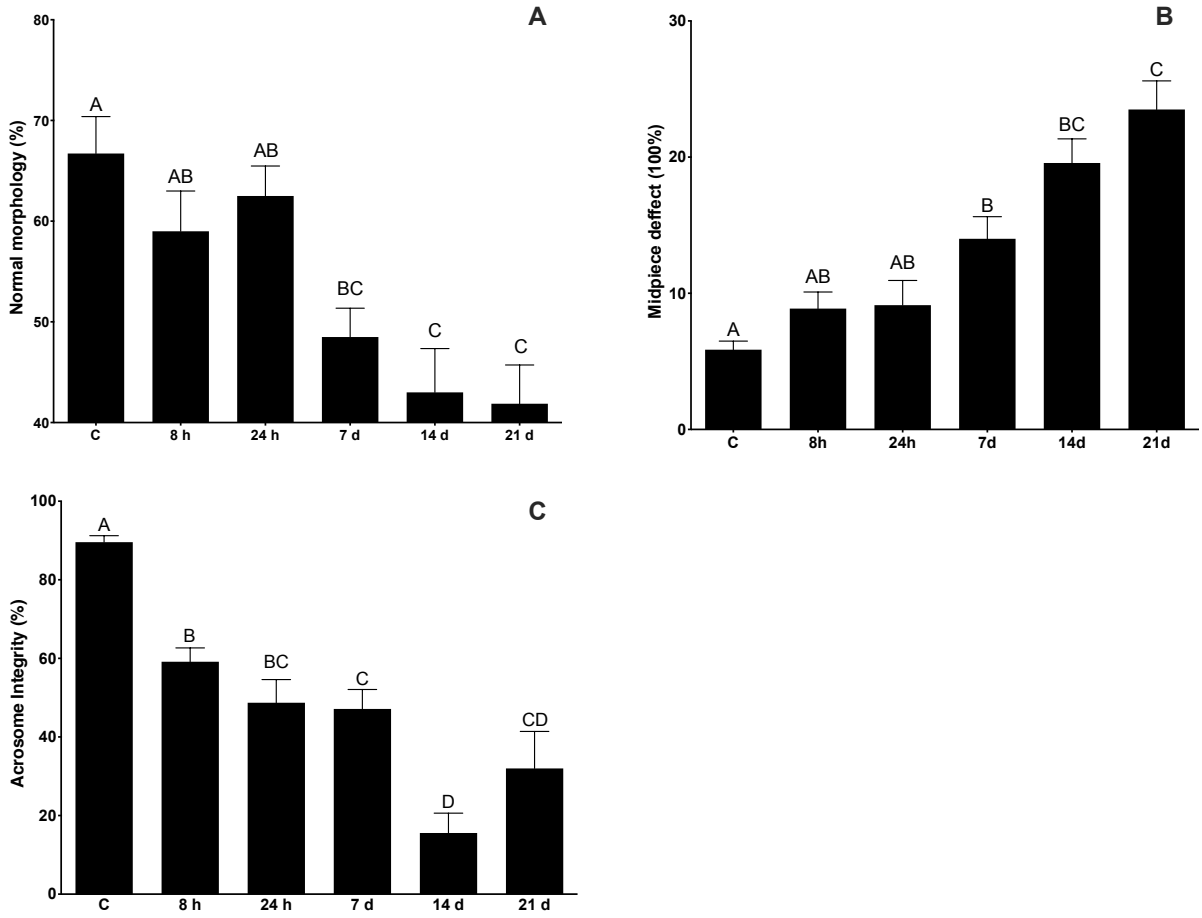
Figure 6.1 Epididymal sperm kinetics (mean ± SEM) in control mice (C) and mice at various intervals after exposure to acute mild testicular warming (40 ºC for 20 min).



[A-C]Within an end point, means without a common superscript differed (P<0.05).

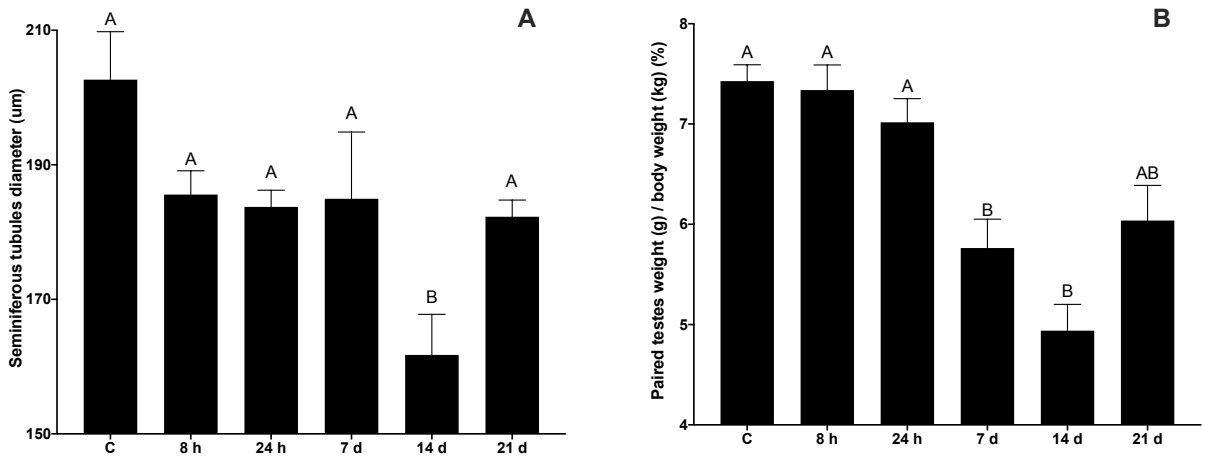
After heat exposure, there were significant reductions in seminiferous tubule diameter on Day 14 and in testicular weight as a percentage of body weight on Days 7 and 14 (Fig. 6.3). Regarding modifications in gene expression, Hsp70 (Hspa1b) and Gpx1 had fold increases (P<0.001) at 24 and 8 h, respectively, with Hspa1b remaining upregulated at 24 h (Table 6.2). Furthermore, StAR peaked at Day 14 (P<0.05) before returning to baseline on Day 21 (Table 6.2). In addition, Trp53 and Casp8 were both upregulated (P<0.05) on Day 14 (Table 2), whereas Bcl-2 had a marked decrease (P<0.05) on Days 7 and 14 (Table 2).

Figure 6.2 Mean (± SEM) percentages for sperm morphology and acrosomal integrity in control mice (C) and mice at various intervals after exposure to acute mild testicular warming (40 ºC for 20 min).



[A-D]Within an end point, means without a common superscript differed (P<0.05).

Figure 6.3 Mean (± SEM) seminiferous tubule diameter and paired testes weight (relative to body weight) in control mice (C) and mice at various at various intervals after exposure to acute mild testicular warming (40 ºC for 20 min).



[A,B]Within an end point, means without acommon superscript differed (P<0.05).

Table 6.2. Mean (± SEM) fold gene expression (2-ΔΔCT) of genes of interest in C57 BL6 mice at 8 and 24 h and at 7, 14 and 21 d after exposure to acute mild testicular warming (40 ºC for 20 min), normalized relative to control (C).

Groups	CON	8 h	24 h	7 d	14 d	21 d
HSPa1b	-	1.940^{B}	10.63^{A}	1.382^{B}	0.3176^{B}	0.4258^{B}
GPX1	-	7.347^{A}	0.1899^{B}	0.03255^{B}	0.5542^{B}	0.1570^{B}
StAr	-	1.332^{B}	1.734^{B}	0.9740^{B}	15.86^{A}	1.264^{B}
Trp53	-	1.027^{B}	1.365^{B}	0.4064^{B}	8.241^{A}	0.6962^{B}
Bcl-2	-	1.523^{A}	0.5725^{AB}	0.3672^{B}	0.2247^{B}	1.508^{A}
Casp8	-	1.007^{B}	0.1675^{B}	0.3469^{B}	4.308^{A}	1.148^{B}

[A,B] Within an end point, means without a common superscript differed (P<0.05).

6.5 Discussion

To our knowledge, due to the strategic sampling schedule, this was the first study using a relatively mild, short-duration heat stress that detected significant impacts on reproductive end points, with concurrent changes in gene expression. Our hypotheses that mild acute heat stress upregulates chaperone and antioxidant systems and upregulates mRNA of Trp53-dependent apoptosis factors belonging to both intrinsic and extrinsic pathways were supported, whereas the hypothesis that it causes early downregulation of StAR mRNA was not supported.

In the present study, our assessments enabled us to relate changes in sperm quality to changes in mRNA. Only a few genes have been described as being up- or downregulated after testicular heat stress in mice, with clear involvement of heat shock proteins (Perez-Crespo et al., 2008), antioxidant systems (Wechalekar et al., 2008; Kastelic et al., 2019) and apoptotic pathways (Wechalekar et al., 2008; Kastelic et al., 2019). Additional pathways have been theorized (Durairajanyagam et al., 2015; Paul et al., 2008; Yin et al., 1997) but not thoroughly tested. Therefore, genes assessed in our study were specifically chosen as they are components of pathways implicated or suspected as being involved in responses to oxidative and heat stress in various physiological systems. Consideration should be given to the fact that our interpretations regarding molecular responses to heat stress were based solely on mRNA data.

Impaired sperm quality and production can be caused by apoptosis following heat stress (Cammack et al., 2009). The upregulation of Trp53 observed in our study is a fundamental marker of activation of the Trp53-dependent apoptotic pathway, which may function as an indicator of sperm quality in mice (Mostafa et al., 2014. The Trp53-dependent apoptotic cascade has two pathways, intrinsic and extrinsic (Durairajanayagam et al., 2015), represented by Bcl-2

and Casp8, respectively, in the present study. Regarding the intrinsic pathway, downregulation of Bcl-2 impairs the balance with Bax in the Bax:Bcl-2 ratio, leading to cell death mediated by release of cytochrome C and activation of caspases (Durairajanayagam et al., 2015; mostafa et al., 2014). Increased temperature and cryptorchidism were described as activators of the pathway in various species (Mostafa et al., 2014; Pagliari et al., 2005). Reduced Bcl-2 is associated with infertility in men; however, a physiological level was positively correlated with better sperm parameters (Mostafa et al., 2014). Regarding the extrinsic pathway, upregulation of Casp8 is positively correlated with apoptosis and is highly associated with previous activation of Fas gene by Trp53 in an upstream manner (Ding et al., 2000). Furthermore, activation of Casp8 was reported in rat testes exposed to 43 ºC for 15 min, with identification of Casp3, 6, 7 and 9 as collaborating agents in the apoptotic cascade (Ding et al., 2000).

Associations described above were likely related to the major impacts on sperm motility observed at 14 and 21 d ($P<0.05$), due to effects on spermatids and spermatocytes, respectively based on epididymal transit time (Rockett et al., 2001); both of these cell types are highly susceptible to apoptosis (Rockett et al.., 2001). Similarly, in a mouse study (Pérez-Crespo et al., 2008) with more intense testicular heat exposure (42 ºC for 30 min), at 14, 21 and 28 d after exposure, there were reductions in both total motility (from ~67% to 28, 8 and 37% respectively) and progressive motility (from ~39% to 5.5, 6.5 and 6.6%). In other studies, when mice were exposed to temperatures of 36-40 ºC for longer intervals (several hours or days), impairments in motility occurred at similar time frames (Wechalekar et al., 2008; Kastelic et al., 2019). In rats exposed to 43 ºC for 15 min, apoptotic markers (*in situ* terminal deoxynucleotidyl transferase-mediated deoxy-UTP end labelling (TUNEL) assay) were highest in spermatids and

spermatocytes (Ding et al., 2000). Based on current findings and cited literature, peak reductions in motility were detected at 14 and 21 d after exposure; therefore, at the time of heat exposure, these sperm would have been at spermatid and spermatocyte stages of development, respectively, with formation and development of the flagellum, which is crucial for both overall and progressive motility (Lehti and Sironen, 2016; Moreno and Schatten, 2000).

Remarkably, percentage intact acrosomes were significantly decreased (by approximately one-third [$P<0.05$]) at 8 h. In another study, exposing bovine sperm *in vitro* to 41 ºC for 3 h caused a five-fold increase in acrosome abnormalities when evaluated using FITC-PSA (Lybaert et al., 2009). In addition, heat stress can induce a premature acrosome reaction (Murase et al., 2007). In mice exposed to 40 ºC for 60 min or to 42 ºC for 30 min, epididymal sperm collected in the first hours after treatment had impaired DNA integrity (Sailer et al., 1997), apparently secondary to formation of ROS (Boe-Hansen et al., 2020).

In our study, the first significant decrease in normal sperm morphology and increase in midpiece defects was observed at 7 d; those sperm would have been in the epididymis at heat exposure (Durairajanayagan et al., 2015). In mice exposed to 40 ºC for 60 min, important sperm morphological abnormalities were observed from 7 to 35 d after exposure (15-50% more abnormalities, $P<0.01$) (Murase et al., 2007). Furthermore, in rams, chronic scrotal insulation (288 d) impaired morphology (50% increase, $P<0.05$)) in cells at 14 d after exposure (Hamilton et al., 2019). Similar results were also observed in cattle subjected to insulation, with ~4 fold increase in mid-piece defects on day 12 ($P<0.05$), plus increased nuclear vacuoles ($P<0.05$) and abnormal sperm head morphology ($P<0.05$) (Mostafa et al., 2014). Based on our findings and supported by the literature, spermatids and spermatocytes were the cell types most susceptible to

heat stress, which was likely caused by Trp53-dependent apoptosis. Furthermore, studies have reported important correlations between apoptosis and abnormal sperm morphology and indications that many cells will be subject to phagocytosis during epididymal transit (Ricci et al., 2002; Zorn et al., 2012).

A major consequence of a reduction in sperm quality is impairment of fertility. Although fertility was not accessed in this study, substantial deleterious effects on sperm kinetics (total and progressive motility), morphology and acrosome integrity, all of which are fundamental for fertilization and ongoing development, would assuredly have reduced fertility. The current findings provided support for previous reports in mice of reduced fertility after exposure to 43 ºC for 20 min (Rocket et al., 2001), with only one litter sired by mature sperm cell that were spermatocytes at the time of exposure ($P<0.05$) or a severe decrease in *in vitro* fertilization rate (from ~90 to 15%) and litter size (from ~7 to 1) after exposure to 42 ºC for 30 min (Paul et al., 2008). Furthermore, at 14 and 23 d after heat exposure to 42 ºC for 30 min, fertility decreased from ~80 to ~20% ($P<0.001$) (Pau et al., 2008).

Additional important aspects were impacts on testicular weight and seminiferous tubule size, with significant decreases at 14 and 7 d, respectively (Fig. 6.3 A and B). In previous studies, reductions in testicular weight were reported as early as 16 h after heat stress (Wechalekar et al., 2008), with either chronic exposure (37-38 ºC ambient temperature for 8 h/d for 3 d; (Wechalekar et al., 2008)), or greater heat intensity (43 ºC water bath for 20 min; (Rocket et al., 2001). Furthermore, similar damage has been reported in other species (Ding et al., 2000; Hikim et al., 2003). The studies described above would, likely have had a greater impact on the testes leading to total ablation of spermatids and spermatocytes (Sertoli cell only

tubules) as observed after exposure of mice to 40 ºC for 12 h (2X with an interval of 12 h), which was observed at 14 and 20 d after treatment [40], leading to a decrease in testicular weight, similar to that observed in this study.

This is apparently the first report of concurrent downregulation of Bcl-2 and upregulation of Casp8 mRNA, implying activation of both intrinsic and extrinsic Trp53-dependent apoptotic pathways after acute, mild testicular heat stress in a murine model. Involvement of these genes in causing germ cell apoptosis would have contributed to reducing seminiferous tubule diameter and testes weight, based on the fundamental contributions of spermatogenesis to both end points. However, other apoptotic mechanisms can be involved in a heat stress episode, as reported (Barth and Bowman, 1994), indicating that more studies are required to fully understand the pathophysiological impacts of heat stress on testes.

Two other important findings were described in the study, namely the upregulation of the chaperone and antioxidant systems shortly after exposure to heat stress, represented in this work by Hspa1b (Hsp70) and GPX1, respectively. Heat Shock Protein 70 (Hsp70), is one of the most important components of the Heat Shock family (Korfanty et al., 2014), fundamental in response to heat stress (Cao et al., 2009; Zhao et al., 2010). In previous studies with chronic testicular heat exposure (26-36 ºC) in rabbits or 43 ºC for 20 min, there were increases in Hsp70 and other Hsps (e.g. 60, 40 and 90) shortly after exposure (Zorn et al., 2012;Korfanty et al., 2014), indicating rapid activation of the chaperone system, similar to the present findings. Furthermore, Glutathione Peroxidase 1 (Gpx1) is one of the most important factors protecting against oxidative damage (mostly ROS related) following heat stress (Paul et al., 2008; Cao et al., 2009; Zhao et al., 2010). Similar to our study, exposing mice to 42 ºC for 20 min lead to 4-fold

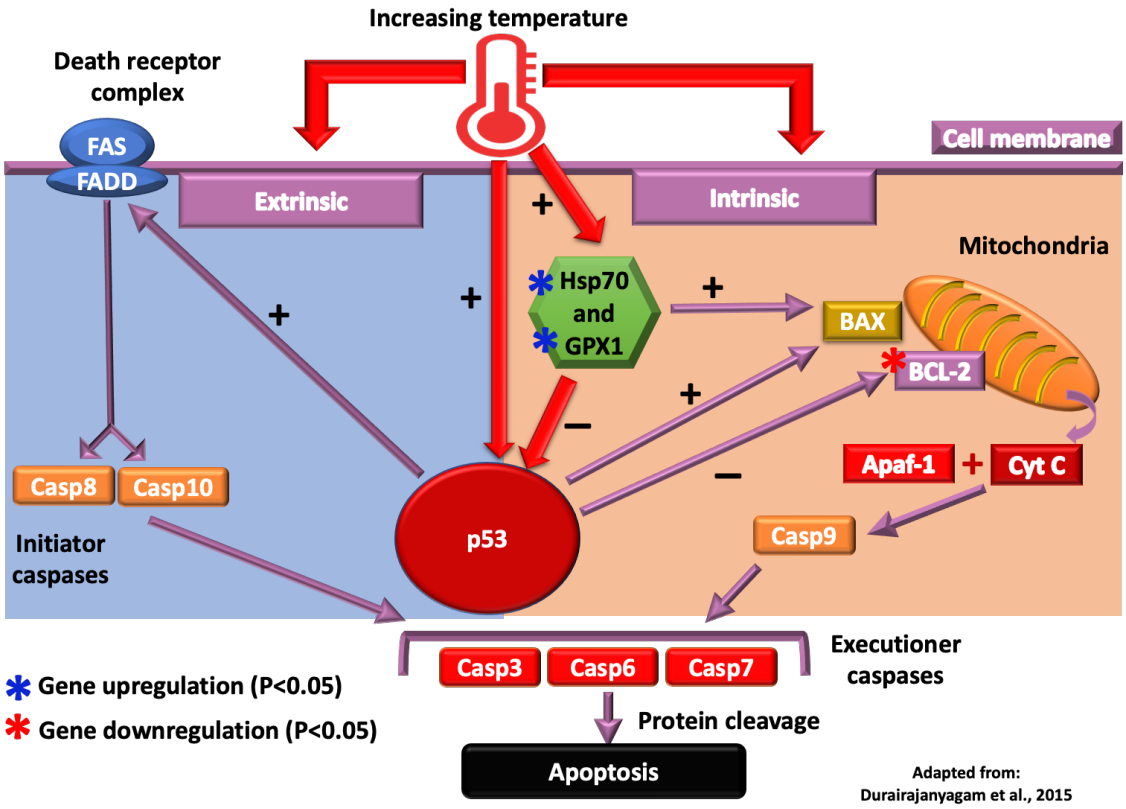
upregulation in the Gpx1 gene at 24 and 48 h after exposure (Paul et al., 2008). However, despite evidence that there was upregulation of protective responses, there were still reductions in sperm quality (Pei et al., 2012).

Lastly, it was noteworthy that Steroidogenic Acute Regulatory protein (StAR), critical for steroidogenesis, including testosterone production (Clark et al., 1996), had a significant peak at 14 d. Although there are reports of decreases in StAR and blood testosterone concentrations soon after heat stress (Au et al., 1983; Bergh and Damber, 1984; Berg et al., 1984; Keel et al., 2009), those studies were either more intense or chronic with respect to insult, whereas the current heat stress was mild and acute. Upregulation of StAR at 14 d was speculated to be a response to demands for increased testosterone to support testicular renewal (Waler, 2009), regaining normal concentrations at 21 d.

6.6 Conclusion

In conclusion, acute mild heat stress profoundly impaired sperm quality and caused reductions in both seminiferous tubule diameter and testicular weight in mice. Regarding the pathogenesis of these changes, we concluded that: 1) based on mRNA, chaperone and antioxidant systems were activated soon after heat exposure; 2) Trp53-dependent intrinsic and extrinsic apoptotic pathways were involved at the mRNA level and expected to have caused testicular cell apoptosis, resulting in deleterious impacts on spermatocytes and spermatids; and 3) mild acute testicular heat stress in mice affected sperm, including abnormal morphology, loss of acrosomes and impaired motility.

Fig. 6.3 Suggested pathways of heat stress impacts and responses in testes (adapted from Durairajanayagam et al., 2015). Heat stress activates the p53 gene, leading to the activation of the extrinsic and intrinsic p53-dependent apoptotic pathways. Regarding the extrinsic pathway, heat stress-induced upregulation of pr53 upregulates the FAS gene, leading to formation of the FAS + FADD (FAS associated death domain) complex, which upregulates initiator caspases Casp8 and 10. In the intrinsic pathway, an activated p53 upregulates BAX and downregulates BCL-2, thereby increasing the BAX-BCL2 ratio, stimulating the release of Cyt C from mitochondria and having it form a complex with Apaf-1 that activates Casp9 (initiator). The initiator caspases, activated through either the extrinsic or intrinsic pathways, promote apoptosis by signaling to executioner caspases (Casp 3,6 and 7), thereby completing the apoptotic process. Furthermore, the chaperone system (Hsp70) and antioxidant system (GPX1) are physiological responses that downregulate p53 function and as consequence, suppress apoptosis. *Fold change (P<0.05) in gene (blue = upregulation and red = downregulation).



Chapter 7: General Discussion

The question regarding how global warming and increased temperature can impair animal reproduction has been intriguing researchers for many years. It is well known that sperm quality, as well as fertility, can be severely impaired after an episode of heat stress. Historically, initial observations in comparative physiology were related to understanding how spermatogenesis took place when considering several testicular positions in various mammals, ranging from internal to ascrotal and to pendular testes (Setchell, 1978). With several studies came the understanding that testes need to be 3-5 ºC below body core temperature for proper testicular function and spermatogenesis to take place.

A research line in the early 1960s and 1970s, led by Professor Setchell, contributed immensely to the understanding of impacts of elevated temperature on testicular function. This research employed pioneering approaches to answer important questions around the theme, most importantly, to theorize about the pathophysiology of heat stress on testicular function. It is important to indicate, however, that very few studies (Waites and Setchell et al, 1964; Setchell et al., 1966,1978,1995; Mieusset et al., 1992) contributed to the development of a dogma accepted for decades; perhaps the most important paper was one published by Waites and Setchell (1964), that concluded that testes function on the brink of hypoxia, and in response to heat stress, there's an increase in testicular metabolic rate but no change in its blood flow or O_2 delivery, which therefore would lead the testes to frank hypoxia. Although these conclusions were based on very few data points, this explanation was widely accepted for several decades.

Furthermore, it is fundamental to reiterate the differences in methodology between the studies performed by Prof. Setchell and those ones described in this thesis work. Whereas we

used flow probes with higher precision and attached on the wall of the testicular artery, constituting a better approach than the utilization of, for example, color Doppler that allows a rough identification of modulation of blood flow through a transcutaneous approach (Barca et al., 2020). The approaches used by Prof. Setchell are now outdated and consisted of the utilization of microspheres (Setchell et al., 1995), tritiated water (Waites and Setchell, 1964) and Kripton^{85} (Setchell et al., 1966), being the last two approaches having inherent risks involving radioactivity. Therefore, the methodology used in this thesis which obtained blood flow data "*in* loco" using high quality measurements constitute a better tool and probably, the lack of blood flow increase observed in Setchell's previous work may be related to poor precision in the measurement methodology.

Since then, low O_2 has usually been described as the main cause behind the pathophysiology of heat stress on testes (Hamilton et al., 2018; Waites and Setchell, 1964). Interestingly, our research group conducted studies using creative approaches to further investigate the topic. The first study (Kastelic et al., 2017) constituted exposure of rams to the following treatments: an insulated versus non-insulated scrotum and 14, 21 or 85% O_2 in inspired air. Interestingly, the impacts on sperm quality observed in the insulated rams were neither replicated by hypoxia nor prevented by hyperoxia, suggesting, therefore, that temperature itself was the main pathophysiological agent of heat stress. A second study (Kastelic et al., 2019) used a similar approach; in a murine model, mice were exposed to 20 versus 36 ºC and 13, 21 or 95% O_2. Once again, no other treatment replicated nor prevented the impact caused by heat stress on the testes, supporting the conclusions of the first study. A third study (Barros-Adwell et al., 2018) in bulls also provided important fundamental knowledge to the questions asked in this thesis work. In that study, there was significantly higher blood flow at 35 vs 5 ºC (~8.2 $\pm$ 0.9

versus 4.9 $\pm$ 0.7 ml/min/100 g testes, respectively), providing direct evidence that testicular blood flow increased in response to increasing temperature.

Therefore, despite the long-standing dogma that hypoxia is the underlying pathogenesis of reductions in sperm motility, morphology, and fertility that occur when testes are warmed, there were indications that this dogma was incorrect. As a consequence, a critical question to be addressed in this thesis was as follows: **Is the long-standing dogma that increased testicular temperature does not cause increased blood flow and the resulting hypoxia is responsible for decreases in sperm motility, morphology and fertility, actually incorrect? In addition to refuting the role of blood flow and hypoxia in the pathogenesis of heat-induced changes in sperm, involvement of the *P53*-dependent intrinsic and extrinsic pathways on the pathophysiology of heat stress on testes and sperm quality were also characterized.**

This thesis research was carefully designed, based on the information briefly reviewed above. As described, when rams and mice were exposed to systemic hypoxia, sperm defects associated with testicular hyperthermia were not replicated. Testicular hypoxia is associated with changes in testicular morphology, as well as spermatogenic arrest and reductions in sperm quality and motility (Farías et al. 2013; Saxena et al., 1995); however, the damage described is usually associated with chronic hypoxia, mostly hypobaric hypoxia, replicating conditions at high altitudes (Verrati et al., 2008) or due to testicular torsion (Loboda et al., 2010) or varicocele (Damsgaard et al., 2008). Therefore, in Chapter 2, we hypothesized that in the testes, as described for other systems (Treml et al., 2018; Harris et al., 2013; Adachi et al., 1976), hypoxia would cause increased testicular blood flow to support the increased testicular metabolic rate with no signals of hypoxia, as systemic O_2 decreased. The experimental model used was rams under general anesthesia, sequentially exposed to 100, 21 and 13% O_2, essentially replicating the

previous experimental conditions used for mice (Kastelic et al., 2019). With the sequential reductions in O_2, there was clear cardiovascular compensation, a significant increase in testicular blood flow and conductance providing increased O_2 that maintained O_2 delivery. This, plus the increase in O_2 extraction, supported the increased metabolic rate, with no signs of hypoxia or impaired metabolism.

The above-described findings indicated that: 1) compensatory mechanisms in the testes maintained increased metabolic demands, even under hypoxic conditions; and 2) testes had a strong capacity to modulate their blood flow, which was described in detail for apparently the first time. In addition to hypoxia, hyperthermia was the other main effect in the 2x2 factorial studies (Kastelic et al., 2018) that were the impetus for this thesis. As discussed above, despite the long-standing dogma that testicular hyperthermia did not increase testicular blood flow, the only evidence for this was a study (Waites and Setchel, 1964) that used only four rams. When testicular temperature was increased from 39 to 42 ºC, there actually was an increase in testicular blood flow, but no statistical difference, probably due to inadequate statistical power. Furthermore, it was noteworthy that no increase in lactate was observed. Many studies have based their methodologies on the previously mentioned work (Hamilton et al., 2018), without verifying that the conclusions were valid. The above-described questions regarding the present state of knowledge and data obtained in Chapter 2 contributed to ideas described in the remainder of the thesis.

In Chapter 3, we used essentially the same ram model that we used in Chapter 2, except that instead of decreasing O_2, we warmed the testes and we tested the hypothesis that under heat stress, there is an increase in testicular blood flow, without signs of hypoxia as testicular temperature increased. Indeed, an elevation in testicular temperature caused significant increases

in testicular blood flow, conductance, O_2 delivery and extraction, all of which supported the elevated testicular metabolic rate, without any signs of hypoxia nor metabolic impairment. These findings supported our hypothesis and contributed to debunk the long-standing dogma that no increase in blood flow occurred under testicular heat stress.

Chapters 2 and 3 really built on the previous studies published by our laboratory (Kastelic et al., 2017,2018,2019; Barros-Adwell et al., 2018), clearly demonstrating that if there is inadequate testicular oxygenation, either due to: 1) systemic hypoxia; or to 2) increased testicular temperature that increases testicular metabolism, increases in both blood flow (to deliver O_2 to the testes) and testicular extraction of O_2 from arterial blood can compensate and sustain aerobic metabolism, with no signs of hypoxia.

The limitations to the model used in Chapters 2 and 3 were that the rams were maintained under general anesthesia, which can substantially alter blood flow. However, application of epidural anesthesia and local anesthetic blocks at incision sites enabled us to use a relatively light plane of anesthesia. Furthermore, given the time and effort required to isolate the blood vessels and install flow probes and catheters and the need to create three sequential conditions (varying oxygenation or testicular temperature), at each condition, blood flow and oxygenation were only measured for 45 min. In a future study, perhaps the ideal model would be to install flow probes and catheters in the animals under anesthesia, then with utilization of appropriate restraining methods, perform measurements in non-anesthetized or lightly sedated animals. In Chapters 2 and 3, a sequential reduction in O_2 or increase in temperature, respectively, were used, in lieu of having the various treatments in a randomized order, as that was the fastest and easiest way to conduct these studies. Furthermore, it is noteworthy that hypoxia (13% O_2 in inspired air) and 40 ^{o}C (testicular temperature), were the final treatments in their respective experiments. This was

done purposefully, as these are quite harsh conditions and likely would have had carry-over effects on the other treatments if used in the beginning of the study.

In Chapters 2 and 3, rams were used as an experimental model, as they are relatively inexpensive and could be brought into Dr. Tyberg's specialized cardiovascular laboratory at the Foothills campus. However, our long-term interest was to understand scrotal/testicular thermoregulation in bulls. In general, there are two distinct biological types of bulls, *Bt*, which are of European origin, and *Bi*, which are native to Asia (although both types are now widely distributed beyond their places of origin). In general, *Bt* cattle are best adapted to relatively temperate climates, whereas *Bi* cattle are much better adapted to warm environments. Differences between *Bi and Bt* regarding thermoregulatory capabilities are well known. For *Bi* bulls, several aspects have been described as advantages regarding temperature modulation, such as skin morpho-physiological aspects including range of hair and skin color and highly efficient sweat-glands (Amakiri et al., 1974). Furthermore, another study compared differences in the vascularity of the testicular cone of *Bi, Bt and* crossbred bulls (Brito et al., 2004). Interestingly, it was observed that better thermoregulatory capability was associated with pendulosity of the scrotum, thinner testicular arteries and closer proximity between the testicular artery and vein in the testicular vascular cone, all parameters important morphophysiological differences between *Bi* when compared to *Bt bulls*, providing additional evidence for fundamental differences between cattle breeds and adding to knowledge regarding testicular thermoregulation.

Based on the above-indicated information and the knowledge obtained in our two previous studies (Chapters 2 and 3) we developed another study. In Chapter 4, we had an opportunity to study not only the response of testicular heat stress in cattle using critical end

points, but also to compare the difference in the response of *Bi* (Nelore) and *Bt* (Angus), representing a thermotolerant vs a thermosensitive breed, respectively.

Our hypotheses were that: 1) testicular blood flow increases in response to elevation of testicular temperature in cattle; and 2) *Bi* bulls have better ability to modulate testicular blood flow and O_2 delivery in response to heat stress than *Bt* bulls. The methodology used in this study was similar to that used in Chapters 2 and 3, with important differences.

In Chapters 2 and 3, in the ram model, the FiO_2 was adjusted (26.14 ± 1.1%) to maintain physiological PaO_2 (93.1 ± 12.7 mmHg, mean ± SD). However, in Chapter 4, an FiO_2 of 98.0 ± 1.12% was used for the cattle, leading to higher O_2 saturation of the blood, which may have introduced confounding factors between studies. Furthermore, the work in Chapter 4 was relatively more difficult, due to the size of the animals and intrinsic challenges with general anesthesia in cattle, as well as the level of instrumentation required.

Therefore, we sought collaborators and a location to enable us to perform the study, as well as a collaborative grant proposal to provide the required financial support. We developed an international partnership with Dr. João Carlos Pinheiro Ferreira at UNESP (São Paulo State University), Brazil, with whom we co-applied and obtained financial support from FAPESP (Research Support Federation of the State of São Paulo). Within UNESP in the campus of Botucatu, we had a wonderful opportunity to conduct our research and we received excellent support and cooperation from the researchers, students and staff.

Data from this study supported the first hypothesis; there were significant increases in testicular blood flow and conductance and an improved O_2 delivery rate, with adequate O_2 availability to support the increased testicular metabolic rate, without indications of hypoxia (no

changes in lactate concentrations) nor any indications of metabolic impairment. Furthermore, the absence of difference in extraction rate ($P>0.05$; Fig. 4.1D), could have been caused by the higher FiO_2 used (98 ± 1.12 %), what would restrain the activation of the higher O_2 extraction state.

Another important difference observed between the studies in Chapters 2 and 3 (rams) and 4 (cattle), was the variation in hemoglobin concentrations and changes throughout the studies. There was an observed decrease ($P<0.05$) in hemoglobin concentrations, with the increase in testicular temperature in Chapter 3; although there was a difference between the *Bi* and *Bt* throughout the study, only a non-significant increase ($P>0.05$) was observed with the increase in the testicular temperature. A very plausible explanation for the differences observed are that rams and bulls have very different volumes of blood, and the multiple sampling required in the study could have contributed to the observed reduction ($P<0.05$). Furthermore, the long anesthetic period, could have caused the decrease in hemoglobin concentrations, due to sequestration of hemoglobin in the spleen or non-splenic sites during anesthesia (Ceylam et al., 2007; Wilson et al., 2004). The increased FiO_2 (98 ± 1.12%) in Chapter 4 (cattle) and the reduction observed in hemoglobin concentrations in Chapter 3 (rams) were confounding factors that contributed to differences in the extraction levels, which increased in Chapter 3 ($P<0.05$), but did not change ($P>0.05$) in Chapter 4.

Furthermore, the above indicated can be further explained when considering two fundamental formulas used to determine O_2 content and O_2 delivery (Table 7.1). To illustrate this point, data from rams in Chapter 3 and from *Bi* bulls in Chapter 4 were compared. Based on the CaO_2 formula (Table 7.1), SaO_2, PaO_2 and hemoglobin concentration are factors that can modulate the CaO_2.

Table 7.1. Formulas for CaO2 and TDO2.

CaO_2 = arterial O_2 content (ml/dl)	CaO_2= (SAO_2 * Hb[] * 1.34) + (0.0031 * PAO_2)
Testicular O_2 delivery (ml/min)	TDO_2 = Q(t) * CaO_2/100

(Caulkett et al., 1996)

The higher FIO_2 used in Chapter 4, besides modulating saturation as indicated above, also had profound impacts on PAO_2 (Tables 7.1 and 7.2). Furthermore, the higher overall hemoglobin concentrations in *Bi* (12.92 ± 0.5) and *Bt* bulls (11.93 ± 0.85 g/dl), when compared to rams (9.66 ± 0.1 g/dl; Chapter 3), also contributed to a higher CaO_2. Both breeds of bulls in Chapter 4 had higher hemoglobin concentrations than rams in Chapter 3, with data from *Bi* bulls used to illustrate the point.

Due to the higher FIO_2 and the higher hemoglobin concentrations in bulls in Chapter 4 when compared to rams in Chapter 3, there was a clear difference in TDO_2 when comparing both studies at 37 and 40 ºC (Table 7.2). More specifically, as described in Fig. 3.3 D, at 40 ºC, the rams entered in a high O_2 extraction state that was not observed in bulls in Chapter 4. At 40 ºC, due to the modulations caused by FIO_2 and hemoglobin concentration, there's roughly a 75% increase in the TDO_2 (Table 7.2), which explains why bulls did not enter a high extraction state and therefore no changes in extraction rate were observed. Therefore, the higher FIO_2 used in Chapter 4 was an important confounding factor and fundamental difference between studies in Chapters 3 and 4.

Table 7.2. TDO2 values at various temperatures for rams and bulls in Chapters 3 and 4.

O_2 delivery (ml/min) at various testicular temperatures (ºC)	Rams (Chapter 3)	*B. indicus* bulls (Chapter 4)
34	1.69 ± 0.19	1.54 ± 0.19
37	1.87 ± 0.17	2.03 ± 0.15
40	2.10 ± 0.13	3.56 ± 0.31

Data are mean ± SD.

However, no significant difference between breeds was observed. Therefore, the well-known thermoresistance of *Bi* could be related to other factors than the vascular response. Possible explanations include the vascular morphophysiological differences described above (Jian et al., 2013; Brito et al. 2004), or perhaps genetic differences that provide better protection at the molecular level. Regardless, the most important finding was that both *Bi and Bt* bulls had similar capabilities to modulate testicular blood flow to address metabolic needs under heat stress exposure.

One of the challenges of the study, similar to that described for Chapters 2 and 3, was the inability to randomize order of treatments, due to the intense nature of the exposure to 40 ºC, as previously indicated in Chapter 3. Furthermore, all bulls underwent all three treatments (35, 37 and 40 ºC). Furthermore, we used two of the important representative breeds of *Bt* and *Bi*, Angus and Nelore, respectively. A continuation of the work described in Chapter 4 could involve utilization of more breeds of both species and expand findings in a breed-specific manner. Furthermore, another approach would be exposure to an individual treatment for a longer interval (ideally a few hours instead of 45 min, as performed). Alternative approaches have been described, such as utilization of Doppler to measure modulations in testicular blood flow using non-anesthetized animals (Barca Junior et al., 2019), However, in our perspective, no other

method exceeds the performance of the flow probes used in Chapters 2 to 4, although they do have a limitation regarding maintaining animals under general anesthesia, but compensate in accuracy and precision of the data obtained.

Chapters 2 to 4 involved measurement of testicular blood flow and O_2 delivery. In those studies, there was very good evidence that in response to either systemic hypoxia or increased testicular temperature, there was increased testicular blood flow, delivering more O_2 to the testis, coupled with greater removal of O_2 from the blood and uptake by the tissues, thereby ensuring adequate provision of O_2 to support tissue needs and absolutely no indications of hypoxia. Therefore, these new findings, in combination with previous studies (mouse and ram papers), provided good evidence that the long-standing dogma that testicular hyperthermia causes testicular hypoxia, is simply incorrect.

Alternatively, the collective evidence was that there were direct effects of testicular hyperthermia that were responsible for the decrease in sperm quality. Therefore, in Chapter 5, the aim was to investigate changes at the molecular level. In previous studies, the involvement of factors related to either intrinsic or extrinsic routes in the *P53*-dependent apoptotic pathway had been described, but concurrent assessment of factors in both routes after heat stress had apparently never been described. Furthermore, studies reported involvement of chaperones and antioxidant molecules in the testicular response (Hamilton et al., 2018; Pei et al., 2012) as well as reductions in testosterone concentrations (Minton et al., 1981; Rhynes and Ewing, 1973), a hormone conferring protection to germ cells undergoing heat exposure.

In Chapter 5, our objective was to further investigate gene modulation and impacts on testosterone concentrations after testicular heat stress. The aim was to investigate how gene expression was modified in cattle after testicular heat stress. The methodology applied was to

expose the bulls to scrotal insulation and subsequently collect testicular samples, so the description of gene modulation in a timely manner could be done, apparently for the first time. We hypothesized that testicular hyperthermia: 1) reduces testicular testosterone concentrations; and 2) alters expression of candidate genes in testes.

Nelore (*Bi*) were used, as they were more readily available than *Bos taurus* bulls (and there was an expectation that the latter bulls would be more readily available in Canada if there was an opportunity to replicate this study). The information obtained in the study was fundamental to provide important insights regarding how *Bi* bulls are impacted by heat stress in relation to gene activity and testosterone concentrations. Increased testicular temperatures caused a severe reduction in testicular testosterone concentrations, with two major fronts of gene modulation, namely: 1) attempted protective response to heat stress through upregulation of *GPX1* (antioxidant system) and Hsp70 (Chaperone system); and 2) how heat stress affected testicular cells through reduction of *StAR* gene and testicular testosterone concentrations and downregulation of BCL-2 which has antiapoptotic activity, associated with the intrinsic route of the *P53*-dependent apoptotic pathway.

The data obtained, including profoundly reduced testicular testosterone concentrations enabled us to elucidate some of the mechanisms involved in the pathway through which: 1) *Bi* bulls responded to heat stress, i.e. increased expression of antioxidant molecules (*GPX1*) and upregulation of chaperones (*HSP*70); and 2) how heat stress damaged testicular cells, including down regulation of *StAR* and BCL-2, fundamental for spermatogenesis and sperm quality and anti-apoptotic activity, respectively.

After the conclusion of the study and availability of results, the intensity of the response was apparent. Unfortunately, due to logistics, only a 48-h exposure was possible. Furthermore, as

bulls had to be castrated for testicular tissue sampling, to conduct a more prolonged study we would either need to use either more bulls or an alternative methodology, such as testicular biopsy through time or even hemicastration, which in our perspective constitute a severe risk to inflammation or non-specific modifications that could be attributed to treatment and not to testicular heating.

A future approach could include a longer sampling period and analysis of more genes, possibly those associated with the P53-dependent apoptotic pathway and steroidogenesis. Therefore a future study, ideally involving both *Bt* and *Bi* bulls, with more frequent sampling, should extend our understanding of testicular gene modulation after heat stress, involvement of the intrinsic and extrinsic routes of the *P53*-dependant pathway and whether there are differences between the species. The investigation should be expanded to more genes, since in other systems when the *P53*-dependent apoptotic pathway was inhibited (Yin et al., 2002) in animals exposed to heat stress, apoptosis was delayed and reduced, but still present, indicating participation of other apoptotic pathways that remain to be identified.

Another interesting finding was related to steroidogenesis. Modulations on RNA levels were identified for the *StAR* gene (downregulation at 24 and 48 h after exposure) with a significant reduction in testicular testosterone concentrations at 48 h versus control bulls. Although *StAR* is fundamentally important in steroidogenesis and a rate-limiting step in the process, it is not the only responsible factor. Therefore, a broader study investigating other enzymes and factors associated with the steroidogenic pathway would certainly produce important information. Although the study produced fundamental advances in the topic, only *B. indicus* bulls were used. Therefore, the next study should include *Bt* bulls or perhaps both *Bt* and *Bi* bulls, to enable a contemporaneous comparison.

Furthermore, another gap to be filled is related to how heat stress exposure of testes during spermatogenesis can impair ability of sperm to participate in fertilization, support embryo development and how it can modulate gene expression in the embryos produced. The literature has several studies available regarding embryos exposed to heat stress (Silva et al., 2013; Eberhardt et al., 2009), but not related to embryos derived from males exposed to testicular hyperthermia, nor the impact on spermatogenic stages (at the time of exposure) on embryo development and gene expression.

A future study involving both species cattle would allow having sperm from *Bi* and *Bt* bulls fertilizing oocytes from each of these two species, generating the following genetic backgrounds (♂x♀): *Bi x Bi; Bi x Bt; Bt x Bt* and *Bt x Bi.* The above-described methodology would allow a thorough investigation on how heat stress impacts males of different species and also species contributions towards heat resistance/susceptibility (untreated females). Developing a gene panel to select heat-resistant sires could be a long-term and important goal, given the profound impact of heat stress on cattle production and reproduction, as well as effects on other species and indeed humans, especially in the context of global warming.

Although the information obtained in Chapter 5 provided great insights in the understanding of the heat stress exposure, the study involved only acute exposure (48 h). Furthermore, more genes of interest and end points remain to be investigated, using a longer sampling period and more animals. To reach that goal, we decided to use a murine model, since mice are very widely used in biomedical research, have a large variety of analytical tools available and are well established for molecular studies. Furthermore, they are much cheaper than cattle, enabling the use of more treatments.

Based on the above described, we pursued a last study (Chapter 6) to complement the findings obtained in the previous chapters. The objectives were to investigate: 1) impacts of mild acute heat stress on sperm and testes; and 2) involvement of *StAR* and P53 genes and P53-dependent intrinsic and extrinsic pathways. We tested the hypotheses that mild acute heat stress: 1) upregulates chaperone (Hsp70) and antioxidant systems (*GPX1*); 2) causes early downregulation of *StAR*; and 3) activates *P53*-dependent apoptosis through both intrinsic (*BCL2*) and extrinsic (*CASP* 8) genes pathways. Results supported all the above-indicated hypotheses, as there was: 1) profound impairment of sperm quality (namely sperm kinetics and morpho-functional aspects); 2) involvement of the chaperone and antioxidant systems (upregulation of *HSP*70 and *GPX1*, respectively); and 3) upregulation of *P53* and modulation of factors associated with the *P53*-dependent apoptotic pathway (intrinsic and extrinsic routes, *BCL2* and *CASP* 8, respectively).

The limitations of Chapter 6 were associated with an inability to complete some of our goals. Our main goal was to extend our findings, going beyond gene modulation to protein expression (Western blot) and localization of protein expression (immunohistochemistry). However, although background studies had been performed, further work was impossible due to COVID19 and closure of most of the university laboratories.

There are important future steps to be taken to promote further understanding of the testicular heat stress pathophysiology. For example, we could search for specific and clear impacts on stages of sperm cell development, with more profound impacts likely on spermatids and spermatocytes, and even could narrow the impact specifically to a cell type. However, in both Chapters 5 and 6, findings were from the testes as a whole, and there is much appeal to pursue cell type-based differences. Therefore, the next steps could be related to assessment of

cell type-specific responses to heat stress, using cell sorting or staining, for example. Another interesting approach would be genetic ablation of factors involved in the response to heat stress and study resulting impacts and modifications.

Furthermore, an interesting possibility is the association of various techniques to the PCR analysis performed in our study, as indicated in Chapter 5, which ideally should have included quantification of protein levels (Western Blot) and immunolocalization of the protein in the testicular architecture, allowing cell-specific protein localization. Another possibility would be utilization of cell sorting based on specific markers for various spermatogenic and testicular cells, enabling further RNA expression and protein quantification analyses. Specifically, gene modulation approaches such as RNA sequencing or utilization of gene panels associated with each pathway observed in the study would contribute to a more complete understanding of the pathophysiological pathway.

It is fundamental to consider individual differences regarding heat stress response; although the obtained data indicated a common line of response, it is clear that the possibility of variation in the response rate is plausible within the same breed or group of individuals. Therefore, a more extensive study with more animals would allow identification of resistant animals within a group of similar individuals and provide important insights regarding morphological and genetic traits associated with heat resistance. Adaptation to the climate, may also play a fundamental aspect on the response to increased heat load. In a very elegant design comparing *Bi* bulls to *Bt* bulls adapted and non-adapted, the *Bi* bulls followed by the adapted *Bt*, had lower rectal temperatures, larger scrotal circumferences and fewer morphologically abnormal sperm (Wildeus and Hammond, 1994), indicating that for future studies, adaptation also have to be investigated. Lastly, another fundamental parameter to be considered, is the

association of trace element deficiencies, which is more common in tropical regions and the impact of testicular hyperthermia, more specifically how that impairs the antioxidant response by GPX1, as element trace deficiencies potentiate the oxidative damage (Shazia et al., 2012).

Another important aspect to be discussed is that regarding impact on the testes, studies including full body exposure to local (testes) heat stress present very similar impacts on sperm quality, spermatogenesis and steroidogenesis in either an acute or chronic exposure (Rocket et al., 2015; Kastelic et al., 2020). Physiologically, full body exposure to heat stress leads to prostration, reduction in feed and water intake and an overall health impact (Rojas-Downing et al., 2017), recent fever episodes also are related to reproductive impairment (Carlsen et al., 2003, while the same is not observed for local testes exposure. To our knowledge, although he heat impact in the testes may differ, the overall outcome in the testes follows the same pattern, more detailed studies will need to be developed to investigate possible confounding factors when the whole body is impacted by an increase in core temperature.

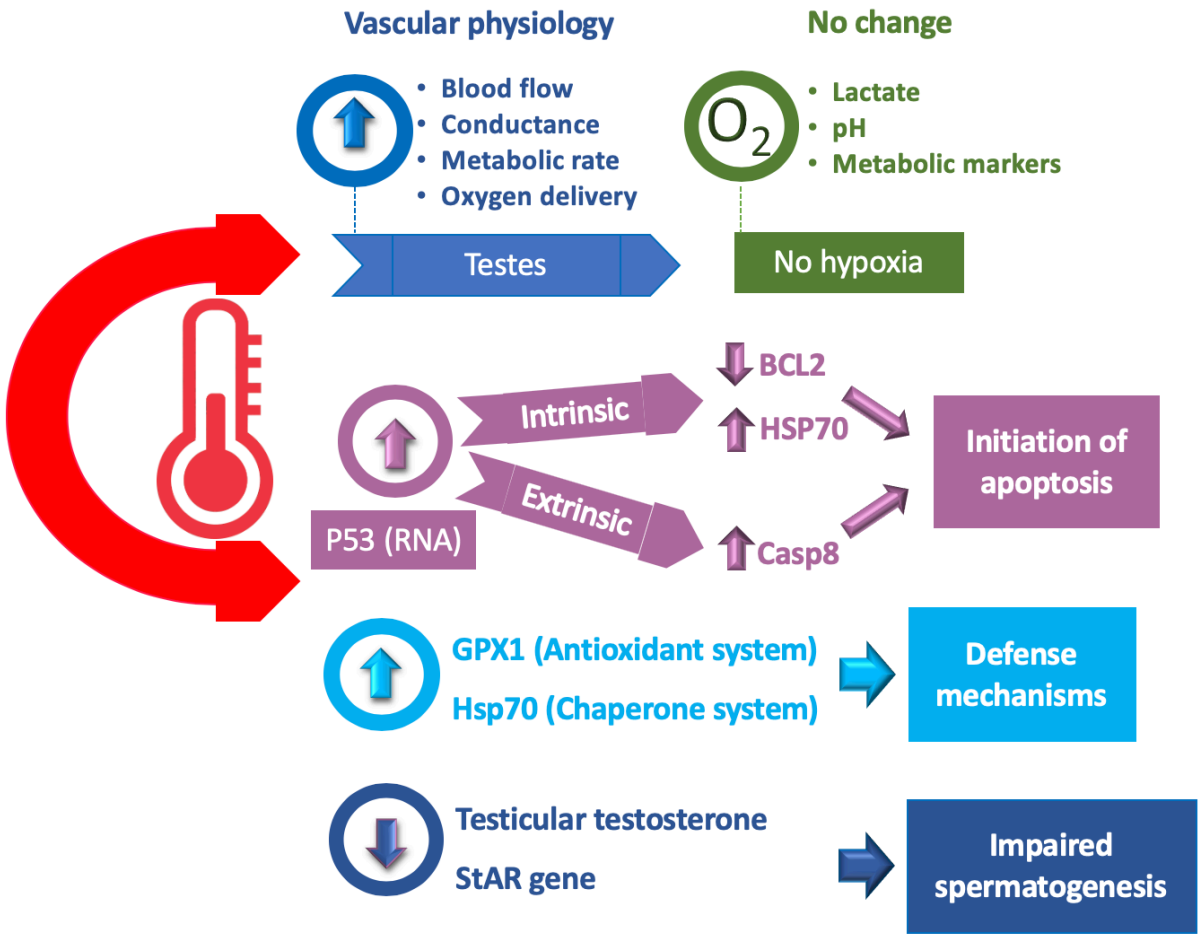
From an overall perspective, much of the data collected in this thesis were novel and assisted in addressing several important questions, to varying extents. When experiments associated with this thesis work were initially proposed, the long-standing dogma, that under heat stress, there is no increase in testicular blood flow and the resulting hypoxia is the main pathogenic agent in the impaired sperm quality and fertility, had not been discarded, although there were in doubt, due to recent studies in our lab. However, Chapters 2, 3 and 4 were key studies that critically tested and ultimately changed the above-described paradigm. Based on the data generated, it is now clear that the there is modulation of testicular blood flow to supply its metabolic needs, without signs of hypoxia or impaired metabolism in rams exposed to high

testicular temperatures or low O_2 concentrations and in *Bi* and *Bt* bulls exposed to high testicular temperatures.

Furthermore, in Chapters 5 and 6, there were indications regarding involvement of the *P53*-dependent apoptotic pathway and the impacts of *StAR* and testosterone concentrations in association with impaired sperm morphology and motility. Involvement of the *P53*-dependent apoptotic pathway as one of the main effectors of cellular apoptosis after heat stress became clear in both murine and bovine models, leading to new knowledge and providing new avenues to study how heat stress can impair testicular functions and spermatogenesis. The overall description of the findings obtained in the previous chapters has been summarized in Figure 7.1, taking into consideration blood flow, O_2 delivery and uptake and the molecular findings obtained in this thesis.

Revisiting the overall aim of this thesis work, we concluded that testicular blood flow and O_2 delivery and uptake increased to sustain tissue oxygenation when animals were exposed to either hypoxia or hyperthermia. The findings contributed to debunk the long-standing dogma stating that no changes in testicular blood flow occur under heat stress, opening a new avenue of research based on impacts of temperature itself. Furthermore, the work presented provide novel information towards understanding the pathophysiology of heat stress on testes and testicular function and for apparently the first time implicate involvement of the intrinsic and extrinsic routes of the *P53*-dependent apoptotic pathways, the activity of the chaperone and antioxidant systems, as well as modulations in *StAR* gene and testosterone concentrations.

Figure 7.1 Proposed overall summary of vascular and molecular response of testes to heat stress.



The results of our studies provided novel information regarding testicular vascular physiology and O_2 dynamics under heat stress and also described several factors associated with the pathophysiology of heat stress in the testes. These findings constitute a new pathway to investigate how heat stress impairs spermatogenesis and fertility in mammals. Lastly, it is expected that the above-described findings will serve as a strong base for new studies on the topic and on development of a new understanding on how heat stress affects reproduction in male mammals.

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APPENDIX A: PERMISSION TO PUBLISH

Chapters 1 to 5 are published (as noted below). As indicated on the websites of each of the three journals, these papers can be included in a graduate student thesis.

- Chapter 1: Rizzoto G and Kastelic JP. A new paradigm regarding testicular thermoregulation in ruminants? Theriogenology 2020:15:147-66.
- Chapter 2: Rizzoto G, Hall C, Tyberg JV, Thundathil JC, Caulkett NA, Kastelic JP. Increased testicular blood flow maintains oxygen delivery and avoids testicular hypoxia in response to reduced oxygen content in inspired air. Scientific Reports 2018;8:10905.
- Chapter 3: Rizzoto G, Hall C, Tyberg JV, Thundathil JC, Caulkett NA, Kastelic JP. Testicular hyperthermia increases blood flow that maintains aerobic metabolism in rams. Reproduction Fertility and Development 2019;31:683-8.
- Chapter 4: Rizzoto G, Ferreira JCP, Mogollón Garcia HD, Teixeira-Neto FJ, Bardella LC, Martins CL, Silva JRB, Thundathil JC, Kastelic JP. Short-term testicular warming under anaesthesia causes similar increases in testicular blood flow in *Bos taurus* versus *Bos indicus* bulls, but no apparent hypoxia. Theriogenology 2020;145:94-9
- Chapter 5: Rizzoto G, Ferreria JCP, Codgnoto VM, Oliveira KC, Mogollón Garcia HD, Pupulim AGR, Teixeira-Neto FJ, Castilho A, Nunes SG, Thundathil JC, Kastelic JP. Testicular hyperthermia reduces testosterone concentrations and alters gene expression in testes of Nelore bulls. Theriogenology, 2020:152;64-8.