Heat stress and antioxidant agents: Effects on gamete development

Dissertation to obtain the Degree of Doctor by:

Maria José Maya Soriano

Under the direction of:

Manel López Béjar

Dpt. de Sanitat i d'Anatomia Animals

PhD program: Animal Medicine and Health

Dpt. of Animal Health and Anatomy

Faculty of Veterinary

Universitat Autònoma de Barcelona

November, 2012

Assessment Committee

Board members

Pedro Lorenzo (Universidad Complutense de Madrid, Spain)

Jordi Miró (Universitat Autònoma de Barcelona, Spain)

Ramiro Alberio (University of Nottingham, UK)

Alternate 1: Ali Fouladi (Royal Veterinary College, UK)

Alternate 2: Xavi Manteca (Universitat Autònoma de Barcelona, Spain)

External evaluators

Rhiannon E. Lloyd (University of Portsmouth, UK)

Cinzia Allegrucci (University of Nottingham, UK)

This doctoral thesis was supported by Spanish Ministry of Science and Innovation, MICINN, Ref. RTA2008–00070-C02–02. MJ Maya-Soriano was supported by a PIF Grant from Universitat Autònoma de Barcelona.

A mis padres y hermana,

por todo su apoyo y comprensión.

(...hacia atrás, ni para coger impulso)

Recordarás algo de lo que leas, bastante de lo que oigas, mucho de lo que veas, y todo lo que hagas.

John Little B.

CONTENTS

LIST OF TABLES

Chapter I:

Chapter II:

Chapter IV:

Chapter V:

Chapter VI:

LIST OF FIGURES

Literature review:

Chapter I:

Chapter II:

Chapter III:

ABSTRACT

The general aim of this thesis has been the study of the effects of high temperatures on gamete development and how the use of antioxidant agents supplemented to the maturation medium and sperm storage/freezing extenders could counteract the negative effects of heat stress and/or the excessive production of reactive oxygen species (ROS), in order to find additional methods to improve fertility during the warm season of the year.

In chapter 1, the influence of heat stress on bovine oocyte maturation were evaluated and compared with overmatured oocytes by the use of a logistic regression. Based on the odds ratio, heat-stressed (41.5ºC, 18-21h of maturation; HSO) and overmatured (28h of maturation; OMO) oocytes were, 14.5 and 5.4 times more likely to show anomalous metaphase II (MII) morphology, and 6.3 and 9.3 times more likely to show cortical granule (CG) distribution pattern IV (aging oocyte), respectively, than control oocytes (38.5ºC; CO). Taking into account both maturation processes, the risk of undergoing anomalous oocyte maturation was 17.1 and 18 times greater in oocytes cultured in HSO and OMO groups, respectively, than those in the CO group. Hence, heat stress proved to be valuable in aging oocytes by advancing nuclear and cytoplasmic processes in a similar form to that of oocyte overmaturation.

The aim of chapter 2 was to compare by a logistic regression the effects of heat stress on bovine oocyte maturation from oocytes collected during the cold (February-March) or warm (May-June) periods of the year. Based on the odds ratio, HSO were 26.83 and 13.01 times more likely to show an anomalous MII morphology and CG pattern IV (aging oocyte), respectively. However, only a significant interaction between season of collection and treatment was found in terms of cytoplasmic maturation, being oocytes collected during the cold season 25.96 times more likely to show an anomalous maturation when exposed to the heat treatment. From this chapter, it can be concluded that exists a higher tolerance to heat stress from oocytes harvested in the warm season compared to those collected in the cold period of the year.

In chapter 3, the presumptive protective effects of antioxidant agents (retinol, retinyl and oleic acid) on maturation medium were evaluated on bovine HSO. Based on a Chi-square test ($P < 0.05$), heat stress affected negatively the MII progression ($P < 0.05$) and produced a premature CG exocytosis ($P < 0.01$). On the other hand, in terms of antioxidant supplementation, retinol allowed to improve the oocyte MII progression under heat stress conditions ($P = 0.031$), although retinyl and oleic acid, at the concentrations used in this study, could not counteract adverse effects of HS. Hence, retinol proved to be valuable in heat-stressed oocytes protecting nuclear maturation.

Chapter 4 aimed to assess the effect of long exposure to summer circadian heat stress cycles (31ºC, 3 hours/day) on sperm parameters and the motile-subpopulation structure of epididymal sperm cells from rabbit bucks, by a PROC GLM procedure. Sperm total motility and progressivity were negatively affected by high temperature ($P < 0.05$), affecting also specific motility parameters ($P < 0.05$). According to motile sperm-subpopulations derived from a FASTCLUS procedure, heat stress significantly increased ratio of less motile subpopulations, although maintaining percentage of the high motile subpopulation. Hence,

the induced changes in sperm motility produced by environmental heat stress are linked to concomitant changes in the proportion of motile sperm-subpopulations of the epididymis, although these changes did not affect the subpopulation with the highest motile epididymal sperm cells.

In the chapter 5, the effect of antioxidant agents (bovine serum albumin, retinol and retinyl) supplemented at different concentrations into a commercial freezing extender was evaluated in order to improve post-thaw rabbit sperm quality since cryopreservation increases ROS levels. In general, all the sperm quality parameters were negatively affected by the cryopreservation process, specially total motility. However, the addition of antioxidant agents did not improve thaw-sperm quality, although retinyl supplementation seems to be toxic. More studies are required in order to find the appropriate antioxidants and their most effective concentrations, which will improve rabbit post-thaw sperm quality.

The aim of the last chapter was assess the effect of high temperatures on bull epididymal sperm cells and how the addition of retinol as antioxidant agent in the storage extender could improve sperm quality parameters. Sperm samples were submitted to four different temperatures (4, 22, 32 and 41.5ºC for 3 hours). Hence, sperm quality parameters are mainly affected by high temperatures (41.5ºC) and the addition of retinol to the storage extender did not show any effect on sperm quality parameters with an exemption of the percentage of altered acrosomes, which was reduced in presence of retinol. Thus, retinol may stabilize sperm acrosomal membrane in situations of oxidative stress due to high temperatures.

RESUMEN

El objetivo principal de esta tesis es el estudio de los efectos de las altas temperaturas en el desarollo gamético y cómo el uso de agentes antioxidantes suplementados a los medios de maduración o mantenimiento/congelación de espermatozoides podría contrarrestar los efectos negativos del estrés térmico y/o de la excesiva producción de radicales libres de oxígeno (ROS), con la finalidad de encontrar métodos adicionales que mejoren la fertilidad durante la época cálida del año.

En el capítulo 1 se ha evaluado la influencia del estrés térmico sobre la maduración de ovocitos bovinos en comparación con los efectos producidos por una sobremaduración ovocitaria mediante regresión logística. En base a las odds ratio, los ovocitos estresados térmicamente (41.5ºC, entre las 18-21h de maduración; HSO) y ovocitos sobremadurados (28h de maduración; OMO) tienen 14.5 y 5.4 veces más probabilidades de presentar metafase II (MII) anómalas, y 6.3 y 9.3 veces más probabilidades de mostrar patrón tipo IV de distribución de gránulos corticales (GC), respectivamente, en comparación con los ovocitos madurados bajo condiciones fisiológicas (38.5ºC). Teniendo en cuenta ambos procesos de maduración, el riesgo de que se produzca una maduración ovocitaria anómala es 17.1 y 18 veces mayor en HSO y OMO, respectivamente, en comparación con el grupo control. Así pues, el estrés térmico produce un envejecimiento ovocitario mediante la aceleración de los procesos nucleares y citoplasmáticos de una forma similar a los que se producen durante la sobremaduración.

El objetivo del segundo capítulo fue comparar mediante regresión logística los efectos del estrés térmico en la maduración ovocitaria según si estos son obtenidos en la época fría (Febrero-Marzo) o cálida (Mayo-Junio) del año. Según las odds ratio, HSO tuvieron 26.83 y 13.01 más probabilidades de presentar una MII anómala y un patrón de GC tipo IV (ovocitos envejecidos), respectivamente. Sin embargo, sólo pudo detectarse la interacción entre la época del año y el tratamiento al que fueron expuestos los ovocitos en cuanto a maduración citoplasmática, siendo los ovocitos obtenidos durante la época fría 25.96 veces más probables de mostrar una maduración anómala cuando se someten a estrés térmico. Así pues, de este segundo capítulo se puede concluir que existe una mayor tolerancia al estrés térmico cuando los ovocitos provienen de la época cálida del año en comparación con los obtenidos en la época fría.

En el capítulo 3, se evaluó el supuesto efecto protector de agentes antioxidantes (retinol, retinyl y ácido oleico) suplementados en el medio de maduración de HSO bovinos. Basándonos en el test Chi-square ($P < 0.05$), el estrés térmico afectó negativamente la progresión nuclear a estadio de MII ($P < 0.05$) y produjo una exocitosis prematura de GC ($P < 0.01$). Por otro lado, en cuanto al suplemento de antioxidantes, retinol mejoró la progresión de los ovocitos a MII cuando estos estaban sometidos a altas temperaturas ($P = 0.031$), aunque retinyl y ácido oleico no pudieron contrarrestar los efectos del estrés térmico a las concentraciones utilizadas en este estudio. Así, retinol parece ser válido protegiendo la maduración nuclear en ovocitos estresados térmicamente.

El objetivo del capítulo 4 fue evaluar el efecto de una exposición prolongada a ciclos circadianos de estrés térmico (31ºC, 3 horas/día) en los parámetros espermáticos y la estructura de las poblaciones

espermáticas móviles de espermatozoides epididimarios de conejo, mediante el procedimiento PROC GLM. La motilidad total y progresividad se vieron afectadas negativamente por las altas temperaturas ($P < 0.05$), afectándose también los parámetros de motilidad específicos ($P < 0.05$). En relación a las poblaciones espermáticas móviles derivadas del procedimiento FASTCLUS, el estrés térmico incrementó la ratio de subpoblaciones menos móviles, aunque los porcentajes de subpoblaciones más móviles se mantuvieron. Así, los cambios en la motilidad espermática inducidos por el estrés térmico ambiental están ligados a cambios en las proporciones de las distintas subpoblaciones espermáticas del epidídimo, aunque estos cambios no afectaron la subpoblación más móvil.

En el capítulo 5, se evaluó el efecto del suplemento de agentes antioxidantes (albúmina sérica bovina, retinol y retinyl) a distintas concentraciones en un medio de congelación comercial como método para mejorar la calidad de los espermatozoides descongelados de conejo, ya que la congelación produce un incremento de los niveles de ROS. Por lo general, todos los parámetros de calidad espermática se vieron afectados negativamente por el proceso de congelación, especialmente la motilidad total. Sin embargo, el hecho de añadir agentes antioxidantes no mejoró la calidad espermática post-congelación, aunque el suplemento con retinyl parece ser tóxico. Por todo ello, es necesario realizar más estudios con el fin de encontrar el antioxidante apropiado y la concentración más efectiva con la cual pueda mejorarse la calidad espermática post-congelación.

El objetivo del último capítulo fue evaluar el efecto de las altas temperaturas sobre los espermatozoides epididmarios de toro y cómo suplementar retinol al medio de mantenimiento como agente antioxidante podría mejorar los parámetros de calidad espermática. Las muestras espermáticas fueron sometidas a cuatro temperaturas diferentes (4, 22, 32 y 41.5ºC durante 3 horas). Los parámetros de calidad espermática se vieron afectados principalmente por las altas temperaturas (41.5ºC). Por otro lado, el suplementar retinol en el medio de mantenimiento no mostró ningún efecto sobre los parámetros de calidad espermática con excepción del porcentaje de acrosomías, el cual se redujo en presencia de retinol. Así pues, el retinol podría estabilizar la membrana acrosomal de los espermatozoides en situaciones de estrés oxidativo debido a las altas temperaturas.

RESUM

L'objectiu principal d'aquesta tesi es l'estudi dels efectes de les altes temperatures en el desenvolupament gamètic i com l'us d'agents antioxidants suplementats als medis de maduració o manteniment/congelació d'espermatozoos podria combatir els efectes negatius de l'estrès tèrmic i/o de l'excés de producció de radicals lliures d'oxigen (ROS), amb la finalitat de trobar mètodes adicionals que millorin la fertilitat a les èpoques càlides de l'any.

En el capítol 1 s'ha avaluat la influència de l'estrès tèrmic sobre la maduració d'oòcits bovins en comparació amb els efectes produïts per una sobremaduració oocitària mitjançant regressió logística. En base a les odds ratio, els oòcits estressats tèrmicament (41.5ºC, entre les 18-21h de maduració; HSO) i oòcits sobremadurats (28h de maduració; OMO) tenen 14.05 i 5.4 vegades més probabilitats de presentar metafase II (MII) anòmales, i 6.3 i 9.3 vegades més probabilitats de mostrar patró tipus IV de distribució de gränuls corticals (GC), respectivament, en comparació amb els oòcits madurats sota condicions fisiològiques (38.5ºC). Considerant tots dos processos de maduració, el risc que es produeixi una maduració oocitària anòmala és 17.1 i 18 vegades major en HSO i OMO, respectivament, en comparació amb el grup control. Així doncs, l'estrès tèrmic produeix un envelliment oocitari mitjançant l'acceleració dels processos nuclears i citoplasmàtics d'una forma silimar als que es produeixen durant la sobremaduració.

L'objectiu del capítol 2 va ser comparar mitjançant regressió logística els efectes de l'etrès tèrmic sobre la maduració oocitària segons si aquests s'obtenen en l'època freda (Febrer-Març) o càlida (Maig-Juny) de l'any. Segons les odds ratio, HSO van tenir 26.83 i 13.01 més probabilitats de presentar una MII anòmala i un patró de GC tipus IV (oòcits envellits), respectivament. No obstant això, només es va poder detectar la interacció entre l'època de l'any i el tractament al què van ser exposats els oòcits en relació a la maduració citoplasmàtica, siguent els oòcits obtinguts durant l'època freda 25.96 vegades més probables de mostrar una maduració anòmala quan es sotmeten a estrès tèrmic. Així doncs, d'aquest segon capítol es pot concloure que existeix una major tolerància a l'estrès tèrmic quan els oòcits provenen de l'època càlida de l'any en comparació amb els obtinguts de l'època freda.

En el capítol 3, es va avaluar el possible efecte protector d'agents antioxidants (retinol, retinyl i àcid oleic) suplementats en el medi de maduració de HSO bovins. Basant-nos en el test Chi-square ($P < 0.05$), l'estrès tèrmic va afectar negativament la progressió nuclear a estadi de MII ($P < 0.05$) i va produir una exocitosi prematura de GC ($P < 0.01$). D'altra banda, segons el suplement d'antioxidants, el retinol va millorar la progressió dels oòcits a MII quan aquests estaven sotmesos a altes temperatures ($P = 0,031$), encara que retinyl i àcid oleic no van poder contrarestar els efectes de l'estrès tèrmic a les concentracions utilitzades en aquest estudi. Així, el retinol sembla vàlid protegint la maduració nuclear en oòcits estressats tèrmicament.

L'objectiu del capítol 4 va ser avaluar l'efecte d'una exposició prolongada a cicles circadiaris d'estrès tèrmic (31ºC, 3 hores/dia) sobre els paràmetres espermàtics i l'estructura de les poblacions

espermàtiques mótils d'espermatozous epididimarios de conill, mitjançant el procediment PROC GLM. La motilitat total i progressivitat es van veure afectades negativament per les altes temperatures ($P < 0.05$), afectant també els paràmetres de motilitat específics ($P < 0.05$). En relació a les poblacions espermàtiques mótils derivades del procediment FASTCLUS, l'estrès tèrmic va incrementar la ratio de subpoblacions menys mótils, encara que els percentatges de les més mótils es van mantenir. Així, els canvis en la motilitat espermàtica induïts per l'estrès tèrmic ambiental estan lligats a canvis en les proporcions de les diferents subpoblacions espermàtiques de l'epidídim, encara que aquests canvis no afecten a la subpoblació més mótil.

En el capítol 5, l'efecte del suplement d'agents antioxidants (albúmina sèrica bovina, retinol i retinyl) a diferents concentracions en un medi de congelació comercial va ser avaluat com a mètode per millorar la qualitat dels espermatozous de conill després de congelar, ja que la congelació produeix un increment dels nivells de ROS. En general, tots els paràmetres de qualitat espermàtica es van veure afectats negativament pel procés de congelació, especialment la motilitat total. No obstant això, afegir agents antioxidants no va millorar la qualitat espermàtica post-congelació, i a més el suplement amb retinyl sembla ser tòxic. Per tot això, cal realitzar més estudis per tal de trobar l'antioxidant apropiat i la concentració més efectiva que pugui millorar la qualitat espermàtica post-congelació en el conill.

L'objectiu de l'últim capítol va ser avaluar l'efecte de les altes temperatures sobre els espermatozous epididmaris de toro i com el suplement del medi de manteniment amb retinol com a agent antioxidant podria millorar els paràmetres de qualitat espermàtica. Les mostres espermàtiques van ser sotmeses a quatre temperatures diferents (4, 22, 32 i 41.5ºC durant 3 hores). Els paràmetres de qualitat espermàtica es van veure afectats principalment per les altes temperatures (41.5ºC) i el suplement amb retinol en el medi de manteniment no va mostrar cap efecte sobre els paràmetres de qualitat espermàtica amb excepció del percentatge d'acrosomíes, que es va reduir en presència de retinol. Així doncs, el retinol podria estabilitzar la membrana acrosomal dels espermatozous en situacions d'estrès oxidatiu degut a les altes temperatures.

INTRODUCTION

INTRODUCTION

One of the main purposes of livestock is the production of meat, milk and eggs, which has grown steadily during the past 40 years (FAO, 2011). The global production derived by is necessary by human population (FAO, 2009).

Cattle are able to convert forage and crop residues, which are useless for human consumption, into edible products (milk and meat). Milk production in Mediterranean countries is based in high industrialization of the process, where only herds with high production per cow are economically rentable (Line, 1986). But, despite this rapid progress in management and genetics, reproductive performance of high producing dairy herds has suffered a dramatic decrease since the mid 1980's (Beam & Butler, 1999; Royal *et al.*, 2000; Lucy, 2001; López-Gatius, 2003). On the other hand, rabbits are valuable for meat production, which is linked to the development of rabbit breeding programs, developed since the 1970's in Spain, which is one of the main producer and consumer countries in Europe (FAO, 2001; Baselga, 2004). Rabbits are also a common laboratory mammal used for experimental studies that are applied to human medicine (Suckow & Douglas, 1997).

It has been described that the most comfortable range of environmental temperatures for production/reproduction of dairy cattle and rabbits is between -0.5° and 20°C (Johnson, 1987) and 12-21ºC (Verga *et al.*, 2007), respectively. However, the Earth system has been changing rapidly over the past several decades due to natural and anthropic factors, and the reported data suggest a significant increase in heat stress pressure on global human population, animals and plants, displacing life from their thermal comfort zone (Lecha, 2007). In accordance, animals in our geographical area are submitted to high temperatures round 20-31 days during the warm months (May-September) and even 4 days during the cold months of the year (October-April; López-Gatius *et al.*, 2006).

Heat stress is one of the major causes of summer low fertility in livestock (Wolfenson *et al.*, 2000) and their impact has been extensively reviewed in dairy cattle (De Rensis & Scaramuzzi, 2003; López-Gatius, 2003; Collier *et al.*, 2006; Roth, 2008). Conception rate is reduced by high environmental temperatures from 40-60% in winter to 10-20% in summer (Wolfenson *et al.*, 2000; Tseng *et al.*, 2004; De Rensis & Scaramuzzi, 2003; Bridges *et al.*, 2005; Ju *et al.*, 2005; Lopez-Gatius 2003). However, in autumn the conception rate is still low (30%) although animals are not submitted to high temperatures anymore (Roth *et al.*, 2000). An explanation is the affectation of antral follicles during the summer (Roth *et al.*, 2001b; De Rensis & Scaramuzzi, 2003; Roth *et al.*, 2008), being necessary 3 or 4 oestrus cycles after high temperatures for fertility restoration (Roth *et al.*, 2001a; Roth *et al.*, 2008).

The preovulatory follicle is sensitive to high environmental temperatures (Ball *et al.*, 1983), and the reactive oxygen species (ROS) derived from are able to provoke abnormal chromosome segregation (Blondin *et al.*, 1997; Roth *et al.*, 2008), anomalies in the development to metaphase II stage (MII) and polar body extrusion, condensation and fragmentation of chromatin, reduction in meiotic spindle diameter (Tseng *et al.*, 2004; Ju and Tseng, 2004), cytoplasmic defects, premature translocation of cortical

granules (Des Torres-Júnior *et al.*, 2008), and in general, an acceleration of oocyte maturation (Schrock *et al.*, 2007). All these alterations suggest that heat stress may premature aging the oocyte, reducing its developmental competence (Payton *et al.*, 2004).

High temperatures also have negative effects on reproductive processes in male, leading to subfertility, temporal or permanent infertility (Setchell, 1998; Lue *et al.*, 1999; Yaeram et al, 2006; Tusell *et al.*, 2011). Animals submitted to heat stress show a reduction in testicular weight (Sailer *et al.*, 1997; Jannes *et al.*, 1998; Rockett *et al.*, 2001), ejaculate volume (Meyerhoeffer *et al.*, 1985) and sperm concentration (De Alba & Riera, 1966; Meyerhoeffer *et al.*, 1985). On the other hand sperm quality is also affected, and an increase in the percentage of dead and abnormal spermatozoa (Casady *et al.*, 1953; Skinner & Louw, 1966; Borg *et al.*, 1993), a loss of motility (Ax *et al.*, 1987; Marai *et al.*, 2002), alterations in chromatin stability (Pérez-Crespo *et al.* 2008; Paul *et al.* 2008) and anomalies in plasmatic and acromosal membrane integrity have been reported (Meyerhoeffer *et al.*, 1985; Borg *et al.*, 1993; Sanocka & Kurpisz, 2004; Roca *et al.*, 2005; Safaa *et al.*, 2008).

High temperatures are also linked to premature embryo loss (Ju *et al.*, 2005), mainly when high temperatures take place at the beginning of the fertile oestrus, at insemination or during the first embryo divisions (Edwards *et al.*, 1997). It has been reported that heat stress produces a reduction in the developmental competence to the blastocyst stage (Edwards & Hansen, 1997; Roth & Hansen, 2004), a reduction in the total number of cells per embryo and an increase in DNA alterations and apoptosis rate, (Paula-Lopez & Hansen, 2002) increasing embryo mortality (Ryan *et al.*, 1992). These alterations are more pronounced as the exposure to heat stress increases in duration (Ealy *et al.*, 1993; Al-Katanani *et al.*, 2002b), although embryo can become more tolerant as preimplantational development proceeds (Paula-Lopes *et al.*, 2002; Rocha *et al.*, 1998).

Hence, heat stress is a common worldwide problem affecting the 60% of the livestock population, which causes big economic losses (Wolfenson *et al.*, 2000). It is the reason why research in the last decades has been focused on improving fertility during the warm months of the year, establishing refrigeration systems for animals in the major sensitive moments (Ealy *et al.*, 1993), using shades (West *et al.*, 2003), aids for oestrus detection (Wolfenson *et al.*, 1995), diet reformulation (Wolfenson *et al.*, 1995) and breeds or cross-breeds more tolerant to heat as *Bos indicus* (West *et al.*, 2003).

The use of antioxidant agents is another interesting way of research in order to improve fertility during summer. It is known that high temperatures induce an increase of ROS levels which are involved in the harmful effects on the integrity and function of cellular membranes (Wolfenson *et al.*, 2000; Hansen *et al.*, 2001). However, ROS are also produced during normal cellular metabolism, and are necessary for some reproductive processes. In those cases, the organism can counteract the excess of ROS by antioxidant enzymes, although deficiencies in the antioxidant system or the excess of ROS that cannot be counteract results in oxidative stress. The oxidative stress causes alterations in lipids, proteins, polysaccharides, DNA and other macromolecules (Miller *et al.*, 1993). According to that, the use of antioxidant agents both *in vivo* as *in vitro* has been checked in order to improve fertility and sperm, oocyte and embryo quality

(Aréchiga *et al.*, 1998b; Tarín *et al.*, 1998; Hansen *et al.*, 2001; Yousef *et al.*, 2003; Lawrence *et al.*, 2004; Marei *et al.*, 2011).

In accordance, the aim of this thesis has been the study of high temperature effects on gamete development and how the use of antioxidant agents supplemented to the maturation medium and sperm storage/freezing extenders could counteract the negative effects of heat stress and/or the excessive production of ROS, in order to find additional methods to improve fertility during the warm season of the year.

AIMS / OBJETIVOS

AIMS

The general aim of this thesis is to figure out the heat stress effects on gamete development, which leads impairments in animal fertility causing high annual economic losses in livestock. Additionally, the current thesis tries to achieve the development of techniques and methods to decrease negative heat stress impact at the cellular level through *in vitro* studies simulating high temperatures done at the central part of the day during the warm periods of the year.

The specific aims are to:

1. Analyze in which way oocyte maturation is affected by heat stress by the development of an *in vitro* model.

2. Find if a seasonal effect exists in oocytes submitted to heat stress conditions by an *in vitro* study.

3. Develop an *in vitro* system to counteract harmful effects of high temperatures by the supplementation of antioxidant agents to the oocyte maturation medium.

4. Determine how epdidymal sperm cells are affected by high temperatures using an *in vivo* and *in vitro* study.

5. Analyze the effect of adding antioxidant agents to the storage/freezing extender in order to counteract the negative effects of heat stress or radical oxygen species derived from cryopreservation on sperm cells.

OBJETIVOS

El objetivo general de esta tesis es profundizar en el conocimiento acerca de los efectos del estrés térmico sobre el desarrollo gamético, que finalmente se expresan en una importante reducción de la fertilidad, causando graves pérdidas económicas en los animales de producción. Por otro lado, también se pretenden desarrollar técnicas o métodos que disminuyan los efectos negativos del estrés térmico mediante el desarrollo de estudios *in vitro* simulando las altas temperaturas que tienen lugar en las horas centrales del día durante el periodo más caluroso del año.

Los objetivos específicos son los siguientes:

1. Analizar de qué forma se ve afectada la maduración ovocitaria bajo condiciones de estrés térmico desarrollando un modelo de investigación *in vitro.*

2. Encontrar si existe efecto estacional en cuanto a la afectación de los ovocitos sometidos a estrés térmico mediante un estudio *in vitro.*

3. Suplementar el medio de maduración *in vitro* de ovocitos con agentes antioxidantes para contrarrestar los efectos negativos del estrés térmico.

4. Analizar de qué forma se ven afectados los espermatozoides epididimarios bajo condiciones de estrés térmico desarrollando modelos de investigación *in vivo* e *in vitro*.

5. Suplementar el medio de mantenimiento/congelación de espermatozoides con agentes antioxidantes para contrarrestar los efectos negativos del estrés térmico o de los radicales libres de oxígeno derivados del proceso de congelación.

LITERATURE REVIEW

1. GLOBAL ECONOMIC SITUATION AND MILK PRODUCTION IN THE LAST DECADES

The profitability of dairy herds greatly depends on fertility. Yet despite rapid worldwide progress in genetics and management of high producing dairy herds, the past five decades and especially since the mid 1980s, have seen a drastic increase in the incidence of reproductive disorders and infertility of dairy cows as milk production increases (Foote, 1996; Beam & Butler, 1999; Butler, 2000; Royal *et al.*, 2000; Lucy, 2001; López-Gatius, 2003) provoking a higher risk of infertility under suboptimal conditions (Rougoor *et al.*, 1999; Fahey *et al.*, 2002; Calus *et al.*, 2005). Hence, as increases in milk production correlates negatively with fertility (Pryce *et al.*, 1998; Rauw *et al.*, 1998; Abdallah & McDaniel, 2000), in recent years the main objective of development in mammalian reproductive technology has been to preserve fertility (Gosden & Nagano, 2002).

It is noteworthy that changes in the genetics and physiology of food animals for increased production are making these animals less adapted to warm environments reducing their thermoregulatory ability in the face of heat stress (Berman *et al.*, 1985) and magnifying the seasonal depression in fertility caused by this factor (Al-Katanani *et al.*, 1998; Roth, 2008). It is especially true for dairy cattle (Pryce *et al.*, 1998; Rauw *et al.*, 1998; Abdallah & McDaniel, 2000) during hot seasons in tropical and subtropical areas (Cavestany *et al.*, 1985; Roth *et al.*, 2002), where it could be commonly find a 3–5h hyperthermic stress under field conditions (Ju & Tseng, 2004).

The low fertility during the hot season is a worldwide problem affecting about 60% of the world dairy cattle population (Wolfenson *et al.*, 2000) and is being commonly associated with high ambient temperatures (Roth *et al.*, 2001b). Hence, investigation through new management practices can improve the health and fertility of dairy cows (Windig *et al.*, 2005). Other species as rabbits are also sensitive to the negative effects of high temperatures with harmful consequences in their reproductive eficiency through the summer season (Finzi, 1987; Kennou, 1990; Yamani, 1990; Finzi, 1991; Berchiche y Lebas, 1994; Morera *et al.*, 1995).

2. HEAT STRESS

2. 1. Climate definition

The climate is a combination of elements that include temperature, humidity, rainfall, air movement, radiation, barometric pressure, and ionization (Johnson, 1987). Climatic zones differ around the world and are dependent on latitude, prevailing winds, evaporative conditions, water availability, elevation, proximity to mountains and other factors (West, 2003).

2. 2. Global Warming

The Earth system has been changing rapidly over the past several decades due to natural and anthropic factors. Studies have stated an increase in the global average surface temperature of about 0.6ºC over the past 20th century. The predictions portend increments over 0.1-0.2ºC per decade (MacCracken *et al.*, 2008), with an increase in medium and maximum temperatures of 1.5-2.1ºC in 2020 (Lecha, 2007). These data suggest a significant increase in heat stress pressure on global human population, animals and plants (Lecha, 2007), displacing life from their thermal comfort zone. The effect of global warming are expected to be larger in the middle-high latitudes than in the tropics, over land areas than oceans, and larger in winter than in summer (MacCracken *et al.*, 2008).

2. 3. What is heat stress?

Heat stress can be defined as any combination of environmental variables, or the sum of forces external to the bodily system of a homeothermic animal, that acts to displace body temperature from its resting state to conditions that are higher than animal's thermoneutral zone (5-20ºC; Lee, 1965; Yousef, 1984). The environmental temperature, radiant energy, relative humidity, and wind speed all contribute to the degree of heat stress (De Rensis & Scaramuzzi, 2003).

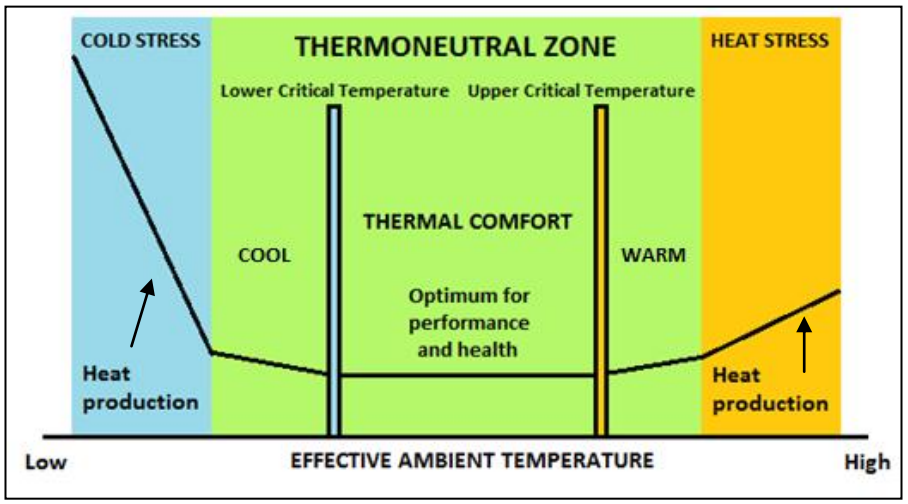
2. 4. Thermoneutral zone

Each homeotherm animal has an optimal ambient temperature zone (Thermoneutral Zone) within which metabolic rate is at minimum (Yousef, 1985; Johnson, 1987). The Thermoneutral Zone (Figure 1) is bounded by the lower critical temperature (LCT) and the upper critical temperature (UCT), which vary and depend upon age, level of production, breed and the degree of acclimatization (Fuquay, 1981; Young, 1981; Shearer & Beede, 1990b).

Thereby, the thermoneutral zone is subdivided into three subzones (Anderson & Bates, 1984; Yousef, 1985):

- **Cool Zone:** the range of ambient temperature where heat production remains minimal. The animal conserves energy by cover and tissue insulation (piloerection and peripheral vasoconstriction) and by heat production induced by cold (shivering).
- **Thermal Comfort Zone:** the range of ambient temperature where optimum productivity, efficiency, and performance is demonstrated.
- **Warm Zone:** the range of ambient temperature where heat production is minimal. The thermoregulatory responses are: a decrease in tissue insulation (vasodilatation) and an increase in heat exchange surface area by changing posture.

Figure 1. Thermal zones in animals (adapted from Yousef, 1985). The ambient temperatures above the UCT activate the thermoregulatory evaporative heat loss processes: increase the blood flow to peripheral tissues, sweating and panting, and decrease feed intake and nutrient absorption (Shearer & Beede, 1990b). Both sweating and panting require the expenditure of considerable amounts of energy, and as a consequence, the animal's heat production will rise above the minimal level in the heat stress zone (Anderson & Bates, 1984).



It has been described that the most comfortable range of environmental temperatures for milk production of dairy cattle is between -0.5° and 20°C (Johnson, 1987), while Berman *et al.* (1985) indicated that the upper critical air temperature at which Holstein cattle may maintain a stable body temperature is 25 to 26°C. This explains why high producing dairy cattle, which were originally bred in Holland and Germany (moderate climate), are less well adapted to the heat stress conditions as they prevail in the subtropical areas. High yielding dairy cows are more susceptible to heat stress than other cows because they have a lower UCT, due to the substantial increase in metabolic heat output that these cows are experiencing due to the high milk production (Kadzere *et al.*, 2002). On the other hand, the comfort zone temperature for productive and reproductive performance in rabbits has been established in 12-21ºC (Marai *et al.*, 1991; Verga *et al.*, 2007).

2. 5. THI index as a predictor of heat stress impact

In order to estimate the thermal stress to which the animal is exposed, it was developed a "temperature humidity index" (THI) (Thom, 1958; 1959) that incorporates the effects of both ambient air and dry bulb temperatures, and relative humidity in a sole index. THI is at present the most used stress index to evaluate the impact of climatic conditions and to asses the effect of heat stress on the livestock production all over the world (Bianca, 1962; Hahn, 1969; McDowell *et al.*, 1979; Fuquay, 1981; Rodriquez *et al.*, 1985; Igono & Johnson, 1990; Ravagnolo *et al.*, 2000; West, 2003; Garcia-Ispierto *et al.*, 2006).

The maximum THI (maximum temperature and minimum humidity) for dairy cattle has been calculated to be 72 (Thom, 1959; Johnson *et al.*, 1962), however, for high producing dairy cows this value has been shifted down to 68 (Finch, 1986; Zimbelman *et al.*, 2009) because of the concurrent rise in metabolic heat production associated with the vast amount of milk synthesis (Hansen, 2007a). Therefore, there is a clear

relationship between maximum THI over the period 21–30 days of gestation and subsequent fetal loss (García-Ispierto *et al.*, 2006), because it is when implantation of the embryo occurs in the cow (Guillomot *et al.*, 1993).

Productive and reproductive performance in rabbits is also impaired with high THI (over 27.8), which implies the beginning of heat stress (Marai *et al.*, 2002; García-Tomás *et al.*, 2008). THI values in rabbits are lower than in cow due to the presence of few sweat glands and their less ability to dissipate the heat excess (Verga *et al.*, 2007). If the temperature is above 25-3OºC, animals exhibit an increase in ear temperature (Lebas *et al.*, 1986; Marai & Habeeb, 1994) and also mortality among the offspring (Rafai and Papp, 1984; Marai *et al.*, 1991). When ambient temperature exceeds 30ºC a significant increase in both rectal and ear temperature occurs (Kamar *et al.,* 1975; Shafie *et d.,* 1982; Abo-Elezz *et al.*, 1984 and Wolfenson & Blum, 1988; Marai & Habeeb, 1994). Thus the appetite is depressed, the productive and reproductive performances are impaired and the resistance to disease is decreased. Above 35ºC, rabbits can no longer regulate their internal temperature and heat prostration sets in (Lebas *et al.,* 1986; Marai & Habeeb, 1994), finishing at 4OºC with a considerable panting and salivation.

2. 6. Relation between ROS and heat stress

Reactive Oxigen Species (ROS) are molecules with one or more unpaired electron(s) (Agarwal *et al.*, 2006). These highly reactive molecules attack the nearest stable molecule to obtain an electron. Subsequently, the targeted molecule becomes a free radical itself and initiates a cascade of events that can ultimately lead to cellular damage.

ROS are formed as necessary by-products during normal enzymatic reactions (Skibba *et al.*, 1991; Sikka *et al.*, 1995; Ikeda *et al.*, 1999; Kim *et al.*, 2005) and represent a broad category of molecules: radicals (i.e. hydroxyl radicals or superoxide anions), nonradicals (i.e. ozone or single oxygen) and oxygen derivatives (Agarwal & Prabakaran, 2005). The presence of physiological ROS levels mediate between gametes and crucial reproductive processes (Aitken, 1997; Attaran *et al.*, 2000) as sperm capacitation, acrosome reaction (Kothari *et al.*, 2010), fertilization, stabilization of the mitochondrial capsule in the mid-piece (Agarwal *et al.*, 2008; Desai *et al.*, 2009; Gonçalves *et al.*, 2010), sperm-oocyte interactions (De Lamirande *et al.*, 1997b), implantation and early embryo development (Sakkas *et al.*, 1998; Lapointe & Bilodeau, 2003).

It is noteworthy that cellular exposure to heat stress increases the production of ROS promoting cellular oxidation events (Skibba *et al.*, 1991; Sikka *et al.*, 1995; Ikeda *et al.*, 1999; Kim *et al.*, 2005), associated with cellular hyperthermia (Skibba & Stadnicka, 1986; Malayer *et al.*, 1990; Ando *et al.*, 1997). However, *in vitro* processes as cryopreservation of sperm cells are also involved in the production of an additional toxic source of ROS (Wang *et al.*, 1997; Bailey *et al.*, 2000; Bilodeau *et al.*, 2000).

The extent of ROS damage to cell systems depends on the balance between their production and removal rates (Guille & Joenje, 1991). If the protective mechanism cannot remove the high amount of ROS or the damage produced by, the organism gets into oxidative stress condition (Agarwal *et al.*, 2003). Due to their

highly reactive nature, ROS can combine readily with other molecules, directly causing oxidation that can lead to structural and functional changes (i.e. DNA and protein modifications or enzyme inactivation) and result in cellular damage (Ribarov & Benov, 1981; Comporti, 1989; De Lamirande & Gagnon, 1995; Wells *et al.*, 1997; Guérin *et al.*, 2001; Agarwal *et al.*, 2005a).

In addition, excess of ROS also attacks gametes, inhibiting sperm-oocyte fusion (Aitken *et al.*, 1993), altering oocyte maturation and oxidative status in oviduct (Matsuzuka *et al.*, 2005), impairing the preimplantation development of embryos, increasing rates of early pregnancy loss (Kao *et al.*, 2008), causing male infertility due to lipid peroxidation in sperm membranes (Sharma & Agarwal, 1996; Wang *et al.*, 2003; Bucak *et al.*, 2010a), decreasing sperm viability and motility (Rao *et al.*, 1989), increasing mid-piece morphological defects, altering sperm capacitation, acrosome reaction, and inducing sperm apoptosis (Sikka, 1996; Gandini *et al.*, 2000; Bansal & Bilaspuri, 2007; Bucak *et al.*, 2010a).

3. ANIMAL MECHANISMS TO COUNTERACT HEAT STRESS

Endothermic animals, as cattle, can maintain a relatively constant body temperature that is independent of the temperature of the surrounding environment. However, when heat production exceeds heat loss to the environment, the heat stress condition is established (Fuquay, 1981). To regulate its body temperature, the animal must dissipate the excessive amount of metabolic heat produced into the surrounding environment (Fuquay, 1981) through different ways of heat loss (Collier *et al.*, 2006): (i) physical ways (i.e. conduction, convection and radiation), (ii) evaporative ways or (iii) both.

The rate of exchange depends on the ability of the environment to accept heat and vapour, and also on the animal production status, color coat, the latitude of the world globe where the animal is located and if it is day or night (Finch, 1986; Shearer & Beede, 1990a; Ealy *et al.*, 1993). The sensible means of heat loss are most effective when THI values are low. However, when THI values increases, the animal relies more on evaporative heat loss (Mclean, 1963; Finch *et al.*, 1982) by increasing sweating and respiratory rates (panting; Stevens, 1981; Maia *et al.*, 2005).

Rabbits in general use body position, breathing rate and peripheral temperature, especially ear temperature, as the three devices to modify heat loss. However, respiration and the ears are the most important dissipation pathways. Marai and Habeeb (1994) indicated that between 0 and 30ºC, latent heat loss is controlled only by altering the breathing rate since rabbits are poor at sweating, a reaction made additionally less effective by the presence of the fur (Marai *et al.*, 2002).

4. CELL MECHANISMS TO COUNTERACT HEAT STRESS

The cellular response to stress is an essential defense mechanism against different stressors as heat stress. Cells exposed to heat shock can undergo an array of biochemical responses for protection from elevated temperature (Edwards & Hansen, 1997). In addition, if the heat stress effect is not lethal (short-term mild heat stress), the cell response documented experimentally is the induction of a thermotolerance process, which protects cells from a subsequent and more severe heat stress (Craig & Gross, 1991; Maresca & Lindquist, 1991; Georgopoulos & Welch, 1993; Li *et al.*, 1995). As an example, when embryos in culture are first exposed to a milder heat stress, they become more resistant (Ryan *et al.*, 1992; Ealy & Hansen, 1994; Arechiga *et al.*, 1995; Arechiga & Hansen, 1998).

4. 1. Heat Shock Protein family (HSPs)

The heat shock response is a rapid and transient gene-expression program (Richter *et al.*, 2010) based on the upregulated synthesis of HSPs to confer cell resistance to different stressors (Kiang & Tsokos, 1998), and thermotolerance (Li & Werb, 1982). However, HSPs also play an essential and physiological role in mammalian fertilization and embryo development.

HSPs are classified into two well characterized families according to their molecular weight and functions:

- **Molecular chaperones** (HSP100s, HSP90s, HSP70s (the major class), HSP60s and small HSP): the predominant class involved in facilitating protein folding and assembly (Grossman *et al.*, 1984).
- **Proteases:** as stabilizer of damaged proteins involved in the prevention of aggregation, allowing an opportunity for repair or degradation (Welch, 1992).

Oocytes are transcriptionally inactive after reaching abouth 110 μm (Hyttel *et al.*, 1997) and does not undergo increased synthesis of HSP70 in response to heat stress (Edwards & Hansen, 1997), making the oocyte sensible to stress (Welch & Suhuan, 1985; Edwards & Hansen, 1996; Sugiyama *et al.*, 2007). In fact, it has been reported that oocytes become more resistant to heat shock after HSP70 mRNA microinjection (Hendrey & Kola, 1991).

However, HSP may serve as a protector in embryo development and be involved in the acquisition of thermal resistance (Hansen, 2007b). HSP70 can be induced as early as the two-cell stage (Edwards & Hansen, 1996; Edwards *et al.*, 1997), but also in compact morulae or blastocysts (Bernardini *et al.*, 2004). The phenomenon has been described more evident as development proceeds (Muller *et al.*, 1985; Ealy & Hansen, 1994; Arechiga *et al.*, 1995; Arechiga & Hansen, 1998; Paula-Lopes & Hansen, 2002). According to this, the inhibition of HSP70 causes a reduction in blastocyst development by an increase in cell death (Matwee *et al.*, 2001).

Furthermore, HSP is also increased in testis from animals submitted to high temperatures, increasing their expression as the exposure time to heat increases (Cao *et al.*, 2009). It has been described that HSP70 is present on the acrosome in ejaculated bovine spermatozoa (Kamaruddin, 1998; Matwee *et al.*, 2001) and serves as a stress protector prior to fertilization (Miller *et al.*, 1992).

4. 2. Antioxidant molecules

Antioxidant molecules are the main defense factors in the cell against oxidative stress induced by ROS (both physiological and abnormal production; Loven, 1988; Sikka, 1996). This system includes enzymes as (Brigelius-Flohe *et al.*, 1994; Yu, 1994; Schwaab *et al.*, 1998; Bilodeau & Mirault, 1999): catalase, superoxide dismutase and glutation peroxidases family (i.e. Glutathione reductase). Phisiological processes produce high levels of ROS (Fisher & Aitken, 1997) that can induce a variety of DNA lesions. Fortunately the testis (Bauche *et al.*, 1994), seminal plasma (Sikka, 1996; Kim & Parthasarathy, 1998; Aitken & Baker, 2004; Sikka, 2004) and oviduct (Miller & Shultz, 1987) antioxidant system complement the DNA repair systems.

4. 3. Growth factors

Growth factors, such as insulin like growth factor-I (IGF-I), may exert also a protective action against a variety of stress by reducing the incidence of spontaneous apoptosis and by decreasing the oxidant effect (Vincent & Feldman, 2002; Jousan & Hansen, 2004; Mauro & Surmacz, 2004).

5. CONSEQUENCES IN THE ANIMAL WHEN HEAT STRESS CANNOT BE COUNTERACT

The reproductive processes in mammals are sensitive to hyperthermia in both males and females. Summer infertility has multifactorial nature, since hyperthermia directly alters and impairs the cellular functions of various tissues of the reproductive system (Wolfenson *et al.*, 2000), but also elicits indirect responses such as redistribution of blood flow among body organs, reduction in food intake or respiratory alkalosis (De Rensis & Scaramuzzi, 2003).

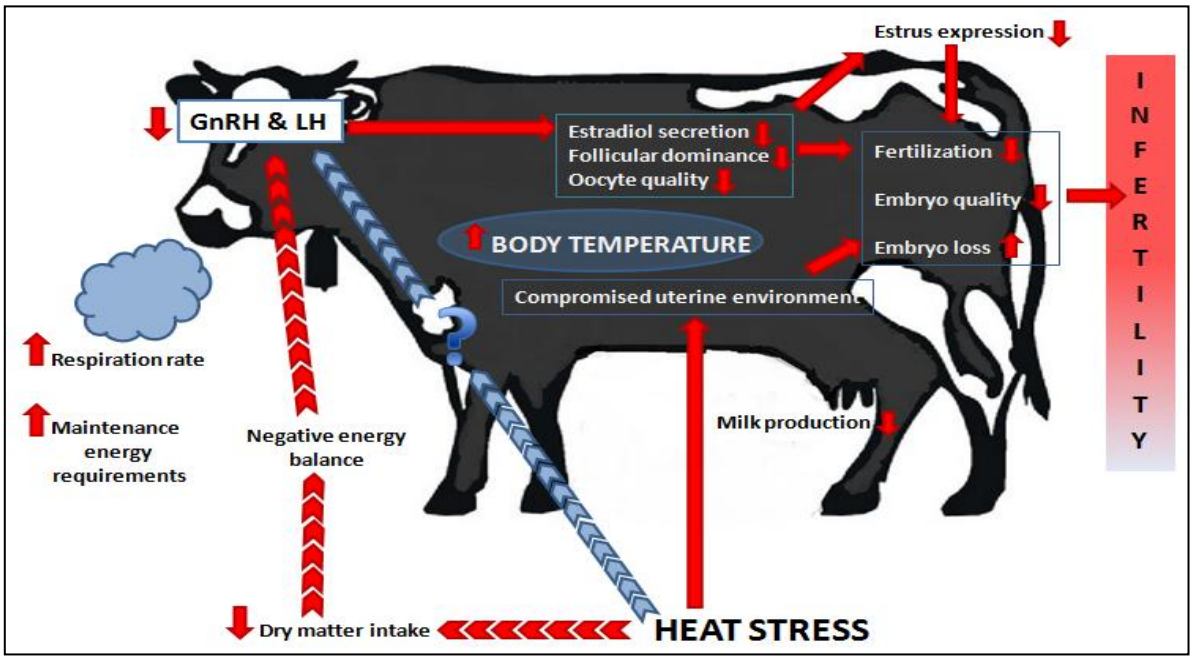
5. 1. Consequences in the female

It is well documented that mammalian females (cow (Monty & Racowsky, 1987; Al-Katanani *et al.*, 2002b), sow (Omtvedt *et al.*, 1971), ewe (Dutt, 1963) and rabbit doe (Marai *et al.*, 2001)) exposed to heat stress have lower fertility during the summer than in any other season (Badinga *et al.*, 1985; Cavestany *et al.*, 1985), with reductions in conception rate, implantation sites, embryo development, litter size, milk production and survival rates (Dobao *et al.*, 1983; Edwards *et al.*, 1968; Omtvedt *et al.*, 1971; Marai *et al.*, 2001; 2002; Zimbelman *et al.* 2009; Kadokawa *et al.*, 2012).

There is not a single mechanism by which heat stress can reduce fertility in livestock females and the problem is due to the accumulation of the effects of several factors (De Rensis & Scaramuzzi, 2003; Figure 2):

- **Direct effects** affecting reproductive organs: alterations in endocrine function (De Rensis & Scaramuzzi, 2003), ovarian follicular dynamics and oocyte maturation (Wolfenson *et al.*, 1995), hormone secretions (Wolfenson *et al.*, 2000), uterine environment (Putney *et al.*, 1988b) and embryo development and survival (Edwards & Hansen, 1997; Rivera & Hansen, 2001).
- **Indirect effects** mediated by changes in the metabolic axis in response to reduced dry matter intake: decline of performance (milk production and growth), reduced activity, increase in respiratory rate, body temperature, peripheral blood flow and sweating (De Rensis & Scaramuzzi, 2003).

Figure 2. A description of the mechanisms generated by heat stress which may affect reproduction in the lactating dairy cow. Heat stress can act in more than one way to reduce fertility in lactating dairy cow. Heat stress can reduce dry matter intake to indirectly inhibit GnRH and LH secretion from the hypothalamo-pituitary system (dashed red lines). However, it is not clear if heat stress can also directly influence the hypothalamo-pituitary system (dashed blue line) to reduce GnRH and LH secretion. Heat stress can directly compromise the uterine environment (solid lines) to cause embryo loss and infertility (modified from De Rensis & Scaramuzzi, 2003).



It has been demonstrated that reproductive variables are significantly impaired during the warm period (Labèrnia *et al.*, 1998; López-Gatius, 2003). High temperatures at insemination time are one of the main factors associated with pregnancy loss up until 90 days of gestation (García-Ispiero *et al.*, 2006), with a significant correlation with early fetal loss (López-Gatius *et al.*, 2002; 2004b). Cows in the warm season of the year are 3.7-5.4 more likely to miscarry compared to the cold period (López-Gatius *et al.*, 2004a),

more specifically, when maternal hyperthermia is experienced between the onset of oestrus and insemination (Putney *et al.*, 1989b), and during the first few cleavage divisions of the early embryo (Dutt, 1963; Putney *et al.*, 1988a; Ealy *et al.*, 1993).

Females submitted to high temperatures experience detrimental effects on follicle development, oocyte physiology (leading to rise in oocytes of reduced competence; Payton *et al.*, 2004; Roth & Hansen, 2004), hormone profiles (Abdel- Sammee and Marai, 1997), oestrus expression, uterine function and blood flow (Abdel- Sammee and Marai, 1997) and embryo viability (increasing embryo mortality; Thatcher & Hansen, 1993), provoking pregnancy losses and a reduced conception rate (Hansen & Arechiga, 1999; Wolfenson *et al.*, 1995; De Rensis & Scaramuzzi, 2003).

5. 1. 1. Rectal temperature

It has been reported that rectal temperatures of cows during the summer are significantly higher than during winter (Al-Katanani *et al.*, 2002b). Usually, cows keep normothermia (38.7ºC; Roth *et al.*, 2001b); whereas rectal temperatures reach or exceed 41ºC due to high temperatures (heat stress; Ealy *et al.*, 1993; Torres-Junior *et al.*, 2008). This effect is also increased due to high milk production which leads high metabolic rates making impossible for the cow to maintain her body temperature at normal levels (Wolfenson *et al.*, 1995; De Rensis & Scaramuzzi, 2003). In those situations, heated environment combined with the body heat produced from metabolic processes produces a gradual increase in core temperature up to 41ºC for up to 4 h before gradually returning to normal (Sugiyama *et al.*, 2007). On the other hand, it has been demonstrated that rabbit body temperature also increases when the environmental temperature exceeds 28ºC (Amici *et al.*, 1995; Franci *et al.*, 1996; Marai *et al.,* 2001).

Elevations in rectal temperature are not without consequence. The higher rectal temperature during summer impairs fertility in dairy cows. Pregnancy rates have been shown to decrease with each 0.5ºC or degree increased in rectal temperature (Ulberg & Burfening, 1967; Gwazdauskas *et al.*, 1973). The explanation is that reproductive tract temperatures rise in parallel to rectal temperature (Gwazdauskas *et al.*, 1973; Roman-Ponce *et al.*, 1981), and cellular function of different tissues of the reproductive tract are harmfully affected (Wolfenson *et al.*, 2000; Hansen *et al.*, 2001) when body temperature increases above the set point by 1.5 to 2 ºC (Gwazdauskas *et al.*, 1973; Thatcher, 1974; Wolfenson *et al.*, 1995). The effects of such increments are a reduction in embryonic development and increased proportion of degenerate/retarded embryos (Putney *et al.*, 1989a; b).

5. 1. 2. Dry matter intake

Hyperthermia associated with heat stress has been proven to reduce dry matter intake (Hansen, 1997b; Jonsson *et al.*, 1997; Fuquay, 1981; Ronchi *et al.*, 2001) by reducing the appetite, enlarging the energy gap between intake and expenditure through maintenance and milk production (Collier *et al.*, 1992; Moore *et al.*, 2005), and also prolonging the period of negative energy balance. In dairy cow, dry matter intake exhibits a significant decline when maximum THI reach 77 (Johnson *et al.*, 1963), although THI as low as 64 (Igono *et al.*, 1992) can cause also a reduction of the normal feed intake (6.5%; Ominski *et al.*, 2002).

Furthermore, the recovery from the negative effects is not immediate and remains depressed some time after the period of heat stress (Holter *et al.*, 1996; 1997).

In the case of rabbits, the primary effect of thermal stress is a reduction in feed intake (Simplicio *et al.*, 1988; Paci *et al.*, 1993; Morrow-Tesch *et al.*, 1994; Franci *et al.*, 1996; Marai *et al.*, 2001) with also an increase in water consumption (Marai *et al.*, 2001). However, a slight increase (1.8%) of the feed intake in short heat exposure has also been observed, which may be attributed to immediate compensatory growth with higher feed intake that followed the reduction in growth under thermal conditions (Abdel-Kafy *et al.*, 2008).

5. 1. 3. Negative energy balance period (NEB)

Metabolic changes associated with NEB, which is present in the high yielding dairy cow at the post partum period under thermoneutral conditions, are enlarged by summer heat stress conditions (Stott & Wiersma, 1976; Badinga *et al.*, 1985; De Rensis *et al.*, 2002). Heat stress aggravates NEB predominantly by the drop in dry matter intake (Collier *et al.*, 1992; Blackshaw & Blackshaw, 1994), leading to a decrease in plasma concentrations of insulin, glucose and IGF-I (altering follicle development; Jolly *et al.*, 1995; Bucholtz *et al.*, 1996; Rabiee *et al.*, 1997; O'Callagan & Boland, 1999), an increase in prolactin plasma concentrations (inhibiting follicular development; (Abilay *et al.*, 1975; Igono *et al.*, 1987; Ronchi *et al.*, 2001; De Rensis & Scaramuzzi, 2003) and non-esterified fatty acid (reducing fertility; Butler, 2001; Lucy *et al.*, 1992; Vanholder *et al.*, 2005; Leroy *et al.*, 2006), which can negatively affect reproduction, as reflected by the reduction in embryo quality following a NEB episode (Leroy *et al.*, 2008).

5. 1. 4. Milk production & Litter size

Lactating dairy cow's productivity declines significantly under heat stress conditions (West, 2003). In fact, milk yield declines when rectal temperature reaches (39°C) (Zimbelman *et al.* 2009; Kadokawa *et al.*, 2012). The estimated milk yield reduction is round 0.32 kg per unit increased in THI (Ingraham *et al.*, 1979), or 1.8 and 1.4 kg for each 0.55°C increase in rectal temperature (Johnson *et al.*, 1963). The same reduction has been described in rabbits. Milk yield in rabbits is significantly lower in summer than in the other seasons (El-Sayiad *et al.*, 1994), being especially lower when temperature reaches 30ºC (Szendro *et al.*, 1998). Daily milk yield was found to be lower by nearly 10% during the hot period of the day (Maertens and De Groote, 1990), by 7.7 g when the temperature rise above 20ºC (Rafai and Papp, 1984) and by 62.29% in summer (48.8g) than in winter (Ayyat and Marai, 1998).

On the other hand, litter size at birth is also lower during summer and highest during winter (Patial *et al.*, 1991; Ayyat and Marai, 1998; Bassuny, 1999; Habeeb *et al.*, 1999), estimating 5.8 kits in summer and 6.6 kits in winter (a reduction of 14%) and being also reduced at 21 days (Bassuny, 1999) and at weaning (at 28-35 days of age; Ayyat and Marai, 1998; Bassuny, 1999). The number of kits born alive is also lower during May and August (McNitt and Moody, 1990).

5. 1. 5. Oestrus alterations

Heat stress reduces the length (Abilay *et al.*, 1975) and intensity (Gangwar *et al.*, 1965) of oestrus expression (Hansen, 1997b; Nobel *et al.*, 1997), and in addition, increase the duration of anooestrus and silent ovulation (Gwazdauskas *et al.*, 1981). It is possible that the major reason is because of the physical lethargy produced by high temperatures, possibly as an adaptative response that limits heat production. Oestrus alterations leads to a reduction in the number of mounts in hot weather compared to cold weather (Pennington *et al.*, 1985). However, some studies pointed that heat stress did not provoke alterations in oestrus expression (Howell *et al.*, 1994; Roth *et al.*, 2000).

5. 1. 6. Conception and pregnancy rate

Climate factors are highly relevant for conception rate, especially during the period 3 days before to 1 day after artificial insemination (AI) in cow (coincident with oocyte maturation and ovulation; Putney *et al.*, 1989b; García-Ispierto *et al.*, 2007). Heat stress at that point has been associated with a decrease in cattle fertility in countries with warm weather (Dutt, 1963; Putney *et al.*, 1989b; Al-Katanani *et al.*, 1999; Al-Katanani *et al.*, 2002b). Moreover, the exposure during the first 3 or 7 days of pregnancy cause a decrease in embryonic viability and development (Dunlap & Vincent, 1973; Putney *et al.*, 1988a), due to the embryo at that point is more sensitive since resistance is adquired as development proceeds (Ealy *et al.*, 1993). Conception rates drops from about 40–60% in cooler months to 10–20% or lower in summer, depending on the severity of the thermal stress (Badinga *et al.*, 1985; Cavestany *et al.*, 1985; Roth *et al.*, 2000; De Rensis *et al.*, 2002; López-Gatius, 2003). Negative effects seem to appear from 75 THI units, although the effects become more obvious from 80 (Hahn, 1981; García-Ispierto *et al.*, 2007). In terms of temperature, heat stress starts at 25ºC (Bitman *et al.*, 1984) but conception rate decreases from 31ºC (García-Ispierto *et al.*, 2007), declining 6.9–12.8% for each 0.5°C increase in uterine temperature (Gwazdauskas *et al.* 1973).

Pregnancy rate is also significantly impaired during the warm months (De Rensis *et al.*, 2002) due to the rise in rectal temperatures (Roth *et al.*, 2000). High temperatures increase the number of pregnancy losses that rise from 2.1 to 12.3% for cows that become pregnant during the warm period which contrasts clearly with the cold period. It seems that cool environment preserves gestation, probably as a reflection of cow well-being (Garcia-Ispierto *et al.*, 2006).

In the case of rabbits, conception rate also decreases with heat stress (Ulberg & Burfening, 1967; Marai *et al.*, 2001). Conception rate is reduced if the ambient temperature is high enough to elevate rectal temperature of females by 1-2ºC. Particularly, severe sustained heat stress of 35°C reduces the conception rate from 66% in the first parity to 33% in the second parity against 100% in winter (Shafie *et al.,* 1984).

5. 1. 7. Negative effects still in autumn

Traditionally, low summer fertility has been associated mainly with the warm months of the year (June-September; 10-20%). However, fertility remains lower still in autumn (October-November; 30%), although cows are no longer exposed to thermal stress (Ron *et al.*, 1984; Badinga *et al.*, 1985; Cavestany *et al.*, 1985; Hansen, 1997b; Wolfenson *et al.*, 2000; Roth *et al.*, 2001a; Zeron *et al.*, 2001).

The delayed effect of summer heat stress on autumn fertility is a transient phenomenon; oocyte quality improves gradually towards the end of the autumn (Roth *et al.*, 2001a). For the present, once the pool of ovarian oocytes is damaged by high temperatures, two or four estrous cycles (about 2 months) are needed for recovery after the end of heat stress (Ron *et al.*, 1984; Roth *et al.*, 2001a). The reason is because small antral follicles take about 40–50 days to develop into large preovulatory follicles (Lussier *et al.*, 1987), although the time needed for recovery of quality may depend on the duration and severity of the thermal stress (Roth *et al.*, 2001a). Accordingly, autumn fertility can be enhanced by removal of impaired follicles by frequent follicle aspiration, leading a more rapid emergence of healthy oocytes (Roth *et al.*, 1999; 2001a).

However, the delayed impairment in fertility during autumn can be explained by more other factors than the oocyte itself. Delayed effects are related also with alterations in follicular dynamics and dominance (Badinga *et al.*, 1994; Wolfenson *et al.*, 1997; Roth *et al.*, 2000), impairments on steroidogenesis (in thecal and granulose cells; Wolfenson *et al.*, 1997; Roth *et al.*, 2001b) and cell-cell communications (granulose-cumulus cells and cumulus-oocyte; Wolfenson *et al.*, 1997; Ambrose *et al.*, 1999).

5. 1. 8. Hormones

Hot environment causes an endocrine imbalance and finally compromise folliculogenesis in dairy cows (Lucy, 2001; De Rensis & Scaramuzzi, 2003) by damaging follicular and luteal cells, reducing steroidogenesis and compromising follicular and oocyte development (Torres-Junior *et al.*, 2008).

Estradiol, Inhibin and Follicular Stimulation Hormone (FSH):

A decrease in plasma estradiol and inhibin concentration is found in heat-stressed cows during the follicular phase (Gwazdauskas *et al.*, 1981; Wilson *et al.*, 1998a; b; Wolfenson *et al.*, 1995), being more pronounced during the first follicular wave (Wolfenson *et al.*, 1995) and even in heated cows in September compared with those heated in July (Badinga *et al.*, 1993). The reason is the extended period of exposure to daily heat stress in late summer (September), which may severely impair follicular function. However, plasma FSH concentrations are higher during the periovulatory period in heat stressed cows; thus, is induced synergically by the low secretion of both estradiol and inhibin, which can not inhibit FSH secretion (Roth *et al.*, 2000; Wolfenson *et al.*, 2000).

Progesterone & Luteinizant Hormone (LH):

The effect of heat stress on plasma progesterone and LH concentration is controversial. Some studies found that heat stress had no effect on progesterone (Wilson *et al.*, 1998b), although, other studies have reported reduced (Wise *et al.*, 1988; Wolfenson *et al.*, 1988; Howell *et al.*, 1994; Jonsson *et al.*, 1997; Wolfenson *et al.*, 1993b), increased (Abilay *et al.*, 1975; Vaught *et al.*, 1977; Trout *et al.*, 1998; De Rensis & Scaramuzzi, 2003) or unchanged (Roth *et al.*, 2000; Guzeloglu *et al.*, 2001) concentrations during summer in dairy cows. In the case of LH unchanged (Gwazdauskas *et al.*, 1981), decreased (Madan & Johnson, 1973; Gwazdauskas *et al.*, 1981; Rosenberg *et al.*, 1982), or increased (Roman-Ponce *et al.*, 1981) leves are also reported. Low concentration of plasma progesterone has consequences for fertility, especially during luteal phase and pre-conception estrous cycle: steroidogenesis alterations in the dominant follicles, abnormal oocyte maturation and endometrial morphology, failure on implantation and early embryonic death (Ahmad *et al.*, 1995; Shaham-Albalancy *et al.*, 1996a; b; Mann *et al.*, 1999).

Prostaglandin & Cortisol:

Heat stress can also affect endometrial prostaglandin secretion (Putney *et al.*, 1989b) leading to premature luteolysis and embryo loss (Vasconcelos *et al.*, 1998). In terms of blood cortisol profile, no alterations by immediate or delayed heat stress have been reported in a study (Torres-Junior *et al.*, 2008).

5. 1. 9. Blood flow redistribution effects on uterine environment and endometrial function

Heat stress leads to a redistribution of blood flow from the visceral organs to the periphery. The resultant decreased perfusion of nutrients and hormones could compromise the ovary blood flow (De Castro & Hansen, 2007), intrauterine environment, endometrial and oviductal function (Gwazdauskas *et al.*, 1975; Roman-Ponce *et al.*, 1978; Hansen & Arechiga, 1999), by a reduction in the production of interferon-ɣ and an increased production of prostaglandin PGF2α from the endometrium (altering corpus luteum maintenance; Wolfenson *et al.*, 2000). All these effects lead to altered cellular function, inhibited or altered embryonic development and sometimes have been linked to early embryonic loss and unsuccessful inseminations (Wolfenson *et al.*, 2000; Rivera & Hansen, 2001).

5. 2. Consequences in the male

Normal testicular function is temperature dependant (Harrison & Weiner, 1948; Ivell, 2007). Testes originate within the abdominal cavity and descend into a scrotum where they are appreciably cooler than the body (2-8ºC below core body temperature; Setchell, 1978; Waites & Setchell, 1990). The importance of thermoregulation in the testis is illustrated by the fact that slight increases in temperature can disrupt spermatogenesis and ultimately cause problems with fertility (Lue *et al.*, 1999; Paul *et al.*, 2008).

High environmental or local temperatures (cryptorchidism or inflammation; Skinner and Louw, 1966; Mieusset *et al.*, 1987; Mieusset *et al.*, 1992b; Lue *et al.*, 2000; Banks *et al.*, 2005) increase testis temperature. The heat production is lost from the testes and scrotum to the environment by sweat glands from scrotal skin (Setchell & Breed, 2006) and the impact of heat stress will depend on the animal ability to dissipate the heat. In the case of pigs, they have a low capacity for increased sweating when the ambient temperature increases from 23 to 34ºC (Stone, B.A. 1981), and rabbits are very susceptible to heat since they have few functional sweat glands (Verga *et al.*, 2007).

The exposure of males to severe heat stress (high THI) adversely affects their reproductive traits (Marai *et al.*, 2002) and takes to subinfertility (McLaren *et al.*, 1994; Jannes *et al.*, 1998; Setchell, 1998; Lue *et al.*, 1999) or a total infertility (Jannes *et al.*, 1998; Setchell, 1998; Yaeram *et al.*, 2006). Hence, the effects of heat can be by direct actions on the testis or by indirect effects (cellular changes in the testis), causing disruption of spermatogenesis and infertility in a variety of species: mouse (Hand *et al.*, 1979), rat (Collins & Lacy, 1969), bull (Casady *et al.*, 1953), boar (Wettemann *et al.*, 1976), ram (Mieusset *et al.*, 1991), rabbit (Elnagar, 2010) and human (Baranski, 1993; Mieusset & Bujan, 1995).

5. 2. 1. Rectal and scrotal skin temperature

Animals placed in a hot environment can undergo hyperthermia and rise in rectal temperature if the evaporation system of the scrotal skin surface cannot keep the scrotal skin cool (Wettemann *et al.*, 1976; Egbunike & Dede, 1979; Wettemann & Desjardins, 1979; Wettemann *et al.*, 1979; Stone, 1982; Meyerhoeffer *et al.*, 1985; Zhu *et al*., 2004); so then, the testis warms up (Setchell & Mieusset, 1996). Particularly, increasing environmental temperature from 10 to 30ºC elevates body temperature from 39.7 to 40.5ºC in rabbits (Johnson *et al.*, 1957). Hence, scrotal skin temperature can rise to values normally found in the abdomen (Stone, 1982; Trudeau & Sanford, 1986) leading to impair normal testicular function (Setchell *et al.*, 1994).

5. 2. 2. Respiratory rate, water consumption and feed intake

Respiratory rate is always increased when animals are submitted to high temperatures as a mechanism to dissipate the heat (rabbit (Shafie *et al.,* 1982; Marai *et al.*, 2002); Johnson *et al.,* 1957), boar (Egbunike & Dede, 1979; Wettemann & Desjardins, 1979; Wettemann *et al.*, 1979; Stone, 1982) and bulls (Meyerhoeffer *et al.*, 1985)). Water consumption is also incremented by animals submitted to high temperatures (Meyerhoeffer *et al.*, 1985; Marai *et al.*, 2002). However, feed intake is reduced (Marai & Habeeb, 1994; Marai *et al.*, 2002). The decrease in food consumption is due to the impairment of appetite as a result of the peripheral thermal receptors stimulation by the environmental temperature which transmits suppressive nerve impulses to the appetite centre in the hypothalamus.

5. 2. 3. Endocrinology

Testosterone is vital for the initiation of spermatogenesis, as well as for sperm maturation (Sharpe *et al.* 1992). The main physiological function of Leydig cells is testosterone production, which is transiently but

severely reduced by heat stress (Liu and Stocco 1997; Murphy *et al.* 2001), so then, spermatogenesis is impaired (Gwazdauskas, 1984). The effects of high temperatures on plasma testosterone concentrations depend largely on how stressful the environment has been to the animal. Testosterone levels declines some days after heat stress and then serum levels return to near normal values due to an acclimatization process (Rhynes & Ewing, 1973; Wettemann & Desjardins, 1979). Therefore, it seems that heat stress exposure causes a transitory decrease in peripheral testosterone concentrations by suppression of the synchronous diurnal rhytm in plasma testosterone levels (Stone & Seamark, 1984).

5. 2. 4. Libido

High temperatures affect reproductive behaviour in males as it does in females. In fact, it exists a decrease in the male libido due to heat, but animals can mate normally during the cooler part of the day (Winfield *et al.*, 1981). When heat stress gets severe enough, it can cause decreased libido in the bull and a decline in semen quality that persists for about two months after the end of heat stress as a result of the damage to the spermatozoa precursor cells (Hansen, 2005). The same tendency was found in male rabbits (Zeidan *et al.*, 1997; Daader and Seleem, 1999). On the other hand, as described in boars and bulls, libido can also be unaffected keeping the ability to ejaculate (Wettemann *et al.*, 1979) with an increase in the ejaculation time (De Alba, J. & Riera, S. 1966; Egbunike & Dede, 1979).

5. 2. 5. Testis weight

It has been widely reported that one of the more obvious effects of acute or chronic exposure to high scrotal temperatures is a decrease in testes weight (Gasinska & Hill, 1990), which reaches a minimum some days after heat shock and usually returns towards normal values some days after (Setchell & Waites, 1972; McLaren *et al.*, 1994; Sailer *et al.*, 1997; Jannes *et al.*, 1998; Rockett *et al.*, 2001). Although, when the heat stress is such severe is difficult to recover it (Sailer *et al.*, 1997). This effect has been described in a wide range of species, such as mice (Sailer *et al.*, 1997; Jannes *et al.*, 1998; Rockett *et al.*, 2001), rat (Aktas & Kanter, 2009) or rabbit (Chou *et al.,* 1974). This reduction suggests degeneration in the germinal epithelium and a partial atrophy in the seminiferous tubules (El-Sherry *et al.,* 1980).

5. 2. 6. Histological changes

Histological changes have been observed in testes so early after exposure to heat (Young, 1927). Pachytene spermatocytes (De Vita *et al.*, 1990; Banks *et al.*, 2005; Paul *et al.*, 2008) and early spermatids (Devkota *et al.*, 2010) are the most easily affected cells by heat. In general, heat stress produce a decline in the average number of round spermatids (coincident with a reduction in sperm number and impaired fertility; Wettemann *et al.*, 1976; Wettemann & Desjardins, 1979), degenerative lesions on seminiferous tubules (Rockett et al, 2001; Devkota *et al.*, 2010) with a consecuent loss of internal pressure (Devkota *et al.*, 2010) and changes in Sertoli cells (Devkota *et al.*, 2010). The impact of effects on male germinal cells depends on the ability to respond to stress by repairing their DNA and their ability to undergo apoptosis or necrosis (Rockett et al, 2001). This effect has been described in a wide range of species (mice: Sailer *et*

al., 1997; Rockett et al, 2001; hamster: Vigodner *et al.*, 2003; rabbit: Zhang *et al.*, 2002; boar: Wettemann & Desjardins, 1979; Malmgren, L. 1989; bull: Karabinus *et al.*, 1997; Devkota *et al.*, 2010).

5. 2. 7. Ejaculate volume and sperm output

Modifications in the ejaculate volume have been described by some authors in a wide range of species. Rabbits semen volume is seasonally influenced (Roca *et al.*, 2005). Seminal plasma volume and gel were significantly lower in boars (Stone, 1982) and bulls (Meyerhoeffer *et al.*, 1985) ejaculates subjected to thermal treatment, although no differences were appreciated by other authors (Johnson & Branton, 1953; McNitt & First, 1970; Wettemann *et al.*, 1976; 1979; Egbunike & Dede, 1979; Malmgren, 1989). Generally, changes in ejaculate volume may be due to a low sperm concentration, a decrease in seminal plasma volume (Macirone and Walton, 1983) or by the hypoactivity of the accessory glands (Zeidan *et al.*, 1997).

Taking into account spermatozoa concentration, season and high temperature effects are inconsistent, although in general, a drastic decrease in sperm output has been described (bull: De Alba & Riera, 1966; Meyerhoeffer *et al.*, 1985; boar: Egbunike & Dede, 1979; Wettemann *et al.*, 1979; Trudeau & Sanford, 1986; ram: Mieusset *et al.*, 1992b; mice: Jannes *et al.*, 1998; rabbit: Marai *et al.*, 2002; Roca *et al.*, 2005).

5. 2. 8. Ejaculate proteins and pH

Seminal plasma is a physiological secretion from multiple glands of the male reproductive tract that plays an important role in the final maturation of the spermatozoa through hormonal, enzymatic and surface-modifying events, and as a vehicle for ejaculated spermatozoa (Mann 1978). Seasonality may also change the secretory function of one or more of the accessory glands, influencing the amount of fluid produced and the chemical composition of the seminal plasma (Strzezek *et al.* 2000; Strzezek 2002). According to that, the protein content in the ejaculate tends to be higher during winter (Trudeau & Sanford, 1986), although semen pH values tend to be lower (Trudeau & Sanford, 1986; Cheon *et al.*, 2002).

5. 2. 9. Fertility

Males exposed to heat can produce sperm which do not produce normal offspring in exposed or unexposed females, due to embryo quality is linked to sperm quality (Dutt & Simpson, 1957; Fowler & Dun, 1966; Howarth, B. 1969; Rathore, A.K. 1970; Mieusset *et al.*, 1992b). So, reduced fertility can result from:

- **Failure in fertilization:** ram (Mieusset *et al.*, 1992b), boar (Wettemann & Bazer, 1985), rats (Setchell *et al.*, 1988) and rabbits (Cummins & Glover, 1970).
- **Embryonic death or abnormalities:** developmental competence affected after normal fertilization (mice (Bellvé, 1972; Zhu *et al.*, 2004), bull (Walters *et al.*, 2006), rabbit (Howarth *et al.*, 1965; Burfening, P.J. & Ulberg, L.C. 1968)).
- **Both:** ram (Rathore, 1970; Mieusset *et al.*, 1991; 1992b), boar (Wettemann & Bazer, 1985), mice (Burfening *et al.*, 1970), rat (Setchell *et al.*, 1988).

Hereby, thermal stress leads to low pregnancy rates (Wettemann et al, 1976; 1979), sperm production with low motility and low ability to attach and penetrate the zona pellucida of the oocyte (low polyspermy; Hendricks *et al.*, 2009), producing embryos with delayed or reduced pronuclear formation (Walters *et al.*, 2006), a reduction in the ability to cleavage and to become blastocysts (Walters *et al.*, 2004; 2005), an increase in apoptosis (Walters *et al.*, 2005) and a decrease in embryo survival (Wettemann et al, 1976; Stone, 1982).

6. CONSEQUENCES IN GAMETES WHEN HEAT STRESS CANNOT BE COUNTERACT

Oxidative insults occur to cells *in vivo* and *in vitro* from exposure to free radicals generated by exogenous agents (i.e., radiation, chemicals, hyperoxia or heat) and endogenous processes such as normal cellular metabolism. Under extreme oxidative conditions cellular injury and death may occur, especially if the antioxidant protective mechanisms of cells are compromised (Chow, 1979; Halliwell *et al.*, 1992).

In the last decade, considerable efforts have been dedicated to shedding light on the heat stress-induced impairment of processes in the reproductive system. *In vivo* and *in vitro* studies are among the means of achieving improved understanding of mechanisms by which thermal stress negatively influences fertility (Wolfenson *et al.*, 2000). However, *in vivo* studies do not allow effective differentiation between direct effects of elevated temperature on the developmental competence of gametes. For that reason, *in vitro* studies have proven beneficial to identify it.

6. 1. CONSEQUENCES IN THE FEMALE GAMETE

6. 1. 1. Oocyte maturation

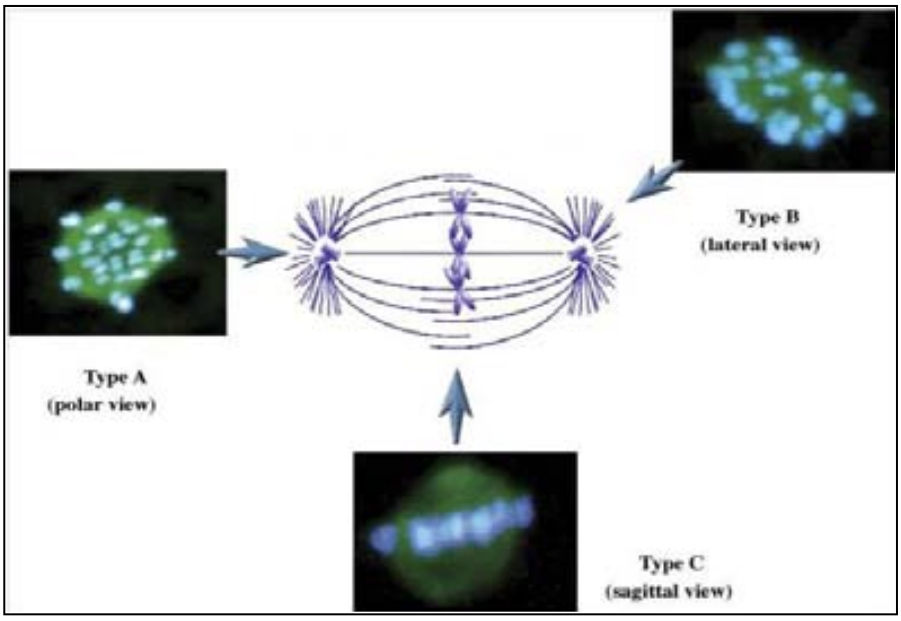
Oocyte enclosed in the follicle needs to undergo maturation to become able to support successful fertilization and embryo development (adquisition of developmental competence; Hyttel *et al.*, 1997; Mermillod *et al.*, 1999; Trounson *et al.*, 2001; Sutton *et al.*, 2003). The process is coincident with the expression of oestrus in the cow. This capacity to sustain early development is intrinsically linked to the process of folliculogenesis. In fact, the follicular environment maintains oocytes in an arrested state of meiosis, at the germinal vesicle (GV) stage (Sutton *et al.*, 2003).

Oocyte maturation involves both the nuclear and cytoplasmic events. While nuclear maturation includes the resumption of the first meiotic division and the progression of meiosis to the metaphase II stage (MII), with a correct dynamics of chromosome separation; cytoplasmic maturation includes a serie of processes as redistribution of cytoplasmic organelles and the storage of mRNA, proteins, and transcription factors, that are necessary for the oocyte to acquire the capacity to support male pronucleus formation, monospermic fertilization, and early embryonic development (Eppig *et al.*, 1996; Meirelles *et al.*, 2004).

Nuclear and cytoplasmic maturation are interlinked events that occur simultaneously at determined times, even though the molecular programming of the cytoplasm may have already started during the phase of oocyte growth. Progression to MII is often completed by 16 to 22h after resumption of meiosis, whereas translocation of cortical granules is noted between 20 to 30h (Wolfenson *et al.*, 1997).

Marked changes in the nucleus are important in reducing number of maternal copies of chromosomes. Nuclear maturation involves the breakdown of the germinal vesicle (GVBD; oocyte becomes transcriptionally quiescent; Hyttel *et al.*, 1997), chromosome condensation and segregation, completion of meiosis I (MI), extrusion of the first polar body, and arrest at MII (Roth & Hansen, 2005), a progress induced by the pre-ovulatory gonadotrophic surge (Sutton *et al.*, 2003). Chromosomes are distributed in relation with the microtubules within the meiotic spindle during all nuclear stages (from MI to MII) and also in the first polar body. For oocytes freshly matured, the meiotic spindles are well organized characterized by a short wide, barrel-shaped structure with its width greater than its length (Ju *et al.*, 2005) with chromosomes aligned along the midline (Roth & Hansen, 2005) (Figure 3).

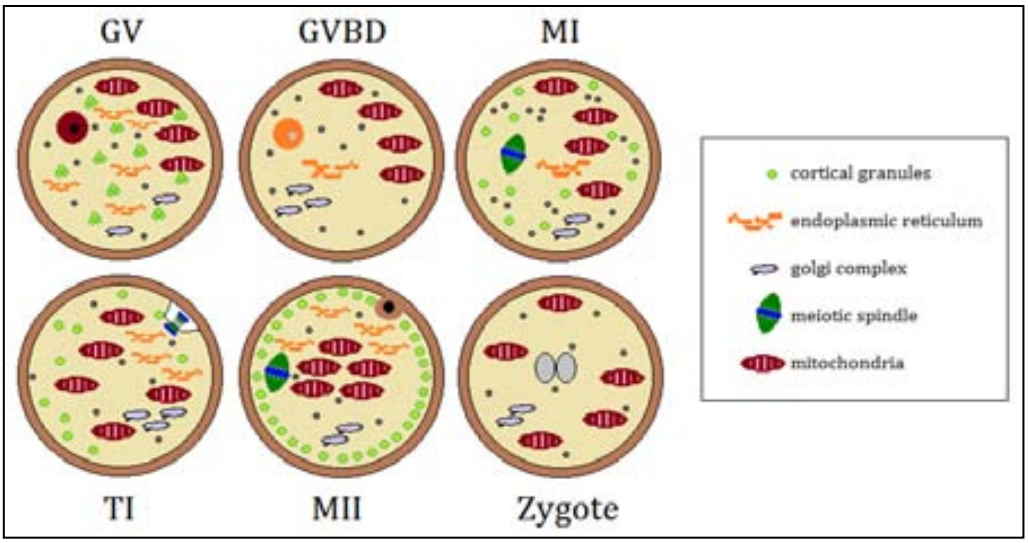
Figure 3. Classification of spindle views in mature bovine oocytes during immunocytochemical examination. Types A and C show the spindle from the polar (top) and sagittal directions, respectively. Type B represents a lateral view (angle, 30–45º, Ju *et al.*, 2005).



On the other hand, the cytoplasmic maturation process can be divided into three main events (Ferreira *et al.*, 2009): (i) redistribution of cytoplasmic organelles, (ii) dynamics of the cytoskeleton and (iii) molecular maturation.

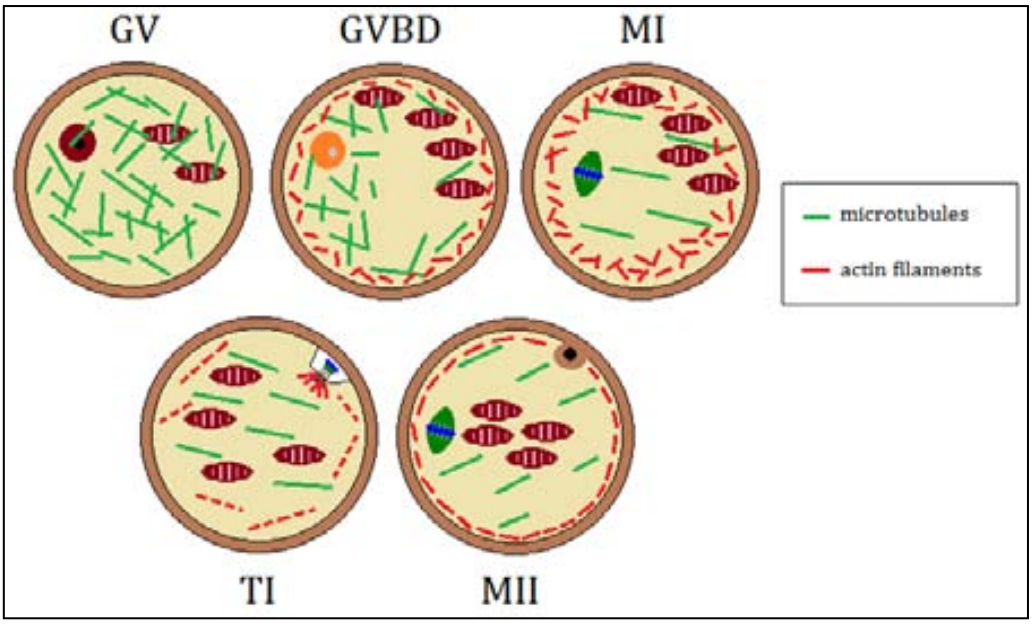
- **Redistribution of cytoplasmic organelles (Figure 4)** through the cytoskeleton network in which the organelles encased by a membrane move and occupy defined positions (Albertini, 1992; Hosoe & Shioya, 1997):
    - Mitochondria: synthesizes the ATP necessary for the synthesis of proteins which supports the completion of subsequent maturation processes and embryo development (Krisher & Bavister, 1998; Stojkovic *et al.*, 2001). The mitochondria moves from a more peripheral position to a more disperse distribution throughout the cytoplasm after the extrusion of the polar body, occupying a central position in the cell (Hyttel *et al.*, 1986; 1997).
    - Cortical granules (CG): exclusively found in oocytes, are derived from the Golgi complex (Ducibella *et al.*, 1994; Hoodbhoy & Talbot, 1994; Wessel *et al.*, 2001) and formed by proteins, structural molecules, enzymes, and glycosaminoglycans. CG are distributed in clusters throughout the cytoplasm of oocytes in the GV stage (Ducibella *et al.*, 1994; Hoodbhoy & Talbot, 1994; Hosoe & Shioya, 1997). At the end of the maturation period (MII stage) the granules are distributed throughout the inner surface close to the plasma membrane (Ducibella *et al.*, 1994; Hoodbhoy & Talbot, 1994; Conner *et al.*, 1997), waiting for the spermatozoon entry and egg activation. Once the spermatozoon enters, the polyspermy is prevented by the exocytosis of cortical granules (cortical reaction; Hosoe & Shioya, 1997) to the perivitelline space as a response to elevated cytoplasmic calcium upon fertilization (Schuel, 1985), modifying the zona pellucida (Yanagimachi, 1994).

Figure 4. Distribution of cytoplasmic organelles during maturation and bovine zygote formation. Nuclear maturation progression and cytoplasmic organelle movement from the immature stage of germinal vesicle to the mature stage of metaphase II and zygote formation (modified from Ferreira *et al.*, 2009).



- **Dynamics of the cytoskeleton (Figure 5):**
  - Microtubules: appears close to the condensed chromatin during GVBD. However, during MI they are forming the meiotic spindle and the metaphase plate in which the chromosomes are arranged in an equatorial manner (Albertini, 1992; Stojkovic *et al.*, 2001). At that stage the metaphase plate is proportionally larger than that formed in MII and the spindle is barrel-shaped and its poles are flattened (Li *et al.*, 2005). During Anaphase I (AI) chromosomes start to separate and therefore a large portion of microtubules placed between the two segregating chromosome sets forming a cone-like triangular structure having a wider and a more tapered shape during Telophase I (TI). The wider portion of the microtubules is linked to the chromosome set destined for extrusion out of the cell, thus forming the first polar body. The tapered portion is associated with the set that will remain in the oocyte and enter MII, again forming the metaphase plate (Li *et al.*, 2005). Finally, during MII the interkinesis takes place as a rapid chromatin condensation process with the disappearance of the microtubules of the meiotic spindle (Fan & Sun, 2004). The metaphase plate reappears soon after this event, although is smaller than in MI by the reduction of chromosome number to half the initial amount (Li *et al.*, 2005).

Figure 5. Dynamics of cytoskeleton filaments during cytoplasmic and nuclear maturation of bovine oocytes (modified from Ferreira *et al.*, 2009).



- Actin filaments: distributed in the cortical area below the oolema (Li *et al.*, 2005) during the transition to AI but are absent among the microtubules, although a relationship exists among them since chromosomes movements depends on processes mediated by actin filaments (Kim *et al.*, 2000; Sun & Schatten, 2006). During AI the spindle elongates and a large quantity of actin filaments can be observed around the chromosomes (Li *et al.*, 2005), and associated to chromosomes and microtubules in TI. Finally, during MII the interkinesis takes place as a rapid chromatin condensation process with the disappearance of the microfilaments (Fan & Sun, 2004).

- **Molecular maturation:** protein synthesis is indispensable for oocyte maturation and also for zygote formation and early embryogenesis which is synthesized by the transcription of ribosomal RNA (rRNA) genes (Cetica *et al.*, 2001; Sirard *et al.*, 2006). During metaphase I protein synthesis in the oocyte is approximately three times greater than that during the GVBD stage. Although, when the cell reaches MII the oocyte exhibits basal levels of mRNA translation (Tomek *et al.*, 2002).

6. 1. 2. Heat stress effects

Cortical granule exocytosis, resumption of meiosis and pronuclear development are considered to be temperature dependent, which their best performance is likely to occur at the 39ºC core body temperature of the bovine (Sugiyama *et al.*, 2007). However, exposing the follicle-enclosed oocyte at the germinal vesicle (GV)-stage to elevated temperatures interferes with oocyte maturation and impairs its ability to become fertilized and form a viable embryo. The effect of maternal hyperthermia on the oocyte may be direct or mediated through the changes in the follicular environment by alterations in follicular fluid content (Wolfenson *et al.*, 1997) or in the surrounding follicular cells (Shimizu *et al.*, 2005). One of the most sensitive stages of cattle oocytes to the elevation of ambient temperature is at the pre-ovulatory stage (Ingraham *et al.*, 1976), as has also been observed in pigs (Tseng, 2004) and sheep (Dutt, 1961). Furthermore, early mammal embryos are also susceptible to damage from ROS produced by elevated temperatures (Johnson & Nasr-Esfahani, 1994).

Heat stress directly affects the dominant follicle and its ability to exert dominance, altering also the oocyte enclosed in (Cavestany *et al.*, 1985; Al-Katanani *et al.*, 2002b). It is noteworthy that one of the major causes of the decline in summer female fertility is due to perturbation in oocyte physiology that results in oocytes of reduced competence to develop embryo (Payton *et al.*, 2004; Roth & Hansen, 2004). Although, embryo development are also directly altered by high temperatures, as described by Putney *et al.* (1989a; b). All the effects depend on the duration and severity of heat stress. These mechanisms ultimately lead to an arrest of the cell cycle, growth and proliferation (Lindquist, 1986; Yost & Lindquist, 1986) and may result in cell death.

Heat stress effects on ovarian follicles

Follicle environment is essential for oocyte growth and development, and it is absolutely dependent on the nurturing capacity of the follicle, in particular by the granulosa cells. Communication between the germ cell and somatic cell compartments of the follicle is essential for normal oogenesis and folliculogenesis (Dong *et al.*, 1996; Simon *et al.*, 1997; Albertini *et al.*, 2001) mainly done by paracrine (Eppig, 2001) and gap-juntional signalling (Eppig, 1991). Usually, the pre-ovulatory follicles are approximately 1.5 ± 2ºC cooler than the ovarian stroma (pig: Hunter *et al.*, 1997; 2000; human: Grinsted *et al.*, 1985; cow: GrØndahl *et al.*, 1996). However, no differences in temperature were observed between the stromal tissue and small antral follicles (GrØndahl *et al.*, 1996; Hunter *et al.*, 1997).

Hyperthermia can directly disrupt follicular function with adverse carry-over effects on its competence (Roth *et al.*, 2000) by altering the follicle or by the metabolic and hormone changes produced by heat stress. All the alterations are reflected in the follicular fluid of the dominant follicle (Shehab-El-Deen *et al.*, 2010) and hence directly affect the developmental capacity of the enclosed oocyte (Leroy *et al.*, 2005) and granulosa cells (Vanholder *et al.*, 2005), incresing apoptosis rate and decreasing embryonic development (Aroyo *et al.*, 2007). However, not only the antral follicles emerged in the follicular wave are affected by high temperatures, also the ovarian pool of small antral follicles (Roth *et al.*, 2000) which extends the negative heat stress effects through autumn.

The heat stress effects on follicle impair the follicle dynamics (Badinga et al., 1993) and developmental competence (Rocha et al., 1998; Al-Katanani et al., 2002b). Follicular growth can be suppressed (Delasota et al., 1993; Gong, 2002) by the compromision of plasma hormone levels (LH, FSH, inhibin, estradiol, IGF-I; Wolfenson *et al.*, 2000; De Rensis & Scaramuzzi, 2003). On the other hand, dominance is also affected by high temperatures (Badinga *et al.*, 1993; Wolfenson *et al.*, 1995; Roth *et al.*, 2000) due to the decrease in size of the first-wave of dominant follicles and the earlier emergence of the second-wave of dominant preovulatory follicle (Badinga *et al.*, 1993; Wolfenson *et al.*, 1995; Roth *et al.*, 2000). Hence, the population of subordinate follicles increases (Badinga *et al.*, 1993) resulting in the ovulation of an aged follicle (Mihm *et al.*, 1999), in follicular co-dominance (Sartori *et al.*, 2004), anovulation or double ovulation (Wolfenson *et al.*, 2000; López-Gatius *et al.*, 2005). These alterations have also potentially adverse effects on the quality of oocytes (Badinga *et al.*, 1993; Roth *et al.*, 2001a; b) and follicular steroidogenesis (Roth *et al.*, 2001a; b; Al-Katanani *et al.*, 2002b).

Heat stress effects on steroidogenesis

Impaired dominant follicle during heat stress also compromise follicle steroidogenic capacity (Faust *et al.*, 1988; Wolfenson et al., 1997) and gonadotropin secretion (Gilad *et al.*, 1993; Wolfenson *et al.*, 1997; Roth *et al.*, 2000). The altered metabolic conditions in the follicular fluid

are known to directly affect granulose cell growth and decrease steroidogenic capacity (Vanholder *et al.*, 2005) wich involve:

- Lower aromatase activity and viability of the granulosa cells (60% of its winter value; Badinga *et al.*, 1993).
- Lower estradiol concentration (50% of that in winter; Badinga *et al.*, 1993; Wolfenson *et al.*, 1997) and higher progesterone (Roth *et al.*, 2001b) in the follicular fluid of dominant follicles (Badinga *et al.*, 1993; Wolfenson *et al.*, 1997) due primarily to a drastic reduction of androstenedione production by theca cells during summer (Wolfenson *et al.*, 1997; Roth *et al.*, 2001b).

Hence, elevated temperature causes follicular cells to luteinize prematurely, accompanied by premature meiotic maturation of the oocyte (Mihm *et al*, 1999), subsequent embryonic development is retarded (Ahmad *et al.*, 1995) and fertility is dramatically compromised (Stock & Fortune, 1993; Mihm *et al.*, 1994). In fact, the steroidogenic effects are lasted until autumn (Wolfenson *et al.*, 1997; Roth *et al.*, 2000; Roth & Hansen, 2004). However, the extent of the heat stress effects is transient and a spontaneous improvement of fertility take place throughout autumn and early winter (Zeron *et al.*, 2001).

Heat stress effects on oocyte (Figure 6)

Although, it is suggested that the recent zygote is most susceptible to maternal heat stress (Dutt, 1961; Sugiyama *et al.*, 2007), the preovulatory bovine oocyte is very sensitive to elevated ambient temperatures (Ingraham *et al.*, 1976; Putney *et al.*, 1988a). Heat stress during the period of follicular growth has the potential to compromise the oocyte, being temperature (Edwards & Hansen, 1996) and duration dependent (Payton *et al.*, 2004). Oocyte quality is then compromised either because of direct actions of elevated temperature on the oocyte or because of alterations in follicular function (Hansen & Arechiga, 1999). After the harmful effects of heat stress it is required a period of two to three estrous cycles for recovery from heat damage and the appearance of competent oocytes (Roth *et al.*, 2001a).

The viability of bovine oocytes is lower in warm versus cold seasons (Monty & Racowsky, 1987; Al-Katanani *et al.*, 2002b), being those oocytes obtained in summer morphologically abnormal displaying a degenerated dark ooplasm, incompletely filled oolemic cavity and dispersed cumulus-cells (Rocha *et al.*, 1998). Furthermore, heat-stress increases the proportion of denuded and/or degenerated cumulus-oocyte complexes (COCs; Torres-Junior *et al.*, 2008).

Taking into account that direct effects on oocyte cannot be studied *in vivo*, the development of *in vitro* studies is indicated, where oocytes are matured 24-48h until reach MII. The use of *in vitro* studies to study heat shock effects facilitates direct determination with the absence of indirect effects on follicles (Gilad *et al.*, 1993; Wolfenson *et al.*, 1997; 2000) or the endometrium (Putney *et al.*, 1988b). The temperatures applied on *in vitro* maturation (IVM) process are based on

visceral temperature, being higher than temperatures within the ovary (GrØndahl *et al.*, 1996; Hunter *et al.*, 1997; 2000). However, this variation in temperature did not alter the developmental rates of bovine oocytes (Shi *et al.*, 1998).

A variety of studies have reported that heat shock impairs both nuclear and cytoplasmic maturation events, such as translocation of cortical granules to the oolemma (Payton *et al.*, 2004), cytoskeleton rearrangement (Roth & Hansen, 2005) and spindle formation (Ju *et al.*, 2005; Roth & Hansen, 2005). As observed during *in vitro* studies, oxidative stress during meiotic maturation may also induce chromosomal errors that prove undetectable in the living oocytes and whose developmental consequences may become manifest after fertilization (Tarín *et al.*, 1996), compromising embryo development. Therefore, continued efforts to identify ovum components perturbed after direct application of heat stress are important, because the oocyte contributes greater than 99% of cytoplasm and half the genetic material to the resulting embryo after fertilization (Schrock *et al.*, 2007).

Exposure of the oocyte to heat stress at the germinal vesicle stage (Payton *et* al., 2004) or during early stages of maturation (Edwards & Hansen, 1997; Roth & Hansen, 2004) results in deleterious effects on the internal organelles, being reduced the oocyte ability for fertilization and further embryonic development. One of the major damages observed in bovine oocytes in response to heat stress conditions are cytoskeleton defects (Gallicano, 2001; Figure 5). The cytoskeleton is mainly composed by microtubuli and microfilaments, and their structure is altered in mature bovine oocytes and two-cell bovine embryos exposed to 41-41.5°C (Tseng et al., 2004). Mild heat stress leads to the reorganization of actin filaments into stress fibers, while severe heat stress results in the aggregation of vimentin or other filament-forming proteins, leading to the collapse of intermediary, actin, and tubulin networks (Welch & Suhan, 1985; Toivola et al., 2010). Moreover, the loss of the correct localization of organelles and a breakdown of intracellular transport processes are observed. The Golgi system and the endoplasmic reticulum (ER) become fragmented under stress conditions and the number of mitochondria and lysosomes decreases (Welch & Suhan, 1985), which lead to a dramatic drop in ATP levels during heat stress (Patriarca & Maresca, 1990). Accordingly, many intracellular processes which are relying on these organelles are negatively affected.

- ***Nuclear maturation:*** The exposure of germinal vesicle oocytes to heat stress reduce the ability of the oocyte to reach the MII stage (Payton *et al.*, 2004). Roth and Hansen (2005) reported a higher proportion of bovine oocytes matured at 38.5ºC reaching MII (characterized by a metaphase plate and formation of the first polar body) than oocytes matured at 40 and 41ºC. Since heat shock reduce the proportion of oocytes that reached MII, there was a corresponding increase in the proportion of heat-shocked oocytes at other stages (MI, AI and TI), in fact, more of the arrested oocytes were at MI for oocytes matured at 41ºC (Roth & Hansen, 2005).

It is known that the meiotic spindle microtubules are susceptible to temperature changes (Aman & Parks, 1994) by affecting the polymerization or depolymerization of meiotic spindle microtubules (Ju *et al.*, 2005). Consequently, the dispersion and segregation of the chromosomes is altered and ultimately can cause chromosomal imbalance and cell death (Ju *et al.*, 2005). The altered or elongated spindle is the predominant pattern on heat-stressed oocytes. Abnormal spindles are characterized by being width sorther than length (width < length), often with reduced size or aberrant microtubules (Ju *et al.*, 2005) and some unaligned chromosomes, or amorphous shape and a large degree of disorganization of chromosomal alignment (Roth & Hansen, 2005). A decrease in spindle sizes is generally observed as the duration of heat shock increase (Ju *et al.*, 2005), being reduced in all dimensions (Ju & Tseng, 2004; Ju *t al.*, 2005). In fact, chromatin starts to aggregate during heat shock, which appear to be correlated with depolymerization of spindle microtubules. These alterations in the spindle microtubules may contribute to altered chromosome separation during fertilization and subsequent embryonic division (Streffer, 1984). These deleterious effects provided a plausible explanation for the low developmental competence observed in heat-stressed bovine oocytes, as well as the low conception rates during warm season (Ju *et al.*, 2005).

- ***Cytoplasmic maturation:*** It has been described that heat stress during maturation affects mainly the cytoplasm rather than the nuclear components of the oocyte, may be due to cytoplasmic maturation is better accomplished *in vivo* than *in vitro* (Wang *et al.*, 2008). Hence, oocyte cytoplasmic maturation is less tolerant to heat stress than nuclear maturation (Wang *et al.*, 2008) since it is impaired at 40ºC and nuclear maturation is not affected until 40.7ºC.

  Cytoplasmic maturation is completed when cortical granules migrate from the Golgi apparatus through the oolema to the vitelline surface, assuming a position 0.4–0.6 μm below the plasma membrane (Ducibella & Buetow, 1994). The translocation of cortical granules to the oolemma after resumption of meiosis occurs via actin microfilaments (Wessel *et al.*, 2002) which are also affected by high temperatures.

  The effect of heat stress on migration of cortical granules is controversial. While an incomplete migration of cortical granules has been described in mouse (Wang *et al.*, 2008), a pre-mature translocation has been described in bovine oocytes (Payton *et al.*, 2004). The incomplete migration of cortical granules in heat stressed oocytes could have been by alterations in microfilament structure (Ju & Tseng, 2004; Rivera *et al.*, 2004). In those cases the peri-cellular actin ring and trans-zonal actin processes are generally absent for heat-stressed oocytes (Roth & Hansen, 2005). Whereas, the pre-mature migration can be explained since heat stress seems to induce premature aging of oocytes (Lawrence *et al.*, 2004; Edwards *et al.*, 2005; Schrock *et al.*, 2007).

On the other hand, mitochondrial congregation around the spindle in the oocyte (Ju & Tseng, 2004; Ju *et al.*, 2005; Roth & Hansen, 2005) and membrane potencial (Soto & Smith, 2009) is also affected by high temperatures. It is suggested that the incomplete congregation of mitochondrial distribution observed in the heat-stressed oocytes could have resulted from a poor mediation of mitochondrial translocation by the microtubules (Ju & Tseng, 2004; Ju *et al.*, 2005; Roth & Hansen, 2005).

- ***Molecular maturation:*** Although studies on molecular events occurring during oocyte maturation under heat stress are scarce, it appeared that protein synthesis is affected by 30 to 50% in heat-stressed oocytes, as reported in mice (Curci *et al.*, 1987) and cattle (Edwards & Hansen, 1996; 1997).

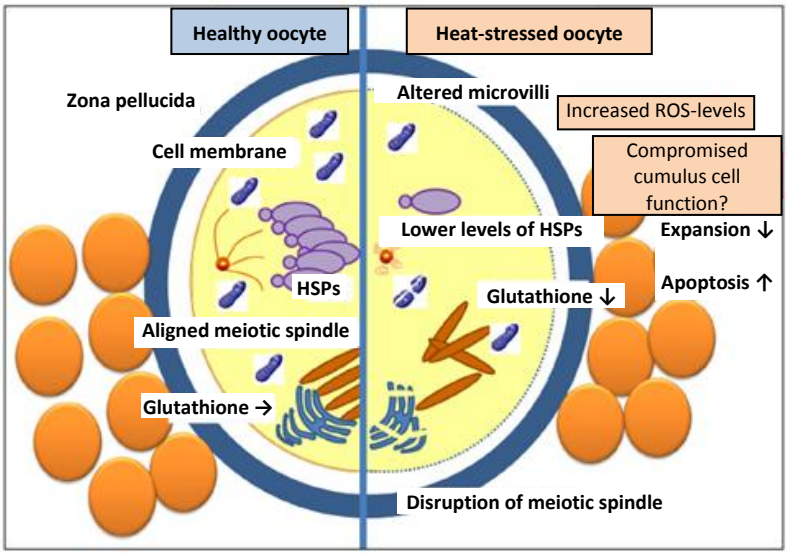
- ***Apoptosis:*** When heat shock takes place during oocyte maturation, apoptosis rate increases (Ju & Tseng, 2004; Roth & Hansen, 2004; 2005; Tseng *et al.*, 2006;), leading to apoptosis in the oocyte (Roth & Hansen, 2004) and cumulus cells, reducing their expansion and associated with reduced developmental competence (Arav & Roth, 2008). Apoptosis events mainly occur by activation of the sphingomyelin pathway, generating the second messenger ceramide (Mathias *et al.*, 1998). Ceramide, in turn, may regulate apoptosis via different signaling cascades (Bcl-2, apoptotic protease-activating factor-1 (Apaf-1), family of cysteine-aspartic acid-specific proteases (caspases)). According to that, heat-stressed oocytes show a higher caspase (Paula-Lopes & Hansen, 2002; Roth & Hansen, 2004) activity compared with freshly oocytes, characterized also by expanded cytoplasm with a small perivitelline space (Roth & Hansen, 2004).

  However, the fact that only a fraction of oocytes exposed to heat stress experienced apoptotic changes points out that some oocytes are better equipped to survive to cellular stress. In accordance, cumulus cells are found to have a critical role in protecting porcine oocytes against oxidative stress-induced apoptosis (Tatemoto *et al.*, 2000) and provide thermoprotection to bovine oocytes (Edwards & Hansen, 1996). Another possibility is that oocytes differ in amounts of HSP70, although oocytes cannot synthesize increased amounts of HSP70 in response to heat shock (Edwards & Hansen, 1996; Edwards *et al.*, 1997).

- ***Aging:*** Previous studies have demonstrated that an increase in temperature may modify the kinetics of maturation (Payton *et al.*, 2003). It has been described that heat-stressed oocytes mature faster than controls (Edwards *et al.*, 2005), inducing a premature aging of the oocytes which have reduced developmental competence (Fissore *et al.*, 2002) as in heat-stressed (Edwards & Hansen, 1996; 1997; Lawrence *et al.*, 2004) oocytes, reducing its fertile lifespan. A premature CG-exocytosis prior to fertilization was reported in early studies and was suggested to be responsible for the enlargement of perivitelline space and altered properties of the zona pellucida (Okada *et al.*, 1993). Taking together, altered kinetics within the ooplasm (premature or accelerated translocation of the cortical granules to the oolemma)

and possible increases in free radical production suggest that a major effect of heat stress may be to ''age'' the oocyte.

Figure 6. Effects of heat stress on the organization of the oocyte at the cellular level (Shehab-el-deen, 2011). Left: an unstressed oocyte, right: oocyte under heat stress. Heat stress leads to damage to the cytoskeleton and as such to poor alignment of the chromosomes (brown filaments). Organelles like the golgi and the endoplasmic reticulum (blue) become fragmented and disassemble. The number and integrity of mitochondria (dark blue) decreases. The synthesis of heat shock proteins (HSPs) decreases (purple). Also, there are changes in the membrane morphology, aggregation of membrane proteins, and an increase in membrane fluidity. Together, all these effects stop cell growth and lead to cell-cycle arrest.



- ***Membrane alterations:*** Seasonal variations in membrane composition have been reported (Zeron *et al.*, 2001). Oocyte membrane is composed by more saturated fatty acids during the warm season than during the cold season of the year, composed mainly by mono- and polyunsaturated fatty acids (i.e. palmitic acid, oleic acid). These alterations in membrane composition are associated with a decrease in oocyte viability and developmental competence.

  There is evidence that heat stress can alter oocyte phospholipid composition (Zeron *et al.*, 2001) by lipid peroxidation and calcium oscillations (Takahashi *et al.*, 2003). In accordance, zona pellucida structure and the microvillus pattern of the oolemma are affected (Ju *et al.*, 1999). The changes that can be observed in membrane morphology are: (i) alteration in the protein:lipid ratio, and (ii) an increased fluidity of membranes (Vigh *et al.*, 2007). These changes led to an increase in membrane permeability, a drop in cytosolic pH and changes in ion homeostasis (Piper *et al.*, 2003). However, it has also been described that heat stress does not compromise membrane integrity (Payton *et al.*, 2003; Lawrence *et al.*, 2004).

- ***Sperm-egg fusion:*** Hirao and Yanagimachi (1978) reported negative effects on sperm–egg fusion and on post-fusion events because of high ambient temperatures. These results imply that fertilization of heat-stressed oocytes is reduced. However, the observed effects conflict with the fact that it has been also described an increase in polyspermy rate by Ju *et al.* (2005).

- ***Development alterations:*** Taken together, these negative effects by high temperatures suggest perturbations occurring in the ooplasm, nucleus, or cumulus cells by heat may compromise continued development of the bovine oocyte (Edwards *et al.*, 2005). Heat shock during the onset of ooestrus and insemination (Ealy *et al.*, 1993; Putney *et al.*, 1989a), or during IVM, reduces developmental competence (Roth & Hansen, 2005; Roth, 2008) as determined by a reduction in cleavage rate (Roth & Hansen, 2004; 2005; Shimizu *et al.*, 2005) and in the percentage of oocytes and cleaved embryos that develop into blastocysts (Edwards *et al.*, 1997; Al-Katanani *et al.*, 2002b; Roth & Hansen, 2004; Payton *et al.*, 2004; Tseng *et al.*, 2006), with an increased proportion of abnormal and retarded embryos (Putney *et al.*, 1989a; Ealy *et al.*, 1993). Blastocyst quality is also decreased by a reduction of total cell number (Sakatani *et al.*, 2004; Matsuzuka *et al.*, 2005). It is consistent with the fact that oocytes harvested from follicles during summer have reduced ability to develop to the blastocyst stage after *in vitro* fertilization than oocytes harvested during winter (Rocha *et al.*, 1998; Al-Katanani *et al.*, 2002). In addition, it is a time dependant process. The oocyte exposition to high temperatures have more pronounced effects on subsequent blastocyst development after fertilization during the first half than during the last 12h of maturation (Edwards *et al.*, 2005; Schrock *et al.*, 2007).

Heat stress effects on cumulus cells

Cumulus cells have a close connection with oocytes during the course of maturation (Eppig, 1982; Buccione *et al.*, 1990) through gap junctions (Moor *et al.*, 1980), which facilitates the transfer of nutrients and molecular signals between oocytes and cumulus cells (Warnes *et al.*, 1977). Gonadotropins, steroids, and other factors from the follicle cells also interact with oocytes to provide essential support for *in vivo* maturation of oocytes (Warnes *et al.*, 1977).

It is generally accepted that cumulus cells support the maturation of oocytes to the MII stage and greatly enhance cytoplasmic maturation (Tanghe *et al.*, 2002), which is responsible for the capacity to undergo normal fertilization and subsequent embryonic development (Tatemoto *et al.*, 2000). Around meiotic resumption, cumulus cells begin to withdraw from the oocyte and there is almost complete loss of gap-junctional communication by the time oocytes reach MI due to a considerable extracellular production of hyaluronic acid by cumulus cells that causes the dispersion of cumulus cells or cumulus expansion (Chen *et al.*, 1990).

However, cumulus cells play also an important role protecting oocytes against irremediable cell damage encompassed by oxidative stress during oocyte maturation, by increasing glutathione content in the oocyte (Tatemoto *et al.*, 2000). The level of antioxidants in the oocyte may be important for the defense against heat stress since it has been shown that addition of an antioxidant (as retinol) could prevent heat-induced reductions in blastocyst development (Lawrence *et al.*, 2004).

During heat stress the surrounding cumulus cells might be affected. Certainly, some of the negative effects of heat stress in reducing ovum development or the ability for fertilization (Zhang *et al.*, 1995) may be mediated through cumulus. Prolonged exposure to heat stress reduces hyaluronic acid production and cumulus cell expansion (Lenz *et al.*, 1983), and also increases DNA damage (Yuan *et al.*, 2008).

Heat stress effects on embryo

Preimplantational embryo development involves processes as cell proliferation, differentiation, and death; all of them tightly regulated by signalling between the embryo and the maternal environment (Ozawa *et al.*, 2002; Matsuzuka *et al.*, 2005). Changes in its environment can affect the embryo, especially during the first cleavage divisions, when most of the embryonic genome is still inactive (Memili & First, 2000) and when systems for regulating osmotic balance are not completely functional (Lane, 2001), making embryos particularly sensitive to certain forms of stress (as heat stress or fever; Ozawa *et al.*, 2002; Sartori *et al.*, 2002). Hence, a rise in body temperature above its regulated set point can compromise the viability of an early developing embryo (Hansen, 2009).

It has been reported that high ambient temperatures between the onset of oestrus (day 1) to the seventh day after oestrus increase the number of abnormal and retarded embryos, and also embryo mortality (Edwards *et al.*, 2001), in artificially inseminated superovulated heifers (Putney *et al.*, 1988a; 1989a). It is a common phenomenon reported in a wide range of species such as bovine (Ingraham & Wager, 1971), ovine (Woody & Ulberg, 1964), porcine (Wildt *et al.*, 1975), rodents (Ozawa *et al.*, 2002). The susceptibility to heat stress increases at the same time that production, so, the harmful effects are more pronounced in dairy cows of high milk production (Sartori *et al.*, 2002).

During the maternal hyperthermia, an increase in ROS concentrations take place coincident with a reduction in antioxidant molecules in embryos (Ozawa *et al.*, 2002). Furthermore, ambient temperature can cause modifications in the physical and chemical biomembrane properties (Zeron *et al.*, 2001), although no alterations have been found in other studies (Edwards & Hansen, 1997). On the other hand, the harmful effects due to heat stress include a decrease in development (Arechiga *et al.*, 1995; Edwards & Hansen, 1997; Rivera & Hansen, 2001) and protein synthesis (Edwards *et al.*, 1995), resulting in a reduced embryo survival rate (Dutt, 1963; Putney *et al.*, 1988a; Ealy *et al.*, 1993). All these effects have been constated by *in vitro* studies,

were oocytes submitted to high temperatures during oocyte maturation showed a reduced cleavage rate and number of embryos which develop to blastocyst stage (Guérin *et al.*, 2001; Al-Katanani *et al.*, 2002b; Roth & Hansen, 2004). On the other hand, embryos submitted to high temperatures also show a reduction in the embryo development to blastocyst stage (Edwards & Hansen, 1997), a decrease in the total number of cells per embryo and an increase in DNA alterations, apoptosis rate (Paula-Lopes & Hansen, 2002; Matsuzuka *et al.*, 2005) and embryo mortality (Ryan *et al.*, 1992; Ozawa *et al.*, 2002), which tend to be more harmful as the severity of heat stress increase, being more vulnerable male than female embryos (James, 1998).

However, effects of heat stress on embryonic survival decrease as embryos proceed through development (Hansen & Arechiga, 1999). This has been shown *in vivo* in sheep (Dutt, 1963), pigs (Tompkins *et al.*, 1967), cattle (Putney *et al.*, 1988a) and rabbits (Wolfenson & Blüm, 1988), and also *in vitro* for cattle (Ealy *et al.*, 1995; Edwards & Hansen, 1997; Ju *et al.*, 1999). The fact that the deleterious effects of maternal heat stress decline as pregnancy proceeds (Ealy *et al.*, 1993) may reflect the acquisition of thermal resistance by the preimplantation embryo as it progresses from the zygote to blastocyst (Edwards & Hansen, 1997). These results suggest that the embryo acquires thermotolerance during development and fertility can be improved during periods of heat stress if cooling is provided for a limited period when embryos are most sensitive to thermal stress (Ealy *et al.*, 1993). In fact, the effects are more severe when high temperatures are applied during *in vitro* fertilization than during *in vitro* maturation (Sugiyama *et al.*, 2007). These results indicate that the oocyte and early embryo undergo a biphasic developmental pattern of resistance to heat shock, with fertilization and cleavage to the two-cell stage being associated with an increase in thermal sensitivity (Edwards & Hansen, 1997). This is followed by morula stage with the restoration of resistance to elevated temperatures. Thus, the embryo acquires one or more thermoprotective responses as embryonic development proceeds. One of the processes that may be involved in developmental acquisition of resistance to heat shock may be stress-induced apoptosis (Paula-Lopes & Hansen, 2002).

Possible explanations why the susceptibility to heat stress effects varies upon development are:

- **Cumulus cells:** may provide protection to the oocyte from elevated temperatures (Edwards & Hansen, 1996). Their loss following fertilization and cleavage may increase thermal sensitivity of two-cell embryos (Chian & Sirard, 1995; Edwards & Hansen, 1996).
- **Increase cell number:** allows a larger embryo survive to the loss of a fraction of its cells.
- **Adquisition of biochemical mechanisms for thermoprotection:** the increase in the synthesis of HSPs (Edwards & Hansen, 1996) limits the deleterious effects of heat stress. While heat shock has no effect on synthesis of either form of HSP70 in oocytes (Edwards & Hansen, 1996; 1997), synthesis of HSP70 molecules is increased by heat shock as early as the 2-cell stage (Edwards & Hansen, 1996; Edwards et al., 1997).

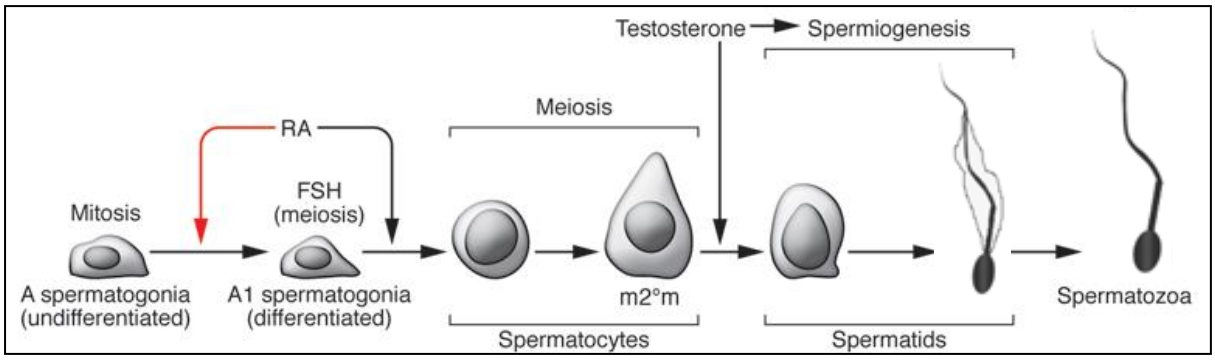
In addition, *in vitro* studies tested found that exposure to a mild heat shock would make embryos more resistant to apoptosis induced by a severe heat shock (Paula-Lopes & Hansen, 2002). The biochemical mechanisms by which mild heat shock prevents apoptosis induced by a more severe heat shock presumably involves HSP70 (Beere & Green, 2001). Therefore, those embryos at stages of development that are capable of heat-induced apoptosis are more resistant to the deleterious effect of heat shock on development. One possibility is that the sensitivity of the early embryo to heat shock is a reflection, at least in part, of the failure of the embryos to undergo heat-induced apoptosis to remove damaged cells from the embryonic lineage (Paula-Lopes & Hansen, 2002).

6. 2. CONSEQUENCES IN THE MALE GAMETE

6. 2. 1. Spermatogenesis

The male germ cell development takes place in testes (Clermont, 1972). Spermatogenesis is a complex biological process which includes changes in diploid cells (spermatogonia) until spermatozoa formation (haploid cells). The process includes three phases: mitotic, meiotic and post-meiotic maturation (spermiogenesis). Spermatogenesis in the meiotic process occurs in spermatocytes, and these cells can be divided into different subpopulations based on their chromatin structure (Russell *et al.*, 1990). Recombination and separation of homologous chromosomes occurs in pachytene spermatocytes during meiosis I and results in the formation of secondary spermatocytes. These cells then proceed through meiosis II, wherein sister chromatids are separated into individual cells. At the completion of meiosis, four haploid gametes, termed round spermatids, result from the division of every spermatocyte. Each round spermatid then undergoes dramatic changes in its cellular morphology (spermiogenesis) to form first an elongating spermatid and finally a spermatozoon (Hogarth & Griswold, 2010; Figure 7). Spermiogenesis takes place in 7 days and includes the morphological modification of round spermatids to spermatozoa, losing cytoplasm and adquiring a flagellum. The spermiogenesis takes several days depending on the species (i.e. 35 days in mice (Paul *et al.*, 2008), 43-44 days in rabbit (Swiestra & Foote, 1965), 72 in man (Paul *et al.*, 2008) or 65 in bull (Saunders, 2003)). During this maturation process the DNA of the germ cell is vulnerable (Robaire & Hales, 2003) and altered cells with damaged DNA are routinely eliminated by apoptosis (Paul *et al.*, 2008).

Figure 7. Expansion of both the undifferentiated (A spermatogonia) and differentiated (A1 spermatogonia) spermatogonial populations occurs by mitosis of these cell types, regulated in part by FSH. The red arrow denotes the required action of vitamin A (RA) in this transition. The subsequent conversion of differentiated spermatogonia to spermatocytes, representing the initiation of meiosis, also requires RA activity. The differentiation of secondary spermatocytes (m2°m) and the process of spermiogenesis (round spermatids to elongated spermatids, then spermatozoa) require testosterone (modified from Hogarth & Griswold, 2010).



6.2.2. Heat stress effects on spermatozoa

Heat stress effects on the testis can be direct on specific cell types within them, but also from indirect effects resulting from the cellular changes occurring in the testis (Setchell, 1998). These effects can be classified at four levels:

- On the testis itself.
- On the number, structure or motility of spermatozoa leaving the testis.
- On the fertilizing ability of the spermatozoa when are apparently normal in appearance and motility.
- On the embryo development from heat-stressed spermatozoa.

It is noteworthy that semen characteristics are not immediately affected by heat. In fact, it will depend on the duration of spermatogenesis, when damaged spermatogenic cells do not enter ejaculates for some time after heat stress. In general, alterations in semen occur about two weeks after heat stress and do not return to normal values until up to eight weeks following the end of the stress (Hansen, 2009).

Sperm quality is directly related with the fertilizing ability of the spermatozoa, and also with the reproductive success (Rodriguez-Martinez & Barth, 2007). The negative effects reported are related with a high exposure of spermatozoa to ROS, which are particularly sensitive to ROS damages mediated by lipid peroxidation because of their high content of polyunsaturated fatty acids (PUFA; Aitken & Clarkson, 1987; Alvarez *et al.*, 1987). In fact, human *in vitro* studies pointed that exposure of sperm to ROS (i.e. hydrogen peroxide) reduces sperm motility, sperm viability, induces acrosme reaction and lipid peroxidation (Fatma *et al.*, 2009; Figure 8). All the ROS generated within semen could produce infertility (Tremellen, 2008). Some of the alterations produced are similar with an "immature" semen picture (i.e. low sperm motility, high numbers of proximal cytoplasmic droplets and abnormal sperm head shapes; Malmgren, 1989).

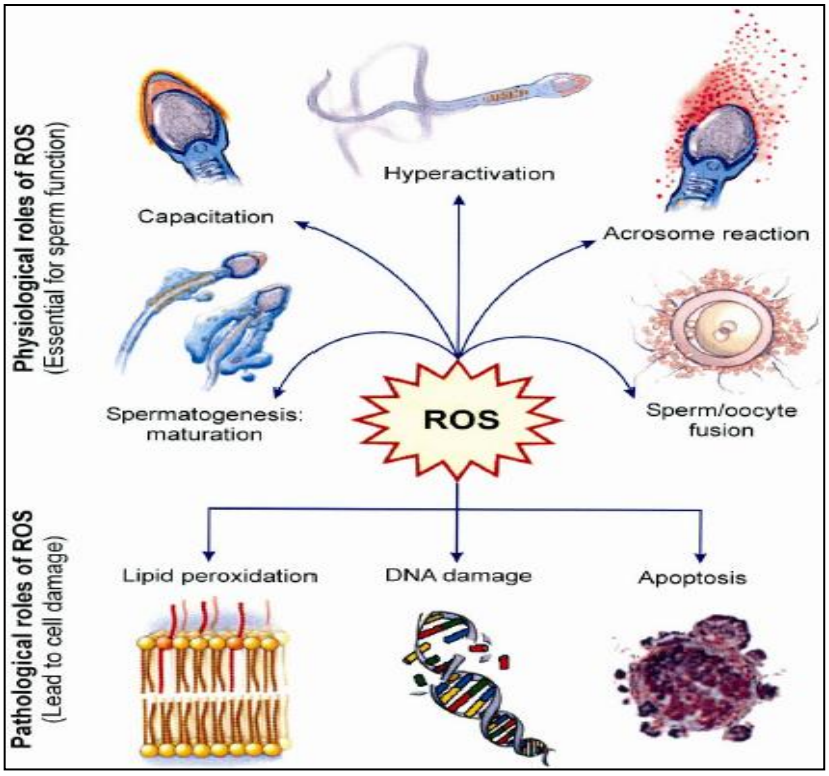


Figure 8. Physiological roles of ROS include maturation, capacitation, hyperactivation, the acrosome reaction and sperm-oocyte fusion. Patohological roles of ROS include lipid peroxidation, DNA damage and apoptosis (Kothari *et al.*, 2010).

Effects on sperm viability

Sperm quality is better during cold season than during warm season, with higher percentages of live sperm (Borg *et al.,* 1993; Daader *et al.*, 1997). Low levels of testosterone induced by high temperatures altered the normal epididymal function producing an increase in number of dead spermatozoa (Mieusset *et al.*, 1992b; Marai *et al.*, 2002; Banks e*t al.*, 2005). As a consequence male fertility decreases. The exposure to high THI had immediate negative effects on sperm viability (Roca *et al.*, 2005; Theau-Clement *et al.*, 1995; Marai *et al.*, 2002), although in general, the increased number of dead spermatozoa show a great rise above two weeks after the highest THI (García-Tomás *et al.*, 2008), and the effects tend to persist between 40 and 60 days after the end of heating (Casady *et al.*, 1953; Skinner & Louw, 1966).

Effects on sperm morphological normalcy

Semen quality varies according to the temperatures among the year. Hence, increased incidence of sperm abnormalities have been reported during the warmer seasons of the year (Ax *et al.*, 1987; Finzi *et al.*, 1995; Daader *et al.*, 1997; Safaa *et al.*, 2008), which are immediately evidencied after actue exposure to high THI (Roca *et al.*, 2005; Theau-Clement *et al.*, 1995; Marai *et al.*, 2002), showing a great drop two weeks later of the highest THI (Moule & Waites, 1963; Smith, 1971; Roca *et al.*, 2005; García-Tomás *et al.*, 2008). The reduction in the percentage of normal sperm does not return to the physiological levels until 6-8 weeks after the end of heat stress (Casady et al, 1953; Skinner & Louw, 1966; Stone, 1981; Malmgren and Larsson, 1984; Meyerhoeffer *et al.*, 1985).

Some of the most frequent defects recorded are proximal (Malmgren, 1989) and distal protoplasmic droplets (Stone, 1982), being higher the proportion of spermatozoa with proximal protoplasmic droplet during the summer months (Vilakazi & Webb, 2004). Heat stress causes also a significant increase of coiled tails (Finzi *et al.*, 1995), decapitated sperm (tailess (Rathore, 1968; Wildeus & Entwistle, 1983) and also pyriform cells (i.e. cells showing posterior constriction of the head region; Vilakazi & Webb, 2004). In accordance, Morrow (1980) indicated that a reduction in fertility may occur in domestic animals when more than 30% of the ejaculated spermatozoa have structural defects.

Effects on sperm acrosomal membrane

The acrosome reaction is an essential event for sperm membrane fusion, being acrosome integrity, as well as enzyme maintenance, essential for fertilization (Õura & Toshimori, 1990; Flesch & Gadella, 2000). The evaluation of the acrosome integrity can also be crucial to predict sperm fertilizing ability (Celeghini *et al.,* 2007). Seasonal changes are described in terms of acrosomal membrane, being the acrosome membrane less altered during winter than in summer season (Meyerhoeffer *et al.*, 1985; Borg *et al.*, 1993; Roca *et al.*, 2005; Safaa *et al.*, 2008). Spermatozoa with changes or abnormalities in sperm acrosomal membrane indicate that have suffered damage by heat stress (Finzi *et al.*, 2010), especially due to the secondary oxidative stress derived from high temperatures (Yin *et al.*, 1997). *In vitro* studies revealed that elevated temperatures little as 40ºC are enough to negatively affect the sperm acrosome and acrosome reaction (Lenz *et al.*, 1983; Malmgren, 1989), causing cell death (Lenz *et al.*, 1983). Abnormalities do not return to physiological levels until 5-8 weeks after the end of heat stress (Wettemann *et al.*, 1979; Meyerhoeffer *et al.*, 1985), although the severity of the effects will depend on the exposition time to heat (Finzi *et al.*, 2010).

Effects on other sperm membranes

Membranes exert a crucial role in the maintenance of sperm fertilizing ability. The plasma membrane is responsible for the maintenance of cell osmotic equilibrium, and its integrity is crucial to sperm survival inside the female reproductive tract (Õura & Toshimori, 1990). However, spermatozoa are especially sensitive to oxidative stress derived from high temperatures due to their high content in PUFA in their membranes, and also by their limitable content in antioxidant molecules (Baker & Aitken, 2005). Sperm plasma membrane can be damaged by ROS which lead to sperm dysfunction resulting in the inability of sperm to penetrate the oocyte (Sanocka & Kurpisz, 2004). Furthermore, sperm plasma membranes damaged show an increase in membrane permeability leading to antioxidant enzymes get out from sperm (Lasso *et al.,* 1994), being described in rabbits (Roca *et al.*, 2005) and boars (Waberski *et al.*, 2010) submitted to high THI.

Effects on sperm motility

Motility is the main factor that allows sperm to reach the oviduct through the cervix and the utero tubal junction (Nakanishi *et al.*, 2004). Season affects sperm motility index, being the lowest motility index recorded in summer (Ax *et al.*, 1987; El-Sherbiny, 1987). Nevertheless, motility increases gradually until reach the highest values during spring (Roca *et al.*, 2005; Safaa *et al.*, 2008). The reduction in progressive motility in presence of high temperatures (Lenzi *et al.*, 1993; Agarwal *et al.*, 1994; Jannes *et al.*, 1998; Armstrong *et al.*, 1999; Roca *et al.*, 2005) is related with the production of ROS (Aitken *et al.,* 1989). However, the exact mechanism through which ROS causes a reduction in motility is not understood. One hypothesis shows that H_2O_2 can diffuse across the membrane into the cells and inhibit the activity of some vital enzymes that controls glucose flux and the intracellular availability of NADPH. This is used as a source of electrons by spermatozoa to fuel the generation of ROS (Aitken *et al.*, 1997). Another hypothesis involves a decrease in axonemal protein phosphorylation and sperm immobilization, both associated with a reduction in membrane fluidity that is necessary for sperm-oocyte fusion (De Lamirande & Gagnon, 1992).

Motility is one of the most sensitive indicators of heat stress. The negative effects on semen quality have been reported for mice (Ren *et al.*, 2006; Pérez-Crespo *et al.*, 2008), rabbits (Roca *et al.*, 2005; Theau-Clement *et al.*, 1995; Marai *et al.*, 2002), rams (Moule & Waites, 1963; Smith, 1971; Mieusset *et al.*, 1992b), boars (McNitt & First, 1970; Stone, 1981; Malmgren & Larsson, 1984) and bulls (Monterroso *et al.*, 1995; Brito *et al.*, 2003), returning to physiological levels 6-8 weeks after the end of heat stress (Heitman & Cockrell, 1984; Meyerhoeffer *et al.*, 1985).

Effects on DNA integrity (Figure 9)

Severe sperm DNA damage is produced during spermatogenesis when oxidative stress due to high temperatures is higher (Karabinus *et al.*, 1997; Sailer *et al.*, 1997. Yin *et al.*, 1997; Banks *et al.*, 2005; Pérez-Crespo *et al.,* 2008; Paul *et al.,* 2008; Kusakabe & Tateno, 2011) even just for a short period of time (Karabinus *et al.*, 1997; Sailer *et al.*, 1997; Banks *et al.,* 2005). The negative effects on DNA will depend on the cell ability to repair their DNA or undergo apoptosis or necrosis (Rockett *et al.*, 2001). Males with sperm DNA damage have low fertility values (Banks *et al.*, 2005) due to lose of sperm fertilizing ability (Aitken *et al.*, 1998). However, some cells can complete their development resulting in motile spermatozoa with DNA damage (Banks *et al.*, 2005), and also keeping their fertilizing ability, being able to reach the oviduct and attach to the zona pellucida (Hourcade *et al.*, 2010). However, in those cases the modifications in DNA not repaired by the oocyte can alter the normal embryo and foetus development (Virro *et al.*, 2004; Aitken & Baker, 2006).

A negative correlation exists between the genetic material in the spermatozoa nucleus and the fertilizing ability, both *in vivo* and *in vitro* (Spano *et al.*, 2005). Sperm DNA is half of the genetic material contributed to the embryo, necessary for fertilization and embryo/foetus development.

The DNA alterations may include: defects on nuclear maturation or condensation, protamine deficiencies, DNA fragmentation or defect in DNA integrity (i.e. single and double strand breaks; Perreault *et al.*, 2003). Abnormalities in chromatin structural integrity are also related with a decrease in sperm viability and morphological abnormalities (Karabinus *et al.*, 1990).

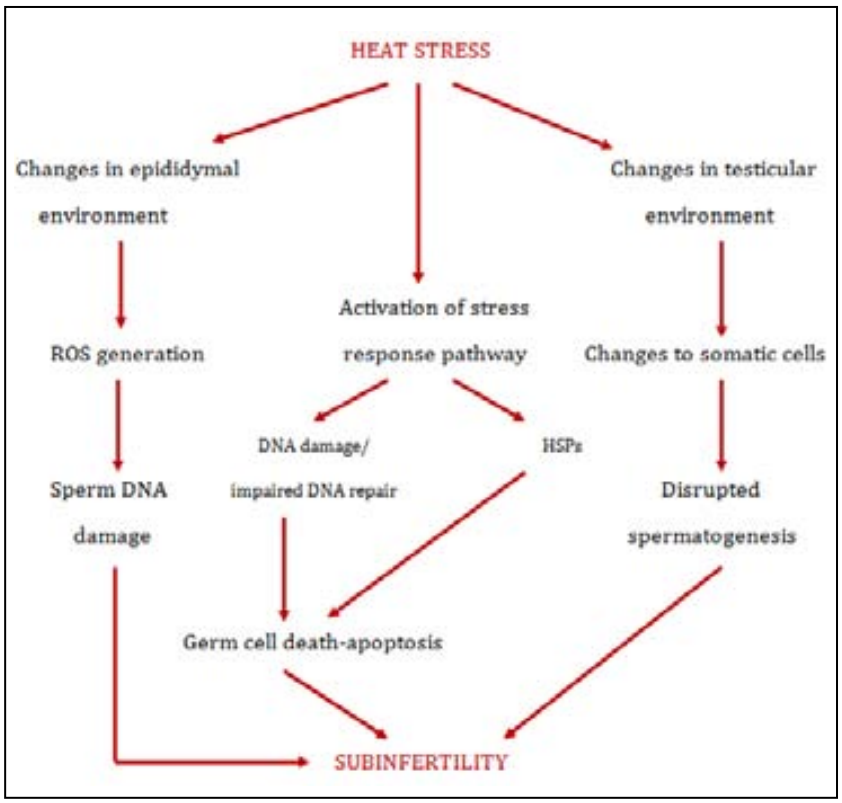


Figure 9. Possible mechanisms involved in male subfertility: heat stress may give rise to lesions in sperm DNA which can result in male subfertility if they escape DNA repair and apoptotic death of affected germ cells (modified from Paul *et al.*, 2008).

Effects on fertilization ability

Developmental competence of the mammalian embryo is dependent on genetic and non-genetic contributions from its parents (Sirard *et al.*, 2006; Baumann *et al.*, 2007), and it is reduced if fertilization is performed by a spermatozoon exposed to heat stress (Hansen, 2009), mainly by DNA damage (Virro *et al.*, 2004). It is clear that heat stress can adversely affect semen quality which could lead to a reduction in the ability to fertilize the ovum (Karabinus *et al.*, 1997) or produce a viable embryo (Mieusset *et al.*, 1992b; Jannes *et al.*, 1998; Yaeram *et al.*, 2006), both leading to a partial or total infertility. It is know that ROS blocks the motility (Aitken *et al.*, 1989; De Lamirande & Gagnon, 1992; Bilodeau *et al.*, 2000), and also decrease sperm-oocyte penetration and block sperm-egg fusion (Aitken *et al.*, 1989; Mammoto *et al.*, 1996). Thus, the use of heat-stressed spermatozoa produce embryos with delayed or reduced pronuclear formation (Walters *et al.*, 2006), cleavage rate (Ulberg & Burfening, 1967; Burfening & Ulberg, 1968; Hendricks *et al.*, 2009) and a reduced ability to become blastocysts with an increase on embryo apoptosis rate (Ulberg & Burfening, 1967; Burfening & Ulberg, 1968; Cozzi *et al.*, 2001; Walters *et al.*, 2004; 2005).

7. HOW TO IMPROVE FERTILITY IN THE WARM SEASON?

7. 1. ENVIRONMENTAL AND NUTRITIONAL MANAGEMENT

In order to improve fertility during the warm season or under conditions of heat stress, some management procedures can be developed, although, conception rate is still pronouncedly below than in winter (Hansen, 1997a; Kadokawa *et al.*, 2012).

7. 1. 1. Shade and fans

To enable animals to maintain normothermia during heat stress in dairy farms, a combination of shade and evaporative cooling systems (sprinkling and ventilation) should be the most common strategy to alleviate the effect of high temperatures (Roth, 2008). The strategy could reduce total heat load from 30 to 50% with a well-designed shade (Bond & Kelly, 1955). It also diminished rectal temperatures and respiratory rates, yielding 10% more milk than cows under no shade environment (Roman-Ponce *et al.*, 1977; Collier *et al.*, 1981; Hansen, 1997a), and improved semen quality, where a significant increase in sperm motility, viability and normalcy was evident (Salah *et al.*, 1992). However, although it is possible to achieve a positive effect on fertility by intensive and frequent use of the sprinkling and ventilation cooling system (Igono *et al.*, 1987; Wolfenson *et al.,* 1988; Bucklin *et al.*, 1991; Huber, 1996; Hansen, 1997a; Collier *et al.*, 2006), results are limited due to the inability of the cooling procedures to totally eliminate hyperthermia during the summer season (Hansen, 1997a; b; Bucklin *et al.*, 1991; Berman & Wolfenson, 1992; Armstrong, 1994; Huber, 1996).

7. 1. 2. Nutritional management

Dry matter intake is reduced by heat stress. In accordance, the reviews of nutritional management for the lactating dairy cow in hot climates (Fuquay, 1981; Collier *et al.*, 1992) appointed to use an appropriate feed supply, including high quality of food (rumen-active fat over concentrate; Collier *et al.*, 2006) an adequate feeding time, mineral supplementation (potassium demand increases; Collier *et al.*, 2006), and readily available cool drinking water to compensate the negative effects of heat stress (García-Ispierto *et al.*, 2006).

7. 1. 3. Aids for oestrus detection

Aids for oestrus detection are important to avoid the increase of the calving-conception interval during the warm months of the year when there is a reduction in oestrus symptoms (Armstrong, 1994). The systems that have been developed include the application of paint to the tailhead (Macmillan *et al.*, 1988), the Heat Watch system (a radiotelemetric pressure transducer to inform abouth the number of times the animal is mounted; Walker *et al.*, 1996) and podometers (to measure increased locomotor activity associated with oestrus; Maatje *et al.*, 1997).

7. 1. 4. Hormonal therapy and timed artificial inseminations protocol

The use of hormones to induce ovulation in a timed artificial insemination protocol improves summer fertility of dairy cows (De Rensis & Scaramuzzi, 2003) by eliminating the need for oestrus detection. These procedures are based on the administration of GnRH or hCG to induce ovulation, followed by a luteolytic dose of prostaglandin F2α 6 to 7 days later and a second treatment with GnRH or hCG 24–60 h after to induce a fertile ovulation (Pursley *et al.*, 1995; Schmitt *et al.*, 1996a;b; Pursley *et al.*, 1997). The principal benefit of these treatments is to induce cyclicity and the development of normal corpora lutea leading to good fertility. Conception rates rise (Ullah *et al.*, 1996; Hansen & Arechiga, 1999) by increasing the number of cows pregnant by 120 days postpartum and reducing the number of days open (Arechiga *et al.*, 1998a; De la Sota *et al.*, 1998; Wolfenson *et al.*, 2000; Almier *et al.*, 2002; De Rensis *et al.*, 2002). However, it is not always successful (Schmitt *et al.*, 1996a).

7. 1. 5. Embryo transfer

Timed embryo transfer can improve pregnancy rates under heat stress conditions if fresh embryos are transferred (Al-Katanami *et al.*, 2002a). Hereby, results can be higher if the procedure is performed 7 days after oestrus, when takes place the most thermosensitive period of development (Ealy *et al.*, 1993; Edwards & Hansen, 1997), due to the harmful effects of heat stress on oocyte quality limit embryonic development (Zeron *et al.*, 2001; Al-Katanani *et al.*, 2002b). Nevertheless, recovery of transferable embryos from superovulated recipients is also reduced by heat stress (Monty & Racowsky, 1987; Putney *et al.*, 1988a), and the use of frozen embryos collected at cool periods of the year or in regions not susceptible to heat stress is needed (Hansen & Arechiga, 1999). Furthermore, the high cost of the procedure must be highlighted, and can be alleviated through the use of *in vitro* fertilization using slaughterhouse material (Hansen & Arechiga, 1999).

7. 1. 6. Genetic selection

Genotype is a major determinant of resistance to heat stress. Cattle breeds of *Bos indicus* origin are more resistant to tropical conditions than breeds that evolved in a temperate climate (*Bos taurus*) due to their superior ability to regulate body temperature (Bennett *et al.*, 1985; Gaughan *et al.*, 1999). *Bos indicus* cattle experience less severe negative effects in response to heat stress (Cartwright, 1955; Johnson, 1965; Skinner & Louw, 1966; Seif *et al.*, 1979; Finch, 1986; Rocha *et al.*, 1998). Taking this into account, a strategy to increase production of dairy cattle in hot climates is the use of breeds that are genetically adapted to the local environment or crosses between European and local breeds, suggesting that selection for lower rectal temperature would improve fertility (Turner, 1982). However, these actions are only useful in areas of the world where high-quality feeds are scarce, the price of milk is low, or where the utilization of European breeds of cattle is impractical (Hansen & Arechiga, 1999).

7. 2. USE OF ANTIOXIDANT AGENTS

The exposure of cows to heat stress reduces the total antioxidant activity in blood plasma, which is negatively associated with THI among lactating cows (Harmon *et al.*, 1997). Hence, the administration of antioxidants (Arechiga *et al.*, 1998a) *in vivo* has been shown to provide slight improvements in pregnancy rates in heat-stressed cows. On the other hand, the addition of antioxidant agents *in vitro* as supplementation of culture media as ROS scavengers has proved improvements in oocyte/embryo culture (Cetica *et al.*, 2001) and sperm quality (Yousef *et al.*, 2003; Bucak *et al.*, 2010a).

7. 2. 1. Enzymatic antioxidants

Enzymatic antioxidants are also known as natural antioxidants; neutralize the excess of ROS and prevent it from damaging the cellular structure (Agarwal & Prabakaran, 2005). Enzymatic antioxidants are composed by:

- Superoxide dismutase (SOD): protects the cell against spontaneous ROS toxicity and lipid peroxidation (Sikka, 1996), and also remove superoxide aninon (O_2) by conversion to H_2O_2 which in turn is removed by catalase and glutathione peroxidase (Meister, 1983). SOD has proven ability protecting spermatozoa (Sikka, 1996; Vallorani *et al.*, 2010) and embryos from oxidative stress (Nonogaki *et al.*, 1992), increasing the proportion of zygotes developed to the expanded blastocyst stage.
- Catalase: controls the oxidative stress in cells mainly resulting from H_2O_2 (Bucak *et al.*, 2007), and also remove O_2 (Sikka, 1996). It has been proved improving sperm quality (Foote, 1967; Maia *et al.*, 2010; Hu *et al.*, 2011).
- Glutathione peroxidase (GPx), Glutathione reductase (GR): play a role in the elimination of hydrogen peroxide (Meister & Anderson, 1983) as demonstrated by protecting spermatozoa from oxidative stress (Irvine, 1996).

7. 2. 2. Nonenzymatic antioxidants

They are also known as synthetic antioxidants or dietary supplements. The body complex antioxidant system is influenced by dietary intake of antioxidants, vitamins, and minerals such as vitamin C, vitamin E, taurine and glutathione (Agarwal *et al.*, 2005a).

- Glutathione (GSH): is a natural antioxidant present in both gametes (Perreault *et al.*, 1988; Yoshida, 1993; De Matos *et al.*, 1996). It plays an important role in protecting cells from oxidative damage (De Matos & Furnus, 2000). It is implicated in cellular resistance to heat stress and oxidative stress (Mitchell *et al.*, 1983; Loven, 1988; Arechiga *et al.*, 1995), and also in the formation of the male pronucleus (Calvin *et al.*, 1986; Yoshida, 1993). It is able to react with many ROS directly (Bucak *et al.*, 2008). GSH supplementation improves semen quality (Monteiro *et al.*, 2009) and their precursor, cysteine, may play an important role as an antioxidant supplement in *in vitro* culture systems (Yoshida, 1993; Bilodeau & Sirard, 2003).

- Cysteine and cysteamine (Cyst): It has been shown to penetrate the cell membrane easily, enhancing the intracellular GSH biosynthesis both *in vivo* and *in vitro* and protecting the membrane lipids and proteins due to indirect radical scavenging properties (Hendin *et al.*, 1999). It has protective effects on sperm (Hendin *et al.*, 1999; Uysal & Bucak, 2007; Tuncer *et al.*, 2010) and oocyte during IVM (De Matos *et al.* 1996; Nabenishi *et al.*, 2011).
- Bovine serum albumin (BSA): is known to eliminate free radicals generated by oxidative stress and protect membrane integrity. It has been useful in protect sperm quality (Uysal & Bucak, 2007).
- Taurine: displayed antioxidative properties by elevating catalase level in close association with SOD concentration (Reddy *et al.*, 2010). It has been used to protect spermatozoa against ROS production (Reddy *et al.*, 2010).
- Carotenoids: such as β-carotene and lycopene are also important components of antioxidant defense. Both protect sperm quality parameters (Uysal & Bucak, 2007) or conception rate (Arechiga *et al.*, 1998a).
- Fatty acids: It has been widely accepted that HS can aggravate the negative energy balance (NEB) during lactation, predominantly due to a drop in dry matter intake (Collier *et al.*, 1992). Moreover, fatty acids in the diet seem to be valuable at improving NEB, having positive effects over the follicle, oocyte and embryo in dairy cows.
- Vitamins: Vitamins A (retinol), E (a-tocopherol), and C (ascorbate) have been reported to act as major antioxidants for protection against diseases and degenerative processes that are caused by oxidative stress (Chaudiere & Ferrari-Illiou, 1999; Wong *et al.*, 2000) as lipid peroxidation (Beconi *et al.*, 1993; Geva *et al.*, 1996; Hsu *et al.*, 1998.). It has been described positive effects using them *in vivo* (Ealy *et al.*, 1994; Arechiga *et al.*, 1998a; b; Castellini *et al.*, 2000; De Rensis & Scaramuzzi, 2003; Yousef *et al.*, 2003) or *in vitro* (Beconi *et al.*, 1993; Olson & Seidel, 2000; Bansal & Bilaspuri, 2007; Córdova *et al.*, 2010; Marques *et al.*, 2010) on sperm quality and also on oocyte/embryo culture protecting them from ROS.

Vitamin A and derivates:

Vitamin A (all-trans retinol; RO) and its metabolites, known as retinoids, are regulators of cell growth, differentiation of many types of cells (Hidalgo *et al.*, 2005), and have ROS scavenger activity (Ikeda *et al.*, 2005). Cows take up vitamin A as naturally contained β-carotene in forages or as a diet supplementation with esterified retinol (NRC Nutrient requirements on Beef -2000- and Dairy -2001- cattle; Gómez *et al.*, 2006).

The derivates from vitamin A have also an important role on reproduction (folliculogenesis, steroidogenesis, oocyte maturation and embryo development) (Gomez *et al.*, 2004; Ikeda *et al.*, 2005; Chiamenti *et al.*, 2010), and their metabolism in cattle reproduction has been reviewed by Ikeda *et al.* (2005). The predominant retinoid is retinyl palmitate, but other retinol esters, such as retinyl oleate, stearate or acetate (retinyl; RI), are also found (Blomhoff & Blomhoff, 2006). Through a series of oxidative reactions, vitamin A generates retinoic acid (RA) and their isomeres

all-trans-RA (ATRA) and 9-cis-RA. Retinol is essential for pregnancy maintenance in mammals, the RA is the most relevant retinoid during vertebrate development (Morris-Kay & Ward, 1999) and the isomere ATRA is the most important retinol metabolite for vertebrate embryogenesis (Morriss-Kay & Ward, 1999; Ross *et al.,* 2000). In the live cell, RA binds to two different kinds of nuclear receptors, namely, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Most of these receptors are present from the oocyte to the hatched blastocyst stage and in cumulus cells (Mohan *et al.*, 2001; 2002).

The vitamin A metabolites are essential for reproduction in both the male and female, as well as for many events in the developing embryo (Clagett-Dame & Knutson, 2011). The oocyte itself is under the retinoid influence within their intrafollicular growth, as the bovine follicular fluid contains RO and β-carotene in proportion to the respective blood contents (Chew *et al.*, 1984; Haliloglu *et al.*, 2002; Hidalgo *et al.*, 2005). These findings are consistent with the stimulation of oocyte development capacity by retinoid in cows (Shaw *et al.*, 1995; Duque *et al.*, 2002), sheep (Eberhardt *et al.*, 1999), gilts (Whaley *et al.*, 2000), and rabbits (Besenfelder e*t al.*, 1996) that had higher blood levels of vitamin A. On the other hand, vitamin A is essential for normal testicular structure and function. The combined actions of FSH, testosterone, and RA are essential for normal mammalian spermatogenesis. RA acts on both Sertoli cells and germ cells and pushes undifferentiated spermatogonia into the differentiation pathway and, eventually, meiotic prophase (Hogarth & Griswold, 2010). Vitamin A is known to be present in rabbit semen (Velazquez *et al.*, 1975) and bull (Gambhir & Ahluwalia, 1975) spermatozoa. Velazquez *et al.* (1975) reported a vitamin A concentration of 3.8 μg/ml in rabbit seminal plasma, and 2.6 μg/10^8 cells in rabbit spermatozoa. This value is markedly higher than in bull spermatozoa (29 ng/10^8 cells; Gambhir & Ahluwalia, 1975). When animals are deprived of vitamin A, their germinal epithelium degenerates and spermatogenesis ceases (Wolbach & Howe, 1925). Moreover, there was a positive correlation between intracellular content of retinol in seminal plasma and sperm motility (Kao *et al.*, 2008), membrane integrity (Roels *et al.*, 1969), and also with sperm normalcy, since lower levels of vitamin A result in higher sperm midpiece abnormalities and coiled tail (Abdulkareema *et al.*, 2005).

The use of retinoids *in vivo* as antioxidant agents has been used both in males and females. It has been reported that dairy cow fertility can be improved at the second artificial insemination by a diet rich in retinol and β-carotenes during the warm months (Arechiga *et al.*, 1998a). However, it has been suggested that a retinoid cumulative effect is needed to obtain an effective concentration in oviduct and uteri (Guerin *et al.*, 2001; Livingston *et al.*, 2004). This effect is due to β-carotene metabolism and location of retinoid receptors, which are present in the COC. Hence, retinoids could counteract HS effects by receptor mediated effects (Lawrence *et al.*, 2004). Developmental competence of the oocyte is enhanced by a RO treatment as reported in the cow (Shaw *et al.*, 1995), sheep (Eberhardt *et al.*, 1999) and gilt (Whaley *et al.*, 2000), as well as in rabbits that have high blood levels of vitamin A (Besenfelder *et al.*, 1996). Vitamin A may

influence embryonic development by advancing resumption of meiosis and altering follicular hormonal environment during follicle maturation (Whaley *et al.*, 2000). In accordance, *in vivo* administration of retinoids has been shown to enhance pre-implantation embryonic development (Shaw *et al.* 1995; Chiamenti *et al.*, 2010) and their quality (Shaw *et al.*, 1995; Eberhardt *et al.*, 1999).

Due to their ROS scavenger activity, retinoids have been used in previous *in vitro* studies, some of them done under heat stress conditions, alleviating the negative effects derived from. The supply of retinoid to oocyte and embryos must be ensured within a physiological range, since both excess and deficiency of retinoid cause abundant teratogenic defects due to its pleiotrophic activity (Lane *et al.*, 1999). Therefore, an improvement in the conception rate (Gómez *et al.*, 2006), a beneficial effect on nuclear (Vahedi *et al.*, 2009; Nasiri *et al.*, 2011; Taheit *et al.*, 2011; Liang *et al.*, 2012) and cytoplasmic competence after oocyte IVM (Duque *et al.*, 2002; Gomez *et al.*, 2003; Hidalgo *et al.*, 2003; Nasiri *et al.*, 2011; Liang *et al.*, 2012), and enhanced embryo development rates to blastocyst have beene reported (Gómez *et al.*, 2003; Lawrence *et al.*, 2004; Livingston *et al.*, 2004; Gómez *et al.*, 2008; Hajializadeh *et al.*, 2008; Rajesh *et al.*, 2010; Atikuzzaman *et al.*, 2011). On the other hand they have been proved to be valuable inhibiting cumulus cell apoptosis during IVM (Deb *et al.*, 2012). However, to our knowledge there are no reports on the use of retinoids in sperm extenders.

Fatty acids:

Fatty acids in the diet seem to be valuable at improving NEB, having positive effects over the follicle, oocyte and embryo in dairy cows. Given the importance of the oxidative status of the oocyte and the fact that bovine oocytes are rich in fatty acids (McEvoy *et al.*, 2000), seasonally increases variation in the fatty-acid profiles of the follicular fluid and oocyte being associated with reduced development of the oocyte (Zeron *et al.*, 2001). In the hot summer, the proportions of saturated fatty acids in oocyte and granulosa cells are higher than those of mono- and polyunsaturated fatty acids, implying reduced oxidative status of the oocyte. It has been reported that oleic acid is present in high concentrations in bovine oocyte membranes during winter and its low concentration in summer can be related to the poor quality of oocytes (Zeron *et al.*, 2001). Zeron Y. *et al.* (2001) also suggested that oleic acid concentration has an important role on temperature oocyte membrane adaptation, and high levels of it have been related with high embryo recovery rates (Leroy *et al.*, 2005; Fouladi-Nashta *et al.*, 2007). According to that, saturated fatty acids (palmitate (C16:0) and stearate (C18:0)) can induce apoptosis (Paumen *et al.*, 1997; Lu *et al.*, 2003) by contrast with the monounsaturated fatty acids (palmitoleate, oleate and linoleate) which were found to prevent the pro-apoptotic effect (Eitel *et al.*, 2002). So, the use of specific fatty acids could improve fertility during warm months.

The use of fatty acids *in vivo* in an attempt to improve fertility has been reported feeding ewes with a diet supplemented with fish oil, increasing the proportion of polyunsaturated fatty acids in

the plasma and cumulus cells and improving oocyte quality as determined by membrane integrity (Zeron *et al.*, 2002). Similarly, lactating dairy cows fed with a diet rich in calcium salts of palm oil increase the number of resultant embryos produced *in vitro* following oocyte aspiration (Fouladi-Nashta *et al.*, 2004). Hence, a high level of feeding (Adamiak *et al.*, 2005) or a high level of fat in the diet (Fouladi-Nashta *et al.*, 2007) improves oocyte developmental competence in cows. There are evidences that suggest that dietary supplementation with omega-3 fatty acids affects reproduction in male (Paulenz *et al.*, 1999; Rooke *et al.*, 2001), by increasing the percentage of sperm with membrane integrity, total motility and progressivity. On the other hand, omega-3 fatty acids have also been reported to be important for sperm membrane integrity, sperm motility and viability, as well as cold sensitivity (Robinson *et al.*, 2006).

The use of fatty acids on *in vitro* studies has revealed controversial results. It is noteworthy that lipid-rich oocytes possess better developmental competence (Jeong *et al.*, 2009; Aardema *et al.*, 2011). However, the exposure to higher concentrations of palmitic or stearic acid during maturation resulted in small number of lipid droplets, whereas oleic acid resulted in more and larger lipid droplets, suggesting better developmental competence (Aardema *et al.*, 2011). Despite of these results, a negative effect of oleic acid on metaphase progression has been described by Jorritsma *et al.* (2004), as a significant decrease in the cleavage rate and subsequent development. However, Leroy *et al.* (2005) described no effects on nucleus of bovine oocytes. On the other hand, the saturated fatty acids palmitic and stearic had a significant inhibitory effect on granulose cell proliferation (Vanholder *et al.*, 2005), inhibiting bovine thecal cell proliferation, viability and progesterone production, whereas oleic acid did not alter these parameters (Vanholder *et al.*, 2006).

Moreover, the treatment of COCs with linoleic acid significantly inhibited cumulus cell expansion and retarded development of the oocytes to the MII stage in a dose-dependent manner. The supplementation to bovine oocytes during maturation alters the molecular mechanisms regulating oocyte maturation and results in a decrease in the percentage of oocytes at MII stage and the inhibition of the subsequent early embryo development (Marei *et al.*, 2010). Hence, linoleic acid induced alterations in mitochondrial distribution and activity as well as increasing ROS levels, which mediate, at least in part, the inhibitory effect on oocyte maturation (Marei *et al.*, 2011). However, an appropriate level of linolenic acid in oocyte maturation medium can induce molecular changes associated with oocyte maturation, leading to an increase in oocyte developmental potential and improving blastocyst quality. Treatment of COCs with it significantly increased the percentage of oocytes at the MII stage resulted in a significantly higher percentage of cleaved embryos, blastocyst rate and better-quality embryos (Marei *et al.*, 2009).

EXPERIMENTAL DESIGN

EXPERIMENTAL DESIGN

In order to get the objectives exposed previously, we designed six different experiments that are shown in different chapters in this thesis:

1. **Chapter I** was designed to analyze in which way bovine oocyte maturation is affected by heat stress by the development of an *in vitro* model. The study was performed by using slaughterhouse bovine ovaries since the *in vivo* experiment would affect farm productivity. However, the *in vivo* effect of high temperatures was evaluated in a preliminary study with rabbit does being affected the oocyte progression to the metaphase II. The number of oocytes obtained from the experimental does was insufficient for a complete paper, although results were presented at the *11th Congreso internacional de la Asociación Española de Reproducción Animal* (2012).

2. **Chapter II** was planned once we get the results about how oocytes are affected by high temperatures, being necessary to find if a seasonal effect exists in bovine oocytes submitted to heat stress conditions, again by an *in vitro* study.

3. **Chapter III** was designed to try counteracting harmful effects derived from high temperatures by supplementing the *in vitro* oocyte maturation medium with the use of several antioxidant agents.

4. **Chapter IV** was performed to figure out high temperature effects on male gametes. For this purpose an *in vivo* study was developed to determine how rabbit epididymal sperm cells are affected by high temperatures. Epididymal sperm cells are more sensitive to high temperatures than ejaculated spermatozoa and not protected by seminal plasma.

5. **Chapter V** was designed to analyze the effect of adding antioxidant agents to the freezing extender in order to improve sperm quality after cryopreservation, since it is affected by reactive oxygen species derived from the process. Ejaculated rabbit spermatozoa were used trying to find a practical application. The use of rabbit samples allowed to avoid the additional freezing-thawing process find by using commercial bovine straws.

6. **Chapter VI** was performed to detect how bovine epididymal sperm cells are affected by high temperatures by using an *in vitro* study, since the use of commercial bovine straws implies additional variable factors to the experiment as the freezing-thawing effect on spermatozoa. Additionally, retinol as an antioxidant agent was used to counteract the negative effects derived from heat stress. Only retinol was assessed as antioxidant supplementation due to the results obtained from previous chapters.

In future experiments additionally to this doctoral thesis, we are planning to perform other experiments to figure out additional points related with heat stress and gamete development:

1. To develop an *in vivo* study to assess heat stress effects on rabbit oocyte maturation.

2. To analyze antioxidant agent supplementation on rabbit epididymal sperm cells to counteract heat stress effects.

3. To analyze cleavage and embryo development by the use of oocytes and sperm cells matured or stored in presence of antioxidant agents and heat stress conditions.

CHAPTERS

I

Does heat stress provoke the loss of a continuous layer of cortical granules beneath the plasma membrane during oocyte maturation?

C. Andreu-Vázquez[a], F. López-Gatius[b], I. García-Ispierto[b], M.J. Maya-Soriano[a], R.H.F. Hunter[c], M. López-Béjar[a]

[a]Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Spain.

[b]Department of Animal Production, Universitat de Lleida, Spain.

[c]Institute of Reproductive Medicine, Hannover Veterinary University, Germany.

Submitted 09.09.2009; accepted 22.12.2009

Zygote, 18 (November), 2010, pp. 293–299.

This study was supported by Spanish Ministry of Science and Innovation, MICINN, Ref. RTA2008–00070-C02–02. Andreu-Vázquez was supported by an FPU Grant from the Spanish Ministry of Science and Education, MCE, Ref.AP2007-01598. Maya-Soriano was supported by a PIF Grant from the Universitat Autònoma de Barcelona.

Abstract

The objective of the present study was to evaluate the influence of heat stress on bovine oocyte maturation. Both nuclear stage and distribution of cortical granules (CG) were simultaneously evaluated in each oocyte. Oocyte overmaturation under standardconditions of culture was also evaluated. For this purpose, logistic regression procedures were used to evaluate possible effects of factors such as heat stress, overmaturation, replicate, CG distribution and metaphase II (MII) morphology on oocyte maturation. Based on the odds ratio, oocytes on heat stressed (HSO) and overmaturated (OMO) oocyte group were, respectively, 14.5 and 5.4 times more likely to show anomalous MII morphology than those matured under control conditions (CO). The likelihood for an oocyte of showing the CG distribution pattern IV (aging oocyte) was 6.3 and 9.3 times higher for HSO and OMO groups, respectively, than for the CO group. The risk of undergoing anomalous oocyte maturation, considering both nuclear stage and distribution of CG was 17.1 and 18 times greater in oocytes cultured in HSO and OMO groups, respectively, than those in the CO group. In conclusion, heat stress proved to be valuable in aging oocytes. Heat stress advanced age for nuclear and cytoplasmic processes in a similar form to that of oocyte overmaturation.

Keywords: Aging, cortical granules, heat stress, oocyte maturation, overmaturation

Introduction

Although molecular studies are now considered especially important (Bhojwani *et al.*, 2006; Fair *et al.*, 2007; Evsikov & Marin de Evsikova, 2009; Siemer *et al.*, 2009) classical concepts concerning the maturation of mammalian oocytes (Szollosi, 1975b; Thibault *et al.*, 1987) still focus on two aspects: nuclear maturation and cytoplasmic maturation (Sirard, 2001; Ferreira *et al.*, 2009) that are usually highly coordinated (Eppig, 1996). Nuclear maturation involves the transition from a germinal vesicle nucleus to a second metaphase arrangement of the chromosomes and formation of a first polar body by the time of ovulation in most species so far studied. Cytoplasmic maturation is expressed as changes in protein composition, but most conspicuously in the redistribution of organelles that are termed the cortical granules (CG) (Szollosi, 1962, 1967). Such granules are, in fact, small vesicles that contain enzymes. During resumption of meiosis, the CG migrate from the Golgi apparatus to close to the vitelline surface, assuming a position 0.4–0.6 μm below the plasma membrane (Ducibella & Buetow, 1994). Only when situated just beneath the plasma membrane can they undergo exocytosis by fusing with the egg membrane. This fusion enables release of the CG contents into the perivitelline space, an important step in membranous maturation and in instigating a block to polyspermy (Szollosi, 1967; Hosoe & Shioya, 1997; Wang *et al.*, 1997).

There have been diverse studies that focussed on the formation and distribution of the CG in mammalian oocytes (Szollosi, 1967, 1978; Flechon, 1970; Thibault *et al.*, 1987; Hosoe & Shioya, 1997; Wang *et al.*, 1997; Wessel *et al.*, 2001, 2002) but none in the present context. In this study on maturation of bovine oocytes, the influence of heat stress on both nuclear stage and distribution of cortical granules has been evaluated in each oocyte. As heat stress seems to induce premature aging of oocytes (Lawrence *et al.*, 2004; Edwards *et al.*, 2005; Schrock *et al.*, 2007) the effect of overmaturation on nuclear and cortical granules distribution features was also evaluated. This research has relevance to bovine reproduction as heat stress has been associated with reduced fertility in many countries (de Rensis & Scaramuzzi, 2003; López-Gatius, 2003) especially when it coincides with the insemination time (Putney *et al.*, 1989; García-Ispierto *et al.*, 2007).

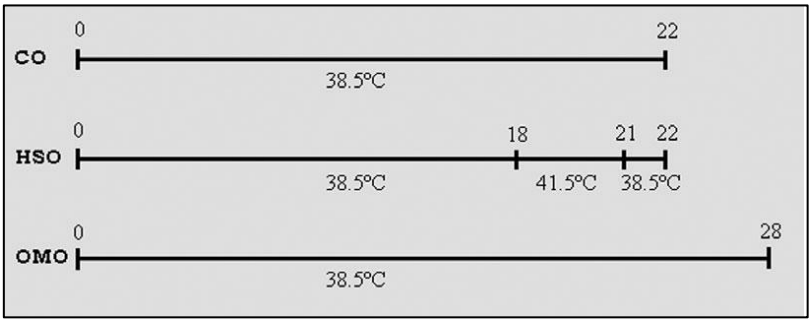
Materials and methods

Experiment design

Three groups were established: (1) control oocytes (CO; n=75), maintained under culture conditions (38.5ºC for 22 h); (2) heat stressed oocytes (HSO; n=80) submitted to the heat treatment; and (3) overmaturated oocytes (OMO; n=20) cultured for 28 h (Fig. 1).

As a 3 h heat treatment mimics hyperthermia real conditions (Tseng *et al.*, 2004) of the 22 h of the *in vitro* maturation (IVM) process, the HSO group was exposed to 41.5ºC during the period of 18 to 21 h of maturation.

Figure 1. A time-line figure illustrating the experimental setup for cumulus–oocyte complexes (COCs) culture and groups of study: CO (control oocytes; n=75), HSO (heat stress oocytes; n=80) and OMO (overmaturation oocytes; n=20).



Chemicals and reagents

All chemicals were purchased from Sigma unless otherwise indicated.

Collection of oocytes

Ovaries recovered from slaughter housed young heifers and placed into Dulbecco's phosphate buffered saline solution (PBS; DPBS 10×; GIBCO-Invitrogen) including 1% (v/v) antibiotic antimycotic solution (AA; 10,000 units/ml penicillin, 10 mg/ml streptomycin and 25 mg/ml amphotericin B) and carried to the laboratory at room temperature. Ovaries were then washed twice in warm sterile PBS and were kept at 37.5ºC until ovarian puncture that took place within 2 h from their recovery. Next, 2 to 8 mm-sized follicles were aspirated using an 18-gauge needle joined to a 5ml syringe. Cumulus–oocyte complexes (COCs) were obtained and placed into working medium (WM; Medium 199 with Earle's salts 25 mM HEPES and NaHCO3, 1% v/v AA solution). Only oocytes enclosed in three or more layers of compact cumulus cells and presenting a homogeneous and translucent ooplasm were selected.

In vitro maturation

Selected COCs were washed twice in WM and once in maturation medium (MM; Medium 199 with Earle's salts, L-glutamine and NaHCO3, supplemented with 20 µg/ml epidermal growth factor, 2 mM sodium pyruvate and 1% v/v AA solution) that had been pre-equilibrated for 3 h at 38.5ºC in 5% (v/v) CO2 in humidified air.

After washing, COCs were randomly allocated to groups of 20–25 and placed into 4-well dishes (Nunc) containing 500 µl of MM. All the processes were performed in a laminar flux booth during approximately 2 h from follicular aspiration to the entrance of COCs to IVM.

COCs were cultured at different temperature conditions for 22 h (CO and HSO groups) or 28 h (OMO group) according to the experimental design in an atmosphere of 5% (v/v) CO2 in humidified air. To reduce variation of temperature during culture, incubation of control and overmaturation groups and heat stress group COCs were performed in two different CO2 incubators. While one incubator was set at

38.5ºC, the other was used for the heat treatment. Both incubators were kept closed during the whole maturation period and their temperature was verified by checking of clinical thermometers that were placed inside of the incubators.

Zona pellucida digestion and oocyte fixation

After 22 h (CO and HSO groups) or 28 h (OMO group) of IVM, COCs were removed from maturation wells and denuded of cumulus cells by pipetting and washed twice in PBS with 0.05% (w/v) of bovine serum albumin (BSA; fraction V BSA). Oocytes were then immersed in PBS containing 0.4% (w/v) pronase for 3 min to dissolve zona pellucida and washed five times in PBS with 0.05% (w/v) BSA. Oocytes were fixed in a PBS solution containing 4% (w/v) of paraformaldehyde for 45min at room temperature and washed five times in PBS with 0.05% (w/v) BSA.

Oocyte permeabilization and cortical granules staining

Oocytes were immersed in a permeabilizing solution of PBS containing 0.3% Triton X100 and 0.05% (w/v) BSA for 5 min at room temperature and washed five times in PBS with 0.05% BSA. Oocytes were then incubated in the dark for 30 min at room temperature into a staining solution of PBS containing 100 µg/ml of fluorescein isothiocyanate-labelled Lens culinaris agglutinin (FITC– LCA) and 0.05% BSA.

Nuclear staining and mounting

Stained oocytes were thoroughly washed in PBS with 0.05% (w/v) BSA to remove excess of FITC–LCA before mounting, and mounted between a pretreated with poly-L-lysine coverslip and a glass slide supported by a washer. The antifade mounting medium contained 4,6-diamidino-2-phenilidole (DAPI; Vectashield; Vector laboratories, Inc.) for counterstaining DNA. The coverslip was sealed with nail polish and preparations were kept at 4ºC and protected from light until examination by fluorescence and laser confocal microscopy.

Nuclear and cytoplasmic maturation evaluation

Nuclear and cytoplasmic maturation status was evaluated for each oocyte. Nuclear content and apical and equatorial section of each oocyte were photographed under UV epifluorescent microscope (Nikon Eclipse TE 2000S) and laser confocal microscope (Leica TCS SP2) respectively.

Cultured oocytes were checked to have reached metaphase II (MII) or not. Normality in MII morphology was furthermore registered according to the modified classification of Tseng (2004) (normal MII: uniform alignment of the chromosomes on the spindle; anomalous MII: nuclear content changed into chromatin-like structure forming condensed aggregates or forming aberrantly distributed chromosomes).

Translocation of CG to the oolemma was used as an indicator of cytoplasmic maturation (Damiani *et al.*, 1996). The distribution of GC was classified into four patterns according the classification of Hosoe &

Shioya (1997) (pattern I: GCs distributed in clusters or large aggregates; pattern II: GC individually dispersed and partially clustered or aggregated; pattern III: GC completely dispersed; pattern IV: no CG).

Statistical analysis

Only oocytes that reached MII nuclear stage were included in the statistical analysis. The following data were recorded for each oocyte: replicate (1–4), group (CO, HSO or OMO), MII morphology (normal MII versus anomalous MII) and CG distribution pattern (I, II, III or IV).

On data from each oocyte, a first logistic regression analysis was performed using MII anomalous morphology as the dependent variable (0 or 1) and replicate, group and CG distribution pattern as independent factors. A second logistic regression analysis was performed using CG distribution pattern IV as dependent variable (0 or 1) and replicate, group and MII morphology as independent factors. A further logistic regression analysis was performed using anomalous oocyte maturation, considering both MII anomalous morphology and GC distribution pattern IV as the dependent variable (0 or 1) and replicate and group as independent factors. All variables above were considered as class variables.

Logistic regressions analyses were performed using the SPSS package, version 17.0 (SPSS Inc.) according to the method of Hosmer & Lemeshow (1987). Basically, this method involves five steps as follows: (1) preliminary screening of all variables for univariate associations; (2) construction of a full model using all the variables found to be significant in the univariate analysis; (3) stepwise removal of non-significant variables from the full model and comparison of the reduced model with the previous model for model fit and confounding; (4) evaluation of plausible two ways interactions among variables; and (5) assessment of model fit using Hosmer–Lemeshow statistics. Variables with univariate associations showing p-values <0.25 were included in the initial model. We continued modelling until all the main effects or interaction terms were significant according to the Wald statistic at $p<0.05$.

Results

Effects of heat stress on nuclear maturation

Of the 175 cultured and evaluated oocytes, four showed immature nuclear stages after IVM and were excluded from the study.

Logistic regression analysis indicated no significant effect of replicate or CG distribution pattern on MII morphology. Table 1 shows the adjusted odds ratios of the variable finally included in the logistic model. No significant interactions were found. Based on the odds ratio, oocytes in HSO and OMO groups were, respectively, 14.5 and 5.4 times more likely to showed anomalous MII morphology after the IVM than those matured under control conditions.

Table 1. Odds ratios of variables included in the final logistic regression model for metaphase II (MII) anomalous morphology.

		MII anomalous morphology				
Factor	**Class**	**n**	**%**	**Odds ratio**	**95% CI[a]**	***p*-value**
Group	Control	8/73	10.9	Reference	-	-
	Heat stress	28/78	35.9	14.5	6.1-34.6	<0.001
	Overmaturation	8/20	40.0	5.4	1.7-17.2	0.004

Likelihood ratio test 48.85; 2 d.f., p < 0.0001. Nagelkerke r^2 = 0.337.

[a]Confidence interval for the odds ratio.

Effects of heat stress on cortical granule distribution

Figure 2 shows laser confocal microscopic images of the equatorial section of FITC–LCA-labelled bovine oocytes showing representative CG distribution patterns and the number of oocytes for each pattern and group of study.

Logistic regression analysis indicated no significant effect of replicate or MII morphology on CG distribution pattern IV. Table 2 shows the adjusted odds ratios of the variable finally included in the logistic model. No significant interactions were found. Based on the odds ratio, the likelihood for an oocyte of showing CG distribution pattern IV was 6.3 and 9.3 times higher for HSO and OMO groups, respectively, than for the CO group.

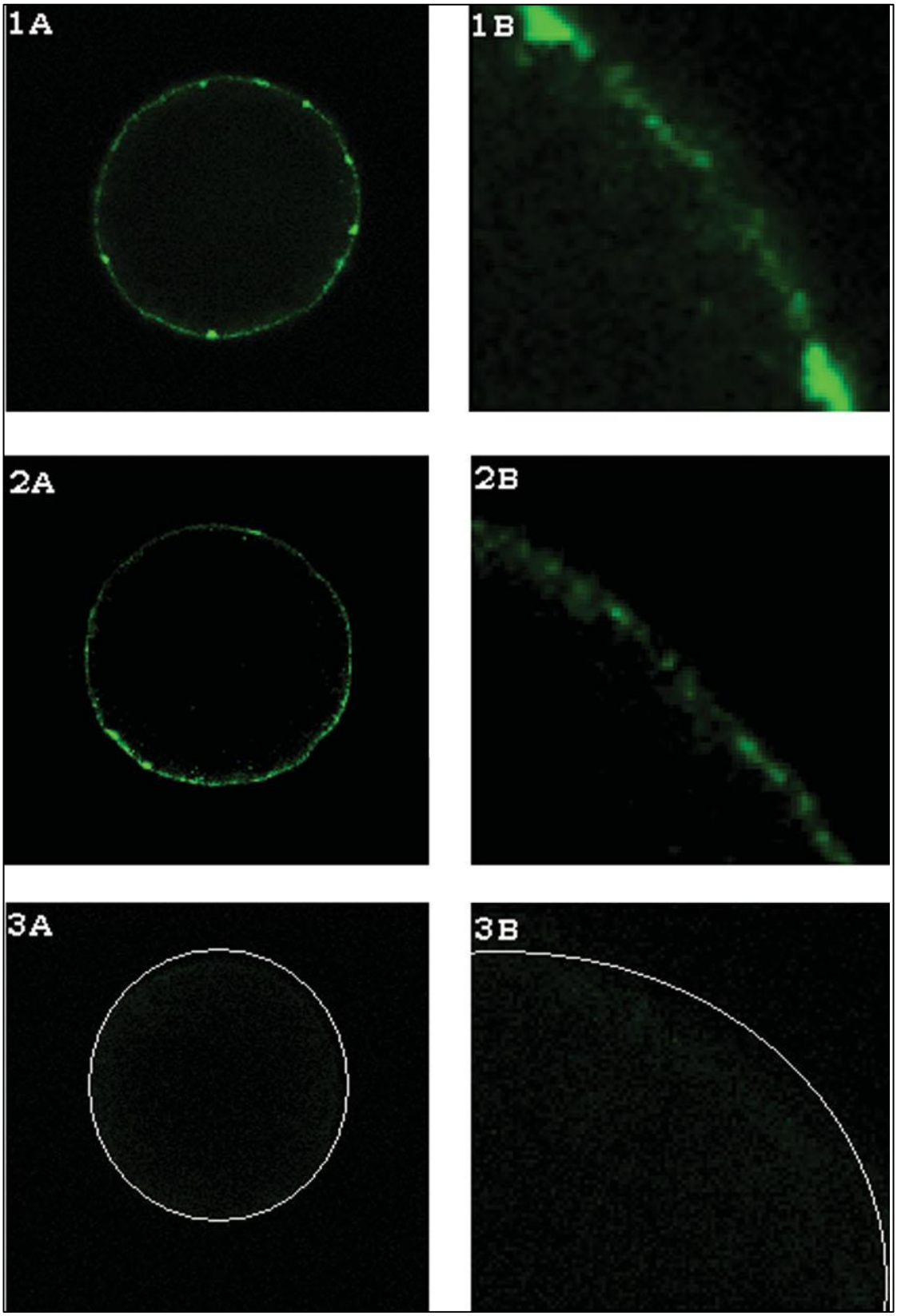
Table 2. Odds ratios of variables included in the final logistic regression model for cortical granules (CG) distribution pattern IV.

		CG distribution pattern IV				
Factor	**Class**	**n**	**%**	**Odds ratio**	**95% CI[a]**	***p*-value**
Group	Control	4/73	5.6	Reference	-	-
	Heat stress	21/78	26.9	6.4	2.1-19.6	0.001
	Overmaturation	7/20	35.0	9.3	2.4-36.3	0.001

Likelihood ratio test 17.88; 2 d.f., p < 0.0001. Nagelkerke r^2 = 0.154.

[a]Confidence interval for the odds ratio.

Figure 2. Laser confocal microscopic images of the equatorial section (A) and its detail (B) of fluorescein isothiocyanate Lens culinaris agglutinin (FITC–LCA)-labelled bovine oocytes showing representative patterns of CG distribution for the different groups: pattern II (1A, 1B: CO=14/73, HSO=12/78, OMO=1/20), pattern III (2A, 2B: CO=54/73, HSO=45/78, OMO=12/20), pattern IV (3A, 3B: CO=14/73, HSO=21/78, OMO=7/20). CO, control oocytes; HSO, heat stress oocytes; OMO, overmaturation oocytes.



Effects of heat stress on oocyte maturation considering both nuclear and cytoplasmic maturation

Logistic regression analysis indicated no significant effect of replicate on anomalous maturation considering both MII anomalous morphology and CG distribution pattern IV. Table 3 shows the adjusted odds ratios of the variable finally included in the logistic model. No significant interactions were found. Based on the odds ratios, the risk of undergo anomalous oocyte maturation was 17.1 and 18 times greater in oocytes cultured in HSO and OMO groups, respectively, than those in CO group.

Table 3. Odds ratios of variables included in the final logistic regression model for anomalous oocyte maturation, considering both anomalous patterns of metaphase II (MII) morphology and cortical granules (CG) distribution pattern.

		MII anomalous morphology and CG distribution pattern IV				
Factor	**Class**	**n**	**%**	**Odds ratio**	**95% CI[a]**	***p*-value**
Group	Control	1/73	1.4	Reference	-	-
	Heat stress	15/78	19.2	17.1	2.0-133.5	0.007
	Overmaturation	4/20	20.0	18.0	1.9-172.0	0.012

Likelihood ratio test 16.45; 2 d.f., $p < 0.0001$. Nagelkerke $r^2 = 0.178$.

[a]Confidence interval for the odds ratio.

Discussion

Heat stress proved to be valuable in aging oocytes. For this purpose nuclear and cytoplasmic maturation for each oocyte were assessed simultaneously. Similar figures of aged oocytes (Szollosi, 1971, 1974, 1975a) were registered for heat stressed and overmaturated oocytes. By using logistic regression procedures, the models were highly explanatory and significant. Three consecutive analyses showed how dramatically heat stress advanced age for nuclear and cytoplasmic processes. Including both nuclear and cytoplasmic maturation measurements, and based on the odds ratio, heat stressed and overmaturated oocytes were 17 and 18 times, respectively, more likely to age than controls, used as reference.

Heat stress during the last period of IVM resulted in a high percentage of oocytes showing a pattern IV of CG distribution. Pattern IV, which is characterized by the loss of the continuous layer of CG, was firstly described in bovine oocytes by Hosoe Shioya (1997) and is the most common pattern of CG distribution for oocytes that had underwent exocytosis after insemination or activation. Although heat stress

advanced oocyte maturation in previous studies (Payton *et al.*, 2004; Edwards *et al.*, 2005), pattern IV of CG distribution related to the final maturation of oocyte was not described. We describe herein how heat treatment or overmaturation can result in a pattern IV of CG distribution in a large number of oocytes. These results suggest that heat stress applied at the end of the period of oocyte maturation can induce CG loss.

As observed in the present study, an anomalous progression to second meiotic metaphase was a feature of heat stressed oocytes. A modified behaviour of the cytoskeleton may be the underlying cause, as it has been described in previous studies (Tseng *et al.*, 2004; Ju *et al.*, 2005; Roth & Hansen, 2005). Nonetheless, other aspects of nuclear behaviour may be modified by temperature stress, such as the rate and normality of chromosomal condensation (Roti, 2008). The behaviour of contractile proteins of the cytoskeleton will be perturbed by inappropriate temperature. Aspects that remain to be clarified are: (1) how much variation in temperature an oocyte can withstand; and (2) whether tolerance to shifts in temperature changes with the stage of meiotic maturation.

Overall, there is the question as to whether tolerance to temperature modifications *in vitro* differs from that in the living animal. There is already a body of evidence indicating that the temperature of preovulatory Graafian follicles is lower than temperature elsewhere in the ovary of rabbits (Grinsted *et al.*, 1980), women (Grinsted *et al.*, 1985), pigs (Hunter *et al.*, 1997, 2000, 2006) and perhaps cattle (Greve *et al.*, 1996). This finding suggests the existence of sensitive systems of temperature regulation *in vivo* that may be difficult to mimic *in vitro*.

A further experiment that would be relevant would be to culture individual Graafian follicles (Baker & Neal, 1972, 1974; Picton *et al.*, 2008) under different protocols of heat stress. Such a study might reveal greater perturbations in oocyte maturation when within a heat stressed follicle.

References

Baker, T.G. & Neal, P. 1972. Gonadotrophin-induced maturation of mouse Graafian follicles in organ culture. In Oogenesis (eds. I.D. Biggers & A.W. Schuetz), pp. 377–96. Baltimore: University Park Press.

Baker, T.G. & Neal, P. 1974. Organ culture of cortical fragments and Graafian follicles from human ovaries. *J Anat,* **117**: 361–371.

Bhojwani, M., Rudolph, E., Kanitz, W., Zuehlke, H., Schneider, F. & Tomek, W. 2006. Molecular analysis of maturation processes by protein and phosphoprotein profiling during *in vitro* maturation of bovine oocytes: a proteomic approach. *Cloning Stem Cells,* **8**: 259–274.

Damiani, P., Fissore, R.A., Cibelli, J.B., Long, C.R., Balise, J.J., Robl, J.M. & Duby, R.T. 1996. Evaluation of developmental competence, nuclear and ooplasmic maturation of calf oocytes. *Mol Reprod Dev,* **45**: 521–534.

Ducibella, T. & Buetow, J. 1994. Competence to undergo normal, fertilization-induced cortical activation develops after metaphase I of meiosis in mouse oocytes. *Dev Biol,* **165**: 95–104.

Edwards, J.L., Saxton, A.M., Lawrence, J.L., Payton, R.R. & Dunlap, J.R. 2005. Exposure to a physiologically relevant elevated temperature hastens *in vitro* maturation in bovine oocytes. *J Dairy Sci,* **88:** 4326–4333.

Eppig, J.J. 1996. Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. *Reprod Fert Dev,* **8**: 485–489.

Evsikov, A.V. & Marin de Evsikova, C. 2009. Gene expression during the oocyte-to-embryo transition in mammals. *Mol Reprod Dev,* **76:** 805–818.

Fair, T., Carter, F., Park, S., Evans, A.C. & Lonergan, P. 2007. Global gene expression analysis during bovine oocyte *in vitro* maturation. *Theriogenology,* **68**: 91–97.

Ferreira, E.M., Vireque, A.A., Adona, P.R., Meirelles, F.V., Ferriani, R.A. & Navarro, P.A. 2009. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology,* **71**: 836–848.

Flechon, J.E. 1970. Glycoprotein nature of cortical granules of rabbit egg: presentation by comparative use of ultrastructural cytochemical technics. *J Microsc,* **9**: 221–242.

García-Ispierto, I., López-Gatius, F., Bech-Sabat, G., Santolaria, P., Yaniz, J.L., Nogareda, C., De Rensis, F. & López-Bejar,M. 2007.Climate factors affecting conception rate of high producing dairy cows in northeastern Spain. *Theriogenology,* **67**: 1379–1385.

Greve, T., Grondahl, C., Schmidt, M. *et al.* 1996. Bovine preovulatory follicular temperature: implications for *in vitro* production of embryos. *Arch Tierzuch,* **39**: 7–14.

Grinsted, J., Blendstrup, K., Andreasen, M.P. & Byskov, A.G. 1980. Temperature measurements of rabbit antral follicles. *J Reprod Fert,* **60**: 149–155.

Grinsted, J., Kjer, J.J., Blendstrup, K. & Pedersen, J.F. 1985. Is low temperature of follicular fluid prior to ovulation necessary for normal oocyte development? *Fertil Steril,* **43**: 34–39.

Hosmer, D.W. & Lemeshow, S. 1987. Applied Logistic Regression. Wiley. New York.

Hosoe, M. & Shioya, Y. 1997. Distribution of cortical granules in bovine oocytes classified by cumulus complex. *Zygote,* **5**: 371–376.

Hunter, R.H., Grondahl, C., Greve, T. & Schmidt, M. 1997. Graafian follicles are cooler than neighbouring ovarian tissues and deep rectal temperatures. *Hum Reprod,* **12**: 95–100.

Hunter, R.H., Bogh, I.B., Einer-Jensen,N., Muller, S. & Greve, T. 2000. Pre-ovulatory graafian follicles are cooler than neighbouring stroma in pig ovaries. *Hum Reprod,* **15**: 273–283.

Hunter, R.H., Einer-Jensen, N. & Greve, T. 2006. Presence and significance of temperature gradients among different ovarian tissues. *Microsc Res Tech,* **69**: 501–507.

Ju, J.C., Jiang, S., Tseng, J.K., Parks, J.E. & Yang, X. 2005. Heat shock reduces developmental competence and alters spindle configuration of bovine oocytes. *Theriogenology,* **64**: 1677–1689.

Lawrence, J.L., Payton, R.R., Godkin, J.D., Saxton, A.M., Schrick, F.N. & Edwards, J.L. 2004. Retinol improves development of bovine oocytes compromised by heat stress during maturation. *J Dairy Sci,* **87**: 2449–2454.

López-Gatius, F. 2003. Is fertility declining in dairy cattle? A retrospective study in northeastern Spain. *Theriogenology,* **60**: 89–99.

Payton, R.R., Romar, R., Coy, P., Saxton, A.M., Lawrence, J.L. & Edwards, J.L. 2004. Susceptibility of bovine germinal vesicle-stage oocytes from antral follicles to direct effects of heat stress *in vitro*. *Biol Reprod,* **71**: 1303–1308.

Picton, H.M., Harris, S.E., Muruvi, W. & Chambers, E.L. 2008. The *in vitro* growth and maturation of follicles. *Reproduction,* **136**: 703–715.

Putney, D.J., Drost, M. & Thatcher, W.W. 1989. Influence of summer heat stress on pregnancy rates of lactating dairy cattle following embryo transfer or artificial insemination. *Theriogenology,* **31**: 765–778.

Rensis, F.D. & Scaramuzzi, R.J. 2003. Heat stress and seasonal effects on reproduction in the dairy cow: a review. *Theriogenology,* **60**: 1139–1151.

Roth, Z. & Hansen, P.J. 2005. Disruption of nuclear maturation and rearrangement of cytoskeletal elements in bovine oocytes exposed to heat shock during maturation. *Reproduction,* **129**: 235–244.

Roti, J.L. 2008. Cellular responses to hyperthermia (40–46ºC): cell killing and molecular events. *Int J Hyperthermia,* **24**: 3–15.

Schrock, G.E., Saxton, A.M., Schrick, F.N. & Edwards, J.L. 2007. Early *in vitro* fertilization improves development of bovine ova heat stressed during *in vitro* maturation*. J Dairy Sci,* **90**: 4297–4303.

Siemer, C., Smiljakovic, T., Bhojwani,M., Leiding, C., Kanitz, W., Kubelka, M. & Tomek, W. 2009. Analysis of mRNA associated factors during bovine oocyte maturation and early embryonic development. *Mol Reprod Dev,* **76**: 1208–1219.

Sirard, M.A. 2001. Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. *Theriogenology,* **55**: 1241–1254.

Szollosi, D. 1962. Cortical granules: a general feature of mammalian eggs? *J Reprod Fert*, **4**: 223–224.

Szollosi, D. 1967. Development of cortical granules and the cortical reaction in rat and hamster eggs. *Anat Rec,* **159**: 431–446.

Szollosi, D. 1971.Morphological changes inmouse eggs due to aging in the fallopian tube. *Am, J, Anat,* **130**: 209–225.

Szollosi, D. 1974. The spindle structure in mammalian eggs: the effect of ageing. Colloque: Les accidents chromosomiques de la reproduction. pp. 241–50.

Szollosi, D. 1975a. Mammalian eggs aging in the fallopian tubes. In Aging Gametes, Their Biology and Pathology. Proceedings of the International Symposium on Aging Gametes, 13–16 June 1973. pp. 98–121. Seattle: Washington, USA.

Szollosi, D. 1975b. Ultrastructural aspects of oocyte maturation and fertilization in mammals. La fecondation. Colloque de la Societe Nationale pour l'Etude de la Sterilite et de la Fecondite. pp. 13–35.

Szollosi, D., Gerard, M., Menezo, Y. & Thibault, C. 1978. Permeability of ovarian follicle: corona cell–oocyte relationship in mammals. *Ann Biol Anim Bioch Biophys,* **18**: 511–521.

Thibault, C., Szollosi, D. & Gerard, M. 1987. Mammalian oocyte maturation. *Reprod Nutr Dev,* **27**: 865–896.

Tseng, J.K., Chen, C.H., Chou, P.C., Yeh, S.P. & Ju, J.C. 2004. Influences of follicular size on parthenogenetic activation and *in vitro* heat shock on the cytoskeleton in cattle oocytes. *Reprod Domest Anim,* **39**: 146–153.

Wang,W., Hosoe,M., Li, R. & Shioya, Y. 1997. Development of the competence of bovine oocytes to release cortical granules and block polyspermy after meiotic maturation. *Dev Growth Differ,* **39**: 607–615.

Wessel, G.M., Brooks, J.M., Green, E., Haley, S., Voronina, E., Wong, J., Zaydfudim, V. & Conner, S. 2001. The biology of cortical granules. *Int Rev Cytol,* **209**: 117–206.

Wessel, G.M., Conner, S.D. & Berg, L. 2002. Cortical granule translocation is microfilament mediated and linked to meiotic maturation in the sea urchin oocyte. *Development,* **129**: 4315–4325.

II

Bovine oocytes show a higher tolerance to heat shock in the warm compared to the cold season of the year.

M.J. Maya-Soriano[a], F. López-Gatius[b], C. Andreu-Vázquez[a], M. López-Béjar[b]

[a]Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Spain.

[b]Department of Animal Production, Universitat de Lleida, Spain.

Submitted 10.11.2010; accepted 10.01.2012

Theriogenology. Accepted for publication.

THE_12268

This study was supported by Spanish Ministry of Science and Innovation, MICINN, Ref. RTA2008–00070-C02–02. Andreu-Vázquez was supported by an FPU Grant from the Spanish Ministry of Science and Education, MCE, Ref.AP2007-01598. Maya-Soriano was supported by a PIF Grant from the Universitat Autònoma de Barcelona.

Abstract

Heat stress is especially harmful for bovine ovarian follicle development and oocyte competence. In this study, we assessed the effects of heat shock on oocyte maturation in oocytes collected during the cold (February-March; n = 114) or warm (May-June; n = 116) periods of the year. In both cases, cumulus-oocyte complexes were matured under control (38ºC) and heat shock conditions (41.5ºC, 18-21h of maturation). For each oocyte, nuclear stage, cortical granule (CG) distribution and steroidogenic activity of cumulus cells were evaluated. Based on the odds ratio, heat-stressed oocytes were 26.83 times more likely to show an anomalous metaphase II (MII) morphology. When matured under heat shock conditions, oocytes obtained in both seasons were similarly affected in terms of nuclear maturation, whereas a seasonal effect was observed on cytoplasmic maturation. For oocytes collected during the cold season, the likelihood to show an anomalous maturation was 25.96 times higher when exposed to the heat treatment than when matured under control conditions. By contrast, oocytes collected during the warm season matured under control or heat shock did not show significant risk of showing an anomalous cytoplasmic maturation. Our findings indicate an increased rate of premature oocytes in response to heat shock as well as a higher tolerance to this stress of oocytes harvested in the warm season compared to those collected in the colder period.

Keywords: Oocyte, heat shock, stress tolerance, cortical granules, cold season, warm season.

Introduction

In dairy cows, the optimum air temperature range for milk production and reproduction is 25-26ºC (Hansen *et al.,* 2001; West, 2003). In northeastern Spain, animals endure high temperatures (> 25ºC) for 20-31 days in each of the warm months (May-September) and up to four days in each of cold months (October-April) (López-Gatius *et al.,* 2006). Nevertheless, due to high genetic selection for milk production, a decline in heat tolerance has recently been observed (Wolfenson *et al.,* 2000). Heat stress is known to cause an increase in rectal temperature and reductions in dry matter intake, milk production, and fertility (Ealy *et al.*, 1993; Wolfenson *et al.,* 1995; West, 2003). Problems related to heat stress have economic repercussions worldwide but especially affect animals in the Northern hemisphere (Wolfenson *et al.,* 2000; Edwards *et al.*, 2005). Conception rates in these animals are lower in summer (June-September) than winter (10-20% vs. 40-60%) (Wolfenson *et al.*, 2000; De Rensis & Scaramuzzi. 2003; Ju *et al.*, 2005).

Fertility effects of heat stress are the outcome of damage to antral follicles produced in warm months (Roth *et al.,* 2000). Thus, after exposure to high temperatures, three to four oestrous cycles are needed to recover fertility (Roth, 2001; García-Ispierto *et al.*, 2007). Heat stress to oocytes is especially harmful three days before and one day after insemination, and affects follicle development and oocyte competence (Hansen *et al.*, 2001; García-Ispierto *et al.*, 2007). Oocyte maturation is usually assessed in terms of nuclear and cytoplasmic events (Sirard, 2001; Ferreira *et al.*, 2009; Andreu-Vázquez *et al.*, 2010), which are usually highly coordinated (Eppig, 1996). In most of the species examined so far, nuclear maturation involves the transition from a germinal vesicle nucleus to a second metaphase arrangement of the chromosomes and formation of a first polar body by the time of ovulation. Cytoplasmic maturation manifests as changes in protein contents, but most conspicuously in the redistribution of organelles such as cortical granules (CG) (Szollosi, 1962; 1967). During resumption of meiosis, the CG migrates from the Golgi apparatus to the vitelline surface, assuming a position 0.4–0.6 μm below the plasma membrane (Ducibella & Buetow, 1994). Only when situated just beneath the plasma membrane can they undergo exocytosis by fusing with the egg membrane. This fusion enables the release of CG contents into the perivitelline space and is an important step in membrane maturation and blockade of polyspermy (Szollosi, 1967; Hosoe & Shioya, 1997; Wang *et al.*, 1997).

Under high temperature conditions, anomalous chromosome segregation and cytoplasmic defects in oocyte maturation have been attributed to a rise in reactive oxygen species (ROS) (Blondin *et al.*, 1997; Schrock *et al.*, 2007; Roth, 2008). Thus, oocytes matured under heat conditions show lower rates of nuclear maturation, chromatin fragmentation, and premature translocation of cortical granules (Ju & Tseng, 2004; Tseng *et al.*, 2004; De S Torres-Junior *et al.*, 2008). A recent study has shown that heat shock causes premature ageing of bovine oocytes (Andreu-Vázquez *et al.*, 2010). Notwithstanding, Ealy *et al.* (1993) and Hansen *et al.* (1999) found that by preincubating oocytes and embryos at slightly higher temperatures than usual, heat adaptation occurred before heat shock could block cell apoptosis.

Heat stress has been linked to reduced fertility in many countries (De Rensis & Scaramuzzi, 2003; López-Gatius, 2003), producing high economic losses. In particular, reproductive problems are observed when

high temperatures coincide with the time of insemination (Putney *et al.*, 1989; García-Ispierto *et al.*, 2007). This study was designed to examine the effects of heat shock on oocyte maturation and to identify possible seasonal differences in oocyte heat tolerance.

Materials and methods

Experimental design

In northern Spain, we can clearly differentiate a warmer (May to September) and colder (October to April) period of the year (López-Gatius *et al.*, 2006). Mean monthly climate variables were similar to those published previously for the area of the study (Santolaria *et al.*, 2010). Briefly, mean temperature (T), maximal T, minimal T, mean relative humidity (RH), minimal RH, mean Temperature-Humidity Index (THI) and maximal THI were 10.3ºC, 16.6ºC, 4.4ºC, 71%, 43%, 50.2 THI and 60.3 THI for the cold period of the study (February-March) and 20.6ºC, 26.9ºC, 14.3ºC, 65%, 37% and 65.6 THI and 72.9 THI, respectively, for the warm period of the study (May-June). Seasonal effects were thus evaluated in bovine oocytes (n = 230) collected in the cold season (CS; February-March; n = 114) or warm season (WS; May-June; n = 116). Two study groups were established as follows: control oocytes (C) cultured at 38.5ºC for 22 hours; and heat-shocked (HS) oocytes subjected 3 hours at the end of the maturation period (from 18 to 21h) to 41.5ºC, simulating the animal rectal temperature during the warmer hours of the day (Tseng *et al.*, 2004). The four groups finally compared were: CCS; HSCS; CWS; HSWS.

Chemicals and reagents

All chemicals were purchased from Sigma (Madrid, Spain) unless otherwise indicated.

Collection of oocytes

Ovaries from heifers recovered at a slaughterhouse were placed in Dulbecco's phosphate buffered saline solution (PBS) supplemented with 1% (v/v) antibiotic/antimycotic solution (AA; 10 000 units Penicillin, 10 mg streptomycin and 25 mg amphotericin B per ml) and transported to the laboratory at room temperature. The mesovarium, oviduct and fat were removed, and the ovaries then washed twice in warm sterile PBS and kept at 37.5ºC until follicle puncture within two hours of ovary recovery. Ovarian follicles (2-8 mm) were aspirated using an 18-gauge needle and 5 ml syringe and placed in working medium (WM; TCM 199-Hepes and 1% v/v AA solution).

In vitro maturation and oocyte preparation for staining

For *in vitro* maturation (IVM), cumulus-oocyte complexes (COCs) larger than 120 µm with three or more layers of compact cumulus cells and a homogeneous translucent ooplasm were selected.

Selected COCs were washed twice in WM and randomly placed in groups of 20-25 in four-well dishes (Nunc Tm 150288; Biocen; Spain) containing 500 µl of maturation medium (MM; TCM 199 supplemented with 20 µg/ml epidermal growth factor, 0.2 mM sodium pyruvate and 1% v/v AA solution).

All procedures took place in a laminar flow cabinet within two hours of follicular puncture.

COCs were cultured according to the experimental design in an atmosphere of 5% (v/v) CO_2 in humidified air. Control and HS oocytes were incubated in two different CO_2 incubators equipped with temperature and humidity probes.

COCs were morphologically assessed for cumulus cell expansion after 22 hours of IVM. Next, the COCs were denuded of cumulus cells by pipetting inside the wells and washing three times in PBS with 0.05% (w/v) bovine serum albumin (BSA Fraction V).

Maturation media were kept in Eppendorfs and recovered after centrifugation (2 500 rpm, 37ºC for 15 minutes) for hormone determinations. Aliquots were kept at -20ºC until analysis.

Denuded oocytes were immersed in PBS containing 0.4% (w/v) pronase E at 37.5ºC for 1-2 minutes until zona pellucida digestion. The oocytes were then washed five times in PBS-BSA 0.05% (w/v) and fixed in a PBS solution containing 4% (w/v) paraformaldehyde (Panreac, PRS 141451.1210) at refrigeration temperature for 30 minutes.

Oocytes were washed three times in PBS-BSA 0.05% and then incubated for 5 minutes in a permeabilizing solution of PBS containing 0.3% Triton X100 and 0.05% (w/v) BSA at room temperature. Next, the oocytes were washed five times in PBS-BSA 0.05%.

Cortical granule staining

Oocytes were incubated under dark conditions for 30 minutes at room temperature in a 100 µg/ml fluorescein isothiocyanate-labeled lens culinaris agglutinin (FITC-LCA) solution and washed five times to eliminate lectin excess.

Nuclear staining and mounting

Oocytes were immersed for 10-20 minutes in the dark at room temperature in a Hoechst 33342 (1 µl/ml; Invitrogen H1399) solution for nuclear staining and then washed three times. The oocytes were mounted between a coverslip and a glass slide treated with poly-L-lysine. The antifade Vectashield mounting medium for fluorescence (Vector H-1000) was used. The coverslip was sealed with nail polish and preparations kept at 4ºC in dark conditions until analysis.

Oocyte evaluation

Nuclear and cytoplasmic maturation were evaluated in each oocyte. Nuclear stages were determined under an UV epifluorescent microscope (Nikon Eclipse TE 2000S) and a laser-confocal microscope (Leica TCS SP2). Metaphase II (MII) was considered the correct nucleus stage after oocyte maturation. Nuclear stages were classified according to the modified classification of Tseng (2004): Anaphase-Telophase I (A-T), normal MII (uniform alignment of the chromosomes on the spindle) or anomalous MII (nuclear contents

appearing as a chromatin-like structure forming condensed aggregates or forming aberrantly distributed chromosomes).

Translocation of CG to the oolema was considered to be an indicator of cytoplasmic maturation (Damiani *et al.*, 1996) and was checked under an UV epifluorescent microscope and a laser-confocal microscope for each oocyte. Four patterns of CG translocation were observed according to Hosoe and Shioya (1997) classification: pattern I (CGs distributed in clusters or large aggregates), pattern II (CGs individually dispersed and partially clustered), pattern III (correct distribution of CGs, completely dispersed in monolayer), pattern IV (no CG).

Hormone determinations

For each study group, the steroidogenic activity of cumulus cells was assessed in the maturation medium. Estradiol and progesterone concentrations were determined after IVM using commercial enzyme immunoassay kits (Neogen Corporation, EIA #402210, EIA #402310, respectively) according to the manufacturer's instructions. Validation of the assay was performed after parallelism and extraction tests. Sensitivities of the estradiol and progesterone assays were 0.03 ng/ml and 0.35 ng/ml, respectively.

Statistical analysis

Only oocytes that reached MII nuclear stage were included in the study. The following data were recorded for each oocyte: replicate (1-5), season of collection (Cold Season versus Warm Season), treament (Control versus Heat Shock), MII morphology (normal MII versus anomalous MII) and CG distribution pattern (I –IV).

The probability of differences between the proportion of oocytes showing anomalous MII and GC distribution pattern IV among replicates was determinate by Chi-square analysis.

According to the results of a previous study (Andreu-Vázquez *et al.*, 2010) two consecutive logistic regressions were performed on data from each oocyte. A first binary logistic regression analysis was performed using MII anomalous morphology as the dependent variable (0 or 1) and season of collection, treatment and their interaction as independent factors. A second was performed using anomalous oocyte maturation, considering both MII anomalous morphology and CG distribution pattern IV, as dependent variable (0 or 1) and replicate, group, season of collection, treatment and their interaction as independent factors. All independent variables were considered as class variables for both analyses.

Logistic regressions analyses were performed using the SPSS package, version 17.0 (SPSS Inc., Chicago, IL, USA) according to the method of Hosmer and Lemeshow (1987). Basically, this method involves five steps as follows: preliminary screening of all variables for univariate associations; construction of a full model using all the variables found to be significant in the univariate analysis; stepwise removal of non-significant variables from the full model and comparison of the reduced model with the previous model for model fit and confounding; evaluation of plausible two ways interactions among variables and assessment of model fit using Hosmer–Lemeshow statistics. Variables with univariate associations showing P values <0.25 were

included in the initial model. We continued modelling until all the main effects or interaction terms were significant according to the Wald statistic at P <0.05.

A T-Student test for unpaired data was performed to evaluate hormone production, using the Welch correction for the groups who did not have equal variances through GraphPad InStat (version 3.01, Windows 95, Graph Pad Software, San Diego, USA). Differences were considered significant at P <0.05.

Results

Effects of heat shock and season of oocyte collection on nuclear maturation

Of the 230 cultured oocytes, only 190 could be evaluated for both nuclear and cytoplasmic maturation processes. Furthermore, four showed immature nuclear stages after IVM and were excluded from the study. Figure 1 shows laser-confocal microscopic images of Hoechst 33342 labelled bovine oocytes showing different nuclear stages.

No differences were detected on the percentage of oocytes showing anomalous MII morphology or CG distribution pattern IV among replicates (p=0.232 and p=0.165). Logistic regression analysis indicated no significant effects of the season of collection or interaction between season and treatment on MII morphology. MII anomalous morphology was registered in 2.2% (2/89) and 38.1% (37/97) of the control and HS oocytes, respectively. Based on the odds ratio, oocytes in HS group were 26.83 times more likely to show anomalous MII morphology than controls (p<0.001; 6.2-115.6 confidence interval for the odds ratio; Nagelkerke R^2 = 0.321).

Effects of heat shock and season of oocyte collection on cytoplasmic maturation

Figure 2 shows laser-confocal microscopic images of the equatorial section of FITC-LCA labelled bovine oocytes showing representative CG distribution patterns and number of oocytes for each pattern and group of study.

Anomalous Cortical Granules (CG) distribution pattern (pattern IV) was registered in 4.5% (4/89) and 37.1% (36/97) of the control and HS oocytes, respectively. Based on the odds ratio, oocytes in HS group were 13.01 times more likely to show CG pattern IV than controls (p<0.001; 4.4-38.8 confidence interval for the odds ratio; Nagelkerke R2= 0.289).

Variables included in the final logistic regression model for anomalous oocyte maturation, considering both anomalous patterns of Metaphase II (MII) morphology and Cortical Granules (CG) distribution pattern are shown in Table 1. According to the odds ratio, the likelihood of an oocyte showing an anomalous maturation was 27.55 times higher for HS group than for the control group. Furthermore, a significant interaction between season of collection and treatment was found. For oocytes collected during the cold season, the likelihood to show an anomalous maturation was 25.96 times higher when exposed to the heat treatment than when matured under control conditions. However, the risk of showing an anomalous maturation was not significantly different between oocytes from HS and C groups when oocytes where collected during the

warm season. The likelihood of showing an anomalous maturation was not different among HS and C groups in the warm and C group in the cold season.

Figure 1. Laser confocal microscopy images of Hoechst 33342 and FITC-LCA-labeled bovine oocytes showing different nuclear stages (blue) after 22h of maturation: (A) Metaphase II (MII) (control=87/89, HS=60/97), (B, C) anomalous MII (control=2/89, HS=37/97). CG are shown in the same pictures (green). Polar bodies are not shown due to the zona pellucida digestion.

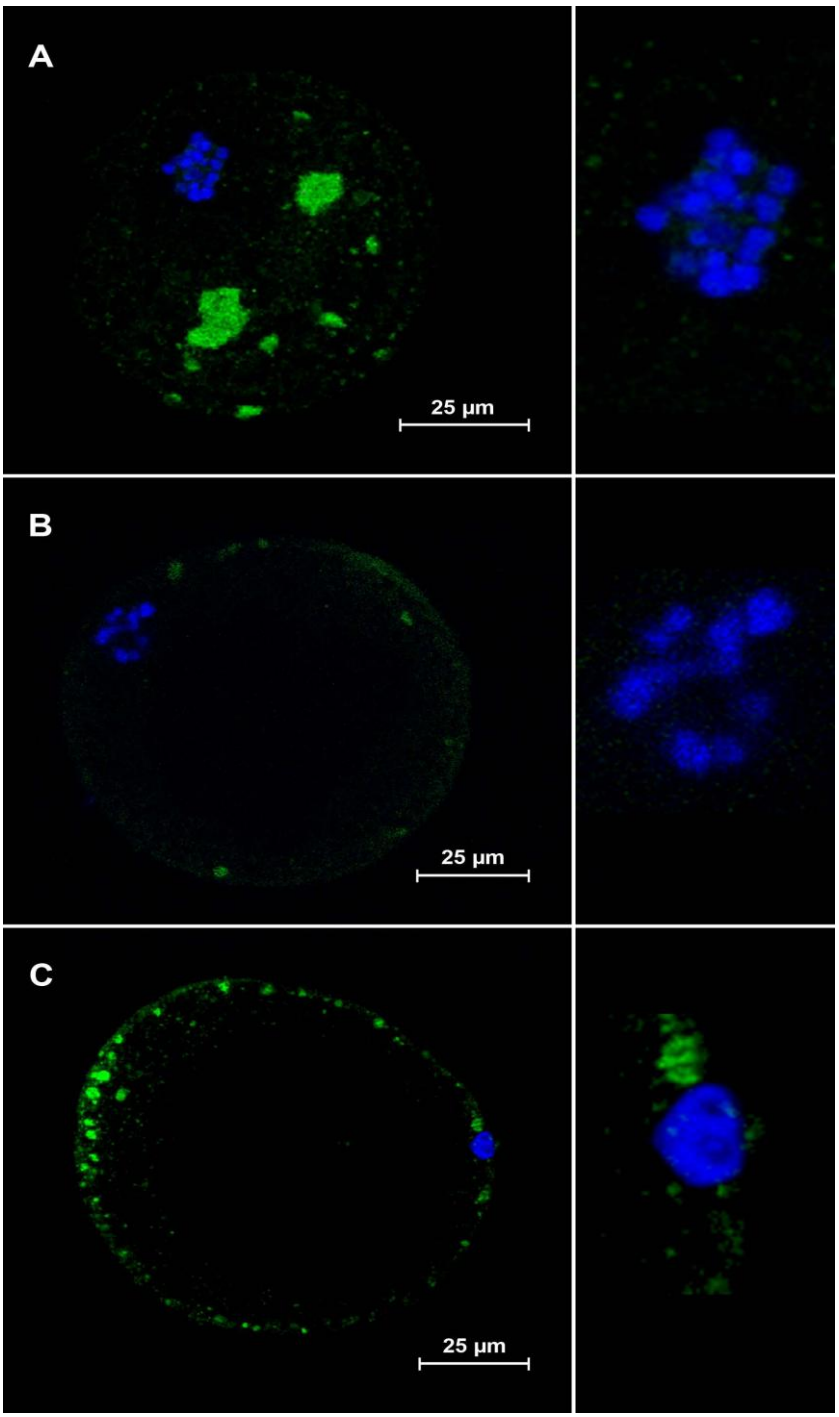


Figure 2. Laser-confocal microscopy images of an equatorial section (A) and detail (B) of FITC-LCA-labeled bovine oocytes showing representative CG distribution patterns (green) for the different groups after 22h of maturation: Pattern II or CGs partially clustered in the oolplasm (1A-B; C=22/89, HS=11/97), Pattern III or CGs dispersed in a monolayer (2A-B; C=64/89, HS=50/97), Pattern IV or no CG (3A-B; C=3/89, HS=36/97).

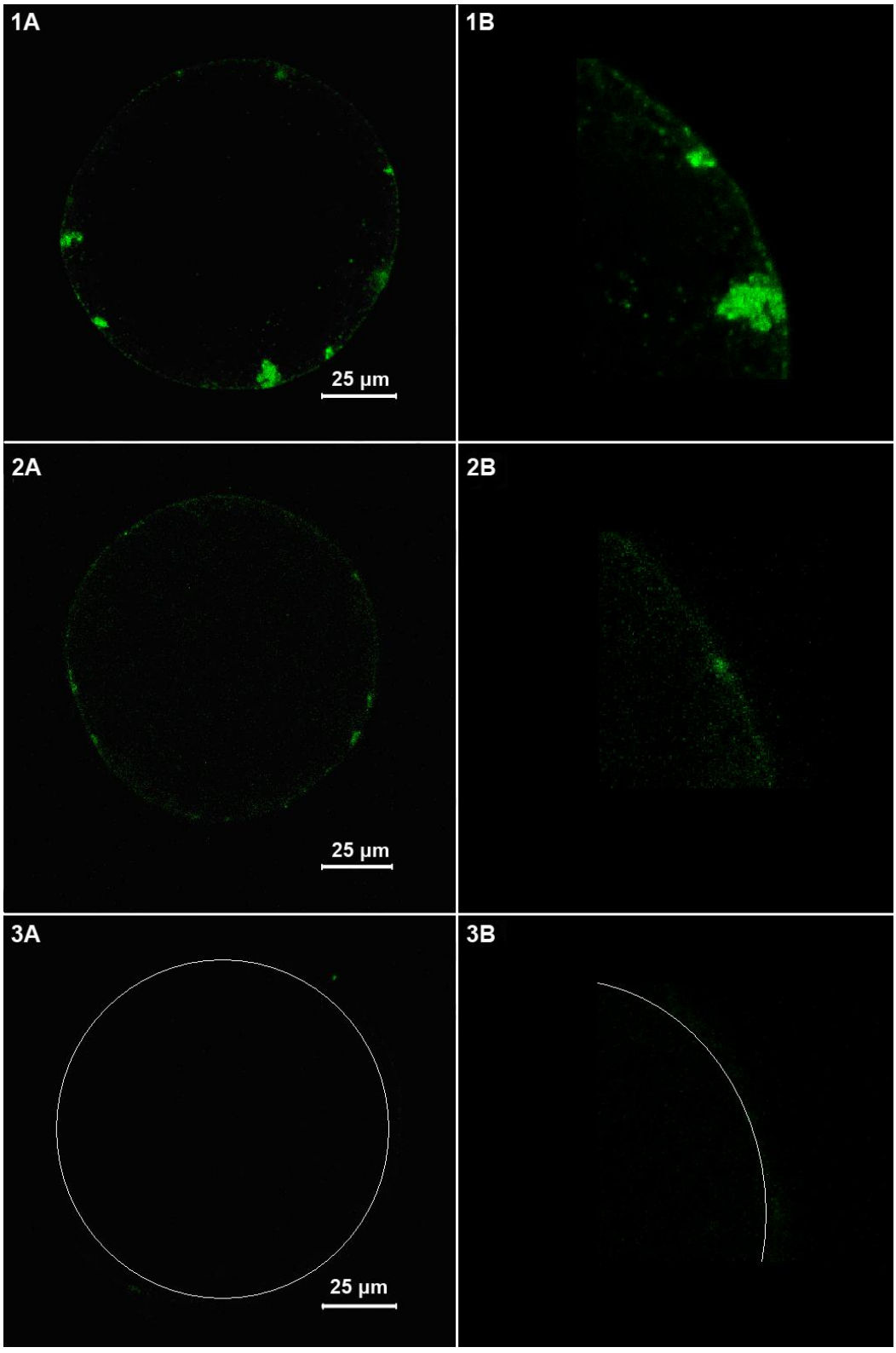


Table 1. Odds ratios of variables included in the final logistic regression model for anomalous oocyte maturation, considering both anomalous patterns of Metaphase II (MII) morphology and Cortical Granules (CG) distribution pattern.

Factor	Class	MII anomalous morphology and CG distribution pattern IV		Odds Ratio	95% CI[a]	p-value
		n	%			
Treatment	Control	2/89	2,2	Reference		
	Heat Shock	26/97	26,8	27,55	6,1 - 125,3	<0,001
Treatment by Season	Control-Cold Season	1/42	2,4	Reference		
	Control-Warm Season	1/47	2,1	-	-	NS
	Heat Shock Cold Season	19/49	38,8	25,96	3,9 - 204,8	0,002
	Heat Shock Warm Season	7/48	14,6	-	-	NS

Likelihood ratio test 33, 140; 1 d.f., P < 0,0001. Nagelkerke R^2 = 0,286

[a]Confidence interval for the odds ratio

Effects of heat shock and season of oocyte collection on steroidogenic activity

No significant differences were observed in estradiol or progesterone contents per oocyte after IVM in the maturation media according to season (cold or warm season) or IVM conditions (Control or HS). However, we detected a trend toward higher estradiol concentrations (P=0.059) available per oocyte in the IVM medium for the two HS groups (HSCS: 34 pg/oocyte; and HSWS: 30 pg/oocyte) than for the control ones (CCS: 25 pg/oocyte; and CWS: 20 pg/oocyte). Progesterone concentrations available per oocyte were 1.4 ng/oocyte in the groups of the cold season (CCS and HSCS) and 0.6-0.8 ng/oocyte in the groups of the warm season (CWS and HSWS).

Discussion

The results of this study indicate that heat shock impaired nuclear and cytoplasmic processes, in agreement with our previous findings (Andreu-Vázquez *et al.*, 2010). In addition, oocytes were found to better tolerate heat shock during the warm period compared to the colder period of the year.

Compared to controls, a significantly greater percentage of heat-shocked oocytes showed an anomalous MII stage and CG distribution pattern IV. Aberrant progression to the second meiotic metaphase stage was recorded in 38% of the heat-shocked oocytes, probably as the result of chromosomal and meiotic spindle alterations, as described by other authors (Payton *et al.*, 2004; Tseng *et al.*, 2004; Roth & Hansen, 2005). Our observation of a high proportion of oocytes showing CG distribution pattern IV, presumptive of premature exocytosis, when exposed to high temperatures has also been described previously (Andreu-Vázquez *et al.*, 2010). Thus, heat shock at the end of the IVM period seems to provoke anomalies compatible with oocyte aging (Andreu-Vázquez *et al.*, 2010). This pattern, characterized by loss of a continuous CG layer, is the most common pattern produced in oocytes after fertilization or activation due to exocytosis. Heat shock could thus be the cause of an advanced state of oocyte maturation (Payton *et al.,* 2004; Edwards *et al.,* 2005) resulting in CG loss (Andreu-Vázquez *et al.*, 2010).

A seasonal effect on the capacity of the oocytes to undergo nuclear maturation (MII percentage) could not be demonstrated. Thus, oocytes collected in both seasons were similarly affected under heat shock conditions. Conversely however, seasonal effects were produced on the cytoplasmic maturation capacity of the oocytes. Hence, oocytes recovered in the cold season were more sensitive to heat shock than those obtained in the warmer months. It therefore seems that bovine oocytes are more sensitive to high temperatures suffered during cold months. The climate change effects of natural and anthropogenic factors have been estimated as a global temperature rise of 0.1-0.2ºC/decade (MacCracken, 2008) and since the 70's, average annual temperatures have tended to rise. This temperature increase is more evident during winter (UNFCCC, 2009). In effect, in our geographical area, acute changes in the temperature-humidity index (THI), which have been linked to early fetal loss, occur more severely during the cool period of the year (Santolaria *et al.*, 2010).

The fact that oocytes obtained in the warm season were better able to adapt to high temperatures in terms of their cytoplasmic maturation could reflect a mechanism of heat shock tolerance. It has been described that a defined and properly applied sublethal stress may induce general adaptation and increase tolerance (Pribenszky *et al.*, 2010). This phenomenon has been described *in vitro* in a study in which bovine embryos were progressively exposed to increasing heat shock (Ealy *et al.*, 1993; Hansen & Arechiga, 1999; Paula-Lopes & Hansen, 2002). Heat tolerance could be related to heat shock protein (HSP) expression, as HSP-70 protects oocytes from apoptotic stimuli that harmfully affect DNA (Paula-Lopes & Hansen, 2002). This mechanism of heat tolerance warrents further investigation in future studies designed to improve oocyte and embryo development in warm regions. It also would be of interest to develop experiments to check if the phenomenon of thermal tolerance can be reproduced *in vivo*.

Several studies have clearly shown a detrimental effect of summer heat stress on oocyte and embryo quality or competence in terms of cleavage and blastocyst formation rates (Hansen *et al.*, 2001; Payton *et al.*, 2004; Edwards *et al.*, 2005; Ju *et al.*, 2005). However, the present study does not show an evident detrimental effect of heat shock on oocyte maturation when the oocytes were recovered during the warm season. The warm season was limited to May-June, which corresponds to early summer in the region of study (Santolaria *et al.*, 2010); it could confirm that oocyte donors were exposed to relatively mild heat stress conditions during May-June, and this might explain why oocyte maturation was not affected by the warm season. We aimed to check the effect of *in vitro* heat shock over oocytes not previously affected by severe *in vivo* heat stress. Furthermore, the study was performed on oocytes collected from non-lactating heifers, strengthening the assumption that the heifers, and their ovaries, were not exposed to deep thermal stress during the warm season. Future experiments are needed to study if oocytes undergoing *in vivo* maturation would be able to develop heat tolerance when cows are exposed to severe heat stress *in vivo*, in August for instance, when their body temperatures can rise to 41-41.5ºC.

In conclusion, heat shock was found to increase the rate of premature oocytes although oocytes collected in the cold season were more sensitive to the negative effects of stress than their counterparts collected in the warm season.

References

Andreu-Vázquez, C., López-Gatius, F., García-Ispierto, I., Maya-Soriano, M.J., Hunter, R.H.F., López-Béjar, M. 2010. Does heat stress provoke the loss of a continuous layer of cortical granules beneath the plasma membrane during oocyte maturation? *Zygote,* **24**: 1-7.

Blondin P, Coenen K, Sirard MA. 1997. The impact of reactive oxygen species on bovine sperm fertilizing ability and oocyte maturation. *J Androl,* **18**(4): 454-460.

Damiani, P., Fissore, R.A., Cibelli, J.B., Long, C.R., Balise, J.J., Robl, J.M. & Duby, R.T. 1996. Evaluation of developmental competence, nuclear and ooplasmic maturation of calf oocytes. *Mol Reprod Dev,* **45:** 521-534.

De Rensis F, Scaramuzzi RJ. 2003. Heat stress and seasonal effects on reproduction in the dairy cow-a review. *Theriogenology,* **60**(6): 1139-1151.

De S Torres-Junior JR, de F A Pires M, de Sa WF, de M Ferreira A, Viana JH, Camargo LS, *et al.* 2008. Effect of maternal heat-stress on follicular growth and oocyte competence in Bos indicus cattle. *Theriogenology,* **69**(2): 155-166.

Ducibella, T. & Buetow, J. 1994. Competence to undergo normal, fertilization-induced cortical activation develops after metaphase I of meiosis in mouse oocytes. *Dev Biol,* **165**: 95-104.

Ealy AD, Drost M, Hansen PJ. 1993. Developmental changes in embryonic resistance to adverse effects of maternal heat stress in cows. *J Dairy Sci,* **76**(10): 2899-2905.

Edwards, J.L., Saxton, A.M., Lawrence, J.L., Payton, R.R. & Dunlap, J.R. 2005. Exposure to a physiologically relevant elevated temperature hastens *in vitro* maturation in bovine oocytes. *J Dairy Sci,* **88**: 4326-4333.

Eppig, J.J. 1996. Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. *Reprod Fert Dev,* **8**: 485-489.

Ferreira, E.M., Vireque, A.A., Adona, P.R., Meirelles, F.V., Ferriani, R.A. & Navarro, P.A. 2009. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology,* **71**: 836-848.

Garcia-Ispierto, I., Lopez-Gatius, F., Bech-Sabat, G., Santolaria, P., Yaniz, J.L., Nogareda, C., De Rensis, F. & Lopez-Bejar, M. 2007. Climate factors affecting conception rate of high producing dairy cows in northeastern Spain. *Theriogenology,* **67**: 1379-1385.

Hansen PJ, Arechiga CF. 1999. Strategies for managing reproduction in the heat-stressed dairy cow. *J Anim Sci*, **77** Suppl 2:36-50.

Hansen PJ, Drost M, Rivera RM, Paula-Lopes FF, al-Katanani YM, Krininger CE, *et al.* 2001. Adverse impact of heat stress on embryo production: causes and strategies for mitigation. *Theriogenology,* **55**(1): 91-103.

Hosmer, D.W. & Lemeshow, S. 1987. Applied logistic regression. Willey. New York.

Hosoe, M. & Shioya, 1997. Y. Distribution of cortical granules in bovine oocytes classified by cumulus complex. *Zygote,* **5**: 371-376.

Ju, J.C., Jiang, S., Tseng, J.K., Parks, J.E. & Yang, X. 2005. Heat shock reduces developmental competence and alters spindle configuration of bovine oocytes. *Theriogenology,* **64**: 1677-1689.

Ju JC, Tseng JK. 2004. Nuclear and cytoskeletal alterations of *in vitro* matured porcine oocytes under hyperthermia. *Mol Reprod Dev,* **68**(1): 125-133.

Lopez-Gatius, F. 2003. Is fertility declining in dairy cattle? A retrospective study in northeastern Spain. *Theriogenology*, **60**: 89-99.

Lopez-Gatius F, Garcia-Ispierto I, Santolaria P, Yaniz J, Nogareda C, Lopez-Bejar M. 2006. Screening for high fertility in high-producing dairy cows. *Theriogenology,* **65**(8): 1678-1689.

MacCracken, MC. 2008. Prospects for future climate change and the reasons for early action. *J Air Waste Manag Assoc,* **58**(6):735-786.

Paula-Lopes FF, Hansen PJ. 2002. Heat shock-induced apoptosis in preimplantation bovine embryos is a developmentally regulated phenomenon. *Biol Reprod,* **66**(4): 1169-1177.

Payton, R.R., Romar, R., Coy, P., Saxton, A.M., Lawrence, J.L. & Edwards, J.L. 2004. Susceptibility of bovine germinal vesicle-stage oocytes from antral follicles to direct effects of heat stress *in vitro*. *Biol Reprod,* **71**: 1303-1308.

Pribenszky C, Vajta G, Molnar M, Du Y, Lin L, Bolund L, *et al.* 2010. Stress for stress tolerance? A fundamentally new approach in mammalian embryology. *Biol Reprod,* **83**(5): 690-697.

Putney, D.J., Drost, M. & Thatcher, W.W. 1989. Influence of summer heat stress on pregnancy rates of lactating dairy cattle following embryo transfer or artificial insemination. *Theriogenology,* **31**: 765-778.

Roth Z. 2008. Heat stress, the follicle, and its enclosed oocyte: mechanisms and potential strategies to improve fertility in dairy cows. *Reprod Domest Anim,* **43**(suppl.2): 238-244.

Roth Z, Arav A, Bor A, Zeron Y, Braw-Tal R, Wolfenson D. 2001. Improvement of quality of oocytes collected in the autumn by enhanced removal of impaired follicles from previously heat-stressed cows. *Reproduction,* **122**(5): 737-744.

Roth Z, Hansen PJ. 2005. Disruption of nuclear maturation and rearrangement of cytoskeletal elements in bovine oocytes exposed to heat shock during maturation. *Reproduction,* **129**(2): 235-244.

Roth Z, Meidan R, Braw-Tal R, Wolfenson D. 2000. Immediate and delayed effects of heat stress on follicular development and its association with plasma FSH and inhibin concentration in cows. *J Reprod Fertil,* **120**(1): 83-90.

Santolaria P, López-Gatius F, García-Ispierto I, Bech-Sàbat G, Angulo E, Carretero T, Sánchez-Nadal JA, Yániz J. 2010. Effects of cumulative stressful and acute variation episodes of farm climate conditions on late embryo/early fetal loss in high producing dairy cows*. Int J Biometeorol,* **54**: 93-98.

Schrock, G.E., Saxton, A.M., Schrick, F.N. & Edwards, J.L. 2007. Early *in vitro* fertilization improves development of bovine ova heat stressed during *in vitro* maturation*. J Dairy Sci,* **90**: 4297-4303.

Sirard, M.A. 2001. Resumption of meiosis: mechanism involved in meiotic progression and its relation with developmental competence. *Theriogenology,* **55**: 1241-1254.

Szollosi, D. 1962. Cortical granules: a general feature of mammalian eggs? *J Reprod Fert,* **4**: 223-224.

Szollosi, D. 1967. Development of cortical granules and the cortical reaction in rat and hamster eggs. *Anat, Rec,* **159**: 431-446.

Tseng, J.K., Chen, C.H., Chou, P.C., Yeh, S.P. & Ju, J.C. 2004. Influences of follicular size on parthenogenetic activation and *in vitro* heat shock on the cytoskeleton in cattle oocytes. *Reprod Domest Anim,* **39**: 146-153.

UNFCCC. The United Nations Climate Change Conference. Copenhagen 2009. (http://unfccc.int/resource/docs/natc/esp_nc5.pdf).

Wang, W., Hosoe, M., Li, R. & Shioya, Y. 1997. Development of the competence of bovine oocytes to release cortical granules and block polyspermy after meiotic maturation. *Dev Growth Differ,* **39**: 607-615.

West JW. 2003. Effects of heat-stress on production in dairy cattle. *J Dairy Sci,* **86**(6): 2131-2144.

Wolfenson D, Roth Z, Meidan R. 2000. Impaired reproduction in heat-stressed cattle: basic and applied aspects. *Anim Reprod Sci,* **60-61**: 535-547.

Wolfenson D, Thatcher WW, Badinga L, Savio JD, Meidan R, Lew BJ, *et al.* 1995. Effect of heat stress on follicular development during the estrous cycle in lactating dairy cattle. Biol Reprod, **52**(5): 1106-1113.

III

Retinol improves in vitro oocyte nuclear maturation under heat stress in heifers.

M.J. Maya-Soriano, E. Taberner, M. López-Béjar

Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Spain.

Submitted 08.11.2011; accepted 12.02.2012; Epub 11.07.2012

Zygote. DOI:10.1017/S0967199412000135

This study was supported by Spanish Ministry of Science and Innovation, MICINN, Ref. RTA2008–00070-C02–02. Maya-Soriano was supported by a PIF Grant from the Universitat Autònoma de Barcelona.

Abstract

Heat stress (HS) is especially harmful for bovine ovarian follicle development and oocyte competence. Furthermore, HS causes premature aging on oocytes due to high levels of reactive oxygen species (ROS), involved in the harmful effects over the oocyte maturation and the steroidogenic activity of follicular cells. In this study, the presumptive protective effects of antioxidant agents on heat-stressed oocytes were evaluated. Heifer oocytes were matured for 22 hours under control (38ºC) and heat stress conditions (41.5ºC at 18-21 h of maturation). For each oocyte, nuclear stage and cortical granules (CG) distribution were evaluated. Steroidogenic activity of cumulus cells was also recorded. The antioxidant agents used in the study were: Retinol (1.43 µg/ml), Retinyl (0.28 µg/ml) and Oleic acid (0.05 mg/ml). Based on a Chi-square test ($P < 0.05$), HS affected negatively the metaphase II (MII) progression and produced a premature CG exocytosis. Retinol allowed to improve the oocyte MII progression. However, retinyl and oleic acid, at the concentrations used in this study, could not counteract adverse effects of HS. A decrease in progesterone and increase in estradiol availability were observed when retinyl and oleic acid were supplemented to the maturation medium, respectively. In conclusion, retinol proved to be valuable in heat-stressed oocytes protecting nuclear maturation.

Key words: Oocyte, heat stress, retinol, retinyl, oleic acid.

Introduction

Heat stress (HS) has been linked to reduced fertility in dairy cows in many countries (De Rensis and Scaramuzzi, 2003; Lopez-Gatius, 2003), which leads to high economic losses. The optimum air temperature range for efficient milk production and reproduction has been established at 25-26ºC (Hansen *et al.*, 2001; West, 2003). However, in northeastern Spain, animals endure high temperatures (> 25ºC) for 20-31 days during the warm months (May-September) and up to four days during cold months (October-April) (Lopez-Gatius *et al.,* 2006). HS is known to affect negatively the animal causing an increase in rectal temperature (up to 41ºC), reductions in dry matter intake, milk production, and fertility (Ealy *et al.,* 1993; Wolfenson *et al.,* 1995; West, 2003; Edwards *et al.*, 2005).

HS affects the ovarian follicles development, the oocyte competence and the embryo development, probably due to high levels of reactive oxygen species (ROS) (Ikeda *et al.,* 1999; De S Torres-Junior *et al.,* 2008; Roth *et al.,* 2008). Their effects are especially harmful three days before and one day after the insemination (Hansen *et al.,* 2001; García-Ispierto *et al.,* 2007). When oocytes are matured under heat conditions show lower rates of nuclear maturation, chromatin fragmentation, and a premature translocation of cortical granules (Ju and Tseng, 2004; Tseng *et al.,* 2004; Andreu-Vázquez *et al.,* 2010), causing premature ageing of bovine oocytes (Andreu-Vázquez *et al.*, 2010).

In cells, ROS levels are normally blocked by antioxidant molecules (Miller *et al.,* 1993). Therefore, in attempt to improve fertility rates in dairy cattle exposed to heat stress conditions, some antioxidants agents such as vitamins and some fatty acids have been used *in vivo*. Moreover, *in vitro* studies have also been developed, focusing on oocyte maturation/embryo culture (Hansen *et al.,* 2001; Zeron *et al.,* 2001; Duque *et al.,* 2002; Gomez *et al.,* 2003; Livingston *et al.,* 2004; Hidalgo *et al.,* 2005; Ikeda *et al.,* 2005; Leroy *et al.,* 2005) or steroidogenic activity of granulosa cells (Wolfenson *et al.,* 2000; Hansen *et al.,* 2001). Some of these studies take into account the oxidative stress produced during normal *in vitro* culture (Rajesh *et al.,* 2010) and some of them the negative heat stress effects (Arechiga *et al.,* 1998; Lawrence *et al.,* 2004; Bilby *et al.,* 2006).

Vitamin A (all-trans retinol) and its metabolites are regulators of cell growth, differentiation of many types of cells (Hidalgo *et al.,* 2005), and have ROS scavenger activity (Ikeda *et al.,* 2005). The derivates from vitamin A have also an important role on reproduction (folliculogenesis, steroidogenesis, oocyte maturation and embryo development) (Gomez *et al.,* 2004; Ikeda *et al.,* 2005; Chiamenti *et al.,* 2010). Due to their ROS scavenger activity, retinoids have been used in previous studies, some of them done under HS conditions. Therefore, an improvement in the conception rate (Gomez *et al.,* 2006), a beneficial effect on cytoplasmic competence after *in vitro* maturation (Duque *et al.,* 2002; Gomez *et al.,* 2003) and on embryo development rates to blastocyst, were reported (Lawrence *et al.,* 2004; Livingston *et al.,* 2004; Fouladi-Nashta *et al.,* 2007).

It has been widely accepted that heat stress can aggravate the negative energy balance (NEB) during lactation, predominantly due to a drop in dry matter intake (Collier *et al.,* 1992). Moreover, fatty acids in

the diet seem to be valuable improving NEB, having positive effects over the follicle, oocyte and embryo in dairy cows. Zeron Y. *et al.* (2001) suggest that oleic acid concentration has an important role on temperature oocyte membrane adaptation, and high levels of it have been related with high embryo recovery rates (Leroy *et al.,* 2005; Fouladi-Nashta *et al.,* 2007). So, the use of fatty acids could improve fertility during warm months.

This study was designed to examine the effect of antioxidant products on heat-stressed oocytes identifying presumptive beneficial effects on *in vitro* oocyte maturation.

Materials and methods

Experimental design

Two study groups were established as follows: control oocytes (C) cultured at 38.5ºC for 22 hours; and heat-stressed (HS) oocytes subjected from 18 to 21 h of maturation at 41.5ºC to simulate environmental conditions of heat stress (Tseng *et al.,* 2004). The effect of antioxidant agent addition to the maturation medium was assessed in three experiments using a different antioxidant agent in each of them: Retinol (RO; n = 185), Retinyl (RI; n = 236) and Oleic acid (OA; n = 134).

Chemicals and reagents

All chemicals were purchased from Sigma (Madrid, Spain) unless otherwise indicated.

Collection of oocytes

Ovaries from heifers recovered at a slaughterhouse were placed in Dulbecco's phosphate buffered saline solution (PBS), that was supplemented with 1 % (v/v) antibiotic/antimycotic solution (AA; 10 000 units Penicillin, 10 mg streptomycin and 25 mg amphotericin B per ml), and transported to the laboratory at room temperature. The mesovarium, oviduct and fat were removed, and afterwards the ovaries were washed twice in warm sterile PBS and kept at 37.5ºC until follicle puncture within two hours of ovary recovery. Ovarian follicles (2-8 mm) were aspirated using an 18-gauge needle and 5 ml syringe and were placed in working medium (WM; TCM 199-Hepes and 1% v/v AA solution).

COCs selection

Only oocytes enclosed in three or more layers of compact cumulus cells and presenting a homogeneous and translucent ooplasm were selected for *in vitro* maturation (IVM).

In vitro maturation

Selected COCs were washed twice in WM and randomly placed in groups of 20-25 in four-well dishes (Nunc Tm 150288; Biocen; Spain) containing 500 µl of maturation medium (MM; TCM 199 supplemented with 20 µg/ml epidermal growth factor, 0.2 mM sodium pyruvate and 1 % v/v AA

solution) and the corresponding supplementation in each experiment. In the first one, RO was used at 1.43 µg/ml (5 µM) diluted in 0'03 % (v/v) pure Ethanol (Panreac 141086.1214). A study group with only Ethanol as supplementation was established to confirm there was no effect of the vehicle over the oocyte. In the rest of experiments, RI was used at 0.28 µg/ml and OA at 0.05 mg/ml (200 µM). All procedures took place in a laminar flow cabinet within two hours of follicular puncture.

COCs were cultured according to the experimental design in an atmosphere of 5 % (v/v) CO2 in humidified air. Control and heat-stressed oocytes were incubated in two different CO_2 incubators equipped with temperature and humidity probes.

COCs were morphologically assessed for cumulus cell expansion after 22 hours of IVM. Afterwards, the COCs were denuded of cumulus cells by pipetting inside the wells and washing three times in PBS with 0.05 % (w/v) bovine serum albumin (BSA Fraction V).

Maturation medium were kept in Eppendorfs and recovered after centrifugation (2 500 rpm, 37ºC for 15 minutes) in order to be used for hormone determinations. Aliquots were kept at -20ºC until analysis.

Cortical granule staining

Denuded oocytes were immersed in PBS containing 0.4 % (w/v) pronase E at 37.5ºC for 1-2 minutes until zona pellucida digestion. The oocytes were then washed five times in PBS-BSA 0.05 % (w/v) and fixed in a PBS solution containing 4 % (w/v) paraformaldehyde (Panreac, PRS 141451.1210) at refrigeration temperature for 30 minutes.

Oocytes were washed three times in PBS-BSA 0.05 % and then incubated for 5 minutes in a permeabilizing solution of PBS containing 0.3 % Triton X100 and 0.05 % (w/v) BSA at room temperature. Afterwards, the oocytes were washed five times in PBS-BSA 0.05 %.

Oocytes were incubated in dark conditions for 30 minutes at room temperature in a 100 µg/ml fluorescein isothiocyanate-labeled lens culinaris agglutinin (FITC-LCA) solution and were washed five times to eliminate lectin excess.

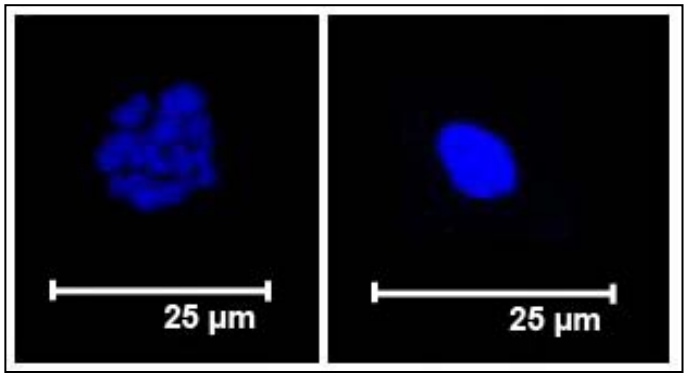
Nuclear staining and mounting

Oocytes were immersed for 10-20 minutes in dark conditions at room temperature in a Hoechst 33342 (1 µl/ml; Invitrogen H1399) solution for nuclear staining and were then washed three times. The oocytes were mounted between a coverslip and a glass slide treated with poly-L-lysine. The antifade Vectashield mounting medium for fluorescence (Vector H-1000) was used. The coverslip was sealed with nail polish and preparations were kept at 4ºC in dark conditions until analysis.

Oocyte evaluation

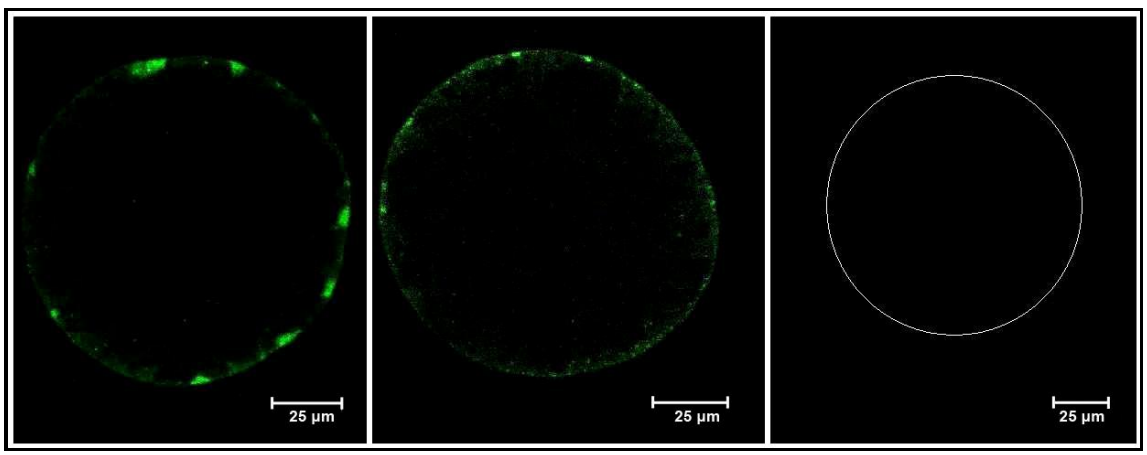
Nuclear and cytoplasmic maturation were evaluated in each oocyte. Nuclear stages were observed under an UV epifluorescent microscope (Nikon Eclipse TE 2000S) and a laser-confocal microscope (Leica TCS SP2). Metaphase II (MII) was considered to be the correct nucleus stage after oocyte maturation. Nuclear stages were classified according to a modified classification by Tseng (Tseng *et al.*, 2004; Andreu-Vázquez *et al.*, 2010): Anaphase-Telophase I (A-T), normal MII (uniform alignment of the chromosomes on the spindle) or anomalous MII (nuclear contents appearing as a chromatin-like structure forming condensed aggregates or forming aberrantly distributed chromosomes; Figure 1).

Figure 1. Laser confocal microscopy images of Hoechst 33342-labeled bovine oocytes showing a Metaphase II (MII) and anomalous MII.



Translocation of CG to the oolema was considered to be an indicator of correct cytoplasmic maturation (Damiani *et al.*, 1996) and was checked under an UV epifluorescent microscope and a laser-confocal microscope for each oocyte. Four patterns of CGs translocation were observed according to Hosoe and Shioya (1997) classification: Pattern I (CGs distributed in clusters or large aggregates), pattern II (CGs individually dispersed and partially clustered), pattern III (correct distribution of CGs, completely dispersed in monolayer), pattern IV (no CG; Figure 2).

Figure 2. Laser-confocal microscopy images of an equatorial section of FITC-LCA-labeled bovine oocytes showing representative CG distribution patterns for the different groups: Pattern II, Pattern III, Pattern IV.



Hormone determinations

For each study group, the steroidogenic activity of cumulus cells was assessed in the maturation medium. Estradiol and progesterone concentrations were determined after IVM using commercial enzyme immunoassay kits (Neogen Corporation, EIA #402210 and EIA #402310 respectively) according to the manufacturer's instructions. Validation test were performed for each assay.

Statistical analysis

Only oocytes that reached MII nuclear stage were included in the statistical analysis. The following data were recorded for each oocyte: replicate (1-7), antioxidant agent (RO or RI or OA), treatment (C versus HS), MII morphology (normal MII versus anomalous MII) and CG distribution pattern (I –IV).

The probability of differences in the proportions of oocytes showing anomalous MII and GC distribution pattern IV among replicates was determinate by Chi-squared analysis.

The results of the nuclear and cytoplasmic maturation were expressed in percentage. The data were analyzed using contingency tables and Pearson's Chi-square statistical test using SPSS (version 15.0 for Windows, SPSS Inc., Chicago, IL., USA). A T-Student test for unpaired data was performed to evaluate hormone production, using the Welch correction for groups who did not have equal variances through GraphPad InStat (version 3.01, Windows 95, Graph Pad Software, San Diego, USA). Significant differences were considered at $P < 0.05$.

Results

Heat stress effects on oocyte maturation

Heat-stressed oocytes showed lower MII percentages ($P < 0.05$) and higher percentage of oocytes with cortical granules pattern IV than the oocytes matured under control conditions ($P < 0.010$). The rise of pattern IV was at the expense of a decrease in the correct pattern III percentage.

Effect of antioxidant agents on oocyte nuclear maturation

When retinol was added to the maturation medium, heat stressed oocytes showed a higher percentage of correct nuclear maturation stage ($P = 0.031$; Figure 3).

Nevertheless, the addition of retinyl or oleic acid to the maturation medium reduced the percentage of MII oocytes regardless the maturation conditions (C group $P = 0.003$, HS group $P = 0.006$; C and HS group $P = 0.000$, respectively; Figure 4 and 5).

Effect of antioxidant agents on cortical granules distribution pattern

A reduction in the percentage of oocytes showing exocytosis pattern (pattern IV) was observed under heat stress conditions (P = 0.010) when retinol was added to the medium (Figure 3). It was linked to an increase in CG pattern II percentage. However, when retinyl was added to the maturation medium, cytoplasmic maturation was significantly altered in heat-stressed oocytes showing a lower GC pattern III percentage (P = 0.002) and a higher exocytosis pattern IV (P = 0.025) (Figure 4). On the other hand, oleic acid supplementation did not significantly affect the cytoplasmic maturation patterns (Figure 5).

Figure 3. Percentage of control and heat-stressed oocytes that reached the MII stage and different CG distribution patterns when retinol was added to the maturation medium. * P < 0.05.

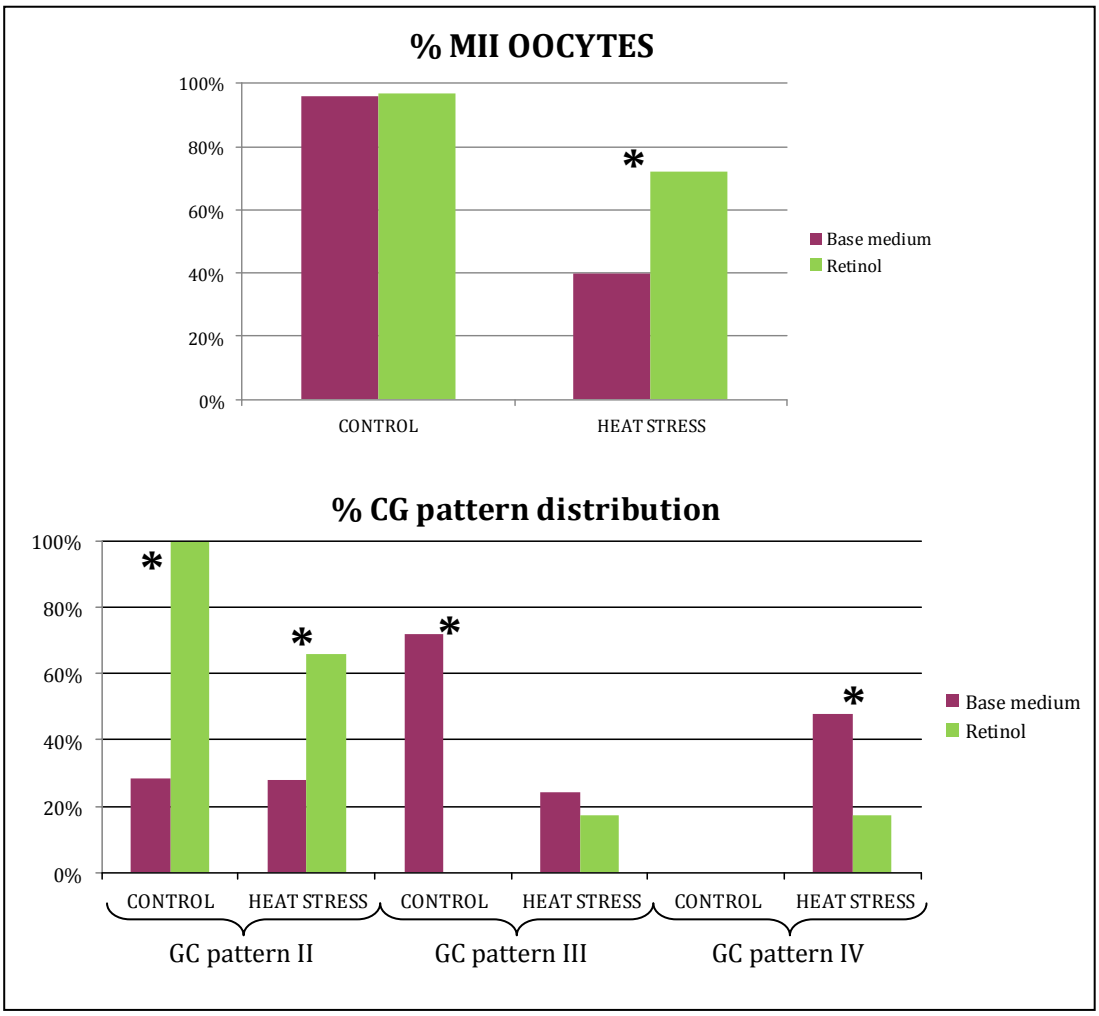


Figure 4. Percentage of control and heat-stressed oocytes that reached the MII stage and different CG distribution patterns when retinyl was added to the maturation medium. * P < 0.05.

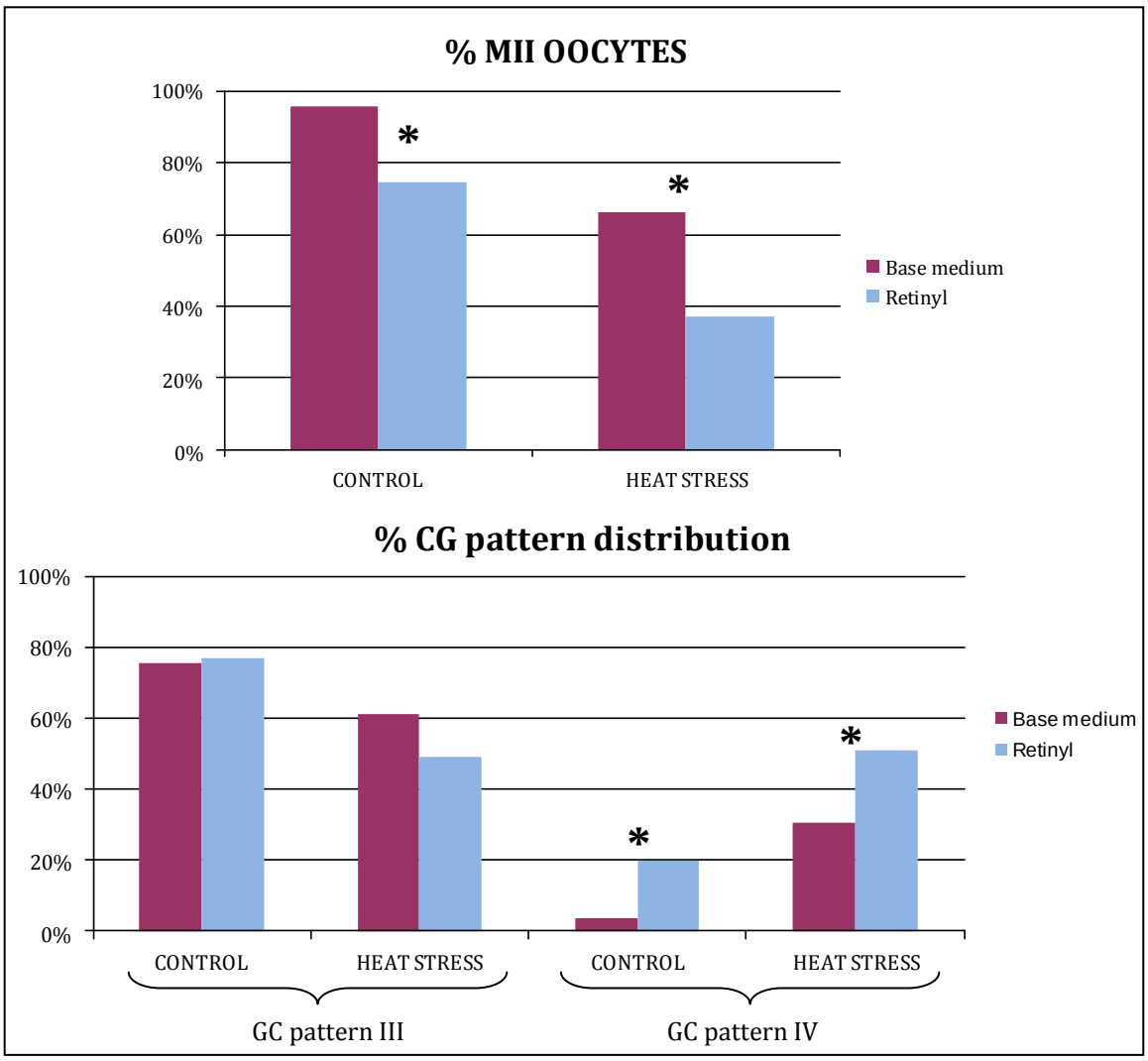
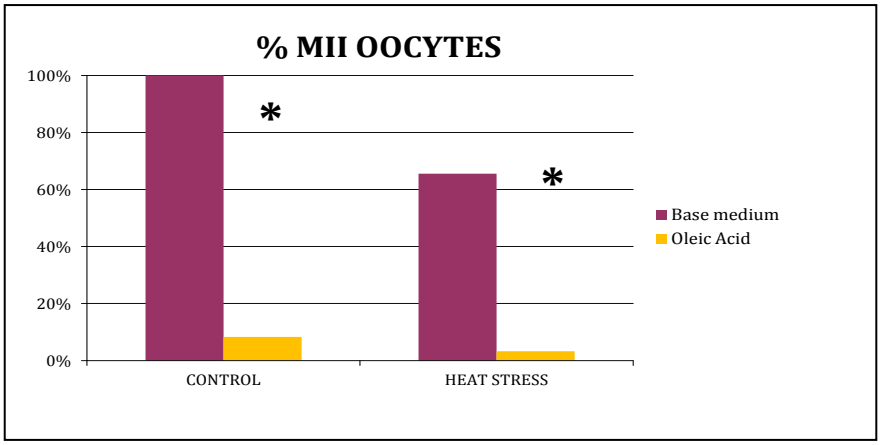
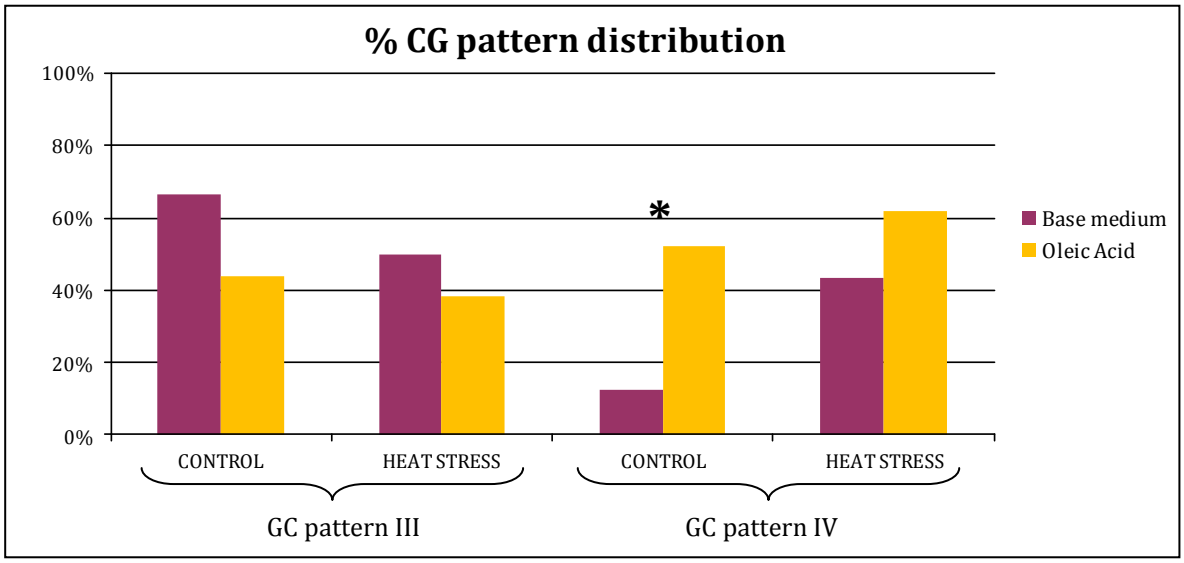


Figure 5. Percentage of control and heat-stressed oocytes that reached the MII stage and different CG distribution patterns when oleic acid was added to the maturation medium. * P < 0.05.





Effect of antioxidant agents on steroidogenic activity

Neither Retinol nor heat stress conditions had effects on steroidogenic activity.

Nevertheless, under physiological conditions, a slight increase on estradiol production (P = 0.0669) and a decrease on progesterone production was observed (P = 0.0275) when retinyl was added to the medium. On the other hand, oleic acid supplementation significantly increased estradiol availability (P = 0.0163), although no effect was found on progesterone.

Discussion

The current study demonstrated that heat stress dramatically affected oocyte maturation at nuclear and cytoplasmic level, in agreement with our previous findings (Andreu-Vázquez *et al.*, 2010). Moreover, heat-stressed oocytes were less affected when retinol was added to the maturation medium, at least at nuclear level.

The greater percentage of heat-stressed oocytes showing an aberrant progression to the second meiotic metaphase stage was probably as a result of chromosomal and meiotic spindle alterations, as described by other authors (Payton *et al.*, 2004; Tseng *et al.*, 2004; Roth and Hansen, 2005; Andreu-Vázquez *et al.*, 2010). The higher proportion of oocytes showing CG distribution pattern IV, presumptive of premature exocytosis, under high temperatures has also been described previously (Andreu-Vázquez *et al.*, 2010). This pattern, characterized by loss of a continuous CG layer, is the most common pattern produced in oocytes after fertilization or activation due to exocytosis. Thus, heat stress at the end of the IVM period seems to cause an advanced state of oocyte maturation (Payton *et al.*, 2004; Edwards *et al.*, 2005), resulting in CG loss, abnormality compatible with oocyte aging (Andreu-Vázquez *et al.*, 2010).

HS affects follicle development and oocyte competence (Hansen *et al.*, 2001; García-Ispierto *et al.*, 2007) due to high levels of reactive oxygen species (ROS) (Ikeda *et al.*, 1999; De S Torres-Junior *et al.*, 2008; Roth *et al.*, 2008). Mammalian cells, including oocytes and early embryos, have evolved several

mechanisms, as antioxidant molecules, to be protected against ROS damage. Antioxidants present in the oocyte, embryo and/or its environment include vitamins as A (retinol), C and E, pyruvate, glutathione (GSH), hypotaurine, taurine, and cysteamine (Guerin *et al.*, 2001).

Some *in vivo* studies described beneficial effects on the use of antioxidant agents or fatty acids in the diet improving fertility during the warm period of the year and also during NEB. It has been reported that dairy cow fertility can be improved at the second artificial insemination by a diet rich in retinol and β-carotenes during the warm months (Arechiga *et al.*, 1998). However, it has been suggested that a retinoid cumulative effect is needed to obtain an effective concentration in oviduct and uteri (Guerin *et al.*, 2001; Livingston *et al.*, 2004). It is due to beta-carotene metabolism and location of retinoid receptors. Those receptors are present in the cumulus oocyte complex, so it is suggested that retinoids could counteract HS effects by receptor mediated (Lawrence *et al.*, 2004). On the other hand, other nutritional management, involving fatty acids, improved some aspects on dairy cow fertility in summer (Bilby *et al.*, 2006).

HS affects harmfully the oocyte maturation at both nuclear and cytoplasmic levels. However, in our study, nuclear oocyte maturation was improved when retinol was added to the maturation medium of heat-stressed oocytes. Vahedi *et al.* (2009) also reported a beneficial effect on bovine nuclear oocyte maturation when all-trans retinoic acid at 1 μM was supplemented *in vitro* at physiological conditions, observing an increase on development rate to MII. Nevertheless, the beneficial effects of retinol are noted only when the effects of heat stress were pronounced enough to reduce continued development of oocytes (Livingston *et al.*, 2004). However, there are controversial results regarding retinol effects on embryo development. Some studies suggested that ovine embryo development to blastocyst could be improved under oxidative stress conditions adding retinol at 6 μM (Rajesh *et al.*, 2010), and also bovine heat-stressed oocytes and embryos, after addition of retinol at 5 μM (Lawrence *et al.*, 2004; Livingston *et al.*, 2004). Nevertheless, these studies take into account the effect on the oocyte through the embryo development but not the retinoid effects done into the oocyte itself. Therefore, in this study we tried to elucidate the effects that retinoids exert into the oocyte itself under heat stress conditions.

On the other hand, a retinol effect on CG was observed in our study, decreasing the exocytosis pattern IV due to HS, but also increasing the CG pattern II oocytes. Although the correct maturation distribution pattern is CG pattern III, the pattern II is considered an immature pattern but not an undesirable distribution. A possible explanation is that retinol could cause a delay effect on CG migration through the oolema, not considering it as a negative effect. It has been described previously that other retinoids improve cytoplasmic maturation of bovine oocytes under physiological conditions (Hidalgo *et al.*, 2003), possibly because retinoids can act as antioxidant agents, regulating the expression of the gonadotrophin receptor, increasing midkine levels (MK; growth differentiation factor) in follicular fluid, suppressing cyclooxygenase synthesis and nitric oxide synthesis in follicle cells, cumulus-granulosa cells and oocytes (Ikeda *et al.*, 2005).

Nevertheless, when other retinoid was used (retinyl) a decrease in the correct oocyte maturation rate was observed, especially on nuclear stage. This effect was seen at both culture conditions. So, retinil seems to be useless to counteract heat stress effects on bovine oocytes, at least at the concentration used in this study. To our knowledge there is no literature about effects of retinyl on bovine oocyte maturation, neither under physiological or heat stress conditions. However, retinyl has been used on embryos and did not show a beneficial effect on development or blastocyst rates (Lima *et al.*, 2004). Although retinoic acid supplementation helps murine embryos to overcome the two-cell block (Hajializadeh *et al.*, 2008), and seems to be valuable in co-cultures of goat embryos with oviductal cells. So, it could suggest that retinyl needs to be metabolised to be effective (Chiamenti *et al.*, 2010).

It has been reported that oleic acid is present in high concentrations in bovine oocyte membrane during winter and its low concentration in summer is related to the poor quality of oocytes (Zeron *et al.*, 2001). Nevertheless, our results showed a negative effect of oleic acid on metaphase progression, as described previously by Jorritsma *et al.* (2004), and did not improve cytoplasmic oocyte maturation under heat stress, at least at the concentration used in this study. However, the addition of oleic acid in the maturation medium seemed to not cause any effect on nucleus of bovine oocytes in a previous study (Leroy *et al.*, 2005). To our knowledge there are no other works taking into account the effect of oleic acid on oocyte cytoplasmatic maturation. It is necessary to take into account that the concentrations used in this study could be not appropriate to improve oocyte maturation under heat stress conditions.

HS affects steroidogenic activity as well as oocyte development. Although no effect was found in hormone production when antioxidant agents were added to the culture medium under HS, some effects where noticed on physiological conditions, except using retinol. Our findings suggested that retinyl supplementation was able to maintain cumulus oophorus cell phenotype, maintaining estradiol secretion, and avoiding its premature luteinisation, thus decreasing progesterone availability for the oocyte. These findings do not correspond with an *in vitro* study in gilts, where an increase in progesterone secretion, but not in estradiol concentration, was reported in those oocytes cultured after an *in vivo* injection of vitamin A before mating (Whaley *et al.*, 2000). Finally, oleic acid supplementation was able to increase estradiol availability per oocyte, as described in other study where estradiol production by granulose cells was also stimulated, but at higher concentrations than used in our study (500µM; Vanholder *et al.*, 2005).

In conclusion, heat stress increased the rate of aged oocytes after *in vitro* maturation, and the use of retinol as supplementation in the maturation medium could be useful improving nuclear maturation in bovine heat-stressed oocytes. However, the addition of retinyl or oleic acid to the medium was not useful to improve maturation of heat-stressed oocytes, at least at the concentrations used in this study. So, further studies are needed in this direction to elucidate the existence of effective concentrations of the assessed antioxidant agents.

References

Andreu-Vázquez, C., López-Gatius, F., García-Ispierto, I., Maya-Soriano, M.J., Hunter, R.H.F., López-Béjar, M. 2010. Does heat stress provoke the loss of a continuous layer of cortical granules beneath the plasma membrane during oocyte maturation? *Zygote,* **18**: 293-299.

Arechiga, C.F., Vazquez-Flores, S., Ortiz, O., Hernandez-Ceron, J., Porras, A., McDowell, L.R., *et al.* 1998. Effect of injection of beta-carotene or vitamin E and selenium on fertility of lactating dairy cows. *Theriogenology,* **50**(1): 65-76.

Bilby, T.R., Block, J., do Amaral, B.C., Sa Filho, O., Silvestre, F.T., Hansen, P.J., *et al.* 2006. Effects of dietary unsaturated fatty acids on oocyte quality and folicular development in lactating dairy cows in summer. *J Dairy Sci,* **89**(10): 3891-3903.

Chiamenti, A., Aguiar Filho, C.R., Freitas Neto, L.M., Chaves, R.M., Paula-Lopes, F.F., Lima, P.F., *et al.* 2010. Effects of retinoids on the *in vitro* development of Capra hircus embryos to blastocysts in two different culture systems. *Reprod Domest Anim,* **45**(5): e68-72.

Collier, R.I., Beede, D.K., Thatcher, W.W., Israel, L.A. and Wilcox, C.J. 1992. Influences of environment and its modifidon on dairy animal health and production. *J Dairy Sci,* **65**: p. 2213.

Damiani, P., Fissore, R.A., Cibelli, J.B., Long, C.R., Balise, J.J., Robl, J.M. & Duby, R.T. 1996. Evaluation of developmental competence, nuclear and ooplasmic maturation of calf oocytes. *Mol Reprod Dev,* **45**: 521-534.

De Rensis, F., Scaramuzzi, R.J. 2003. Heat stress and seasonal effects on reproduction in the dairy cow-a review. *Theriogenology,* **60**(6): 1139-1151.

De S Torres-Junior, J.R., de F A Pires, M., de As, W.F., de M Ferreira, A., Viana, J.H., Camargo, L.S., *et al.* 2008. Effect of maternal heat-stress on follicular growth and oocyte competence in Bos indicus cattle. *Theriogenology,* **69**(2): 155-166.

Duque, P., Diez, C., Royo, L., Lorenzo, P.L., Carneiro, G., Hidalgo, C.O., *et al.* 2002. Enhancement of developmental capacity of meiotically inhibited bovine oocytes by retinoic acid. *Hum Reprod,* **17**(10): 2706-2714.

Ealy, A.D., Drost, M., Hansen, P.J. 1993. Developmental changes in embryonic resistance to adverse effects of maternal heat stress in cows. *J Dairy Sci,* **76**(10): 2899-2905.

Edwards, J.L., Saxton, A.M., Lawrence, J.L., Payton, R.R. & Dunlap, J.R. 2005. Exposure to a physiologically relevant elevated temperature hastens *in vitro* maturation in bovine oocytes. *J Dairy Sci,* **88**: 4326-4333.

Fouladi-Nashta, A.A., Gutierrez, C.G., Gong, J.G., Garnsworthy, P.C., Webb, R. 2007. Impact of dietary fatty acids on oocyte quality and development in lactating dairy cows. *Biol Reprod,* **77**(1): 9-17.

García-Ispierto, I., López-Gatius, F., Bech-Sabat, G., Santolaria, P., Yaniz, J.L., Nogareda, C., De Rensis, F. & López-Bejar, M. 2007. Climate factors affecting conception rate of high producing dairy cows in northeastern Spain. *Theriogenology,* **67**: 1379-1385.

Gomez, E., Royo, L.J., Duque, P., Carneiro, G., Hidalgo, C., Goyache, F., *et al.* 2003. 9-cis-retinoic acid during *in vitro* maturation improves development of the bovine oocyte and increases midkine but not IGF-I expression in cumulus-granulosa cells. *Mol Reprod Dev,* **66**(3): 247-255.

Gomez, E., Rodriguez, A., Goyache, F., Diez, C., Jose Royo, L., Moreira, P.N., *et al.* 2004. Retinoid-dependent mRNA expression and poly-(A) contents in bovine oocytes meiotically arrested and/or matured *in vitro*. *Mol Reprod Dev,* **69**(1): 101-108.

Gomez, E., Caamano, J.N., Rodriguez, A., De Frutos, C., Facal, N., Diez, C. 2006. Bovine early embryonic development and vitamin A. *Reprod Domest Anim,* **41**(Suppl 2): 63-71.

Guerin, P., El Mouatassim, S., Menezo, Y. 2001. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update,* **7**(2): 175-189.

Hajializadeh, N., Babaei, H., Nematollahi-Mahani, S.N. and Azizollahi, S. 2008. The development of mouse early embryos *in vitro* in fribroblasts and cumulus cells co-cultures supplemented with retinoic acid. *Iranian J Vet Res*,**9** (1, 22): 1-8.

Hansen, P.J., Drost, M., Rivera, R.M., Paula-Lopes, F.F., Al-Katanani, Y.M., Krininger, C.E. *et al.* 2001. Adverse impact of heat stress on embryo production: causes and strategies for mitigation. *Theriogenology,* **55**(1): 91-103.

Hidalgo, C.O., Diez, C., Duque, P., Facal, N., Gomez, E. 2003. Pregnancies and improved early embryonic development with bovine oocytes matured *in vitro* with 9-cis-retinoic acid. *Reproduction,* **125**(3): 409-416.

Hidalgo, C., Diez, C., Duque, P., Prendes, J.M., Rodriguez, A., Goyache, F., *et al.* 2005. Oocytes recovered from cows treated with retinol become unviable as blastocysts produced *in vitro*. *Reproduction,* **129**(4): 411-421.

Hosoe, M. & Shioya, Y. 1997. Distribution of cortical granules in bovine oocytes classified by cumulus complex. *Zygote,* **5**: 371-376.

Ikeda, M., Kodama, H., Fukuda, J., Shimizu, Y., Murata, M., Kumagai, J. & Tanaka, T. 1999. Role of radical oxygen species in rat testicular germ cell apoptosis induced by heatstress. *Biol Reprod,* **61**: 393–399.

Ikeda, S., Kitagawa, M., Imai, H., Yamada, M. 2005. The roles of vitamin A for cytoplasmic maturation of bovine oocytes. *J Reprod Dev,* **51**(1): 23-35.

Jorritsma, R., Cesar, M.L., Hermans, J.T., Kruitwagen, C.L., Vos, P.L., Kruip, T.A. 2004. Effects of non-esterified fatty acids on bovine granulosa cells and developmental potential of oocytes *in vitro*. *Anim Reprod Sci,* **81**(3-4): 225-235.

Ju, J.C., Tseng, J.K. 2004. Nuclear and cytoskeletal alterations of *in vitro* matured porcine oocytes under hyperthermia. *Mol Reprod Dev,* **68**(1): 125-133.

Lawrence, J.L., Payton, R.R., Godkin, J.D., Saxton, A.M., Schrick, F.N., Edwards, J.L. 2004. Retinol improves development of bovine oocytes compromised by heat stress during maturation. *J Dairy Sci,* **87**(8): 2449-2454.

Leroy, J.L., Vanholder, T., Mateusen, B., Christophe, A., Opsomer, G., de Kruif, A. *et al.* 2005. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes *in vitro*. *Reproduction,* **130**(4): 485-495.

Lima, P.F., Oliveira, M.A., Goncalves, P.B., Montagner, M.M., Reichenbach, H.D., Weppert, M. *et al.* 2004. Effects of retinol on the *in vitro* development of Bos indicus embryos to blastocysts in two different culture systems. *Reprod Domest Anim,* **39**(5): 356-360.

Livingston, T., Eberhardt, D., Edwards, J.L., Godkin, J. 2004. Retinol improves bovine embryonic development *in vitro*. *Reprod Biol Endocrinol,* **21**: 2:83.

López-Gatius, F. 2003. Is fertility declining in dairy cattle? A retrospective study in northeastern Spain. *Theriogenology,* **60**: 89-99.

López-Gatius, F., García-Ispierto, I., Santolaria, P., Yaniz, J., Nogareda, C., López- Bejar, M. 2006. Screening for high fertility in high-producing dairy cows. *Theriogenology,* **65**(8): 1678-1689.

Miller, J.K., Brzezinska-Slebodzinska, E., Madsen, F.C. 1993. Oxidative stress, antioxidants, and animal function. *J Dairy Sci,* **76**(9): 2812-2823.

Payton, R.R., Romar, R., Coy, P., Saxton, A.M., Lawrence, J.L. & Edwards, J.L. 2004. Susceptibility of bovine germinal vesicle-stage oocytes from antral follicles to direct effects of heat stress *in vitro*. *Biol Reprod,* **71**: 1303-1308.

Rajesh, N., Shankar, M.B. and Deecaraman, M. 2010. Effect of vitamin A supplementation at different gaseous environments on *in vitro* development of pre-implantation sheep embryos to the blastocyst stage. *Animal,* **4**: 11, 1884-1890.

Roth, Z., Hansen, P.J. 2005. Disruption of nuclear maturation and rearrangement of cytoskeletal elements in bovine oocytes exposed to heat shock during maturation. *Reproduction,* **129**(2): 235-244.

Roth, Z., Aroyo, A., Yavin, S., Arav, A. 2008. The antioxidant epigallocatechin gallate (EGCG) moderates the deleterious effects of maternal hyperthermia on follicle-enclosed oocytes in mice. *Theriogenology,* **70**: 887–897.

Tseng, J.K., Chen, C.H., Chou, P.C., Yeh, S.P. and Ju, J.C. 2004. Influences of follicular size on parthenogenetic activation and *in vitro* heat shock on the cytoskeleton in cattle oocytes. *Reprod Domest Anim,* **39**: 146-53.

Vahedi, V., Zeinoaldini, S., Kohram, H. and Farahavar, A. 2009. Retinoic acid effects on nuclear maturation of bovine oocytes *in vitro*. *African J Biotechnol,* **8**(16): 3974-3978.

Vanholder, T., Leroy, J.L.M.R., Van Soom, A., Opsomer, G., Maes, D., Coryn, M., de Kruif, A. 2005. Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation *in vitro*. *Anim Reprod Sci,* **87**: 33–44.

West, J.W. 2003. Effects of heat-stress on production in dairy cattle. *J Dairy Sci,* **86**(6): 2131-2144.

Whaley, S.L., Hedgpeth, V.S., Farin, C.E., Martus, N.S., Jayes, F.C., Britt, J.H. 2000. Influence of vitamin A injection before mating on oocyte development, follicular hormones, and ovulation in gilts fed high-energy diets. *J Anim Sci,* **78**(6): 1598-1607.

Wolfenson, D., Thatcher, W.W., Badinga, L., Savio, J.D., Meidan, R., Lew, B.J., *et al.* 1995. Effect of heat stress on follicular development during the estrous cycle in lactating dairy cattle. *Biol Reprod,* **52**(5): 1106-1113.

Wolfenson, D., Roth, Z., Meidan, R. 2000. Impaired reproduction in heat-stressed cattle: basic and applied aspects. *Anim Reprod Sci,* **60-61**: 535-547.

Zeron, Y., Ocheretny, A., Kedar, O., Borochov, A., Sklan, D., Arav, A. 2001. Seasonal changes in bovine fertility: relation to developmental competence of oocytes, membrane properties and fatty acid composition of follicles. *Reproduction,* **121**(3): 447-454.

IV

Daily exposure to summer temperatures affects epididymal sperm cells in an in vivo rabbit model.

Maya-Soriano[a] M.J., Taberner[a] E., Sabés-Alsina[a] M., Ramon[b] J., Rafel[b] O., Tussell[b] Ll., Piles[b] M., López-Béjar[a] M.

[a]Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Spain.

[b]Unitat de Cunicultura, Institut de Recerca i Tecnologia Agroalimentàries, Caldes de Montbuí, Barcelona, Spain

Submitted 01.09.2012

Reproduction. Submitted for publication.

This study was supported by Spanish Ministry of Science and Innovation, MICINN, Ref. RTA2008–00070-C02–02. Maya-Soriano was supported by a PIF Grant from the Universitat Autònoma de Barcelona.

Abstract

High temperatures have negative effects on sperm quality leading to temporary or permanent sterility. The aim of the study was to assess the effect of long exposure to summer circadian heat stress cycles on sperm parameters and the motile-subpopulation structure of epididymal sperm cells from rabbit bucks. Twelve White New Zealand rabbit bucks were exposed to 17ºC (control group) or to summer circadian heat stress cycles (31ºC, 3 hours/day; heat stress group). Spermatozoa were flushed from the epididymis and assessed for sperm quality parameters at recovery. Sperm total motility and progressivity were negatively affected by high temperature in the heat stress group ($P < 0.05$). Heat stress also negatively affected motility parameters (VCL, VSL, VAP, STR, LIN, WOB and BCF; $P < 0.05$, but not ALH). According to motile sperm-subpopulations, heat stress significantly increased ratio of less motile subpopulations, although maintaining percentage of the high motile subpopulation. In conclusion, our results suggest that the induced changes in sperm motility produced by environmental heat stress are linked to concomitant changes in both the specific motility parameters and the proportion of motile sperm-subpopulations of the epididymis. However, these changes did not affect the subpopulation with the highest motile epididymal sperm cells, which it is still conserved despite the detrimental effect of heat stress.

Key Words: Summer circadian heat stress cycles, rabbit, spermatozoa, epididymis, motile sperm-subpopulation.

Introduction

The Earth system has been changing rapidly over the past several decades due to natural and anthropic factors. Studies have stated an increase in the global average surface temperature of about 0.6ºC over the past 20th century. The predictions presage increments over 0.1 to 0.2ºC per decade (MacCracken, 2008), with an increase in medium and maximum temperatures of 1.5 to 2.1ºC in 2020 (Lecha, 2007). These data suggest a significantly increase in heat stress pressure on global human population, animals and plants (Lecha, 2007), displacing life from their thermal comfort zone. Effects of global warming are expected to be larger in the middle-high latitudes than in the tropics, over land areas than oceans, and in winter than in summer (MacCracken, 2008). Heat stress is known to alter the physiology of livestock, reducing male and female reproduction and production, and increasing mortality (Hoffmann, 2010). In males, high temperatures have negative effects on libido and sperm quality leading to sub-fertility, temporary or permanent sterility (Setchell, 1998a; Lue *et al.*, 1999; Yaeram et al, 2006; Tusell *et al.*, 2011). Similar effects have been reported on human reproduction, where seasonally high temperatures increase the incidence of low fertilization rates, due to spermatogenesis and ovulation suppression, as well as a reduction in early embryo survival (Bronson, 1995).

Heat stress effects on male fertility or their semen characteristics have been widely evaluated. The whole body heating approach is one of the most physiological ways for studying the effect of high temperatures on the testis (Setchell, 1998a), but it can also be analyzed by local heating (cryptorchidism, scrotal insulation or immersion in a water bath; Setchell, 1998a). In ruminants and pigs, the most common technique to study heat stress effects *in vivo* is scrotal insulation (Malmgren, 1989; Vogler *et al.*, 1991, 1993; Mieusset *et al.*, 1992; Karabinus *et al.*, 1997; Walters et al, 2005, 2006), although, some studies have also been developed in climatic chambers (De Alba and Riera, 1966; Skinner and Louw, 1966; Rathore, 1968; Wettemann *et al.*, 1976; Wettemann and Desjardins, 1979; Cameron and Blackshow, 1980; Heitman *et al.*, 1984). On the other hand, rodents are usually submitted to high temperatures by immersing body and testes in water bath (Sailer *et al.*, 1997; Jannes *et al.*, 1998; Setchell *et al.*, 1998b; Rockett *et al.*, 2001; Banks *et al.*, 2005; Pérez-Crespo *et al.*, 2008), and also in climatic chambers (Zhu *et al.*, 2004; Yaeram *et al.*, 2006). In addition, in rabbits the effects of high temperatures have been assessed by observation of seasonal effects (cool vs. warm seasons of the year; Marai *et al.*, 2002; Roca *et al.*, 2005; Safaa *et al.*, 2008), although there exist a report using a climate chamber at 30ºC for 22 hours per day (Finzi *et al.*, 1995). Rabbits are an important livestock species in Mediterranean countries, but it is also a common laboratory mammal used for reproductive toxicological studies that are applied to human medicine. Rabbits are very sensitive to high temperatures since they have few functional sweat glands limiting their ability in eliminating excess body heat when the environmental temperature is high.

General effects of heat stress in males encountered in those studies are a decrease in testis weight, ejaculate volume, spermatozoa concentration, total sperm output, motility, acrosome integrity, sperm chromatin stability, fertility rate and pregnancy rate. Moreover, they have also described an increase in rectal temperature, respiratory rate, and the proportion of dead and abnormal spermatozoa, embryonic

loss and sex ratio distortion. However, all these studies have limited value since they evaluate seasonal effects or simulate unreal situations as local heating and constant exposure to high temperatures along the day in a climatic chamber. The results obtained from those studies should be analyzed with caution, because they are ignoring other factors as the ability of the scrotal skin to sweat or animal physiological, metabolic and endocrinological reactions that can counteract or diminish heat stress effects on male reproductive traits. According to that, this study try to evaluate the high temperature effects on male reproductive traits, simulating as possible the real environmental conditions which animals are submitted to in our latitudes during the warm months of the year.

Furthermore, previous studies usually take into account motility values based on subjective observations. Usually, the subjective scales reported are different between studies and are not comparable. In our study, we evaluate sperm movements using an objective system (CASA) to analyze sperm motility and takes into account specific sperm parameters. In addition, Quintero-Moreno *et al.* (2007) described the existence of specific, motile sperm-subpopulation structures in rabbit fresh ejaculates. These subpopulations have been found before in other species such as boar, dog, horse, donkey and human (Thurston *et al.*, 1999; Rigau *et al.*, 2001; Quintero-Moreno *et al.*, 2003; Buffone *et al.*, 2004; Miró *et al.*, 2005). However, no reports exist analyzing the motile subpopulations of sperm samples from epididymal origin. Sperm-subpopulations are composed by a group of sperm with varying degrees of structural and functional differentiation, which results in subpopulations of different quality. It is believed that only a very small percentage of sperm is able to achieve fertilization (Holt and Van Look, 2004; Holt, 2005). Hence, such structure could suggest the existence of a relationship between changes in the concrete subpopulation structure of an ejaculate and its fertilizing ability (Hirai *et al.*, 2001; Quintero-Moreno *et al.*, 2003). In accordance to that, it is important to elucidate the changes on motile sperm-subpopulation induced by high temperatures to find if fertilization ability can be or not altered.

The aim of the study was to assess the effect of long exposure to *in vivo* summer circadian heat stress cycles, based on data from 3 previous summer seasons, on motile sperm parameters and sperm motile-subpopulation structure of epididymal sperm cells from rabbit bucks.

Material and methods

Chemicals and reagents

All chemicals were purchased from Sigma (Madrid, Spain) unless otherwise indicated.

Animals and samples collection

Animals used in this study came from the Caldes line (Gómez *et al.*, 2002), which is selected for growth rate during the fattening period. Twelve White New Zealand rabbit bucks (180 days old and from different litters) individually housed in commercial wire cages were divided in two buildings. Six males were housed at 17ºC mean temperature (control group) and the rest of males at 17ºC, 18 hours a day, and at 31ºC the rest 3 hours (heat stress group), simulating a summer circadian heat stress cycle. Mean,

minimum and maximum summer temperature values were obtained from 3 previous summers in our geographical area (NE Spain). Animals were also submitted to 60% of relative humidity and cycles of 16h day-light. The experimental animals were part of a study of the effect of heat stress exposure over behavioral and physiological features, and 2 ejaculates per male were collected weekly. At the end of the study (more than a year) animals were euthanized at approximately 2 years old. Epididymal spermatozoa were recovered after flushing cauda epididymis and vas deferens with 10ml of Gent A diluent® (Minitüb, Tiefenbach, Germany). At recovery, sperm quality parameters were assessed.

Analysis of sperm quality parameters

Sperm concentration and total sperm number were determined after counting in a haemocytometer chamber (Neubauer improved). Percentages of viability, morphological abnormalities and intact acrosomes were determined by using the eosin-nigrosin staining. This technique shows viable spermatozoa as being those with a uniform, white stain in all of the cells, whereas the presence of a pinkish stain was indicative of non-viable sperm cells (Bamba, 1988). All determinations were performed after analyzing a minimum of 200 spermatozoa/sample through optical microscope (magnification: 1000x). Motility and progressivity was analyzed by a computer assisted sperm analysis (CASA; Integrated Sperm Analysis System, V1.0, Proiser S.L., Valencia, Spain). Five-microliter aliquots of pre-warmed samples were placed on a warmed (37ºC) slide and covered with a 22 mm2 coverslip. The analysis was based upon the study of 25 consecutive, digitalized photographic images obtained from a single field at a magnification of 100x on a dark field. These 25 consecutive photographs were taken in a time lapse of 1 s, which implied a velocity of image-capturing of one photograph every 40 ms. Four to five separate fields were taken for each sample. The motility descriptors obtained after CASA analyses were:

Curvilinear velocity (VCL): The mean path velocity of the sperm head along its actual trajectory (units: um/s).

- Linear velocity (VSL): The mean path velocity of the sperm head along a straight line from its first to its last position (units: um/s).
- Mean velocity (VAP): The mean velocity of the sperm head along its average trajectory (units: um/s).
- Linearity coefficient (LIN): (VSL/VCL) x100 (units: %).
- Straightness coefficient (STR): (VSL/VAP) x100 (units: %).
- Wobble coefficient (WOB): (VAP/VCL) x100 (units: %).
- Mean amplitude of lateral head displacement (ALH): The mean value of the extreme side-to-side movement of the sperm head in each beat cycle (units: um).
- Frequency of head displacement (BCF): The frequency with which the actual sperm trajectory crosses the average path trajectory (units: Hz).

Finally, total motility was defined as the percentage of spermatozoa which showed a VAP above 10 μm/s, and progressivity as the percentage of spermatozoa which showed a VAP above 50 μm/s and 70% of STR.

Statistical analysis

Data were processed by using the Statistical Analysis Systems Package (SAS. SAS/STAC Software. Cary, NC, USA: SAS Inst. Inc.; 2000). Normality of data distributions was assessed by the Shapiro–Wilks Test, which is included in the UNIVARIATE procedure. Afterwards, the FASTCLUS clustering procedure included in the SAS package was used to separate motile spermatozoa into specific subpopulations. The FASTCLUS procedure performs a disjointed cluster analysis based on Euclidean distances computed from one or more quantitative parameters. In the current study, these variables were the different sperm motility parameters measured by the CASA system. Spermatozoa were divided into clusters such that every observation belonged to a single cluster. Sperm cells that shared similar motility characteristics were assigned to the same cluster, whereas differing spermatozoa were assigned to different clusters. A PROC GLM procedure was applied to evaluate significant differences among clusters ($P < 0.05$) and the LSMEANS procedure was applied to list these differences. Finally, another general linear model (the PROC GLM routine) was used to test for significant differences in sperm parameters ($P < 0.05$) among the samples subjected to different group of temperature conditions. The LSMEANS procedure was used to identify significant differences. The total number of spermatozoa analyzed following this protocol was 2,298 (1168 and 1130 from control and heat stress group, respectively).

Results

Effect of circadian heat stress cycles on epididymal sperm cells

At recovery, percentages of viability, morphological abnormalities and intact acrosomes were similar for both groups of study, without significant differences (Table 1). There were also no differences in sperm concentration and total sperm number (136.7 and 130.3 millions sperm cells/ml and 1167.3 and 1166 millions of sperm cells for control and HS groups, respectively). However, sperm total motility and progressivity were negatively affected in sperm samples from the heat stress group ($P = 0.048$ and $P = 0.0030$, respectively).

These alterations were also associated with significant changes in motility parameters (Table 2). Thus, heat stress group showed significant decreases ($P < 0.0001$) in VCL, VSL, VAP, STR, LIN and WOB. The heat stress group had also higher values of frequency of head displacement (BCF; $P < 0.0001$). No significant differences were detected for the mean amplitude of lateral head displacement (ALH) between groups.

Table 1. Means ± SEM of the sperm quality analysis of epididymal sperm samples from control and heat stress groups.

	Control	Heat Stress
Viability (%)	78.26 ± 3.28[a]	77.09 ± 3.46[a]
Total motility (%)	78.53 ± 1.37[a]	55.63 ± 14.57[b]
Progressivity (%)	38.23 ± 2.70[a]	20.07 ± 7.17[b]
Altered acrosomes (%)	17.87 ± 4.24[a]	29.35 ± 7.12[a]
Morphological abnormalities (%)	37.09 ± 2.35[a]	42.43 ± 14.93[a]

Note: a-b Different superscripts in a row indicate significant differences.

Table 2. Means ± SEM of the motility parameters of epididymal sperm samples from control and heat stress groups.

	Control	Heat Stress
VCL (µm/s)	102.24 ± 1.11[a]	93.80 ± 1.26[b]
VSL (µm/s)	36.59 ± 0.68[a]	28.68 ± 0.76[b]
VAP (µm/s)	54.44 ± 0.70[a]	45.55 ± 0.86[b]
LIN (%)	37.18 ± 0.64[a]	29.74 ± 0.64[b]
STR (%)	64.38 ± 0.71[a]	59.14 ± 0.77[b]
WOB (%)	54.44 ± 0.52[a]	47.77 ± 0.55[b]
ALH (µm)	4.22 ± 0.05[a]	4.30 ± 0.06[a]
BCF (Hz)	8.85 ± 0.12[a]	7.50 ± 0.13[b]

Note: a-b Different superscripts in a row indicate significant differences.

Effect of circadian heat stress cycles on motile sperm-subpopulation structure

The FASTCLUS procedure detected four subpopulations of motile sperm cells. Percentages of motile sperm-subpopulations obtained in the control and heat stress groups are shown in Graphic 1. Table 3 shows the mean values for each motility variable in each subpopulation. According to that, motile sperm-subpopulations were characterized as follows:

CONTROL GROUP

- Subpopulation 1: This subpopulation was defined by overall high values of velocity (VCL, VSL and VAP), low values of linearity (LIN), and high frequency of head displacement (BCF). This subpopulation accounted for the 26.69% of all motile sperm cells.
- Subpopulation 2: This subpopulation was also characterized by overall high values of velocity and high frequency of head displacement. However, it was also characterized by high values of linearity. Subpopulation 2 was made up of the highest percentage of cells, since it included 32.08% of all motile sperm.
- Subpopulation 3: This subpopulation showed middle velocity, low linearity and middle frequency of head displacement. Cells included in this subpopulation accounted for 22.67% of all motile sperm.
- Subpopulation 4: Finally, this subpopulation included sperm with middle-to-low velocity, high linearity and high frequency of head displacement. This subpopulation had the lowest percentage of motile sperm including only a 18.56% of the total motile-sperm subpopulation.

HEAT STRESS GROUP

- Subpopulation 1: This subpopulation was defined as the corresponding one described for the control group. The percentage of spermatozoa included in this subpopulation was 25.52% of all motile sperm.
- Subpopulation 2: This subpopulation was also characterized as the corresponding one described for the control group. The percentage of motile sperm included in this subpopulation was the lowest including 15.73% of the total motile sperm-subpopulation.
- Subpopulation 3: This subpopulation showed middle velocity, low linearity and middle-to-low frequency of head displacement. Subpopulation 3 was made up of the highest percentage of cells, since it included 33.77% of all of motile sperm.
- Subpopulation 4: Finally, this subpopulation included sperm with low velocity, middle linearity and middle-to-low frequency of head displacement. The cells included in this subpopulation accounted for 24.97% of all motile sperm.

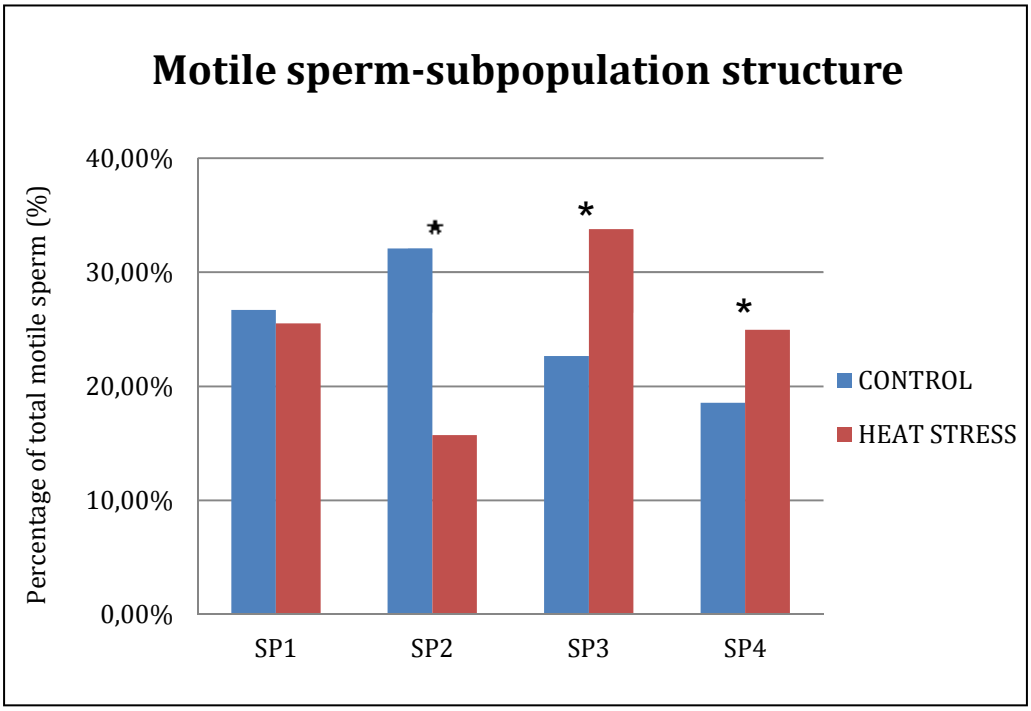
Significant differences were found regarding subpopulation structure between both groups of study. Taking into account these results, circadian heat stress cycles significantly decreased ($P < 0.05$) the percentage of subpopulation 2 (high velocity) by increasing the two less motile subpopulations (3 and 4).

However, samples from the heat stress group kept high percentages of the highest motile subpopulation (25.52 vs. 26.69% of sperm cells in subpopulation 1 for heat stress and control group, respectively).

Table 3. Motile sperm-subpopulations determined in rabbit buck samples from control and heat stress groups. Results are expressed as means ± S.E.M. Different superscripts between rows in the same subpopulation indicates significant ($P < 0.05$) differences.

	Subpopulation 1		**Subpopulation 2**	
	Control	**Heat Stress**	**Control**	**Heat Stress**
VCL (μm/sec)	134.78±1.28[a]	137.70±1.48[a]	119.70±1.16[a]	113.58±1.88[a]
VSL (μm/sec)	25.81±0.68[a]	36.17±0.78[b]	62.71±0.62[a]	68.65±1.00[b]
VAP (μm/sec)	59.15±0.78[a]	64.32±0.90[b]	76.71±0.71[a]	81.05±1.15[a]
LIN (%)	19.59±0.69[a]	26.68±0.79[b]	53.98±0.63[a]	61.66±1.01[b]
STR (%)	44.68±0.90[a]	58.44±1.05[b]	81.69±0.82[a]	84.94±1.33[a]
WOB (%)	44.51±0.72[a]	47.03±0.83[a]	65.78±0.66[a]	72.51±1.06[b]
ALH (μm)	5.85±0.07[a]	6.16±0.08[a]	4.28±0.06[a]	3.87±0.10[a]
BCF (Hz)	9.02±0.21[a]	8.97±0.24[a]	10.69±0.19[a]	10.36±0.31[a]
	Subpopulation 3		**Subpopulation 4**	
	Control	**Heat Stress**	**Control**	**Heat Stress**
VCL (μm/sec)	66.81±1.38[a]	78.84±1.29[b]	68.55±1.53[a]	56.70±1.50[b]
VSL (μm/sec)	12.08±0.73[a]	11.62±0.68[a]	36.89±0.81[a]	18.92±0.79[b]
VAP (μm/sec)	25.78±0.84[a]	29.42±0.78[a]	44.17±0.93[a]	25.82±0.91[b]
LIN (%)	19.30±0.74[a]	14.27±0.69[b]	55.27±0.82[a]	33.69±0.80[b]
STR (%)	47.62±0.98[a]	38.86±0.91[b]	83.28±1.08[a]	71.02±1.06[b]
WOB (%)	40.55±0.78[a]	37.59±0.73[a]	66.11±0.86[a]	46.70±0.84[b]
ALH (μm)	3.36±0.07[a]	4.20±0.07[b]	2.83±0.08[a]	2.81±0.08[a]
BCF (Hz)	5.93±0.23[a]	5.63±0.21[a]	9.00±0.25[a]	6.72±0.25[b]

Figure 1. Mean ± SEM of proportion of motile sperm-subpopulations (SPi for i=1,...,4) in control and heat stress groups. (*) Indicate significant differences (P < 0.05) between groups of study within subpopulation.



Discussion

Our results clearly demonstrated the harmful effects of *in vivo* circadian heat stress cycles on epididymal sperm quality, especially on sperm motility and sperm motile-subpopulation structure.

It has been described that testis temperature rises up when animals are placed in a hot environment and have problems in evaporation of sweat from the scrotal skin (Setchell and Mieusset, 1966). Consequently, histological abnormalities are found in the testis, where the most susceptible cells to heat are spermatids and spermatocytes (Banks *et al.*, 2005; Paul *et al.*, 2008; Devkota *et al.*, 2010).

High temperature impairs semen quality as it has been reported in a variety of species (bull, ram, boar or mice). In all cases, one or more of the following sperm parameters are affected, usually several weeks after the high temperature period: an increase in dead spermatozoa, morphological abnormalities and acrosomal alterations, and a decrease in sperm concentration and motility (Skinner and Louw, 1966; Wettemann *et al.*, 1976; Wettemann and Desjardins, 1979; Jannes *et al.*, 1998; Pérez-Crespo *et al.*, 2008; Safaa *et al.*, 2008). In rabbits, seasonal effects on male reproduction have been usually studied through seminal parameters. It has been established that in summer and beginning of autumn males exposed to acute high temperatures show impairments on sperm concentration, total number of spermatozoa per ejaculate, sperm motility, and variables related to viability and sperm normalcy, compared to winter (Panella and Castellini, 1990; Theau-Clément *et al.*, 1995; Alvariño, 2000; Marai *et al.*, 2002; Nizza *et al.*,

2003; Roca *et al.*, 2005; Safaa *et al.*, 2008). However these effects can be explained also by other season factors than temperature, due to they are not using contemporaneous animals submitted to identical environmental and management conditions with the exception of temperature, as it has been done in our study.

It has been reported that rabbits exposed to chronic heat stress can develop thermotolerance, an adaptation process to high temperatures (Finzi *et al.*, 1988; 1995; Welch, 1993), as seen in rabbit embryos (Makarevich *et al.*, 2007). According to that, it is not surprising to find no significant differences in sperm parameters other than motility or progressivity, due to our experimental animals were submitted to circadian heat stress cycles for a long period of time. A possible explanation to that is that animals even submitted to high temperatures in a climatic chamber, had some hours per day with low temperature coinciding with the night when they fed, allowing them to recovery from the high temperatures. On the other hand, the adaptation process can be explained by the submission to high temperatures at the beginning of the animal's reproductive life (180 days old in our study).

Our results demonstrate the presence of specific, four well-defined sperm subpopulations in rabbit epididymal sperm cells as those reported previously in ejaculates (Quintero-Moreno *et al.*, 2007), which can be easily defined by their motility characteristics. The subpopulations most present in the epididymal sperm samples are coincident with those showing the highest values of motility, including high velocity (VCL, VSL and VAP) and linearity. The motile sperm structure of the other two subpopulations is characterized as those with middle-lower motility.

As described hereby, heat stress negatively affected total sperm motility and progressivity. Furthermore, it is shown that motility changes induced by heat stress are linked to changes in both the specific sperm parameters and the percentages of each motile sperm-subpopulations present in rabbit epididymal sperm cells. The motile sperm-subpopulation structure in heated animals was affected by increasing the proportion of middle-lower motile subpopulations. However, it is noteworthy that these changes did not affect the percentage of one of the highest motile sperm-subpopulation present in rabbit bucks. This could indicate that an overall subpopulation structure is preserved under chronic high temperatures in order to maintain the general function of the ejaculate and their fertilizing ability.

To our knowledge this is the first report taking in to account the effect of heat stress on motile sperm-subpopulations structure. Seasonality effect on sperm-subpopulations has been only studied in male goats (Abdelwahab *et al.*, 2006), rams (Bravo *et al.*, 2011) and deer (Martínez-Pastor *et al.*, 2005). Further research is needed in order to find differences in the effect on the sperm-motile subpopulation structure between acute and chronic exposure to circadian heat stress cycles. This would elucidate if the sperm-motile subpopulation structure is adaptable to heat stress or just maintained in time regardless on environmental conditions.

In conclusion, to our knowledge this is the first report determining effects of heat stress applied *in vivo* on motile sperm-subpopulation structure from epididymal sperm cells in rabbits. In conclusion, our results seem to indicate that the sperm motility changes induced by environmental heat stress are linked to

concomitant changes in both the specific motility parameters and the proportion of motile sperm-subpopulations of the epididymis. However, these changes did not affect the subpopulation with the highest motile epididymal sperm cells, which it is still conserved despite the detrimental effect of heat stress.

References

Abdelwahab, D., Rivera, M.M., Rodríguez-Gil, J.E., Rigau, T., 2006. Seasonality affects on sperm motility kinematic parameters of Murciano-Granadina bucks. *Reprod Domest Anim*, **41**(Suppl.2): 103.

Alvariño, J.M.R., 2000. Reproductive performance of male rabbits. Proc. 7th World Rabbit Congr, 13-35.

Bamba, K., 1988. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. *Theriogenology*, **29**: 1245–1251.

Banks, S., King, S.A., Irvine, D.S., Saunders, P.T., 2005. Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa. *Reproduction*, **129**: 505–514.

Bravo, J.A., Montanero, J., Calero, R., Roy, T.J., 2011. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Ile de France rams. *Anim Reprod Sci*, **129**: 22–29.

Bronson, F.H., 1995. Seasonal variation in human reproduction: environmental factors. *Q Rev Biol*, **70**(2): 141-164.

Buffone, M.G., Doncel, G.F., Marín Briggiler, C., Vazquez-Levin, M.H., Calamera, J.C., 2004. Human sperm subpopulations: relationship between functional quality and protein tyrosine phosphorylation. *Hum Reprod*, **19**(1): 139-146.

Cameron, R.D., Blackshow, A.W., 1980. The effect of elevated ambient temperature on spermatogenesis in the boar. *J Reprod Fertil*, **59**(1): 173–179.

De Alba, J., Riera, S., 1966. Sexual maturity and spermatogenesis under heat stress in the bovine. *Anim Prod*, **8**: 137-144.

Devkota, B., Sadaki, M., Matsui, M., Takahashi, K.I., Matsuzaki, S., Koseki, T., *et al.*, 2010. Effects of scrotal insulation and pathological lesions on α-smooth muscle actin (SMA) and vimentin in the bull testes. *J Reprod Dev*, **56**(2): 187-190.

Finzi, A., Morera, P., Kuzminsky, G., 1988. Acclimation and repeatability of thermotolerance parameters in rabbit. In: *Proc. 4th World Rabbit Congr*, **2**: 419-426.

Finzi, A., Morera, P., Kuzminsky, G., 1995. Sperm abnormalities as possible indicators of rabbit chronic heat stress. *World Rabbit Sci*, **3**(4): 157-161.

Gómez, E.A., Rafel, O., Ramon, J., 2002. The Caldes strain (Spain). *Options Méditerr B Études et Rech*, **38**: 193-198.

Heitman, H. Jr., Cockrell, J.R., Morrison, S.R., 1984. Cycling ambient temperature effect on boar semen. *Anim Prod*, **38**: 129-132.

Hirai, M., Boersma, A., Hoeflich, A., Wolf, E., Foll, J., Aumuller, R. *et al.*, 2001. Objectively measured sperm motility and sperm head morphometry in boars (Sus scrofa): relation to fertility and seminal plasma growth factors. *J Androl*, **22**: 104–110.

Hoffmann, I., 2010. Climate change and the characterization, breeding and conservation of animal genetic resources. *Anim Genet*, **41**(Suppl.1): 32–46.

Holt, W.V., 2005. Is quality assurance in semen analysis still really necessary? A spermatologist's viewpoint. *Hum Reprod*, **20**: 2983–2986.

Holt, W.V., Van Look, K.J., 2004. Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory tests of semen quality. *Reproduction*, **127**: 527–535.

Jannes, P., Spiessens, C., Van der Auwera, I., D'Hooghe, T., Verhoeven, G., Vanderschueren, D., 1998. Male subfertility induced by acute scrotal heating affects embryo quality in normal female mice. *Hum Reprod*, **13**: 372–375.

Karabinus, D.S., Volger, C.J., Saacke, R.G., Evenson, D.P., 1997. Chromatin structural changes in sperm after scrotal insulation of Holstein bulls. *J Androl*, **18**: 549–555.

Lecha, L.B., 2007. IPCC (2007). Fourth Assessment Report. Cambridge, Cambridge University Press.

Lue, Y.H., Hikim, A.P., Swerdloff, R.S., Im, P., Taing, K.S., Bui, T., Leung, A., Wang, C., 1999. Single exposure to heat induces stage-specific germ cell apoptosis in rats: role of intratesticular testosterone on stage specificity. *Endocrinology*, **140**: 1709–1717.

MacCracken, M.C., Moore, F., Topping, J.C. Jr., 2008. Sudden and Disruptive Climate Change: Exploring the Real Risks and How We Can Avoid Them. Earth scan: London, UK.

Makarevich, A.V., Olexikova, L., Chrenek, P., Kubovicova, E., Freharova, K., Pivko, J., 2007. The effect of hyperthermia *in vitro* on vitality of rabbit preimplantation embryos. *Physiol Res*, **56**: 789-796.

Malmgren, L., 1989. Experimentally induced testicular alterations in boars: morphology changes in mature and peripubertal boars. *Zentralbl Veterinarmed A*, **36**(6): 411-420.

Marai, I.F.M., Habeeb, A.A.M., Gad, A.E., 2002. Reproductive traits of male rabbits as affected by climatic conditions, in the subtropical environment of Egypt. *Anim Sci*, **75**: 451-458.

Martínez-Pastor, F., Díaz-Corujo, A.R., Anel, E., Herraez, P., Anel, L., De Paz, P., 2005. Post mortem time and season alter subpopulation characteristics of Iberian red deer epididymal sperm. *Theriogenology*, **64**: 958–974.

Mieusset, R., Quintana Casares, P., Sanchez Partida, L.G., Sowerbutts, S.F., Zupp, J.L., Setchell, B.P., 1992. The effects of heating the testes and epididymides of rams by scrotal insulation on fertility and embryonic mortality in ewes inseminated with frozen semen. *J Reprod Fertil*, **94**: 337-343.

Miró, J., Lobo, V., Quintero-Moreno, A., Medrano, A., Peña, A., Rigau, T., 2005. Sperm motility patterns and metabolism in Catalonian donkey semen. *Theriogenology*, **63**: 1706–1176.

Nizza, A., Di Meo, C., Taranto, S., 2003. Effect of collection rhythms and season on rabbit semen production. *Reprod Domest Anim*, **38**: 436-439.

Panella, F., Castellini, C., 1990. Fattori ambientali e genetici che influiscono sulle caratteristiche del seme di coniglio. *Riv Coniglic*, **8**: 39-41.

Paul, C., Murray, A.A., Spears, N., Saunders, P.T., 2008. A single, mild, transient scrotal heat stress causes DNA damage, subfertility and impairs formation of blastocysts in mice. *Reproduction*, **136**: 73-84.

Pérez-Crespo, M., Pintado, B., Gutiérrez-Adán, A., 2008. Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ratio in mice. *Mol Reprod Dev*, **75**: 40–47.

Quintero-Moreno, A., Miró, J., Rigau, T., Rodríguez-Gil, J.E., 2003. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. *Theriogenology*, **58**: 1973–1990.

Quintero-Moreno, A., Rigau, T., Rodríguez-Gil, J.E., 2007. Multivariate cluster analysis regression procedures as tolos to identify motile sperm subpopulations in rabbit semen and to predict semen fertility and litter size. *Reprod Dom Anim*, **42**: 312-319.

Rathore, A.K., 1968. Effects of high temperature on sperm morphology and subsequent fertility in Merino sheep. *Proc Aust Soc Anim Prod*, **7**: 270-274.

Rigau, T., Farré, M., Ballester, J., Mogas, T., Peña, A., Rodríguez-Gil, J.E., 2001. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. *Theriogenology*, **56**: 801–815.

Roca, J., Martínez, S., Orengo, J., Parrilla, I., Vázquez, J.M., Martínez, E.A., 2005. Influence of constant long days on ejaculate parameters of rabbits reared under natural environment conditions of Mediterranean area. *Livest Prod Sci*, **94**: 169-177.

Rockett, J.C., Mapp, F.L., Garges, J.B., Luft, J.C., Mori, C., Dix, D.J., 2001. Effects of hyperthermia on spermatogenesis, apoptosis, gene expression, and fertility in adult male mice. *Biol Reprod*, **65**: 229–239.

Safaa, H.M., Emarah, M.E., Saleh, N.F.A., 2008. Seasonal effects on semen quality in Black Baladi and white New Zealand rabbit bucks. *World Rabbit Sci*, **16**: 13–20.

Sailer, B.L., Sarkar, L.J., Bjordahl, J.A., Jost, L.K., Evenson, D.P., 1997. Effects of heat stress on mouse testicular cells and sperm chromatin structure*. J Androl*, **18**: 294-301.

Setchell, B.P., 1998a. The Parkes Lecture. Heat and the testis. *J Reprod Fertil*, **114**: 179–194.

Setchell, B.P., Ekpe, G., Zupp, J.L., Surani, M.A., 1998b. Transient retardation in embryo growth in normal female mice made pregnant by males whose testes had been heated. *Hum Reprod*, **13**: 342–347.

Setchell, B.P., Mieusset, R., 1996. Testis thermoregulation, in: Hamamah, S. and Mieusset, R. (eds), Male Gametes Production and Quality. INSERM., Paris, 65-81.

Skinner, J.D., Louw, G.N., 1966. Heat stress and spermatogenesis in Bos indicus and Bos taurus cattle*. J Appl Physiol*, **21**: 1784-1790.

Theau-Clément, M., Michel, N., Esparbié, J., Bolet, G., 1995. Effects of artificial photoperiods on sexual behaviour and sperm output in the rabbit. *Anim Sci*, **60**: 143-149.

Thurston, L.M., Watson, P.F., Holt, W.V., 1999. Sources of variation in the morphological characteristics of sperm subpopulation objectively assessed by a novel automated sperm morphology analysis system. *J Reprod Fertil*, **117**: 271–280.

Tusell, L., David, I., Bodin, L., Legarra, A., Rafel, O., López-Bejar, M. *et al.*, 2011. Using the product threshold model for estimating separately the effect of temperature on male and female fertility. *J Anim Sci*, **89**: 3983-3995.

Vogler, C.J., Bame, J.H., De Jarnette, J.M., McGilliard, M.L., Saacke, R.G., 1993. Effects of elevated testicular temperature on morphology characteristics of ejaculated spermatozoa in the bovine. *Theriogenology*, **40**: 1207-1219.

Vogler, C.J., Saacke, R.G., Bame, J.H., De Jarnette, J.M., McGilliard, M.L., 1991. Effects of scrotal insulation on viability characteristics of cryopreserved bovine semen. *J Dairy Sci*, **74**: 3827-3835.

Walters, A.H., Eyestone, W.E., Saacke, R.G., Pearson, R.E., Gwazdauskas, F.C., 2005. Bovine embryo development after IVF with spermatozoa having abnormal morphology. *Theriogeneology*, **63**: 1925-1937.

Walters, A.H., Saacke, R.G., Pearson, R.E., Gwazdauskas, F.C., 2006. Assessment of pronuclear formation following *in vitro* fertilization obtained after thermal insulation of the testis. *Theriogeneology*, **65**: 1016-1028.

Welch, W.J., 1993. Come le cellule reagiscono allo stress. *Le Scienze*, **51**(299): 50-57.

Wettemann, R.P., Desjardins, C., 1979. Testicular function in boars exposed to elevated ambient temperature. *Biol Reprod*, **20**: 235-241.

Wettemann, R.P., Wells, M.E., Omtveldt, I.T., Pope, C.E., Turman, E.J., 1976. Influence of elevated ambient temperature on reproductive performance of boars. *J Anim Sci*, **42**: 664-669.

Yaeram, J., Setchell, B.P., Maddocks, S., 2006. Effect of heat stress on the fertility of male mice *in vivo* and *in vitro*. *Reprod Fertil Dev*, **18**: 647-653.

Zhu, B., Walker, S.K., Oakey, H., Setchell, B.P., Maddocks, S., 2004. Effect of paternal heat stress on the development *in vitro* of preimplantation embryos in the mouse. *Andrologia*, **36**: 384–394.

V

Absence of beneficial effects on rabbit sperm cell cryopreservation by several antioxidant agents.

Maya-Soriano[a] M.J., Taberner[a] E., Sabés-Alsina[a] M, Piles[b] M, López-Béjar[a] M.

[a]Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Spain.

[b]Unitat de Cunicultura, Institut de Recerca i Tecnologia Agroalimentàries, Caldes de Montbuí, Barcelona, Spain

Submitted 02.07.2012

Zygote. Submitted for publication.

This study was supported by Spanish Ministry of Science and Innovation, MICINN, Ref. RTA2008–00070-C02–02. Maya-Soriano was supported by a PIF Grant from the Universitat Autònoma de Barcelona.

Abstract

The generation of reactive oxygen species associated to cryopreservation can be responsible for mammalian sperm damage and limitable value of stored semen in artificial insemination. The aim of this study was to assess several antioxidant agents supplemented in a commercial freezing extender (Gent B®) in order to improve post-thaw rabbit sperm quality. Ejaculates of twenty-six White New Zealand rabbit bucks were collected, evaluated and frozen using a conventional protocol. Antioxidant agents were supplemented at different concentrations: Bovine Serum Albumin (BSA; 5 mg/ml, 30 mg/ml, 60 mg/ml), Retinol (RO; 50 μM, 100 μM, 200 μM) and Retinyl (RI; 0.282 μg/ml, 2.82 μg/ml). Percentages of viability, morphological abnormalities and intact acrosomes were determined by using the eosin-nigrosin staining. Motility and progressivity were analyzed by a computer assisted sperm analysis (CASA). In general, all the sperm quality parameters were negatively affected by the cryopreservation process, the total motility being the most affected one. The addition of antioxidant agents did not improve thaw-sperm quality. Furthermore, for RI groups a significant decrease in sperm quality parameters was recorded. In conclusion, rabbit sperm quality is negatively affected by the cryopreservation process. To our knowledge this is the first report using these antioxidants as supplementation in rabbit freezing extender. BSA and RO at concentrations used in the study did not improve sperm quality parameters after thawing whereas RI supplementation seems to be toxic. More studies are required in order to find the appropriate antioxidants and their most effective concentrations, which will improve rabbit post-thaw sperm quality.

Key Words: Spermatozoa, rabbit, bovine serum albumin, retinol, retinyl.

Introduction

Artificial insemination (AI) in rabbits is widely extended in Mediterranean countries, performed mainly by cooled semen due to the low fertility and prolificacy rates achieved with cryopreserved sperm cells (Viudes de Castro *et al.*, 1999; Roca *et al.*, 2000; Lopez-Gatius *et al.*, 2005).

During cryopreservation, mammalian sperm cells can suffer physical and chemical stress derived from low temperatures, cryoprotectans, ice formation and osmolarity alterations (Watson, 2000). In fact, less than 50% of the sperm cells which survive keep their fertilizing ability (Holt, 2000; Watson, 2000; Prathalingam *et al.*, 2006; Waterhouse *et al.*, 2006). The generation of reactive oxygen species (ROS) induced by the cryopreservation process (Griveau and Le Lannou, 1997; Calamera *et al.*, 2001; Neild *et al.*, 2003) and the reduction in the antioxidant enzyme activities in semen after a cycle of freezing-thawing (Bilodeau *et al.*, 2000; Chatterjee and Gagnon, 2001; Bucak *et al.*, 2008; Martí *et al.*, 2008) can be responsible for mammalian sperm damage (Griveau and Le Lannou, 1997; Calamera *et al.*, 2001; Neild *et al.*, 2003) and limitable value of stored semen in AI (La Falci *et al.*, 2011). The oxidative stress derived from the overproduction of ROS induces a sublethal damage which results in a reduction of spermatozoa life span, loss of motility, viability, antioxidant enzyme activity, *in vivo* fertilizing ability, alteration on acrosome and plasma membrane integrity, and DNA damage (Aitken *et al.*, 1998; Vishwanath and Shannon, 2000; Medeiros *et al.*, 2002; Sanacka and Kurpisz 2004). However, the negative effects produced on sperm cells will vary between species depending on the initial sperm plasma membrane composition (Holt, 2000) since the plasma membrane is the primary site of damage induced by cryopreservation (Hammerstedt *et al.*, 1990, Parks and Graham, 1992, Holt and North, 1994; Watson, 1995).

Differences in fatty acid composition and sterol levels have been associated with tolerance to cold-shock and cryopreservation (Parks & Lynch, 1992; White, 1993). In general, mammalian sperm cells can be divided in two groups according to their susceptibility to cold temperatures. Sperm cell membranes composed of a high content of polyunsaturated fatty acids (PUFA) and low cholesterol levels (i.e. bull, boar or ram) make membranes susceptible to peroxidative damage, compared with those with low PUFA and high cholesterol levels (i.e. dog, human), making these species more resistant to cold shock (Bailey *et al.*, 2000, La Falci *et al.*, 2011). Rabbit sperm plasma membrane presents a high cholesterol:phospholipid ratio and a low ratio of PUFA:saturated fatty acid in phospholipids (Darin-Bennet and White, 1977, Castellini *et al.*, 2006), giving the membrane structure of intermediate fluidity. This composition makes rabbit sperm cells quite resistant to cold shock (Darin-Bennet and White, 1977). The high concentrations of PUFA within the lipid structure require efficient antioxidant systems to defend against peroxidative damage produced by ROS (Alvarez and Storey, 1989; Aitken and Fisher, 1994; La Falci *et al.*, 2011). However, the protective antioxidant systems in spermatozoa are primarily of cytoplasmic origin, which is mostly discarded during the terminal stages of differentiation (Bucak *et al.*, 2010), and it is also reduced by the storage protocol (Lasso *et al.*, 1994; Bilodeau *et al.*, 2000) being insufficient in preventing lipid peroxidation (LPO) during the freeze‑thawing process (Aurich *et al.*, 1997; Storey, 1997). Consequently,

sperm cells are unable to resynthesize their membrane components (Michael *et al.*, 2007), which leads to structural damage (Sinha *et al.*, 1996) and subsequent sperm dysfunction (Alvarez and Storey, 1989; Aitken and Fisher, 1994; Storey, 1997).

Antioxidant molecules could reduce the impact of oxidative stress, and thus improve semen quality after thawing. In fact, it was stated that supplementation of antioxidants (i.e. glutathione, cysteine, lipid-soluble vitamins) improved sperm quality (even post-thawing) in boar (Funahashi and Sano, 2005; Szczesniak-Fabianczyk *et al.*, 2006), bull (Bilodeau *et al.*, 2001; Bucak *et al.*, 2010; Tuncer *et al.*, 2010), ram (Maxwell and Stojanov, 1996; Bucak *et al.*, 2008; Anghel *et al.*, 2009), stallion (Dennniston *et al.*, 2000; Baumber *et al.*, 2005), dog (Neagu *et al.*, 2010) and rabbit (Castellini *et al.*, 2000; Yousef *et al.*, 2003) sperm cells. However, antioxidants were not beneficial in other studies (Dennniston *et al.*, 2000; Marco-Jimenez *et al.*, 2006). Antioxidants play an important role in scavenging ROS (Baumber *et al.*, 2000) and there are many varieties of antioxidants agents that could be used in this matter, without eliminating completely ROS, because oxidative mechanisms play an important role in the physiological control of mammalian sperm functions as well (sperm capacitation or sperm-egg fusion; Aitken and Fisher, 1994; Griveau and Le Lannou, 1997; Saleh and Agarwal, 2002; Aitken *et al.*, 2004; Ford, 2004; Bennetts and Aitken, 2005; Agarwal *et al.*, 2006).

Although rabbit cryopreserved sperm is not used for commercial purposes at present, it is a valuable laboratory animal, and there is a need for reliable methods of rabbit sperm resource banking (Foote and Carney, 2000). Furthermore, sperm cryopreservation can be an alternative to the transport of animals between countries (Liu *et al.*, 2007) or to the preservation of endangered rabbit breeds (Bolet *et al.*, 2000). However, the methods of freezing semen require further improvements.

The aim of this study was to test bovine serum albumin (BSA), retinol and retinyl as antioxidant agents supplemented at different concentrations in a commercial freezing extender in order to improve rabbit post-thawing sperm quality.

Material and methods

Chemicals and reagents

All chemicals were purchased from Sigma (Madrid, Spain) unless otherwise indicated.

Experimental design

The extender used in the study was the commercial Gent B® (Control group; Minitüb, Tiefenbach, Germany), containing egg yolk and glycerol. The effect of antioxidant agent addition to the commercial extender sperm quality after freezing-thawing was assessed in three different experiments:

- Gent B with Bovine Serum Albumin (BSA) supplemented at 3 different concentrations: 5 mg/ml (BSA 1), 30 mg/ml (BSA 2), 60 mg/ml (BSA 3).

- Gent B with Retinol (RO) at 3 different concentrations: 50 µM (RO 1), 100 µM (RO 2), 200 µM (RO 3).
- Gent B with Retinyl (RI) at 3 different concentrations: 0.282 µg/ml (RI 1), 2.82 µg/ml (RI 2).

Animals and samples collection

The animals used in this study came from the Caldes line (Gómez *et al.*, 2002), which is selected for growth rate during the fattening period. Twenty-six White New Zealand rabbit bucks (180 days old and from different litters) individually housed in commercial wire cages were submitted to 17ºC as mean temperature, 60% of relative humidity and cycles of 16h day-light. An ejaculate per male was manually collected using the gloved-hand method and analyzed to guarantee the quality and the homogeneity of the ejaculates. Immediately after collection, the ejaculated semen was diluted (1:2; v/v) in the commercial extender Galap liquid diluent (IMV Technologies). Semen samples with the presence of calcium carbonate deposits and urine were discarded. The ejaculates underwent standard analysis for volume and sperm concentration. Part of the diluted semen was sent at room temperature to the laboratory within 30 min. Once in the laboratory, the samples were kept in an incubator at 37ºC until sperm quality analysis were done.

Sperm cell cryopreservation

Semen was centrifuged at 2000 rpm for 10min at 25ºC in a programmable refrigerated centrifuge (Hermle Z300K). Afterwards supernatants were discarded and each pellet was re-extended with the different freezing extenders achieving a final concentration of 100 x 106 spermatozoa/mL. Diluted sperm samples were then packaged into 0,25mL straws and immediately cooled to 4ºC for 2h. Subsequently, straws were exposed 10 min to liquid nitrogen (N2) vapours and then plunged into liquid N2 (-196ºC) for storage. Frozen samples were stored in liquid N2 for at least 2 months. Thawing process was performed then by an immediate immersion of the straws in a water bath at 37ºC for 30s. Straws were carefully wiped and opened, and samples were evaluated to determine post-thaw sperm quality parameters on a warmed platina.

Sperm cell quality parameters

Percentages of viability, morphological abnormalities and intact acrosomes were determined by using the eosin-nigrosin staining. This technique shows viable spermatozoa as being those with a uniform, white stain in all of the cells, whereas the presence of a pinkish stain was indicative of non-viable sperm cells (Bamba, 1988). All determinations were performed after analyzing a minimum of 200 spermatozoa/sample through an optical microscope (magnification: 1000x). Motility and progressivity were analyzed by a computer assisted sperm analysis (CASA; Integrated Sperm Analysis System, V.1.2., Proiser S.L., Valencia, Spain). Five-microliter aliquots of pre-warmed samples were placed on a warmed (37ºC) slide and covered with a 22 mm2 coverslip. The analysis was based upon the study of 25 consecutive, digitalized photographic images obtained from a single field at a magnification of 100x on a dark field. These 25 consecutive photographs were taken in a time lapse of 1 s, which implied a velocity of

image-capturing of one photograph every 40 ms. Four to five separate fields were taken for each sample. The motility descriptors obtained after CASA analyses were:

- Curvilinear velocity (VCL): The mean path velocity of the sperm head along its actual trajectory (units: um/s).
- Linear velocity (VSL): The mean path velocity of the sperm head along a straight line from its first to its last position (units: um/s).
- Mean velocity (VAP): The mean velocity of the sperm head along its average trajectory (units: um/s).
- Linearity coefficient (LIN): (VSL/VCL) x100 (units: %).
- Straightness coefficient (STR): (VSL/VAP) x100 (units: %).
- Wobble coefficient (WOB): (VAP/VCL) x100 (units: %).
- Mean amplitude of lateral head displacement (ALH): The mean value of the extreme side-to-side movement of the sperm head in each beat cycle (units: um).
- Frequency of head displacement (BCF): The frequency with which the actual sperm trajectory crosses the average path trajectory (units: Hz).

Finally, total motility was defined as the percentage of spermatozoa which showed a VAP above 10 μm/s, and progressivity as the percentage of spermatozoa which showed a VAP above 50 μm/s and 70% of STR.

Statistical analysis

Data were processed by using the Statistical Analysis Systems Package (SAS. SAS/STAC Software. Cary, NC, USA: SAS Inst. Inc.; 2000). Normality of data distributions was assessed by the Shapiro–Wilks Test, which is included in the UNIVARIATE procedure. A PROC GLM procedure was applied to test for significant differences in sperm parameters ($P < 0.05$) among the samples subjected to different groups of study. The LSMEANS procedure was used to identify significant differences. The total number of spermatozoa analyzed following this protocol was 9521 (4195, 2031 and 3295 from BSA, RO and RI groups, respectively).

Results

Effect of cryopreservation on post-thawing sperm cell quality

In accordance to the sperm quality parameters, significant differences were found between fresh ejaculates and post-thawed sperm cells (Table 1). In general, all the quality parameters were negatively affected by the cryopreservation process. The total motility was the most affected parameter by the freezing-thawing protocol (74.07% vs. 4.86%, for fresh and sperm cells frozen with Gent B respectively).

Table 1. Means ± SEM of the sperm quality analysis from fresh and thawed ejaculates.

	Viability (%)	Total motility (%)	Progressivity (%)	Altered acrosomes (%)	Morphological abnormalities (%)
Fresh	77.0 ± 2.2[a]	74.1 ± 3.9[a]	30.1 ± 2.8[a]	27.4 ± 3.1[a]	24.2 ± 2.4[a]
Gent B	36.5 ± 2.6[b,d]	4.9 ± 0.6[b]	2.7 ± 0.4[b]	67.7 ± 2.3[b]	18.9 ± 1.7[a]
BSA 1	39.6 ± 4.3[b,d]	6.3 ± 0.8[b]	4.0 ± 0.6[b]	68.7 ± 3.1[b]	19.8 ± 2.0[a]
BSA 2	42.3 ± 5.1[b,d]	4.5 ± 0.9[b,c]	3.0 ± 0.7[b,c]	64.8 ± 3.3[b]	20.5 ± 2.5[a]
BSA 3	45.4 ± 4.6[b]	6.5 ± 1.3[b]	4.8 ± 1.2[b]	68.4 ± 4.1[b]	21.6 ± 4.6[a]
RO 1	34.7 ± 4.0[b,d]	4.1 ± 1.0[b,c]	1.1 ± 0.4[b,c]	62.4 ± 2.8[b]	15.2 ± 2.7[a]
RO 2	34.6 ± 5.2[b,d]	2.7 ± 1.0[b,c]	1.1 ± 0.4[b,c]	66.1 ± 4.6[b]	14.6 ± 3.3[a]
RO 3	36.6 ± 3.7[b,d]	1.6 ± 0.5[b,c]	0.8 ± 0.3[b,c]	66.3 ± 3.9[b]	15.0 ± 1.7[a]
RI 1	27.0 ± 2.3[d]	2.3 ± 0.5[b,c]	1.6 ± 0.4[b,c]	72.8 ± 3.9[b]	20.7 ± 1.9[a]
RI 2	9.5 ± 2.0[c]	0.2 ± 0.1[c]	0.1 ± 0.1[c]	92.5 ± 2.6[c]	20.8 ± 2.9[a]

Note: a-d Different superscripts in a column indicate significant differences.

Alterations in total motility and progressivity were also associated with significant changes in specific motility parameters (Table 2). Thus, specific motility parameters were decreased in general after cryopreservation process except STR and ALH. Only normalcy, those sperm cells without any morphological abnormality (i.e. head abnormality, coiled-tail, tailless), was not affected by the process.

Table 2. Means ± SEM of the specific motility parameters from fresh and thawed ejaculate.

	VCL (µm/s)	VSL(µm/s)	VAP (%)	LIN (%)
Fresh	109.6 ± 0.5^{a}	44.3 ± 0.3^{a}	76.7 ± 0.4^{a}	43.9 ± 0.3^{a}
Gent B	84.7 ± 1.7^{b}	29.7 ± 0.8^{b,c}	44.7 ± 0.9^{b,c}	37.8 ± 0.9^{b}
BSA 1	92.8 ± 2.9^{b,c}	34.1 ± 1.4^{b}	49.8 ± 1.6^{b}	38.9 ± 1.4^{a,b}
BSA 2	90.5 ± 3.5^{b,c}	33.4 ± 1.7^{b,c}	47.7 ± 1.8^{b,c}	40.1 ± 1.8^{a,b,c}
BSA 3	86.5 ± 3.1^{b,c}	32.9 ± 1.4^{b,c}	44.5 ± 1.7^{b,c}	40.6 ± 1.4^{a,b}
RO 1	89.7 ± 4.5^{b,c}	24.0 ± 1.6^{c}	46.2 ± 2.4^{b,c}	30.1 ± 1.5^{c,d}
RO 2	96.6 ± 5.9^{a,c}	28.7 ± 2.4^{b,c}	47.8 ± 3.1^{b,c}	33.3 ± 2.2^{b,c,d}
RO 3	103.6 ± 6.0^{a,b,c}	35.2 ± 3.3^{a,b,c}	56.9 ± 3.3^{b}	36.3 ± 3.1^{a,b,c}
RI 1	69.4 ± 3.5^{e}	28.3 ± 1.7^{b,c}	36.4 ± 1.9^{c,d}	40.6 ± 1.7^{a,b}
RI 2	28.1 ± 8.4^{d}	13.1 ± 4.1^{b,c}	17.4 ± 5.4^{d}	22.6 ± 7.2^{d}

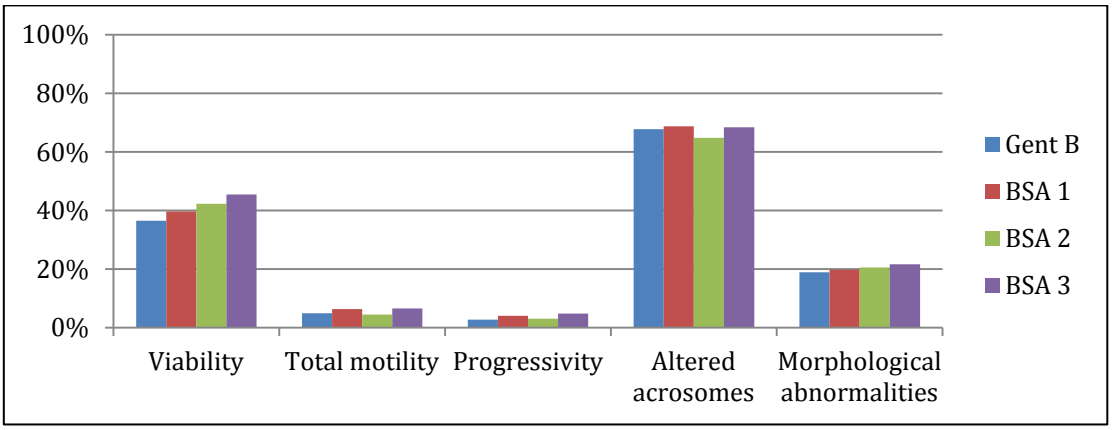
	STR (%)	WOB (%)	ALH (µm)	BCF (Hz)
Fresh	59.7 ± 0.3^{a}	70.9 ± 0.2^{a}	3.70 ± 0.02^{a,b}	9.4 ± 0.1^{a}
Gent B	68.5 ± 1.1^{b,c}	53.7 ± 0.6^{b}	3.71 ± 0.08^{a,b}	9.7 ± 0.2^{a,b,c}
BSA 1	69.4 ± 1.8^{b,c}	54.5 ± 0.9^{b}	4.04 ± 0.14^{a,b,c}	9.6 ± 0.4^{a,b,c}
BSA 2	71.7 ± 2.4^{b,c}	53.7 ± 1.2^{b}	3.89 ± 0.18^{a,b,c}	10.5 ± 0.4^{a,b,c}
BSA 3	76.0 ± 1.7^{c}	52.4 ± 1.1^{b}	3.68 ± 0.15^{a,b}	10.5 ± 0.4^{b}
RO 1	56.0 ± 2.7^{a}	52.7 ± 1.1^{b}	4.16 ± 0.20^{b,c}	7.8 ± 0.4^{c}
RO 2	62.8 ± 2.9^{a,b}	50.8 ± 1.6^{b}	4.43 ± 2.73^{c}	8.9 ± 0.6^{a,c}
RO 3	63.1 ± 4.5^{a,b,c}	56.1 ± 1.9^{b}	4.73 ± 0.32^{c}	9.5 ± 0.7^{a,b,c}
RI 1	75.2 ± 1.9^{b,c}	51.9 ± 1.5^{b}	2.97 ± 0.14^{e}	9.8 ± 0.5^{a,b}
RI 2	34.8 ± 10.1^{d}	29.7 ± 9.1^{c}	1.11 ± 0.35^{d}	4.7 ± 1.6^{d}

Note: a-e Different superscripts in a column indicate significant differences.

Effect of BSA on thawed quality parameters

In general, the addition of BSA to the freezing extender was not more beneficial than the use of Gent B alone. However, in terms of viability, the percentages were higher as the BSA concentration increases, although without significant differences (Figure 1). In terms of altered acrosomes and normalcy, no differences were found between groups of study. Hence, similar sperm quality was obtained by using or not BSA as supplementation.

Figure 1. Semen quality parameters with the addition of BSA to the freezing extender compared with no supplemented samples.



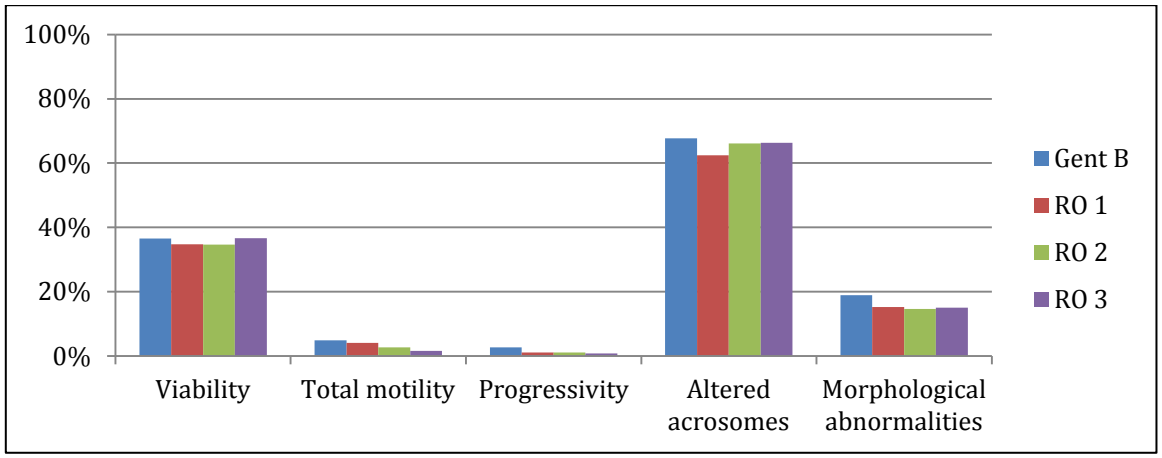
Taking into account total motility and progressivity, the best results were found when BSA at 5 and 30 mg/ml concentrations were present in the freezing extender. Nevertheless, significant differences were not detected once more. Hence, similar results in sperm motility are expected by both using BSA or not. According to that, changes in specific motility parameters after cryopreservation varied according to BSA supplementation (Table 2). In general, all BSA concentrations showed higher parameters of sperm velocity (as represented by VCL, VSL, VAP), LIN and STR and also BCF. However, no significant differences were detected.

Effect of RO on thawed quality parameters

RO supplementation did not show a beneficial effect in comparison with the use of Gent B without antioxidant addition. Significant differences were not found in terms of viability, acrosome integrity or normalcy (Figure 2).

However, according to sperm movements, the supplementation of RO decreased sperm total motility and progressivity as its concentrations increased (Figure 2), although the findings were not significant. Therefore, improvements are not expected by using or not RO in the extender. According to these results the changes in specific motility parameters after cryopreservation varied also according to supplementation (Table 2). RO concentrations showed higher values of sperm velocity, although the best results were detected using RO at 200 µM concentration (higher values of VCL, VSL, VAP, WOB and ALH).

Figure 2. Semen quality parameters with the addition of RO to the freezing extender compared with no supplemented samples.

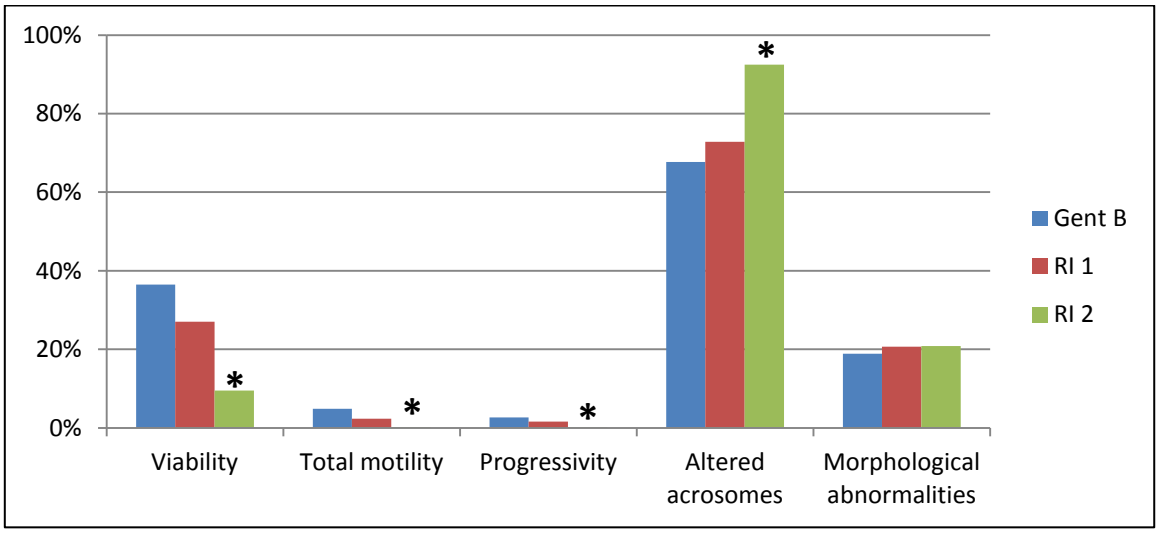


Effect of RI on thawed quality parameters

The supplementation of RI to the freezing extender implied no significant differences in sperm parameters when RI at 0.282 µg/ml concentration was used. However, the use of RI at 2.82 µg/ml concentration significantly decreased sperm quality comparing it with the use of the commercial extender without additives.

In general, supplementation of RI had negative effect on all sperm quality parameters except normalcy (Figure 3), although only RI 2 showed significant differences. Low values were found in terms of viability and acrosome integrity (9.50% vs. 36.49%; 7.48% vs. 32.3%, respectively) between RI 2 and Gent B groups.

Figure 3. Semen quality parameters with the addition of RI to the freezing extender compared with no supplemented samples.



Note: Significant differences (P < 0.05) between RI 2 and commercial extender (*).

In terms of total motility and progressivity, the lowest values obtained were also detected in those samples frozen in presence of RI at 2.82 µg/ml concentration (Table 1). According to that, changes in specific motility parameters after cryopreservation were found (Table 2), and in general, low values were found by using both concentrations in comparison to the control samples remarking that the worst values were always found in RI 2 group.

Discussion

Our results clearly demonstrated the harmful effects of cryopreservation process on rabbit sperm quality, especially on sperm motility and progressivity. However, the use of the antioxidant agents in an attempt to improve thaw-sperm quality was inadequate to achieve significant results, at least at the concentrations used in this study.

The ROS derived from the freezing process affects sperm membrane integrity leading to a decrease of sperm quality and consequently their fertilizing ability. The high content of unsaturated fatty acids on sperm membranes (White, 1993; Buhr *et al.*, 1994) makes the spermatozoa particularly sensitive to oxidative stress by ROS. As a result, lipid peroxidation increases resulting in loss of viability (Aitken *et al.*, 1998; Vishwanath and Shannon, 2000; Medeiros *et al.*, 2002) reduced sperm motility (de Lamirande and Gagnon, 1992; Baumber *et al.*, 2000; Guthrie and Welch, 2006), loss of acrosomal and plasma membrane integrity (Aitken *et al.*, 1998; Vishwanath and Shannon, 2000; Medeiros *et al.*, 2002) and leeds to DNA fragmentation (Aitken *et al.*, 1998; Vishwanath and Shannon, 2000; Medeiros *et al.*, 2002; Bennetts and Aitken, 2005). Rabbit sperm membranes have lower levels of PUFA (Darin-Bennet and White, 1977; Castellini *et al.*, 2006) making these cells less sensitive to oxidative damage, although reduction of sperm quality is still significant as shown in the present study, with a dramatic decrease on total sperm motility and progressivity. Our results are in accordance with Moce and Vicente (2009) who reviewed rabbit cryopreservation studies remarking that the freezing-thawing cycle causes decreases of the percentages of motile and live sperm cells and sperm with intact acrosomes.

Although cryopreserved sperm is not used for commercial purposes at present, there is a need for reliable methods of rabbit sperm resource banking. It can facilitate animal genetic diffusion between countries (Liu *et al.*, 2007), and it can be also useful for preserving endangered rabbit breeds (Bolet *et al.*, 2000). The values of sperm quality after cryopreservation observed in our study are significantly lower than those found in other species. The reason is that there are many extenders and protocols developed for rabbit sperm cryopreservation as reviewed by Moce and Vicente (2009). There is no consensus about the best extender and protocol for rabbit sperm cell cryopreservation. Finally, each research group uses their own protocol and extender, in our case a commercial one that may not have an appropriate composition for rabbit sperm cryopreservation. This may explain why if rabbit sperm is more resistant to cold shock than other species (Darin-Bennet and White, 1977; Castellini *et al.*, 2006), the sperm quality parameters in our study are so critically affected.

In an attempt to minimise the negative effects of all these stressors, research efforts have been focused on the optimization of cryopreservation methods, trying to find the best freezing extender composition. Since spermatozoa and seminal plasma have a limited antioxidant capacity (Aurich *et al.*, 1997; Storey, 1997), the effort to decrease ROS overproduction due to freezing process by the use of antioxidant agents as quenchers of ROS has been studied in a wide range of species ((i) human: Sinclair, 2000; (ii) ram: Maxwell and Stojanov, 1996; Upreti *et al.*, 1998; Baumber *et al.*, 2005; Uysal and Bucak, 2007; Anghel *et al.*, 2009; Maia *et al.*, 2010; (iii) bull: Beconi *et al.*, 1991; 1993; Chen *et al.*, 1993; Foote *et al.*, 1993; Bilodeau *et al.*, 2001; Bucack *et al.*, 2010; Tuncer *et al.*, 2010; (iv) boar: Funahashi and Sano, 2005; Szczesniak-Fabianczyk *et al.*, 2006; (v) dog: Michael *et al.*, 2007; Eulenberger *et al.*, 2009; Neagu *et al.*, 2010; (vi) buffalo: El-Kon, 2011; (vii) stallion: Aurich *et al.*, 1997; Baumber *et al.*, 2000). The aim of using antioxidant treatments should not be the complete ROS elimination since oxidative mechanisms play an important role in the physiological control of mammalian sperm functions (Aitken and Fisher, 1994; Griveau and Le Lannou, 1997; Saleh and Agarwall, 2002; Aitken *et al.*, 2004; Ford, 2004; Bennetts and Aitken, 2005; Agarwal *et al.*, 2006). There are many varieties of antioxidants that could be used in this matter. Unfortunately controversial results have been described. It is therefore necessary to adjust the extender composition, cryoprotectants and cryoprotectant concentrations, as well as the cryopreservation protocol for each single species or even breeds. In rabbits, antioxidants have been specially used to improve semen quality by adding them in the diet (especially vitamin E and C; Yousef *et al.*, 2003; Castellini et al, 2000). Hence, to our knowledge there is no literature about the addition of antioxidant agents on freezing extenders trying to improve rabbit post-thaw quality.

Bovine serum albumin has been used in some studies as an antioxidant agent improving sperm quality. It is known their function eliminating free radicals generated by oxidative stress (Lewis *et al.*, 1997), and the protection of membrane integrity of sperm cells from heat shock during freezing-thawing of canine semen (Uysal *et al.*, 2005). It has also been used to enhance motility and viability of ram sperm cells following cryopreservation process (Matsuoka *et al.*, 2006), being 20mg/ml the concentration more beneficial detected (Uysal and Bucak, 2007). This reported concentration is similar to those used in our investigation. However, our results did not show significant findings, and only quantitative records in terms of viability. As reported by Uysal and Bucak (2007) lower concentrations did not protect sperm cells from lipid peroxidation, although in our study higher concentration (60mg/ml) was insufficient to improve sperm quality. In accordance to that higher concentrations may be needed in rabbit sperm since progressive improvements were found in viability as BSA concentration increases. Further investigations in that way are needed to elucidate the best BSA concentration for rabbit sperm supplementation in freezing extender.

To our knowledge it is the first study using retinol and retinyl as supplementation for freezing extenders in order to improve thaw-sperm quality. They had been used to improve the quality of oocytes and embryos with significantly good results (Lima *et al.*, 2004; Livingstone *et al.*, 2004; Hajializadeh *et al.*, 2008; Vahedi *et al.*, 2009; Rajesh *et al.*, 2010). Some precursors of them, such as beta-carotene and lycopene, are known to be important components of antioxidant defence against lipid peroxidation in

living cells (Agarwal *et al.*, 2005) and protecting plasma membrane against lipid peroxidation (Di Mascio *et al.*, 1989). However, it should be pointed out that the concentrations used in our study were not sufficient to alleviate the harmful effects of cryopreservation, and even retinyl may be toxic for sperm cells since a drastic decrease in sperm quality has been recorded as concentration increases. So, further investigations in the use of retinol are needed to find the best concentration, mainly by using lower concentrations since the sperm quality decreases as retinol presence in the extender increases. Although, the use of retinyl should be avoided as our results demonstrate.

In conclusion, rabbit sperm quality is negatively affected by the cryopreservation process and it could be improved by the used of some antioxidant agents supplemented in the freezing extender. To our knowledge this is the first report using these antioxidants as supplementation in rabbit freezing extender. BSA and retinol did not improve sperm quality parameters after thawing at concentrations used in our study. However, retinyl supplementation seems to be toxic for rabbit sperm (and is not recommendable). More studies are needed in order to find the appropriate antioxidants and to define the most effective concentrations, which will improve post-thaw quality.

References

Agarwal, A., Prabakaran, S., Allamaneni, S. (2006). What an andrologist / urologist should know about free radicals and why. *Urology*, **67**: 2–8.

Agarwal, A., Prahakaran, S.A., Said, T.M. (2005). Prevention of oxidative stress injury to sperm. *J Androl*, **26**: 653-660.

Aitken, R.J., Fisher, H. (1994). Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bio-assays*, **16**: 259‑267.

Aitken, R.J., Gordon, E., Harkiss, D., Twigg, J.P.I., Milne, P., Jennigs, Z., Irvine, D.S. (1998). Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod*, **59**: 1037‑1046.

Aitken, R.J., Ryan, A.L., Baker, M.A., McLaughlin, E.A. (2004). Redox activity associated with the maturation and capacitation of mammalian spermatozoa. *Free Radic Biol Med*, **36**(8): 994–1010.

Alvarez, J.G., Storey, B.T. (1989). Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res*, **23**: 77‑90.

Anghel, A., Zamfirescu, S., Coprean, D., Sogorescu, E. (2009). The effects of cystein, bovine serum albumin and vitamin E on the calitative parameters of frozen-thawed ram semen. *Annals of RSCB*, **14**(2): 97-103.

Aurich, J.E., Schonherr, U., Hoppe, H., Aurich, C. (1997). Effect of antioxidants on motility and membrane integrity of chilled-stored stallion semen, *Theriogenology*, **48**: 185–192.

Bailey J.L., Bilodeau, J.F., Cormier, N. (2000). Semen Cryopreservation in Minireview Domestic Animals: A Damaging and Capacitating Phenomenon. *J Androl*, **21**(1): 1-7.

Bamba, K. (1988). Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. *Theriogenology*, **29**: 1245–1251.

Baumber, J., Ball, B.A., Gravance, C.G., Medina, V., Davies-Morel, M.C.G. (2000). The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. *J Androl*, **21**: 895-902.

Baumber, J., Ball, B.A., Linfor, J.J. 2005. Assessment of the cryopreservation of equine spermatozoa in the presence of enzyme scavengers and antioxidants. *Am J Vet Res*, **66**: 772-779.

Beconi, M.T., Affranchino, M.A., Schang, L.M., Beorlegui, N.B. (1991). Influence of antioxidants on SOD activity in bovine sperm. *Biochem Int*, **23**: 545–553.

Beconi, M.T., Francia, C.R., Mora, N.G., Affranchino, M.A. (1993). Effect of natural antioxidants on frozen bovine semen preservation. *Theriogenology*, **40**: 841–851.

Bennetts, L.E., Aitken, J.R. (2005). A comparative study of oxidative DNA damage in mammalian spermatozoa. *Mol Reprod Dev*, **71**: 77–87.

Bilodeau, J.F., Blanchette, S., Gagnon, I.C., Sirard, M.A. (2001). Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology*, **56**: 275‑286.

Bilodeau, J.F., Chatterjee, S., Sirard, M.A., Gagnon, C. (2000). Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev*, **55**: 282–288.

Bolet, G., Brun, J.M., Monnerot, M., Abeni, F., Arnal, C., Arnold, J., *et al.* (2000). Evaluation and conservation of European rabbit (Oryctolagus cuniculus) genetic resources. First results and inferences. *World Rabbit Sci*, **8**(A): 281–315.

Bucak, M.N., Atessahin, A., Yüce, A. (2008). Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Rumin Res*, **77**: 128‑134.

Bucak, M.N., Tuncer, P.B., Sanözkan, S., Baspinar, N., Taspinar, M., Coyan, K., Bilgili, A., Akalin, P.P., Büyükleblebici, S., Aydos, S., Ilgaz, S., Sunguroglu, A., Oztuna, D. (2010). Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters. *Cryobiology*, **61**(3): 248-253.

Buhr, M.M., Curtis, E.F., Kakuda, N. (1994). Effect of boar sperm cryopreservation on the composition and behaviour of head membrane lipids. *Cryobiology*, **31**: 224‑238.

Calamera, J.C., Fernandez, P.J., Buffone, M.G., Acosta, A.A., Doncel, G.F. (2001). Effects of long-term *in vitro* incubation of human spermatozoa: functional parameters and catalase effect. *Andrologia*, **33**: 79–86.

Castellini, C., Lattaioli, P., Bernardini, M., Dal Bosco, A. (2000). Effect of dietary α-tocopheryl acetate and ascorbic acid on rabbit semen stored at 5ºC. *Theriogenology*, **54**: 523-525.

Castellini, C., Cardinali, R., Dal Bosco, A., Minelli, A., Camici, O. (2006). Lipid composition of the main fractions of rabbit semen. *Theriogenology*, **65**: 703–712.

Chatterjee, S., Gagnon, C. (2001). Production of reactive oxygen species by spermatozoa undergoing cooling, freezing and thawing. *Mol Reprod Dev*, **59**: 452‑458.

Chen, Y., Foote, R.H., Brockett, C.C. (1993). Effect of sucrose, trehalose, hypotaurine, taurine, and blood serum on survival of frozen bull sperm. *Cryobiology*, **30**: 423–431.

Darin-Bennet, A., White, I.G. (1977). Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold shock. *Cryobiology*, **14**: 466–470.

De Lamirande, E., Gagnon, C. (1992). Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. *J Androl*, **13**: 379‑386.

Denniston, D.J., Squires, E.L., Bruemmer, J.E., Brinsko, S.P., McCue, P.M., Graham, J.K. (2000). Effect of antioxidants on the motility and viability of cooled stallion spermatozoa. *J Reprod Fertil Suppl*, **56**: 121-126.

Di Mascio, P., Kaiser, S., Sies, H. (1989). Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch Biochem Biophys*, **274**: 532-538.

El-Kon, I. (2011). Testing Usability of Bovine Serum Albumin (BSA) for Preservation of Egyptian Buffalo Semen. *American-Eurasian J Agric & Environ Sci*, **11**(4): 495-502.

Eulenberger, K., Schäfer-Somi, S., Aurich, C. (2009). Effect of Different Concentrations of Ascorbic Acid on Motility, Membrane Integrity and Chromatin Status of Frozen-Thawed Canine Spermatozoa Within Six Hours of Storage at 37ºC. *Reprod Dom Anim*, **44** (Suppl. 2): 354–358.

Foote, R.H., Carney, E.W. (2000). The rabbit as a model for reproductive and developmental toxicity studies. *Reprod Toxicol*, **14**: 477–493.

Foote, R.H., Chen, Y., Brockett, C.C., Kaproth, M.T. (1993). Fertility of bull spermatozoa frozen in whole milk extender with trehalose, Taurine, or blood serum. *J Dairy Sci*, **76**: 1908‑1913.

Ford, W.C.L. (2004). Regulation of sperm function by reactive oxygen species. *Hum Reprod Update*, **10**(5): 387–399.

Funahashi, H., Sano, T. (2005). Select antioxidants improve the function of extended boar semen stored at 10°C. *Theriogenology*, **6**: 1605-1616.

Griveau, J.F., Le Lannou, D. (1997). Reactive oxygen species and human spermatozoa: physiology and pathology. *Int J Androl*, **20**: 61–69.

Gómez, E.A., Rafel, O., Ramon, J., (2002). The Caldes strain (Spain). *Options Méditerr B Études et Rech*, **38**: 193-198.

Guthrie, H.D., Welch, G.R. (2006). Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *J Anim Sci*, **84**: 2089‑2100.

Hajializadeh, N., Babaei, H., Nematollahi-Mahani, S.N., Azizollahi, S. (2008). The development of mouse early embryos *in vitro* in fibroblasts and cumulus cells co-cultures supplemented with retinoic acid. *Iranian J Vet Res*, **9**(No. 1, Ser. No. 22).

Hammerstedt, R.H., Graham, J.K., Nolan, J.P. (1990). Cryopreservation of mammalian sperm: what we ask them to survive*. J Androl*, **11**: 73–88.

Holt, W.V. (2000). Fundamental aspects of sperm cryobiology: the importance of species and individual differences. *Theriogenology*, **53**: 47–58.

Holt, W.V., North, R.D. (1994). Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of plasma membrane damage in cryopreserved ram spermatozoa. *Biol Reprod*, **51**: 414–424.

La Falci, V.S.N., Yrjo-Koskinen, A.E., Fazeli, A., Holt, W.V., Watson, P.F. (2011). Antioxidant combinations are no more beneficial than individual components in combating ram sperm oxidative stress during storage at 5ºC. *Anim Reprod Sci*, **129**: 180-187.

Lasso, J.L., Noiles, E.E., Alvarez, J.G., Storey, B.T. (1994). Mechanism of superoxide dismutase loss from human sperm cells during cryopreservation*. J Androl*, **15**: 255–265.

Lewis, S.E.M., Sterling, E.S.L., Young, I.S., Thompson, W. (1997). Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. *Fertil Steril*, **67**: 142-147.

Lima, P.F., Oliveira, M.A., Goncalves, P.B., Montagner, M.M., Reichenbach, H.D., Weppert, M., *et al.* (2004). Effects of retinol on the *in vitro* development of Bos indicus embryos to blastocysts in two different culture systems. *Reprod Domest Anim*, **39**: 356–360.

Liu, E., Kitajima, S., Wiese, E., Reifenberg, K., Morimoto, M., Watanabe, T., Fan, J. (2007). Re-establishment of complement C6-deficient rabbit colony by cryopreserved sperm transported from abroad. *Exp Anim*, **56**: 167–171.

Livingston, T., Eberhardt, D., Edwards, J.L. & Godkin, J. (2004). Retinol improves bovine embryonic development *in vitro*. *Reprod Biol Endocrinol*, **2**: 83.

Lopez-Gatius, F., Sances, G., Sancho, M., Yaniz, J., Santolaria, P., Gutierrez, R., Nuñez, M., Nuñez, J., Soler, C. (2005). Effect of solid storage at 15 ◦C on the subsequent motility and fertility of rabbit semen. *Theriogenology*, **64**: 252–260.

Maia, M. da S., Bicudo, S.D., Sicherle, C.C., Rodello, L., Gallego, I.C. 2010. Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in extenders with antioxidants. *Anim Reprod Sci*, **122**(1-2): 118-123.

Marco-Jiménez, F., Lavara, R., Vicente, J.S., Viudes-de-Castro, M.P. (2006). Cryopreservation of rabbit spermatozoa with freezing media supplemented with reduced and oxidised glutathione. *Cryoletters*, **27**(4): 261-268.

Martí, E., Marti, J.I., Mui˜no-Blanco, T., Cebrián-Pérez, J.A. (2008). Effect of the cryopreservation process on the activity and immunolocalization of antioxidant enzymes in ram spermatozoa. *J Androl*, **29**: 459–467.

Matsuoka, T., Imai, H., Kohno, H., Fukui, Y. (2006). Effects of bovine serum albumine and trehalose in semen diluents for improvement of frozen-thawed ram spermatozoa. *J Reprod Develop*, **52**: 675-683.

Maxwell, W.M.C., Stojanov, T. (1996). Liquid storage of ram semen in the absence or presence of some antioxidants. *Reprod Fert Develop*, **32**: 353-360.

Medeiros, C.M., Forell, F., Oliveira, A.T., Rodrigues, J.L. (2002). Current status of sperm cryopreservation: why isn't it better? *Theriogenology*, **57**: 327‑344.

Michael, A., Alexopoulos, C., Pontiki, E., Hadjipavlou-Litina, D., Saratsis, P., Boscos, C. (2007). Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. *Theriogenology*, **68**: 204–212.

Moce, E., Vicente, J.S. (2009). Rabbit sperm cryopreservation: A review. *Anim Reprod Sci*, **110**: 1–24.

Neagu, V.R., García, B.M., Sandoval, C.S., Rodríguez, A.M., Ferrusola, C.O., Fernández, L.G., Tapia, J.A., Peña, F.J. (2010). Freezing dog semen in presence of the antioxidant butylated hydroxytoluene improves postthaw sperm membrane integrity. *Theriogenology*, **73**(5): 645-650.

Neild, D.M., Gabella, B.M., Chaves, M.G., Miragaya, M.H., Colenbrander, B., Aguero, A. (2003). Membrane changes during different stages of a freeze-thaw protocol for equine semen cryopreservation. *Theriogenology*, **59**: 1693–1705.

Parks, J.E., Graham, J.K. (1992). Effects of cryopreservation procedures on sperm membranes. *Theriogenology*, **38**: 209–222.

Parks, J.E., Lynch, D.V. (1992). Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. *Cryobiology*, **29**: 255–266.

Prathalingam, N.S., Holt, W.V., Revell, S.G., Mirczuk, S., Fleck, R.A., Watson, P.F. (2006). Impact of antifreeze proteins and antifreeze glycoproteins on bovine sperm during freezethaw. *Theriogenology*, **66**: 1894–1900.

Rajesh, N., Shankar, M.B. & Deecaraman, M. (2010). Effect of vitamin A supplementation at different gaseous environments on *in vitro* development of pre-implantation sheep embryos to the blastocyst stage. *Animal*, **4,11**: 1884-1890.

Roca, J., Martinez, S., Vazquez, J.M., Lucas, X., Parrilla, I., Martinez, E.A., (2000). Viability and fertility of rabbit spermatozoa diluted in Tris-buffer extenders and stored at 15 ◦C. *Anim Reprod Sci*, **64**: 103–112.

Saleh, R.A., Agarwal, A. (2002). Oxidative stress and male infertility: from research bench to clinical practise*. J Androl*, **23**(6): 737–752.

Sanocka, D.M., Kurpisz, M. (2004): Reactive oxygen species and sperm cells. *Reprod Biol Endocrinol*, **2**: 12-18.

Sarıözkan, S., Bucak, M.N., Tuncer, P.B., Ulutas, P.A., Bilgen, A. (2009). The influence of cysteine and taurine on microscopic-oxidative stress parameters and fertilizing ability of bull semen following cryopreservation, *Cryobiology*, **58**: 134–138.

Sinclair, S. (2000). Male infertility: nutritional and environmental considerations. *Alternatives Med Rev*, **5**: 28–38.

Sinha, M.P., Sinha, A.K., Singh, B.K., Prasad, P.L. (1996). The effect of glutathione on the motility, enzyme leakage and fertility of frozen goat semen. *Theriogenology*, **41**: 237-243.

Storey, B.T. (1997). Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa, *Mol Hum Reprod*, **3**: 203–213.

Szczesniak-Fabianczyk, B., Bochenek, M., Smorag, Z., Silvestre, M.A. (2006). Effect of antioxidants added to boar semen extender on the semen survival time and sperm chromatin structure. *Reprod Biol*, **3**: 81-87.

Tuncer, P.B., Bucak, M.N., Büyükleblebici, S., Sarıözkan, S., Yeni, D., Eken, A., Akalın, P.P., Kinet, H., Avdatek, F., Fidan, A.F., Gündoğan, M. (2010). The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen. *Cryobiology*, **61**(3): 303-307.

Upreti, G.C., Jensen, K., Munday, R., Duganzich, D.M., Vishwanath, R., Smith, J.F. (1998). Studies on aromatic amino acid oxidase activity in ram spermatozoa: role of pyruvate as an antioxidant. *Anim Reprod Sci*, **51**: 275–287.

Uysal, O., Bucak, N. (2007). Effects of Oxidized Glutathione, Bovine Serum Albumin, Cysteine and Lycopene on the Quality of Frozen-Thawed Ram Semen. *Acta Vet Brno*, **76**: 383-390.

Uysal, O., Korkmaz, T., Tosun, H. (2005). Effect of bovine serum albumine on freezing of canine semen. *Indian Vet J*, **82**: 97-98.

Vahedi, V., Zeinoaldini, S., Kohram, H. & Farahavar, A. (2009). Retinoic acid effects on nuclear maturation of bovine oocytes *in vitro*. *African J Biotechnol*, **8**: 3974-3978.

Vishwanath, R., Shannon, P. (2000). Storage of bovine semen in liquid and frozen state, *Anim Reprod S*ci, **62**: 23‑53.

Viudes de Castro, M.P., Vicente, J.S., Lavara, R., (1999). Effet du nombre de spermatozoides sur la fertilite de la semence conserve 24 heures chez le lapin. *Ann Zootech*, **48**: 407–412.

Waterhouse, K.E., Hofmo, P.O., Tverdal, A. and Miller R.R. Jr. (2006). Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. *Reproduction*, **131**: 887–894.

Watson, P.F. (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fertil Dev*, **7**: 871–891.

Watson, P.F. (2000). The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci*, **60–61**: 481–492.

White, I.G. (1993). Lipids and calcium uptake of sperm in relation to cold shock and preservation: a review. *Reprod Fertil Dev*, **5**: 639–658.

Yousef, M.I., Abdallah, G.A., Kamel, K.I. (2003). Effect of ascorbic acid and Vitamin E supplementation on semen quality and biochemical parameters of male rabbits. *Anim Reprod Sci*, **76**: 99–111.

VI

Retinol stabilizes acrosome membrane in heat-shocked bull spermatozoa.

Maya-Soriano MJ, Taberner E, Sabés-Alsina M, López-Béjar M.

Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona, Spain.

Submitted 13.08.2012

Theriogenology. Submitted for publication.

This study was supported by Spanish Ministry of Science and Innovation, MICINN, Ref. RTA2008–00070-C02–02. Maya-Soriano MJ was supported by a PIF Grant from the Universitat Autònoma de Barcelona, Barcelona, Spain.

Abstract

High temperatures have negative effects on sperm quality leading to temporary or permanent sterility. The aim of the study was to assess the effect of high temperatures on epididymal sperm cells in comparison with other temperatures (scrotal, environmental and refrigeration temperatures). The effect of the addition of retinol as an antioxidant agent in order to improve sperm quality parameters was also evaluated. Testes from ten bulls were collected from a slaughterhouse. Spermatozoa were flushed from the caudal epididymis and deferent duct and assessed for sperm quality parameters at recovery. Afterwards, sperm samples were exposed to one of four different temperatures (4, 22, 32 and 41.5ºC for 3 hours) in presence or absence of retinol in the storage extender. Percentages of viability and morphological abnormalities were determined by using the eosin-nigrosin staining. Acrosome integrity and sperm plasma membrane integrity were assessed by fluorescence PSA-lectin staining and the hypo-osmotic swelling test (HOST), respectively. Motility and progressivity were analyzed by a computer assisted sperm analysis (CASA). Sperm quality parameters were mainly affected by high temperatures (41.5ºC). The addition of RO to the storage extender did not show any effect on sperm quality parameters. However, the percentage of spermatozoa with altered acrosome was significantly reduced when retinol was present in the extender under heat stress conditions (41.5ºC). In conclusion, retinol may stabilize sperm acrosomal membrane in situations of oxidative stress due to high temperatures.

Key Words: calves, epididymal spermatozoa, retinol, heat stress.

Introduction

The Earth system has been changing rapidly over the past several decades due to natural and anthropic factors. Studies have stated an increase in the global average surface temperature of about 0.6ºC over the past 20th century, being predicted increments over 0.1 to 0.2ºC per decade (MacCracken *et al.*, 2008), with an increase in medium and maximum temperatures of 1.5 to 2.1ºC in 2020 (Lecha, 2007). These data suggest a significantly increase in heat stress pressure on global human population, animals and plants (Lecha, 2007), displacing life from their thermal comfort zone. In accordance, animals in our geographical area are submitted to high temperatures round 20-31 days during the warm months (May-September) and even 4 days during the cold months of the year (October-April; López-Gatius *et al.*, 2006). Heat stress is known to alter the physiology of livestock, reducing male and female reproduction and production, and increasing mortality (Hoffmann, 2010).

General effects of heat stress in male have been described as an increase in rectal temperature and respiratory rate (Stone, 1982; Meyerhoeffer *et al.*, 1985; Zhu *et al.*, 2004), and negative effects on production and reproduction leading to sub-fertility, temporary or permanent sterility (Setchell, 1998; Lue *et al.*, 1999; Yaeram et al, 2006; Tusell *et al.*, 2011). It has also been described a decrease in testis weight (Sailer *et al.*, 1997; Jannes *et al.*, 1998; Rockett *et al.*, 2001), ejaculate volume (Meyerhoeffer *et al.*, 1985), spermatozoa concentration and total sperm output (De Alba & Riera, 1966; Meyerhoeffer *et al.*, 1985), fertility and pregnancy rate (Hansen, 2009; Hendricks *et al.*, 2009). Moreover, the direct effects of high temperatures reported on spermatozoa are a decrease in the percentage of live and morphologically normal spermatozoa (Casady *et al.*, 1953; Skinner & Louw, 1966; Borg *et al.*, 1993), a loss of motility (Ax *et al.*, 1987; Marai *et al.*, 2002), alterations in acrosome and plasma membrane integrity (Meyerhoeffer *et al.*, 1985; Borg *et al.*, 1993; Sanocka & Kurpisz, 2004; Roca *et al.*, 2005; Safaa *et al.*, 2008), sperm chromatin stability (Pérez-Crespo *et al.* 2008; Paul *et al.* 2008), embryonic loss (Wettemann *et al.*, 1976; Stone, 1982; Walters *et al.*, 2005) and sex ratio distortion (Hendricks *et al.*, 2009).

It is noteworthy that cellular exposure to heat stress increases the production of reactive oxygen species (ROS) promoting cellular oxidation events (Ikeda *et al.*, 1999; Kim *et al.*, 2005). Sperm cell membranes composed of a high content of polyunsaturated fatty acids (PUFA) and low cholesterol levels (i.e. bull, boar or ram) make membranes susceptible to peroxidative damage, compared with those with low PUFA and high cholesterol levels (i.e. dog, human, rabbit), making these species more resistant to cold shock (Bailey *et al.*, 2000, La Falci *et al.*, 2011). The high concentrations of PUFA within the lipid structure require efficient antioxidant systems to defend against peroxidative damage produced by ROS (Aitken & Fisher, 1994; La Falci *et al.*, 2011). However, the protective antioxidant systems in spermatozoa are primarily of cytoplasmic origin, which is mostly discarded during the terminal stages of differentiation (Bucak *et al.*, 2010), and it is also reduced by the storage protocol (Lasso *et al.*, 1994; Bilodeau *et al.*, 2000). Consequently, sperm cells are unable to resynthesize their membrane components (Michael *et al.*, 2007), which leads to structural damage (Sinha *et al.*, 1996) and subsequent sperm dysfunction (Storey, 1997).

The administration of antioxidants agents *in vivo* has been shown to provide slight improvements in pregnancy rates in heat-stressed cows (Arechiga *et al.*, 1998). On the other hand, the addition of antioxidant agents *in vitro* as supplementation of culture media as ROS scavengers has proved improvements in oocyte/embryo culture (Córdova *et al.*, 2010; Marei *et al.*, 2011; Liang *et al.*, 2012). Hence, antioxidant molecules could reduce the impact of oxidative stress, and thus improve semen quality (Yousef *et al.*, 2003; Szczesniak-Fabianczyk *et al.*, 2006; Bucak *et al.*, 2010; Tuncer *et al.*, 2010). There are many varieties of antioxidant agents that could be used to improve sperm quality under oxidative events, without eliminating completely ROS, because oxidative mechanisms play an important role in the physiological control of mammalian sperm functions as well (sperm capacitation or sperm-egg fusion; Aitken *et al.*, 2004; Agarwal *et al.*, 2006).

The aim of the study was to assess the direct effect of high temperatures on epididymal spermatozoa of bulls simulating the conditions reported during summer season, and also the use of retinol as an antioxidant agent for improving sperm quality after the oxidative stress produced by heat stress.

Material and methods

Chemicals and reagents

All chemicals were purchased from Sigma (Madrid, Spain) unless otherwise indicated.

Animals and samples collection

Testes from ten bulls (12-30 months old) recovered at a slaughterhouse were transported to the laboratory at room temperature within 15 min since the slaughter. Once in the laboratory, testes were measured and dissected. Epididymal spermatozoa were recovered after flushing cauda epididymis and vas deferens with 10ml of Kenney medium (Kenney *et al.*, 1975), composed mainly by milk and glucose. At recovery, sperm quality parameters were assessed.

Experimental design

After initial sperm cell evaluation samples were centrifuged at 2500 rpm for 10 min at 25ºC (Hermle Z300K) and re-suspended with the storage extender. Four study groups were then established as follows: refrigeration temperature (4ºC; RT), environmental temperature (22ºC; ET), scrotal temperature (32ºC; ST) and rectal temperature simulating that reached during the central part of the day in a hot summer day (41.5ºC; HT; Tseng *et al.*, 2004). On the other hand, the same four groups of study were established with 6 µM retinol (all-trans-retinol; RO) as antioxidant agent added into the storage extender (RT + RO, ET + RO, ST + RO, HT + RO).

Analysis of sperm quality parameters

Sperm concentration and total sperm number were determined after counting in a haemocytometer chamber (Neubauer improved). Percentages of viability and morphological abnormalities were

determined by using the eosin-nigrosin staining. This technique shows viable spermatozoa as being those with a uniform, white stain in all of the cells, whereas the presence of a pinkish stain was indicative of non-viable sperm cells (Bamba, 1988). Acrosome integrity was assessed by fluorescence PSA-lectin staining. Sperm plasma membrane functional integrity was assessed by the use of the hypo-osmotic swelling test (HOST) as reported by Gholami *et al.* (2010). All determinations were performed after analyzing a minimum of 200 spermatozoa/sample through optical microscope (magnification: 1000x). Motility and progressivity was analyzed by a computer assisted sperm analysis (CASA; Integrated Sperm Analysis System, V1.2, Proiser S.L., Valencia, Spain). Five-microliter aliquots of pre-warmed samples were placed on a warmed (37ºC) slide and covered with a 22 mm2 coverslip. The analysis was based upon the study of 25 consecutive, digitalized photographic images obtained from a single field at a magnification of 100x on a dark field. These 25 consecutive photographs were taken in a time lapse of 1 s, which implied a velocity of image-capturing of one photograph every 40 ms. Four to five separate fields were taken for each sample. The motility descriptors obtained after CASA analyses were:

- Curvilinear velocity (VCL): The mean path velocity of the sperm head along its actual trajectory (units: um/s).
- Linear velocity (VSL): The mean path velocity of the sperm head along a straight line from its first to its last position (units: um/s).
- Mean velocity (VAP): The mean velocity of the sperm head along its average trajectory (units: um/s).
- Linearity coefficient (LIN): (VSL/VCL) x100 (units: %).
- Straightness coefficient (STR): (VSL/VAP) x100 (units: %).
- Wobble coefficient (WOB): (VAP/VCL) x100 (units: %).
- Mean amplitude of lateral head displacement (ALH): The mean value of the extreme side-to-side movement of the sperm head in each beat cycle (units: um).
- Frequency of head displacement (BCF): The frequency with which the actual sperm trajectory crosses the average path trajectory (units: Hz).

Finally, total motility was defined as the percentage of spermatozoa which showed a VAP above 10 μm/s, and progressivity as the percentage of spermatozoa which showed a VAP above 50 μm/s and 70% of STR.

Statistical analysis

Data were processed by using the Statistical Analysis Systems Package (SAS. SAS/STAC Software. Cary, NC, USA: SAS Inst. Inc.; 2000). Normality of data distributions was assessed by the Shapiro–Wilks Test, which is included in the UNIVARIATE procedure. A general linear model (the PROC GLM routine) was used to test for significant differences in sperm parameters ($P < 0.05$) among the samples subjected to different group of study. The LSMEANS procedure was used to identify significant differences. The total number of spermatozoa analyzed following this protocol was 10,565 (2031, 2541, 2534, 2317 and 1142 from samples at recovery, RT, ET, ST, HT groups, respectively).

Results

Effect of temperature on epididymal sperm cells

Sperm quality parameters were mainly affected by high temperatures (41.5ºC) but not by the incubation at scrotal, environmental or refrigeration temperatures (Table 1). In terms of viability, the percentages of live spermatozoa decreased after incubation at the different temperatures used in this study, being significantly lower in those samples incubated at 41.5ºC (Figure 1). All groups of study showed significant lower values of total motility and progressivity compared with fresh samples, although the most significant decrease was observed again by 41.5ºC samples. Comparing altered acrosomes, the tendency was similar to those parameters with the fresh values reported before. Altered acrosome percentages increased in all treatment groups compared, being significantly higher in those samples incubated at 32ºC and even higher at 41.5ºC (Figure 2). The percentage of membrane integrity was also negatively affected by temperatures, being significantly lower for groups stored at 4 and 32ºC, and even lower in 41.5ºC groups (Figure 1).

Figure 1. Sperm cells after HOST and eosin-nigrosin staining. Presence of live and dead cells (in pink) and spermatozoa with coiled and non-coiled tail (altered membrane; arrow).

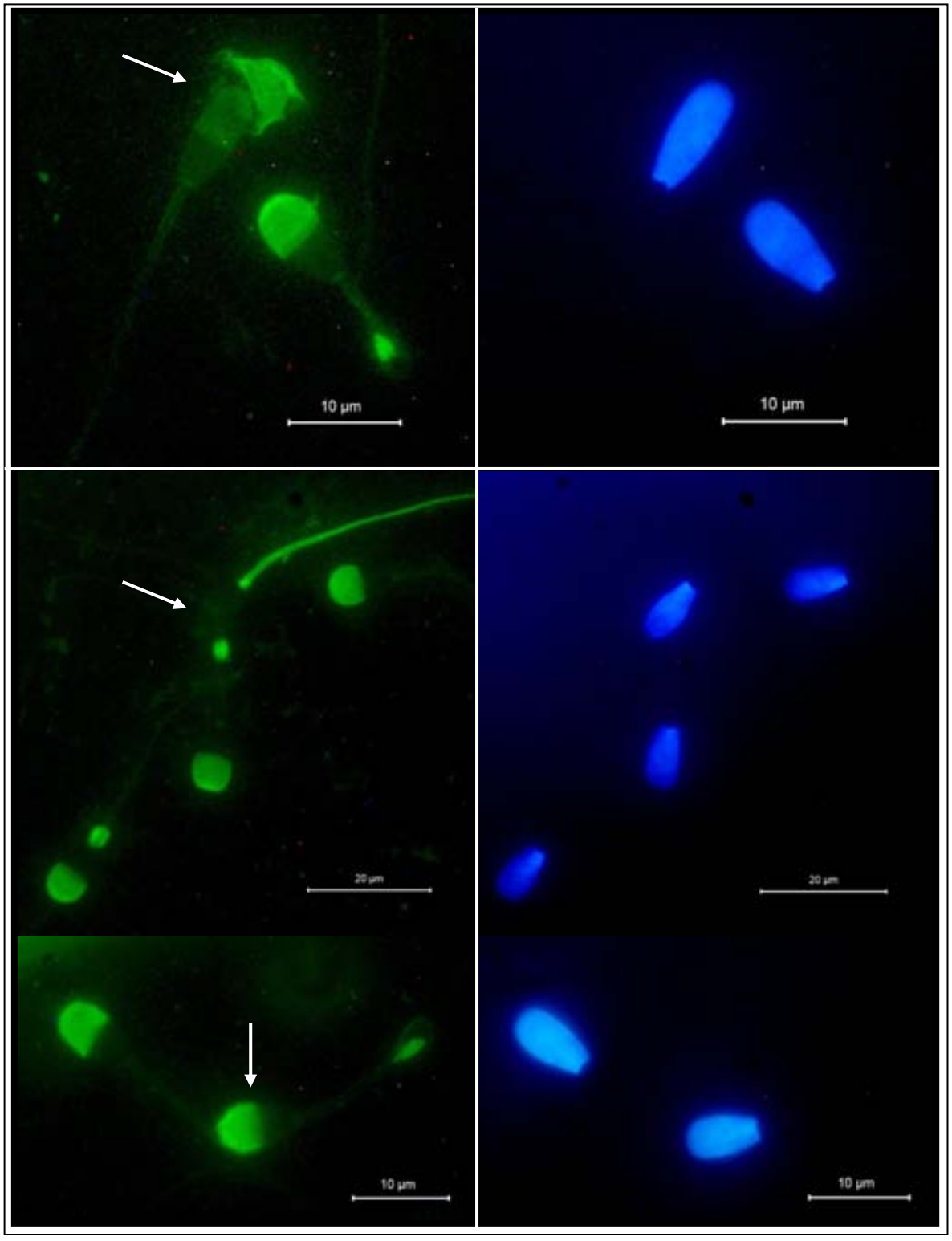
However, no differences were detected in terms of morphological abnormalities, although when we classified each abnormality, significant differences were found in distal droplets, folded and coiled tails. Fresh samples showed higher percentages of distal droplets than any other group of study. On the other hand, more folded tails were detected in all of the study groups. Finally, coiled tails significantly increased when spermatozoa were incubated at high temperatures (41.5ºC).

Table 1. Means ± SEM of the sperm quality analysis of epididymal sperm samples from fresh, and study groups.

	Fresh	**HT**	**HT + RO**	**ST**	**ST + RO**
Viability (%)	85.3 ± 1.4[a]	69.0 ± 2.6[b]	75.8 ± 2.3[b,c]	81.7 ± 2.4[a,c]	82.7 ± 1.5[a,c]
Total motility (%)	71.2 ± 3.0[a]	17.9 ± 2.2[b]	24.5 ± 4.0[b]	46.9 ± 4.5[c]	46.6 ± 3.7[c]
Progressivity (%)	48.9 ± 3.5[a]	9.3 ± 1.3[b]	15.8 ± 3.6[b]	31.1 ± 3.4[c]	31.8 ± 3.3[c]
Altered acrosomes (%)	3.5 ± 0.6[a]	14.2 ± 1.5[b]	9.2 ± 1.2[c]	7.3 ± 1.1[c,d]	6.5 ± 0.9[a,c,d]
Membrane integrity (%)	90.8 ± 0.8[a]	65.3 ± 3.3[b]	72.3 ±3.4[b,c]	79.6 ± 1.7[c,d]	79.8 ± 1.9[c,d]
Morphological abnormalities (%)	70.4 ± 4.1[a]	68.6 ± 6.5[a]	76.9 ± 4.9[a]	69.5 ± 4.5[a]	68.8 ± 4.1[a]
Head abnormalities (%)	0.6 ± 0.2[a]	0.9 ± 0.3[a]	0.6 ± 0.2[a]	0.5 ± 0.2[a]	0.7 ± 0.2[a]
Proximal droplets (%)	5.7 ± 1.8[a]	4.7 ± 1.6[a]	4.2 ± 1.6[a]	5.3 ± 2.0[a]	4.1 ± 1.4[a]
Distal droplets (%)	49.2 ± 6.9[a]	4.3 ± 0.9[b]	3.2 ± 0.7[b]	5.5 ± 1.1[b]	3.9 ± 1.0[b]
Folded tail (%)	2.9 ± 0.6[a]	34.8 ± 4.2[b]	47.8 ±3.6[b]	45.3 ± 4.5[b]	48.5 ± 4.3[b]
Coiled tail (%)	4.1 ± 0.9[a]	17.7 ± 3.1[b]	15.0 ± 2.3[b]	5.6 ± 1.0[a]	6.7 ± 1.1[a]
Tailles (%)	7.3 ± 2.4[a]	5.7 ± 2.1[a]	5.7 ± 2.0[a]	6.8 ± 2.8[a]	4.4 ± 1.4[a]
Mid-piece abnormalities (%)	0.5 ± 0.2[a]	0.6 ± 0.2[a]	0.4 ± 0.2[a]	0.5 ± 0.2[a]	0.4 ± 0.2[a]
	Fresh	**ET**	**ET + RO**	**RT**	**RT + RO**
Viability (%)	85.3 ± 1.4[a]	81.9 ± 2.3[a,c]	83.9 ± 1.7[a,c]	85.0 ± 1.5[a]	85.2 ± 1.3[a]
Total motility (%)	71.2 ± 3.0[a]	49.8 ± 4.1[c]	46.8 ± 3.9[c]	48.8 ± 4.3[c]	46.3 ± 4.6[c]
Progressivity (%)	48.9 ± 3.5[a]	29.8 ± 2.5[c]	29.1 ± 2.4[c]	30.3 ± 3.3[c]	28.4 ± 4.0[c]
Altered acrosomes (%)	3.5 ± 0.6[a]	5.1 ± 0.6[a,d]	5.4 ± 0.6[a,c,d]	5.3 ± 0.6[a,c,d]	4.8 ± 0.7[a,d]
Membrane integrity (%)	90.8 ± 0.8[a]	83.0 ± 1.9[a,d]	83.1 ± 1.6[a,d]	82.1 ± 2.0[c,d]	83.5 ± 2.3[a,d]
Morphological abnormalities (%)	70.4 ± 4.1[a]	66.5 ± 3.3[a]	65.2 ± 3.6[a]	68.4 ± 2.4[a]	67.3 ± 2.9[a]
Head abnormalities (%)	0.6 ± 0.2[a]	0.8 ± 0.1[a]	0.7 ± 0.3[a]	0.5 ± 0.1[a]	0.9 ± 0.2[a]
Proximal droplets (%)	5.7 ± 1.8[a]	6.1 ± 1.8[a]	6.4 ± 1.8[a]	5.8 ± 2.0[a]	5.6 ± 2.2[a]
Distal droplets (%)	49.2 ± 6.9[a]	5.6 ± 1.0[b]	4.7 ± 1.2[b]	6.2 ± 2.0[b]	4.1 ± 0.9[b]
Folded tail (%)	2.9 ± 0.6[a]	43.3 ± 4.1[b]	43.0 ± 4.5[b]	46.3 ± 4.3[b]	46.9 ± 4.9[b]
Coiled tail (%)	4.1 ± 0.9[a]	4.4 ± 0.9[a]	4.6 ± 0.6[a]	4.1 ± 0.9[a]	4.1 ± 0.9[a]
Tailles (%)	7.3 ± 2.4[a]	6.1 ± 1.7[a]	5.5 ± 2.1[a]	4.9 ± 1.7[a]	5.2 ± 1.8[a]
Mid-piece abnormalities (%)	0.5 ± 0.2[a]	0.2 ± 0.1[a]	0.3 ± 0.1[a]	0.4 ± 0.2[a]	0.6 ± 0.2[a]

Note: a-d Different superscripts in a row indicate significant differences.

Figure 2. Sperm cells with intact and altered (arrow) acrosome membrane after FITC-PSA lectin staining (green fluorescence) and DAPI nuclear staining (blue fluorescence).



Motility and progressivity differences can be explained by specific motility parameters (Table 2). VCL, VSL and VAP decreased significantly in all study groups compared with fresh samples, being more accused in samples stored at 41.5ºC. LIN percentages decreased significantly only at high temperatures (41.5ºC). STR parameter also decreased significantly except for 32ºC groups. WOB increased significantly for all study groups, except at 41.5ºC where WOB parameter significantly decreased. ALH decreased significantly in all study groups and even more at 41.5ºC. Finally, BCF parameter decreased significantly in 4 and 22 groups, being more accused at 41.5ºC.

Table 2. Means ± SEM of the motility parameters of epididymal sperm samples from fresh and study groups.

	Fresh	**HT**	**HT + RO**	**ST**	**ST + RO**
VCL (μm/s)	112.5 ± 1.0^{a}	62.9 ± 1.8^{b}	72.1 ± 1.7^{c}	91.1 ± 1.4^{d}	85.7 ± 1.3^{d,e}
VSL (μm/s)	67.4 ± 0.9^{a}	30.4 ± 1.2^{b}	37.3 ± 1.1^{c}	54.8 ± 1.1^{d}	51.1 ± 1.0^{d,e,f}
VAP (μm/s)	86.0 ± 0.9^{a}	40.0 ± 1.2^{b}	45.4 ± 3.6^{b}	69.0 ± 1.1^{c}	63.7 ± 1.1^{d}
LIN (%)	58.2 ± 0.5^{a,d}	51.7 ± 1.3^{b}	53.5 ± 0.9^{b,c}	59.2 ± 0.7^{a}	58.8 ± 0.7^{a}
STR (%)	76.4 ± 0.5^{a,c}	72.4 ± 1.2^{b,d}	77.8 ± 0.9^{a}	76.0 ± 0.7^{a,c}	77.9 ± 0.6^{a}
WOB (%)	74.8 ± 0.4^{a}	67.5 ± 1.1^{b}	66.8 ± 0.8^{b}	76.4 ± 0.6^{c,d}	74.5 ± 0.6^{a,c}
ALH (μm)	3.52 ± 0.03^{a}	2.45 ± 0.07^{b}	2.68 ± 0.06^{b,c}	2.85 ± 0.05^{c}	2.75 ± 0.04^{c}
BCF (Hz)	9.4 ± 0.1^{a}	7.8 ± 0.2^{b}	9.5 ± 0.2^{a}	8.9 ± 0.1^{a,c}	9.5 ± 0.13^{a}
	Fresh	**ET**	**ET + RO**	**RT**	**RT + RO**
VCL (μm/s)	112.5 ± 1.0^{a}	91.2 ± 1.3^{d}	89.6 ± 1.3^{d,f}	84.9 ± 1.3^{e,f}	82.7 ± 1.3^{e}
VSL (μm/s)	67.4 ± 0.9^{a}	53.0 ± 1.1^{d,e}	49.7 ± 0.9^{e,f}	51.0 ± 1.0^{d,e,f}	48.3 ± 1.0^{f}
VAP (μm/s)	86.0 ± 0.9^{a}	70.6 ± 1.1^{c}	66.5 ± 1.0^{c,d}	66.4 ± 1.1^{c,d}	62.3 ± 1.0^{d}
LIN (%)	58.2 ± 0.5^{a,d}	56.2 ± 0.7^{c,d}	55.0 ± 0.7^{b,c,e}	58.0 ± 0.7^{a,d,e}	56.8 ± 0.7^{a,c,d,e}
STR (%)	76.4 ± 0.5^{a,c}	72.1 ± 0.7^{b,d}	73.0 ± 0.7^{b,d}	74.1 ± 0.7^{c,d}	74.2 ± 0.7^{c,d}
WOB (%)	74.8 ± 0.4^{a}	77.5 ± 0.5^{d}	74.7 ± 0.5^{a,c}	77.6 ± 0.5^{d}	75.7 ± 0.5^{a,c,d}
ALH (μm)	3.52 ± 0.03^{a}	2.86 ± 0.04^{c}	2.84 ± 0.04^{c}	2.68 ± 0.04^{b,c}	2.69 ± 0.04^{b,c}
BCF (Hz)	9.4 ± 0.1^{a}	8.6 ± 0.1^{c}	9.2 ± 0.1^{a}	8.6 ± 0.1^{c}	9.0 ± 0.1^{a,c}

Note: a-f Different superscripts in a row indicate significant differences.

Effect of the addition of retinol to the storage extender

The addition of RO to the storage extender showed variable results (Table 1). In terms of viability, total motility, progressivity, membrane integrity and morphological abnormalities no significant differences were detected by the use of RO. However, higher percentages of live, motile and progressive spermatozoa

with also higher membrane integrity percentages were detected in 41.5 groups supplemented with RO compared with no supplemented groups. Altered acrosome percentages decreased significantly when RO was present in the storage extender at high temperatures (41.5ºC), being those values more similar to those obtained at lower temperatures (4, 22, 32ºC).

Although no differences were detected in terms of total motility and progressivity, some differences were found observing specific motility parameters (Table 2). VCL and VSL increased significantly in presence of RO when spermatozoa were stored at high temperatures (41.5ºC). However, in terms of VAP no significant differences were detected at 41.5 + RO, but decreased in 32 + RO groups. STR percentages were significantly higher when RO was present at high temperatures (41.5ºC). Moreover, the WOB parameter decreased significantly when RO was present at 22ºC. In terms of BCF, the presence of RO significantly increased the parameter at 22 and 41.5ºC. Finally, in accordance of LIN and ALH no significant differences were observed.

Discussion

Our results clearly demonstrated the harmful effects of high temperatures on epididymal sperm quality, and the fact that the presence of retinol in the storage extender may be valuable stabilizing the sperm acrosomal membrane.

It has been described that high temperatures impair semen quality as it has been reported in a variety of species. The ROS derived from heat stress affects sperm membrane integrity leading to a decrease of sperm quality and consequently their fertilizing ability. The decision to use epididymal spermatozoa in our study was done by the fact that sperm from the caudae epididyma is more sensitive to the effects of ROS (Fernández-Santos *et al.*, 2009), being easily to mimic the conditions on the field with just 3 hours of incubation.

The high content of PUFA on bull sperm membranes (Parks & Lynch, 1992) makes the spermatozoa particularly sensitive to oxidative stress by ROS. As a result, lipid peroxidation increases resulting in loss of viability (Medeiros *et al.*, 2002) reduced sperm motility (Baumber *et al.*, 2000; Guthrie & Welch, 2006), loss of acrosomal and plasma membrane integrity (Aitken *et al.*, 1998; Medeiros *et al.*, 2002) and leading to DNA fragmentation (Bennetts & Aitken, 2005). Our results are consistent with all the harmful effects reported in the literature. Sperm submitted to 41.5ºC showed a decrease in all the sperm quality parameters. However, it is noteworthy that the storage of sperm at other temperatures (scrotal, environmental and refrigeration temperature) during 3 hours did not modify the quality of sperm with the exception of the percentages of motility and progressivity. These results are in accordance with those reported by Monterroso *et al.* (1995) and Bansal & Bilaspuri (2009), where the bull sperm motility was reduced after 2-3 hours of incubation.

In an attempt to minimize the negative effects of ROS overproduction due to high temperatures the use of antioxidant agents as quenchers of ROS has been studied in a wide range of species (Baumber *et al.*, 2000; Bilodeau *et al.*, 2001; Szczesniak-Fabianczyk *et al.*, 2006; Bucack *et al.*, 2010; Maia *et al.*, 2010; Neagu *et*

al., 2010; Tuncer *et al.*, 2010). The aim of using antioxidant treatments should not be the complete ROS elimination since oxidative mechanisms play an important role in the physiological control of mammalian sperm functions (Bennetts & Aitken, 2005; Agarwal *et al.*, 2006). There are many types of antioxidant agents that could be used in this matter. Unfortunately, controversial results have been described. In bulls, antioxidants have been specially used to improve semen post-thaw quality by addition into the freezing extender (Beconi, 1993; Foote *et al.*, 2002; Andrabi *et al.*, 2008; El-Sheshtawy *et al.*, 2008; O'Flaherty *et al.*, 2009; Bucak *et al.*, 2010; Reddy, 2010), but it is necessary to adjust the extender composition, cryoprotectants and cryoprotectant concentrations, as well as the cryopreservation protocol for each single species or even breeds. To our knowledge there is no literature about the addition of antioxidant agents on storage extenders trying to improve bull sperm quality under heat stress conditions.

Vitamin A (all-trans retinol) and its metabolites, known as retinoids, are regulators of cell growth, differentiation of many types of cells (Hidalgo *et al.*, 2005), and have ROS scavenger activity (Ikeda *et al.*, 2005). Vitamin A is essential for normal testicular structure and function (Hogarth & Griswold, 2010) being present in human, rabbit and bull spermatozoa (Gambhir & Ahluwalia, 1975; Velazquez *et al.*, 1975; Virji *et al.*, 1981). In the bovine spermatozoa 90% of the total vitamin A is present in the acrosomal membrane (Gambhir & Ahluwalia, 1975). There was a positive correlation between retinol content in seminal plasma and sperm motility (Kao *et al.*, 2008), membrane integrity (Roels *et al.*, 1969), and also with sperm normalcy, since lower levels of vitamin A result in higher sperm midpiece abnormalities and coiled tail (Abdulkareema *et al.*, 2005). In our study all parameters, with the exception of normalcy, were improved by the addition of retinol to the storage extender at high temperatures, although only in a quantitative manner. An explanation would be that the concentration used in the study was unable to counteract all the negative effects derived from ROS. The fact that retinol had no effect on the other storage temperatures may support the fact that retinol, as an antioxidant agent and ROS quencher, only is effective when high amounts of ROS are present. However, our study reveals that retinol significantly reduce the percentage of spermatozoa with altered acrosomes when they are submitted to heat stress conditions, which are consistent with the fact that vitamin A at physiological concentrations stabilizes membranes (Roels *et al.*, 1969), and some precursors of vitamin A are known to be important components of antioxidant defense against lipid peroxidation in living cells (Agarwal *et al.*, 2005) and protecting plasma membrane against lipid peroxidation (Di Mascio *et al.*, 1989).

Retinoids have been used in previous *in vitro* studies with oocytes and embryos, some of them done under heat stress conditions, alleviating the negative effects derived from. A beneficial effect on nuclear (Vahedi *et al.*, 2009; Nasiri *et al.*, 2011; Liang *et al.*, 2012; Maya-Soriano *et al.*, 2012; Tahei *et al.*, 2012) and cytoplasmic competence after oocyte IVM (Gomez *et al.*, 2003; Nasiri *et al.*, 2011; Liang *et al.*, 2012) and embryo development rates were reported (Lawrence *et al.*, 2004; Rajesh *et al.*, 2010; Atikuzzaman *et al.*, 2011). However, to our knowledge, it is the first study using retinol as supplementation for sperm storage extenders in order to improve sperm quality. It should be pointed out that the concentrations used in our study were not sufficient to alleviate all the harmful effects of high temperatures, although have been

proved to be valuable for stabilizing the sperm acrosomal membrane at the same concentrations as used for oocytes or embryos. So, further investigations in the use of retinol are needed to find if there exists a concentration able to improve all sperm quality parameters.

In conclusion, to our knowledge this is the first report using retinol as supplementation for sperm storage extender. Our results seem to indicate that retinol may stabilize sperm acrosomal membrane in situations of oxidative stress due to high temperatures.

References

Abdulkareema, T.A., Al-Habobyb, A.H., Al-Mjameia, S.M., Hobia, A.A. 2005. Sperm abnormalities associated with vitamin A deficiency in rams. *Small Rumin Res*, **57**(1): 67-71.

Agarwal, A., Prabakaran, S., Allamaneni, S. 2006. What an andrologist / urologist should know about free radicals and why. *Urology*, **67**(1): 2–8.

Agarwal, A., Prahakaran, S.A., Said, T.M. 2005. Prevention of oxidative stress injury to sperm. *J Androl,* **26**(6): 653-660.

Aitken, R.J., Fisher, H. 1994. Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bio-assays,* **16**(4): 259‑267.

Aitken, R.J., Gordon, E., Harkiss, D., Twigg, J.P.I., Milne, P., Jennigs, Z., Irvine, D.S. 1998. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod,* **59**(5): 1037-1046.

Aitken, R.J., Ryan, A.L., Baker, M.A., McLaughlin, E.A. 2004. Redox activity associated with the maturation and capacitation of mammalian spermatozoa. *Free Radic Biol Med,* **36**(8): 994–1010.

Andrabi, S.M.H., Ansari, M.S., Ullah, N., Afzal, M. 2008. Effect of non-enzymatic antioxidants in extender on post-thaw quality of buffalo (Bubalus bubalis) Bull spermatozoa. *Pakistan Vet J,* **28**(4): 159-162.

Arechiga, C.F., Staples, C.R., McDowell, L.R., Hansen, P.J. 1998. Effects of timed insemination and supplemental beta-carotene on reproduction and milk yield of dairy cows under heat stress. *J Dairy Sci,* **81**(2): 390–402.

Atikuzzaman, M., Koo, O.J., Kang, J.T., Kwon, D.K., Park, S.J., Kim, S.J., Limas Gomez, M.N., Oh, H.J., Hong, S.G., Jang, G., Lee, B.C. 2011. The 9-cis retinoic acid signaling pathway and its regulation of prostaglandin-endoperoxide synthase during *in vitro* maturation of pig cumulus cell-oocyte complexes and effects on parthenogenetic embryo production. *Biol Reprod,* **84**(6): 1272-1281.

Ax, R.L., Gilbert, G.R., Shook, G.E. 1987. Sperm in Poor Quality Semen from Bulls During Heat Stress Have a Lower Affinity for Binding Hydrogen-3 Heparin1. *J Dairy Sci,* **70**(1): 195‑200.

Bailey, J.L., Bilodeau, J.F., Cormier, N. 2000. Semen Cryopreservation in Minireview Domestic Animals: A Damaging and Capacitating Phenomenon. *J Androl,* **21**(1): 1-7.

Bamba, K. 1988. Evaluation of acrosomal integrity of boar spermatozoa by bright field microscopy using an eosin-nigrosin stain. *Theriogenology,* **29**(6): 1245–1251.

Bansal, A.K., Bilaspuri, G.S. 2009. Antioxidant effect of vitamin E on motility, viability and lipid peroxidation of cattle spermatozoa under oxidative stress. *Anim Sci,* **27**(1): 5-14.

Baumber, J., Ball, B.A., Gravance, C.G., Medina, V., Davies-Morel, M.C.G. 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. *J Androl,* **21**(6): 895-902.

Beconi, M.T., Francia, C.R., Mora, N.G., Affranchino, M.A. 1993. Effect of natural antioxidants on frozen bovine semen preservation. *Theriogenology,* **40**(4): 841–851.

Bennetts, L.E., Aitken, J.R. 2005. A comparative study of oxidative DNA damage in mammalian spermatozoa. *Mol Reprod Dev,* **71**(1): 77–87.

Bilodeau, J.F., Blanchette, S., Gagnon, I.C., Sirard, M.A. 2001. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology,* **56**(2): 275‑286.

Bilodeau, J.F., Chatterjee, S., Sirard, M.A., Gagnon, C. 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev,* **55**(3): 282–288.

Borg, K.E., Lunstra, D.D., Christenson, R.K. 1993. Semen characteristics, testicular size, and reproductive hormone concentrations in mature Duroc, Meishan, Fengjing, and Minzhu boars. *Biol Reprod,* **49**(3): 515-521.

Bucak, M.N., Tuncer, P.B., Sanözkan, S., Baspinar, N., Taspinar, M., Coyan, K., Bilgili, A., Akalin, P.P., Büyükleblebici, S., Aydos, S., Ilgaz, S., Sunguroglu, A., Oztuna, D. 2010. Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters. *Cryobiology,* **61**(3): 248-253.

Casady, R.B., Myers, R.M., Legates, J.E. 1953. The effect of exposure to high ambient temperature on spermatogenesis in the dairy bull. *J Dairy Sci,* **36**: 14-23.

Córdova, B., Morató, R., Izquierdo, D., Paramio, T., Mogas, T. 2010. Effect of the addition of insulin-transferrin-selenium and/or L-ascorbic acid to the *in vitro* maturation of prepubertal bovine oocytes on cytoplasmic maturation and embryo development. *Theriogenology,* **74**(8): 1341–1348.

De Alba, J., Riera, S. 1966. Sexual maturity and spermatogenesis under heat stress in the bovine. *Anim Prod,* **8**: 137-144.

Di Mascio, P., Kaiser, S., Sies, H. 1989. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch Biochem Biophys,* **274**(2): 532-538.

El-Sheshtawy, R.I., El-Sisy, G.A., El-Nattat, W.S. 2008. Use of Selected Amino Acids to Improve Buffalo Bull Semen Cryopreservation. *Global Veterinaria*, **2**(4): 146-150.

Fernández-Santos, M.R., Domínguez-Rebolledo, A.E., Esteso, M.C., Garde, J.J., Martínez-Pastor, F. 2009. Refrigerated Storage of Red Deer Epididymal Spermatozoa in the Epididymis, Diluted and with Vitamin C Supplementation. *Reprod Domest Anim,* **44**(2): 212–220.

Foote, R.H., Brockett, C.C., Kaproth, M.T. 2002. Motility and fertility of bull sperm in whole milk extender containing antioxidants. *Anim Reprod Sci,* **71**(1-2): 13-23.

Gambhir, K.K., Ahluwalia, B.S. 1975. Vitamin A in bovine sperm acrosomes. *J Reprod Fertil,* **43**(1): 129-132.

Gholami, H., Chamani, M., Towhidi, A., Fazeli, M.H. 2010. Improvement of Semen Quality in Holstein Bulls during Heat Stress by Supplementing Omega-3 Fatty Acids. *World Academy of Science, Engineering and Technology,* **68**: 939-945.

Gomez, E., Royo, L.J., Duque, P., Carneiro, G., Hidalgo, C., Goyache, F., *et al.* 2003. 9-cis-Retinoic acid during *in vitro* maturation improves development of the bovine oocyte and increases midkine but not IGF-I expression in cumulus–granulosa cells. *Mol Reprod Dev,* **66**(3): 247–255.

Guthrie, H.D., Welch, G.R. 2006. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *J Anim Sci,* **84**(8): 2089‑2100.

Hansen, P.J. 2009. Effects of heat stress on mammalian reproduction. *Phil Trans R Soc B,* **364**(1534): 3341-3350.

Hendricks, K.E., Martins, L., Hansen, P.J. 2009. Consequences for the bovine embryo of being derived from a spermatozoon subjected to post-ejaculatory aging and heat shock: development to the blastocyst stage and sex ratio. *J Reprod Dev,* **55**(1): 69-74.

Hidalgo, C., Diez, C., Duque, P., Prendes, J.M., Rodriguez, A., Goyache, F., *et al.* 2005. Oocytes recovered from cows treated with retinol become unviable as blastocysts produced *in vitro*. *Reproduction,* **129**(4): 411–421.

Hoffmann, I. 2010. Climate change and the characterization, breeding and conservation of animal genetic resources. *Anim Genet,* **41**(Suppl1): 32–46.

Hogarth, C.A., Griswold, M.D. 2010. The key role of vitamin A in spermatogenesis. *J Clin Invest,* **120**(4): 956–962.

Ikeda, M., Kodama, H., Fukuda, J., Shimizu, Y., Murata, M., Kumagai, J., Tanaka, T. 1999. Role of radical oxygen species in rat testicular germ cell apoptosis induced by heat stress. *Biol Reprod,* **61**(2): 393-399.

Ikeda, S., Kitagawa, M., Imai, H., Yamada, M. 2005. The roles of vitamin A for cytoplasmic maturation of bovine oocytes. *J Reprod Dev,* **51**(1): 23–35.

Jannes, P., Spiessens, C., Van der Auwera, I., D'Hooghe, T., Verhoeven, G., Vanderschueren, D. 1998. Male subfertility induced by acute scrotal heating affects embryo quality in normal female mice. *Hum Reprod,* **13**(2): 372–375.

Kao, S.H., Chao, H.T., Chen, H.W., Hwang, T.I.S., Liao, T.L., Wei, Y.H. 2008. Increase of oxidative stress in human sperm with lower motility. *Fertil Steril,* **89**(5): 1183–1190.

Kenney, R.M., Bergman, R.V., Cooper, W.L., Morse, G.W. 1975. Minimal contamination techniques for breeding mares: technique and preliminary finding. *Proc Am Assoc Equine Pract*, 327–336.

Kim, H.J., Kang, B.S., Park, J.W. 2005. Cellular defense against heat shock-induced oxidative damage by mitochondrial NADP+-dependent isocitrate dehydrogenase. *Free Radical Res,* **39**(4): 441-448.

La Falci, V.S.N., Yrjo-Koskinen, A.E., Fazeli, A., Holt, W.V., Watson, P.F. 2011. Antioxidant combinations are no more beneficial than individual components in combating ram sperm oxidative stress during storage at 5ºC. *Anim Reprod Sci,* **129**(3-4): 180-187.

Lasso, J.L., Noiles, E.E., Alvarez, J.G., Storey, B.T. 1994. Mechanism of superoxide dismutase loss from human sperm cells during cryopreservation. *J Androl,* **15**(3): 255–265.

Lawrence, J.L., Payton, R.R., Godkin, J.D., Saxton, A.M., Schrick, F.N., Edwards, J.L. 2004. Retinol Improves Development of Bovine Oocytes Compromised by Heat Stress During Maturation. *J Dairy Sci,* **87**(8): 2449–2454.

Lecha, L.B. 2007. IPCC 2007. Fourth Assessment Report. Cambridge, Cambridge University Press.

Liang, S., Kang, J., Liu, J.X., Suo, L., Yue, L., Wang, W., Yin, X.J. 2012. The influence of 9-cis-retinoic acid on nuclear and cytoplasmic maturation and gene expression in canine oocytes during *in vitro* maturation. *Theriogenology,* **77**(6): 1198-1205.

Lopez-Gatius, F., Garcia-Ispierto, I., Santolaria, P., Yaniz, J., Nogareda, C., Lopez-Bejar, M. 2006. Screening for high fertility in high-producing dairy cows. *Theriogenology,* **65**(8): 1678-1689.

Lue, Y.H., Hikim, A.P., Swerdloff, R.S., Im, P., Taing, K.S., Bui, T., Leung, A., Wang, C. 1999. Single exposure to heat induces stage-specific germ cell apoptosis in rats: role of intratesticular testosterone on stage specificity. *Endocrinology,* **140**(4): 1709–1717.

MacCracken, M.C., Moore, F., Topping, J.C. Jr. 2008. Sudden and Disruptive Climate Change: Exploring the Real Risks and How We Can Avoid Them. Earth scan: London, UK.

Maia, Mda. S., Bicudo, S.D., Sicherle, C.C., Rodello, L., Gallego, I.C. 2010. Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in extenders with antioxidants. *Anim Reprod Sci,* **122**(1-2): 118-123.

Marai, I.F.M., Habeeb, A.A.M., Gad, A.E. 2002. Reproductive traits of male rabbits as affected by climatic conditions, in the subtropical environment of Egypt. *Anim Sci,* **75**: 451-458.

Marei, W.F., Wathes, C., Fouladi-Nashta, A.A. 2011. Differential effects of linoleic and alpha-linolenic fatty acids on spatial and temporal mitochondrial distribution and activity in bovine oocytes. *Reprod Fertil Devel,* **24**(5): 679-690.

Maya-Soriano, M.J., Taberner, E., López-Béjar, M. 2012. Retinol improves *in vitro* oocyte nuclear maturation under heat stress in heifers. *Zygote* (*in press*).

Medeiros, C.M., Forell, F., Oliveira, A.T., Rodrigues, J.L. 2002. Current status of sperm cryopreservation: why isn't it better? *Theriogenology,* **57**(1): 327‑344.

Meyerhoeffer, D.C., Wettemann, R.P., Coleman, S.W., Wells, M.E. 1985. Reproductive Criteria of Beef Bulls during and after Exposure to Increased Ambient Temperature. *J Anim Sci,* **60**(2): 352-357.

Michael, A., Alexopoulos, C., Pontiki, E., Hadjipavlou-Litina, D., Saratsis, P., Boscos, C. 2007. Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. *Theriogenology,* **68**(2): 204–212.

Monterroso, V.H., Drury, K.C., Ealy, A.D., Edwards, J.L., Hansen, P.J. 1995. Effect of heat shock on function of frozen/thawed bull spermatozoa. *Theriogenology,* **44**(7): 947-961.

Nasiri, E., Mahmoudi, R., Bahadori, M.H., Amiri, I. 2011. The Effect of Retinoic Acid on *In vitro* Maturation and Fertilization Rate of Mouse Germinal Vesicle Stage Oocytes. *Cell Journal,* **13**(1): 19-24.

Neagu, V.R., García, B.M., Sandoval, C.S., Rodríguez, A.M., Ferrusola, C.O., Fernández, L.G., Tapia, J.A., Peña, F.J. 2010. Freezing dog semen in presence of the antioxidant butylated hydroxytoluene improves postthaw sperm membrane integrity. *Theriogenology,* **73**(5): 645-650.

O'Flaherty, C., Beconi, M., Beorlegui, N. 1997. Effect of natural antioxidants, superoxide dismutase and hydrogen peroxide on capacitation of frozen-thawed bull spermatozoa. *Andrologia*, **29**(5): 269-275.

Parks, J.E., Lynch, D.V. 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. *Cryobiology,* **29**(2): 255–266.

Paul, C., Murray, A.A., Spears, N., Saunders, P.T. 2008. A single, mild, transient scrotal heat stress causes DNA damage, subfertility and impairs formation of blastocysts in mice. *Reproduction,* **136**(1): 73-84.

Pérez-Crespo, M., Pintado, B., Gutiérrez-Adán, A. 2008. Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ratio in mice. *Mol Reprod Dev,* **75**(1): 40–47.

Rajesh, N., Shankar, M.B., Deecaraman, M. 2010. Effect of vitamin A supplementation at different gaseous environments on *in vitro* development of pre-implantation sheep embryos to the blastocyst stage. *Animal,* **4**(11): 1884-1890.

Reddy, N.N.S., Mohanarao, J.G., Atreja, S.K. 2010. Effects of adding taurine and trehalose to a tris-based egg yolk extender on buffalo (Bubalua bubalis) sperm quality following cryopreservation. *Anim Reprod Sci,* **119**(3-4): 183-190.

Roca, J., Martínez, S., Orengo, J., Parrilla, I., Vázquez, J.M., Martínez, E.A. 2005. Influence of constant long days on ejaculate parameters of rabbits reared under natural environment conditions of Mediterranean area. *Lives Prod Sci,* **94**: 169-177.

Rockett, J.C., Mapp, F.L., Garges, J.B., Luft, J.C., Mori, C., Dix, D.J. 2001. Effects of hyperthermia on spermatogenesis, apoptosis, gene expression, and fertility in adult male mice. *Biol Reprod,* **65**(1): 229–239.

Roels, A., Anderson, R., Lui, N.S.T., Shah, D.O., Trout, M.E. 1969. Vitamin A and Membranes. *Am J Clin Nutr,* **22**(5): 1020-1032.

Safaa, H.M., Emarah, M.E., Saleh, N.F.A. 2008. Seasonal effects on semen quality in Black Baladi and white New Zealand rabbit bucks. *World Rabbit Sci,* **16**: 13–20.

Sailer, B.L., Sarkar, L.J., Bjordahl, J.A., Jost, L.K., Evenson, D.P. 1997. Effects of heat stress on mouse testicular cells and sperm chromatin structure. *J Androl,* **18**(3): 294-301.

Sanocka, D., Kurpisz, M. 2004. Reactive oxygen species and sperm cells. *Reprod Biol Endocrinol,* **23**(2): 12.

Setchell, B.P. 1998. The Parkes Lecture. Heat and the testis. *J Reprod Fertil,* **114**: 179–194.

Sinha, M.P., Sinha, A.K., Singh, B.K., Prasad, P.L. 1996. The effect of glutathione on the motility, enzyme leakage and fertility of frozen goat semen. *Theriogenology,* **41**(3): 237-243.

Skinner, J.D., Louw, G.N. 1966. Heat stress and spermatogenesis in Bos indicus and Bos taurus cattle. *J Appl Physiol,* **21**(6): 1784-1790.

Stone, B.A. 1982. Heat induced infertility of boars: The inter-relationship between depressed sperm output and fertility and an estimation of the critical air temperature above which sperm output is impaired. *Anim Reprod Sci,* **4**(4): 283–299.

Storey, B.T. 1997. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Mol Hum Reprod,* **3**(3): 203–213.

Szczesniak-Fabianczyk, B., Bochenek, M., Smorag, Z., Silvestre, M.A. 2006. Effect of antioxidants added to boar semen extender on the semen survival time and sperm chromatin structure. *Reprod Biol,* **3**(1): 81-87.

Tahaei, L.S., Eimani, H., Yazdi, P.E., Ebrahimi, B., Fathi, R. 2011. Effects of retinoic acid on maturation of immature mouse oocytes in the presence and absence of a granulosa cell co-culture system. *J Assist Reprod Genet,* **28**(6): 553-558.

Tseng, J.K., Chen, C.H., Chou, P.C., Yeh, S.P., Ju, J.C. 2004. Influences of follicular size on parthenogenetic activation and *in vitro* heat shock on the cytoskeleton in cattle oocytes. *Reprod Domest Anim,* **39**(3): 146–153.

Tuncer, P.B., Bucak, M.N., Büyükleblebici, S., Sarıözkan, S., Yeni, D., Eken, A., Akalın, P.P., Kinet, H., Avdatek, F., Fidan, A.F., Gündoğan, M. 2010. The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen. *Cryobiology,* **61**(3): 303-307.

Tusell, L., David, I., Bodin, L., Legarra, A., Rafel, O., López-Bejar, M. *et al.* 2011. Using the product threshold model for estimating separately the effect of temperature on male and female fertility. *J Anim Sci,* **89**(12): 3983-3995.

Vahedi, V., Zeinoaldini, S., Kohram, H., Farahavar, A. 2009. Retinoic acid effects on nuclear maturation of bovine oocytes *in vitro*. *African J Biotechnol,* **8**: 3974-3978.

Velazquez, A., Rosado, A. Jr., Rosado, A. 1975. Participation of vitamin A in the maturation of rabbit spermatozoa. *Int J Fertil*, **20**(3): 151-155.

Virji, N., Vahlquist, A., Eliasson, R. 1981. Vitamin A in human semen. *Experientia,* **37**(12): 1267-1268.

Walters, A.H., Eyestone, W.E., Saacke, R.G., Pearson, R.E., Gwazdauskas, F.C. 2005. Bovine embryo development after IVF with spermatozoa having abnormal morphology. *Theriogeneology,* **63**(7): 1925-1937.

Wettemann, R.P., Wells, M.E., Omtveldt, I.T., Pope, C.E., Turman, E.J. 1976. Influence of elevated ambient temperature on reproductive performance of boars. *J Anim Sci,* **42**(3): 664-669.

Yaeram, J., Setchell, B.P., Maddocks, S. 2006. Effect of heat stress on the fertility of male mice *in vivo* and *in vitro*. *Reprod Fertil Dev,* **18**(6): 647-653.

Yousef, M.I., Abdallah, G.A., Kamel, K.I. 2003. Effect of ascorbic acid and Vitamin E supplementation on semen quality and biochemical parameters of male rabbits. *Anim Reprod Sci,* **76**(1-2): 99–111.

Zhu, B., Walker, S.K., Oakey, H., Setchell, B.P., Maddocks, S. 2004. Effect of paternal heat stress on the development *in vitro* of preimplantation embryos in the mouse. *Andrologia,* **36**(6): 384–394.

DISCUSSION

DISCUSSION

The aim of this thesis has been the study of the effects of high temperatures on gamete development and how the use of antioxidant agents could counteract the negative effects of heat stress and/or the excessive production of ROS.

The current thesis demonstrated that heat stress impaired bovine oocyte maturation at both nuclear and cytoplasmic level, in agreement with previous findings (Payton *et al.*, 2004; Tseng *et al.*, 2004; Edwards *et al.,* 2005; Roth and Hansen, 2005). These negative effects are important since the nuclear and cytoplasmic status of the oocyte at the time of fertilization is the major determining factor of the developmental program of the resultant embryo (Hansen, 2002).

High temperatures *in vitro* simulating those rectal temperatures obtained in dairy cattle during the central part of the day in summer (exceeding 41ºC; Putney *et al.*, 1988b; 1989a; Ealy *et al.*, 1993; Wolfenson *et al.*, 1993a), reveal a greater percentage of oocytes showing an aberrant progression to the second meiotic metaphase stage (MII) and CG distribution pattern IV. The alterations found on the nuclear maturation, such as expanded or condensed nucleus, were probably as a result of chromosomal and meiotic spindle alterations, as described by other authors (Payton *et al.*, 2004; Tseng *et al.*, 2004; Roth and Hansen, 2005). On the other hand, alterations on CG migration by high temperatures lead oocytes to achieve a distribution pattern presumptive of premature exocytosis characterized by the loss of a continuous CG layer. This is the most common pattern produced in oocytes after fertilization or activation due to exocytosis. Hence, heat shock could be the cause of an advanced state of oocyte maturation (Payton *et al.,* 2004; Edwards *et al.,* 2005) resulting in CG loss. The premature CG exocytosis prior to fertilization was reported in early studies and was suggested to be responsible for the enlargement of perivitelline space and for altering the properties of the zona pellucida (Okada *et al.*, 1986; 1993).

Although, the best way to study direct effects of high tempeartures on oocytes could be through *in vitro* studies, avoiding the presence of indirect effects (Putney *et al.*, 1988b; Gilad *et al.*, 1993; Wolfenson *et al.*, 1997; 2000), all the *in vitro* studies found in the literature should be taken with caution because they did not mimic the physiological conditions in which cows are submitted in the field. Some of them applied high temperatures for just 30-60 min (Ryan *et al.*, 1992; Ju *et al.*, 1999), for the first 12 h of IVM (Edwards *et al.*, 2005; Roth & Hansen, 2004; 2005; Schrock *et al.*, 2007) or the majority for the whole 24h of IVM (Lenz *et al.*, 1983; Soto & Smith, 2009). A most appropriate *in vitro* model would be applying around 3 hours simulating those high temperatures of the central part of the day. The exposure to this interval of high temperatures at the end of the oocyte maturation period would be more demonstrative of the resistance to heat shock of the oocyte, since the preovulatory oocyte seems to be the most sensitive stage to heat stress (Ingraham *et al.*, 1976; Putney *et al.*, 1988b).

As observed in the present thesis, an anomalous progression to MII was a feature of heat stressed oocytes. A plausible explanation could be that high temperatures modify cytoskeleton behaviour (Tseng *et al.*, 2004; Ju *et al.*, 2005; Roth & Hansen, 2005) since nuclear maturation is dependent upon extensive

rearrangement of the cytoskeleton (Gallicano, 2001; Shin & Kim, 2003). Nonetheless, other aspects of nuclear behaviour may be modified by temperature stress, such as the rate and normality of chromosomal condensation (Roti, 2008). On the other hand, the high percentage of oocytes showing a pattern IV of CG distribution was firstly described in bovine oocytes by Hosoe & Shioya (1997). Although heat stress advanced oocyte maturation in previous studies (Payton *et al.*, 2004; Edwards *et al.*, 2005), CG pattern IV related to the final maturation of oocyte was not described.

Our current research confirms the fact that heat stress proved to be valuable in aging oocytes, in accordance with previous findings where heat stress at the end of the IVM period seems to cause an advanced state of oocyte maturation (Payton *et al.*, 2004; Edwards *et al.*, 2005). Similar figures of aged oocytes (Szollosi, 1971; 1974; 1975) were registered for heat stressed and overmaturated oocytes. The analyses showed how dramatically heat stress advanced age for nuclear and cytoplasmic processes as overmaturation did, since aged oocyte were 17 and 18 times more likely in heat stressed and overmaturated oocytes than control group, respectively.

Once it has been proved that high temperatures affects oocyte maturation by aging them, seasonal effect must be evaluated since the viability and quality of bovine oocytes and embryos is lower in warm versus cold seasons (Monty & Racowsky, 1987; Al-Katani *et al.*, 2002b). The present thesis tried to find if high temperatures affects in a different way oocytes obtained from different seasons of the year, since global warming effects are going to be more evident during winter (MacCracken, 2008), being animals in our geographical area submitted to high temperatures round 4 days during the cold months of the year (López-Gatius *et al.*, 2006). Hence, seems that bovine oocytes are more sensitive to high temperatures suffered during cold months, and oocytes from the warm season tolerate better heat shock. However, a seasonal effect on the capacity of the oocytes was only found on cytoplasmic maturation, whereas the effect on undergoing nuclear maturation (MII percentage) could not be demonstrated.

The fact that oocytes obtained in the warm season were better able to adapt to high temperatures in terms of their cytoplasmic maturation could reflect a mechanism of heat shock tolerance. It has been described that a defined and properly applied sublethal stress may induce general adaptation and increase tolerance to subsequent stress (Pribenszky *et al.*, 2010). This phenomenon has been described *in vitro* in studies in which bovine embryos were progressively exposed to increasing heat shock (Ealy *et al.*, 1993; Hansen & Arechiga, 1999; Paula-Lopes & Hansen, 2002). Heat tolerance could be related to HSP expression, since HSP70 protects oocytes from apoptotic stimuli that harmfully affect DNA (Paula-Lopes & Hansen, 2002). This mechanism of heat tolerance warrents further investigation in future studies designed to improve oocyte and embryo development in warm regions.

The use of antioxidant agents have been studied in the current thesis in oocyte IVM with a variety of results.

Retinyl used as supplementation in IVM medium showed a harmful effect on oocyte maturation under physiological conditions by decreasing the correct progression of the nucleus to the MII stage. Furthermore, it was stated that this retinoid is useless in counteract heat stress effects on bovine oocytes,

at least at the concentrations and conditions used in this thesis. To our knowledge it is the first time that retinyl is used for IVM. However, there is literature in their use on embryos development, with no beneficial effects on cleavage or blastocyst rates (Lima *et al.*, 2004), although its supplementation helped murine embryos to overcome the two-cell block (Hajializadeh *et al.*, 2008), and seemed to be valuable in goat embryos when co-culture with oviductal cells, suggesting that retinyl needs to be metabolized to be effective (Chiamenti *et al.*, 2010). On the other hand, the presence of retinyl in IVM is able to maintain cumulus oophorus cell phenotype, maintaining estradiol secretion, and avoiding its premature luteinisation, thus decreasing progesterone availability for the oocyte.

Oleic acid was another antioxidant agent evaluated in the present thesis. It has been reported that oleic acid is present in high concentrations in bovine oocyte membrane during winter and its low concentration in summer is related to the poor quality of oocytes (Zeron *et al.*, 2001). Nevertheless, a negative effect of oleic acid on metaphase progression confirms the negative effect described previously by Jorritsma *et al.* (2004), although as described by Leroy *et al.* (2005), their addition did not cause any effect on the nucleus. This fatty acid proved to be unvaluable in counteract heat stress effects on bovine oocyte maturation, and even to be harmful for nuclear progression under control conditions. However, it is necessary to take into account that the concentrations used in this study could be not appropriate for oocyte maturation and even to improve it under heat stress conditions. On the other hand, the presence of oleic acid in IVM medium increased estradiol availability per oocyte, as described in other study (Vanholder *et al.*, 2005) but at higher concentrations than those used in our study.

However, our thesis clearly demonstrated that heat-stressed oocytes were less affected when retinol was added to the maturation medium, at least at nuclear level. The beneficial effect of retinol on the nucleus progression to MII has also been reported by Vahedi *et al.* (2009) at physiological conditions. In terms of high temperatures, retinol proved to be valuable improving development of oocytes (Livingston *et al.*, 2004). On the other hand, retinol has also been used on embryo studies, being valuable improving ovine embryo development to blastocyst under oxidative stress (Rajesh *et al.*, 2010), and also bovine heat-stressed oocytes and embryos (Lawrence *et al.*, 2004; Livingston *et al.*, 2004), being the concentrations used 5-6 μM. Nevertheless, these studies take into account the effect on the oocyte through the embryo development but not the retinoid effects done into the oocyte itself. Therefore, in this thesis we tried to elucidate the effects that retinoids exert into the oocyte itself under heat stress conditions.

On the other hand, it has been described previously that other retinoids improve cytoplasmic maturation of bovine oocytes under physiological conditions (Hidalgo *et al.*, 2003). In our study, the presence of retinol produce a delay effect on CG migration by increasing the CG pattern II, decreasing the exocytosis pattern IV due to heat stress. It is not the correct pattern of cytoplasmic maturation (III) and although it is considered an immature pattern it is not considered an undesirable distribution.

In terms of sperm quality, our results clearly demonstrated the harmful effects of *in vivo* and *in vitro* high temperatures on rabbit and bovine epididymal sperm quality, respectively. The decision to use

epididymal spermatozoa in our study was done by the fact that sperm from the caudae epididyma is more sensitive to the effects of ROS (Fernández-Santos *et al.*, 2009).

It has been reported in a variety of species that high temperatures impair semen quality by an increase in the percentage of dead spermatozoa, morphological abnormalities, acrosomal alterations, and a decrease in sperm motility (Skinner and Louw, 1966; Wettemann *et al.*, 1976; Wettemann and Desjardins, 1979; Jannes *et al.*, 1998; Pérez-Crespo *et al.*, 2008; Safaa *et al.*, 2008).

Despite of all these reported effects, only motility and progressivity were affected by *in vivo* circadian high temperatures cycles in our experimental rabbits. A possible explanation is that the other sperm quality parameters were not affected by the development of thermotolerance (Finzi *et al.*, 1988; 1995; Welch, 1993) due to they were exposed to chronic heat stress. This phenomenon has also been reported in rabbit embryos (Makarevich *et al.*, 2007). Experimental animals may develop thermotolerance because they were able to recover from high temperatures at night, coinciding with low temperatures and the feeding time, or because the submission to high temperatures was at the beginning of the animal's reproductive life (180 days old).

The present thesis first demonstrates the existence of four well-defined sperm subpopulations in rabbit epididymal sperm cells as those reported in ejaculates (Quintero-Moreno *et al.*, 2007), which can be easily defined by their motility characteristics. As described hereby, motility changes due to heat stress are linked to changes in the percentages of each motile sperm subpopulations by increasing the proportion of middle-lower motile subpopulations. However, these changes did not affect the percentage of one of the highest motile sperm subpopulation present in rabbit bucks, indicating that an overall subpopulation structure is preserved under chronic high temperatures in order to maintain the general function of the ejaculate. In fact, preliminary studies support this hipothesis since fertility and prolificacy were not affected when the ejaculates of these rabbit bucks were used in artificial inseminations, suggesting that a percentage of enough high motile spermatozoa able to fertilize always exists (unpublished data). Although, further research is needed to elucidate if sperm-motile subpopulation structure is adaptable to heat stress or just maintained in time regardless on environmental conditions by acute heat stress studies.

Trying to avoid the indirect effects that could induce thermotolerance in our *in vivo* rabbit model, it has been evaluated the direct effects of high temperatures on bull epdiddymal sperm cells by the development of an *in vitro* model comparing with other three reference temperatures (scrotal, environmental and refrigeration temperatures). The high content of PUFA on bull sperm membranes (Parks & Lynch, 1992) makes the spermatozoa particularly sensitive to oxidative stress by ROS. The impairment of quality of sperm cells exposed to 41.5ºC are consistent with all the harmful effects reported in the literature: loss of viability (Medeiros *et al.*, 2002), reduced sperm motility (Baumber *et al.*, 2000; Guthrie & Welch, 2006) and loss of acrosomal and plasma membrane integrity (Aitken *et al.*, 1998; Medeiros *et al.*, 2002).

Since antioxidant agents could act as quenchers of ROS, their use in an attempt to minimize the negative effects of high temperatures has been studied in a wide range of species (Baumber *et al.*, 2000; Bilodeau *et al.*, 2001; Szczesniak-Fabianczyk *et al.*, 2006; Bucak *et al.*, 2010b; Maia *et al.*, 2010; Neagu *et al.*, 2010; Tuncer *et al.*, 2010). In bulls, antioxidants have been specially used to improve semen post-thaw quality by addition into the freezing extender (Beconi, 1993; Foote *et al.*, 2002; Andrabi *et al.*, 2008; El-Sheshtawy *et al.*, 2008; O'Flaherty *et al.*, 2009; Bucak *et al.*, 2010b; Reddy, 2010). However, to our knowledge there is no literature about the addition of antioxidant agents on storage extenders trying to improve bull sperm quality under heat stress conditions.

For this purpose retinol was selected to improve sperm quality since vitamin A is essential for normal testicular structure and function (Hogarth & Griswold, 2010) and some precursors of vitamin A are known to be important components of antioxidant defense against lipid peroxidation in living cells (Agarwal *et al.*, 2005b) and protecting plasma membrane against lipid peroxidation (Di Mascio *et al.*, 1989). To our knowledge, it is the first study using retinol as supplementation for sperm storage extenders in order to improve sperm quality. It was described a positive correlation between retinol content in seminal plasma and sperm motility (Kao *et al.*, 2008), membrane integrity (Roels *et al.*, 1969), and also with sperm normalcy, since lower levels of vitamin A result in higher sperm midpiece abnormalities and coiled tail (Abdulkareema *et al.*, 2005). However, all sperm quality parameters, with the exception of morphological normalcy, were only improved in a quantitative manner by the addition of retinol to the storage extender at high temperatures. Although, our study reveals that retinol significantly reduce the percentage of spermatozoa with altered acrosomes when they are submitted to heat stress conditions, which are consistent with the fact that vitamin A at physiological concentrations stabilizes membranes (Roels *et al.*, 1969), being specially present in acrosomal membrane (Gambhir & Ahluwalia, 1975).

On the other hand, the fact that retinol had no effect on the other storage temperatures may support the hypothesis that retinol, as an antioxidant agent and ROS quencher, only is effective when high amounts of ROS are present. It should be pointed out that the concentration used in our study were not sufficient to alleviate all the harmful effects of high temperatures, although have been proved to be valuable for stabilizing the sperm acrosomal membrane at the same concentrations as used for oocytes and embryos. So, further investigations in the use of retinol are needed to find if there exists a concentration able to improve all sperm quality parameters.

Our thesis also demonstrated the harmful effects of cryopreservation process on rabbit sperm quality, especially on sperm motility and progressivity due to the high levels of ROS derived from the freezing process. However, the use of the antioxidant agents in an attempt to improve thaw-sperm quality was inadequate to achieve significant results, at least at the concentrations and conditions used in this study. Although cryopreserved sperm is not used for commercial purposes at present, there is a need for reliable methods of rabbit sperm resource banking. It can facilitate animal genetic diffusion between countries

(Liu *et al.*, 2007), and it can also be useful preserving endangered rabbit breeds (Bolet *et al.*, 2000). Hence, in an attempt to minimise the negative effects of ROS on sperm quality the use of antioxidant agents as quenchers of ROS has been studied.

The use of retinoids as antioxidant agents improving thaw-sperm quality is first described in the current thesis. Some precursors of them, such as β-carotene and lycopene, are known to be important components of antioxidant defence against lipid peroxidation in living cells (Agarwal *et al.*, 2005b) and protecting plasma membrane against lipid peroxidation (Di Mascio *et al.*, 1989). They had also been proved to be useful improving oocyte and embryo quality with significant good results (Lima *et al.*, 2004; Livingston *et al.*, 2004; Hajializadeh *et al.*, 2008; Vahedi *et al.*, 2009; Rajesh *et al.*, 2010). However, the concentrations of retinol used in our thesis had no effect on sperm quality after thawing and even retinyl concentrations used were toxic for sperm cells since a drastic decrease in sperm quality was recorded as concentration increased. So, the use of retinyl should be avoided in freezing extenders of rabbit sperm cells.

On the other hand, BSA has been used in the current thesis since it is able to eliminate ROS (Lewis *et al.*, 1997), protect sperm membrane integrity (Uysal *et al.*, 2005), and enhance motility and viability of sperm cells after cryopreservation process (Matsuoka *et al.*, 2006). The more beneficial concentration reported in the literature is at a concentration of 20mg/ml (Uysal and Bucak, 2007), but at that concentration, BSA did not improve post-thawing sperm quality in our study. It should be needed higher concentrations for rabbit sperm cells since progressive improvements were found in viability as BSA concentration increased, and taking into account that Uysal and Bucak (2007) described no effects in protecting sperm cells from lipid peroxidation in the use of concentrations below 60mg/ml in ram.

CONCLUSIONS / CONCLUSIONES

CONCLUSIONS

1. Heat stress impairs bovine oocyte maturation at both nuclear and cytoplasmic level by increasing the percentage of oocytes showing an aberrant progression to the second meiotic metaphase stage and cortical granule premature exocytosis.

2. Oocytes collected in the cold season are more sensitive to the negative effects of heat stress than their counterparts collected in the warm season, especially at cytoplasmic level.

3. Retinol as an antioxidant agent supplied in the oocyte maturation medium is useful for improving nuclear maturation in bovine heat-stressed oocytes.

4. The chronic exposure to *in vivo* circadian high temperature cycles proved to be harmful for rabbit epididymal sperm motility and progressivity. Accordingly, motile sperm-subpopulation structure is also modified by high temperatures by increasing less-motile subpopulations, although these changes do not affect the highest motile sperm-subpopulation. On the other hand, high temperatures reduce the percentages of viability, motility, progressivity and cause the loss of acrosomal and plasma membrane integrity on bovine epididymal sperm cells.

5. Retinol is proved to be valuable stabilizing the acrosome membrane in bovine epididymal sperm cells exposed to heat stress. However, the use of retinyl as radical oxygen species quencher during rabbit cryopreservation process should be avoided since it is toxic for sperm, at least under our experimental conditions.

CONCLUSIONES

1. El estrés térmico afecta negativamente la maduración ovocitaria en bovino tanto a nivel nuclear como citoplasmático mediante el incremento del porcentaje de ovocitos con progresión anómala al estadío de metafase II y exocitosis prematura de gránulos corticales.

2. Los ovocitos obtenidos en la época fría del año son más sensibles a los efectos negativos del estrés térmico que aquellos obtenidos durante la época cálida, especialmente a nivel citoplasmático.

3. El retinol como agente antioxidante en el medio de maduración ovocitario es útil mejorando la maduración nuclear en ovocitos bovinos estresados térmicamente.

4. La exposición crónica *in vivo* a ciclos circadianos de altas temperaturas afecta negativamente la motilidad y progresividad de los espermatozoides epididimarios de conejo. Además, la estructura de subpoblaciones espermáticas también se ve afectada por las altas temperaturas incrementando las menos mótiles aunque sin afectar demasiado a una de las más mótiles. Por otro lado, las altas temperaturas sobre los espermatozoides epididimarios bovinos reducen los porcentajes de viabilidad, motilidad y progresividad, alterando también la integridad de la membrana acrosomal y plasmática.

5. El retinol es válido como estabilizador de la membrana acrosomal en espermatozoides epididimarios bovinos sometidos a estrés térmico. Sin embargo, el uso de retinyl como quelante de radicales libres durante el proceso de congelación de espermatozoides de conejo debe evitarse por ser tóxico, al menos bajo nuestras condiciones experimentales.

REFERENCES

References

Aardema, H., Vos, P.L.A.M., Lolicato, F., Roelen, B.A.J., Knijn, H.M., Vaandrager, A.B., Helms, J.B., Gadella, B.M. 2011. Oleic Acid Prevents Detrimental Effects of Saturated Fatty Acids on Bovine Oocyte Developmental Competence. *Biol Reprod*, **85**(1): 62–69.

Abdallah, J.M., McDaniel, B.T. 2000. Genetic parameters and trends of milk, fat, days open, and body weight after calving in North Carolina experimental herds. *J Dairy Sci,* **83**(6): 1364-1370.

Abdel-Kafy, E.M., Ali, W.A.H., Hoda, A.S., Azoz, A.A.A. 2008. Effect of short heat exposure, balanced feed restriction and acetic acid supplement at post weaning on growth and thermoregulation in growing rabbits during hot season. 9th World Rabbit Congress, 507-511.

Abdulkareema, T.A., Al-Habobyb, A.H., Al-Mjameia, S.M., Hobia, A.A. 2005. Sperm abnormalities associated with vitamin A deficiency in rams. Small Rum Res, **57**(1): 67-71.

Abilay, T.A., Johnson, H.D., Madam, L.M. 1975. Influence of environmental heath on peripheral plasma progesterone and cortisol during the bovine oestrus cycle. *J Dairy Sci,* **58**: 1836–1840.

Adamiak, S.J., Mackie, K., Watt, R.G., Webb, R., Sinclair, K.D. 2005. Impact of Nutrition on Oocyte Quality: Cumulative Effects of Body Composition and Diet Leading to Hyperinsulinemia in Cattle. *Biol Reprod*, **73:** 918–926.

Agarwal, A., Gupta, S., Sharma, R.K. 2005a. Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol,* **3**: 1-32.

Agarwal, A., Ikemoto, I., Loughlin, K.R. 1994. Relationship of sperm parameters with levels of reactive oxygen species in semen specimens. *J Urol*, **152**: 107-110.

Agarwal, A., Makker, K., Sharma, R. 2008. Clinical relevance of oxidative stress in male factor infertility: an update. *Am J Reprod Immunol,* **59**(1): 2-11.

Agarwal, A., Prabakaran, S.A. 2005. Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. *Indian J Exp Biol,* **43**(11): 963-974.

Agarwal, A., Prabakaran, S., Allamaneni, S. 2006. What an andrologist/urologist should know about free radicals and why. *Urology*. **67**(1): 2-8.

Agarwal, A., Prahakaran, S.A., Said, T.M. 2005b. Prevention of oxidative stress injury to sperm. *J Androl,* **26**: 653-660.

Agarwal, A., Saleh, R.A. and Bedaiwy, M.A. 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril*, **79**(4): 829–843.

Ahmad, N., Schrick, F.N., Butcher, R.L., Inskeep, E.K. 1995. Effect of persistent follicles on early embryonic losses in beef cows. *Biol Reprod,* **52**: 1129–1135.

Aitken, R.J. 1997. Molecular mechanisms regulating human sperm function. *Mol Hum Reprod,* **3**: 169-173.

Aitken, R.J., Baker, M.A. 2004. Oxidative stress and male reproductive biology. *Reprod Fertil Dev,* **16**: 581–588.

Aitken, R.J., Baker, M.A. 2006. Oxidative stress, sperm survival and fertility control. *Mol Cell Endocrinol*, **250**: 66–69.

Aitken, R.J., Clarkson, J.S. 1987. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil,* **81**: 459-469.

Aitken, R.J., Gordon, E., Harkiss, D., Twigg, J.P., Milne, P., Jennings, Z., Irvine, D.S. 1998. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod*, **59**: 1037-1046.

Aitken, J.R., Clarkson, J.S., Fishel, S. 1989. Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biol Reprod*, **40**: 183-197.

Aitken, R.J., Buckingham, D., Harkiss, D. 1993. Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. *J Reprod Fertil,* **97**: 441–450.

Aitken, R.J., Fisher, H.M., Fulton, N., Gomez, E., Knox, W., Lewis, B., et al. 1997. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. *Mol Reprod Dev*, **47**: 468-482.

Aitken, R.J., Harkiss, D., Buckingham, D. 1993. Relationship between iron-catalysed lipid peroxidation potential and human sperm function. *J Reprod Fertil,* **98**: 257-65.

Aktas, C., Kanter, M. 2009. A morphological study on Leydig cells of scrotal hyperthermia applied rats in short-term. *J Mol Hist,* **40**: 31–39.

Albertini, D.F. 1992. Cytoplasmic microtubular dynamics and chromatin organization during mammalian oogenesis and oocyte maturation. *Mutat Res,* **296**(1-2): 57–68.

Albertini, D.F., Combelles, C.M., Benecchi, E., Carabatsos, M.J. 2001. Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction*. **121**: 647-653.

Al-Katanami, Y.M., Drost, M., Monson, R.L., Rutledge, J.J., Krininger III, C.E., Block, J. 2002a. Pregnancy rates following timed embryo transfer with fresh or vitrifed in vitro produced embryos in lactating dairy cows under heat stress condition. *Theriogenology.* **58**: 171–82.

Al-Katanani, Y.M., Paula-Lopes, F.F., Hansen, P.J. 2002b. Effect of season and exposure to heat stress on oocyte competence in Holstein cows. *J Dairy Sci,* **85**: 390–396.

Al-Katanani, Y.M., Webb D.W. and Hansen, P.J. 1998. Factors affecting seasonal variation in non-return rate of lactating dairy cows. *J Dairy Sci,* **81**(Suppl. 1): 217 (Abstract).

Al-Katanani, Y.M., Webb, D.W. and Hansen, P.J. 1999. Factors affecting seasonal variation in 90-day nonreturn rate to first service in lactating Holstein cows in a hot climate. *J Dairy Sci,* **82**(12): 2611-2616.

Almier, M., De Rosa, G., Grasso, F., Napolitana, F., Bordi, A. 2002. Effect of climate on the response of three oestrus synchronisation techniques in lactating dairy cows. *Anim Reprod Sci,* **71**: 157–168.

Alvarez, J.G. & Storey, B.T. 1992. Evidence for increased lipid peroxidation damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *J Androl,* **13**: 232–241.

Alvarez, J.G., Touchstone, J.C., Blasco, L., Storey, B. 1987. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa: superoxide dismutase as a major enzyme protectant against oxygen toxicity. *J Androl,* **8**: 338-348.

Aman, R.R., Parks, J.E. 1994. Effects of cooling and rewarming on the meiotic spindle and chromosomes of in vitro-matured bovine oocytes. *Biol Reprod,* **50**(1): 103-110.

Ambrose, J.D., Guzeloglu, A., Thatcher, M.J., Kassa, T., Diaz, T. and Thatcher, W.W. 1999. Long-term follicular dynamics and biochemical characteristics of dominant follicles in dairy cows subjected to heat-stress. *J Reprod Fertil Suppl,* **54**: 503–504 (Abstract).

Anderson, J.F., & Bates, D.W. 1984. Environmental-Management in Animal Agriculture - Curtis, SE. *J Am Vet Med Assoc,* **184**(5): 592-592.

Ando, M., Katagiri, K., Yamamoto, S., Wakamatsu, K., Kawahara, I., Asanuma, S., et al. 1997. Age-related effects of heat stress on protective enzymes for peroxides and microsomal monooxygenase in rat liver. *Environ Health Perspect,* **105**: 727–733.

Andrabi, S.M.H., Ansari, M.S., Ullah, N., Afzal, M. 2008. Effect of non-enzymatic antioxidants in extender on post-thaw quality of Buffalo (*Bubalus bubalis*) bull spermatozoa. *Pakistan Vet J*, **28**(4): 159-162.

Arav, A., Zeron, Y., Leslie, S.B., Behboodi, E., Anderson, G.B., Crowe, J.H. 1996. Phase transition temperature and chilling sensitivity of bovine oocytes. *Cryobiology*, **33**: 589–599.

Arav, A., Roth, Z. 2008. Do chilling injury and heat stress share the same mechanism of injury in oocytes? *Mol Cell Endocrinol*, **282**: 150–152.

Arechiga, C.F., Ealy, A.D., Hansen, P.J. 1995. Evidence that glutathione is involved in thermotolerance of preimplantation murine embryos. *Biol Reprod,* **52**: 1296–1301.

Arechiga, C.F. & Hansen, P.J. 1998. Response of preimplantation murine embryos to heat shock as modified by developmental stage and glutathione status. *In Vitro Cell Dev Biol Anim*, **34**: 655-659.

Arechiga, C.F., Staples, C.R., McDowell, L.R., Hansen, P.J. 1998a. Effects of timed insemination and supplemental beta-carotene on reproduction and milk yield of dairy cows under heat stress. *J Dairy Sci,* **81**: 390–402.

Arechiga, C.F., Vazquez-Flores, S., Ortiz, O., Hernandez-Ceron, J., Porras, A., McDowell, L.R., Hansen, P.J. 1998b. Effect of injection of beta-carotene or vitamin E and selenium on fertility of lactating dairy cows. *Theriogenology,* **50**: 65–76.

Armstrong, D.V. 1994. Heat stress interaction with shade and cooling. *J Dairy Sci,* **77**: 2044–2050.

Armstrong, J.S., Rajasekaran, M., Chamulitrat, W., Gatti, P., Hellstrom, W.J., Sikka, S.C. 1999. Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. *Free Radic Biol Med*, **26**: 869-880.

Aroyo, A., Yavin, S., Arav, A., Roth, Z. 2007. Maternal hyperthermia disrupts developmental competence of follicle-enclosed oocytes: in vivo and ex vivo studies in mice. *Theriogenology*. **15**: 1013–1021.

Atikuzzaman, M., Koo, O.J., Kang, J.T., Kang, J.T., Kwon, D.K. Park, S.J., Kim, S.J., Limas Gomez, M.N., Oh, H.J., Hong, S.G., Jang, G., Lee, B.C. 2011. The 9-cis retinoic acid signaling pathway and its regulation of prostaglandin-endoperoxide synthase 2 during in vitro maturation of pig cumulus cell-oocyte complexes and effects on parthenogenetic embryo production. *Biol Reprod,* **84**(6): 1272-1281.

Attaran, M., Pasqualotto, E., Falcone, T., Goldberg, J.M., Miller, K.F., Agarwal, A. and Sharma, R.K. 2000. The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. *Int J Fertil Womens Med,* **45**: 314–320.

Ax, R.L., Gilbert, G.R., Shook, G.E. 1987. Sperm in Poor Quality Semen from Bulls During Heat Stress Have a Lower Affinity for Binding Hydrogen-3 Heparin1. *J Dairy Sci,* **70**(1): 195-200.

Ayyat, M.S., Marai, I.F.M. 1998. Evaluation of application of the intensive rabbit production system under the subtropical conditions of Egypt. *J World Rabbit. Sci,* **6**: 213–217.

Badinga, L., Collier, R.J., Thatcher, W.W., Wilcox, C.J. 1985. Effects of climatic and management factors on conception rate of dairy cattle in subtropical environment. *J Dairy Sci,* **68**: 78–85.

Badinga, L., Thatcher, W.W., Diaz, T., Drost, M., Wolfenson, D. 1993. Effect of environmental heat stress on follicular development and steroidogenesis in lactating Holstein cows. *Theriogenology,* **39**: 797–810.

Badinga, L., Thatcher, W.W., Wilcox, C.J., Morris, G., Entwistle, K. and Wolfenson, D. 1994. Effect of season on follicular dynamics and plasma concentrations of oestradiol-17β, progesterone and luteinizing hormone in lactating Holstein cows. *Theriogenology,* **42**: 1263–1274.

Bailey, J., Bilodeau, J.F., Cormier, N. 2000. Semen Cryopreservation in Minireview Domestic Animals: A Damaging and Capacitating Phenomenon. *J Androl*, **21**(1): 1-7.

Baker, M.A., Aitken, R.J. 2005. Reactive oxygen species in spermatozoa: methods for monitoring and significance for the origins of genetic disease and infertility. *Reprod Biol Endocrinol*, **3**: 67.

Ball, G.D., Leibfried, M.L., Lenz, R.W., Ax, R.L., Bavister, B.D., First, N.L. 1983. Factors affecting successful in Vitro fertilization of bovine follicular oocytes. *Biol Reprod,* **28**: 717-725.

Banks, S., King, S.A., Irvine, D.S. and Saunders, P.T. 2005. Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa. *Reproduction,* **129**: 505-514.

Bansal, A.K., Bilaspuri, G.S. 2007. Effect of ferrous ascorbate on *in vitro* capacitation and acrosome reaction in cattle bull spermatozoa. *Anim Sci Report*, **1**(2): 69–77.

Baranski, B. 1993. Effects of the workplace on fertility and related reproductive outcomes. *Environ Health Perspect,* **101**(Suppl 2): 81–90.

Baselga, M. 2004. Genetic improvement of meat rabbits. Programmes and diffusion. Proc. 8th World Rabbit Congr, **1**: 1-13.

Baserga, R. 1999. The IGF-I receptor in cancer research. *Exp Cell Res,* **253**(1): 1-6.

Bassuny, S.A. 1999. Performance of doe rabbits and their weanlings as affected by heat stress and their alleviation by nutritional means, under Egyptian conditions. *Egyptian J Rabbit Sci,* **9**: 61–72.

Bauche, F., Fouchard, M., Jegou, B. 1994. Antioxidant system in rat testicular cells. *FEBS Lett,* **349**: 392–396.

Baumann, C.G., Morris, D.G., Sreenan, J.M., Leese, H.J. 2007. The quiet embryo hypothesis: molecular characteristics favouring viability. *Mol Reprod Dev*, **74**: 1345-1353.

Baumber, J., Ball, B.A., Gravance, C.G., Medina, V., Davies-Morel, M.C.G. 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. *J Androl*, **21**: 895-902.

Beam, S.W., Butler, W.R. 1999. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. *J Reprod Fertil Suppl,* **54**: 411–424.

Beconi, M.T., Francia, C.R., Mora, N.G., Affranchino, M.A. 1993. Effect of natural antioxidants on frozen bovine semen preservation. *Theriogenology*, **40**: 841‑851.

Beere, H.M., Green, D.R. 2001. Stress management—heat shock protein-70 and the regulation of apoptosis. *Trends Cell Biol,* **11**: 6-10.

Behrman, H.R. & Aten, R.F. 1991. Evidence that peroxide blocks hormone-sensitive cholesterol transport into mitochondria of rat luteal cells. *Endocrinology,* **128**: 2958–2966.

Behrman, H.R., Kodaman, P.H., Preston, S.L., Gao, S. 2001. Oxidative stress and the ovary. *J Soc Gynecol Investig,* **8**: S40–42.

Bellvé, A.R. 1972. Viability and survival of mouse embryos following parental exposure to high temperature. *J Reprod Fert,* **30**: 71-81.

Bennett, I.L., Finch, V.A., Holmes, C.R. 1985. Time spent in shade and its relationship with physiological factors of thermoregulation in three breeds of cattle. *Appl Anim Behav Sci*, **13**: 227–236.

Berman, A., Folman Y., Kaim M., Mamen M., Hertz Z., Wolfensen D., Arieli A. and Graber Y. 1985. Upper critical temperature and forced ventilation effects for high yielding dairy cows in a subtropical climate. *J Dairy Sci*, **68**: 1488-1495.

Berman, A., Wolfenson, D. 1992. Environmental modifications to improve production and fertility. In: Van Horn, H.H., Wilcox, C.J. Eds.., Large Dairy Herd Management. American Dairy Science Association, Champaign, IL, pp. 126–134.

Bernardini, C., Fantinati, P., Zannoni, A., Forni, M., Tamanini, C., Bacci, M.L. 2004. Expression of HSP70/HSC70 in swine blastocysts: effects of oxidative and thermal stress. *Mol Reprod Dev,* **69**: 303–307.

Besenfelder, U.L., Solti, J., Seregi, M., Müller, M., Brem, G. 1996. Different roles for B-carotene and vitamin A in the reproduction of rabbits. *Theriogenology*, **45**: 1583–1591.

Bianca, W. 1962. Relative importance of dry and wet-bulb temperatures in causing heat stress in cattle. *Nature,* **195**: 251–260.

Bilodeau, J.F., Blanchette, S., Gagnon, I.C., Sirard, M.A. 2001. Thiols prevent H2O2-mediated loss of sperm motility in cryopreserved bull semen. *Theriogenology*, **56**: 275‑286.

Bilodeau, J.F., Chatterjee, S., Sirard, M.A., Gagnon, C. 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev,* **55**: 282–288.

Bilodeau, J.F., Mirault, M.E. 1999. Increased resistance of GPx-1 transgenic mice to tumor promoter-induced loss of glutathione peroxidase activity in skin. *Int J Cancer,* **80**: 863–867.

Bilodeau, J.F., Sirard, M.A. 2003. Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. *Theriogenology*, **59**: 939-949.

Bitman, J., Lefcourt, A., Wood, D.L., Stroud, B. 1984. Circadian and ultradian temperature rhythms of lactating dairy cows. *J Dairy Sci,* **67**(5): 1014-1023.

Blackshaw, J.K. & Blackshaw, A.W. 1994. Heat-Stress in Cattle and the Effect of Shade on Production and Behavior. *Aust J Exp Agr,* **34**(2): 285-295.

Block, J., Chase, C.C., Hansen, P.J. 2002. Inheritance of resistance of bovine preimplantation embryos to heat shock: relative importance of the maternal vs. paternal contribution. *Mol Reprod Dev,* **63**: 32-37.

Blomhoff, R., Blomhoff, H.K. 2006. Overview of Retinoid Metabolism and Function. *J Neurobiol*, **66**: 606-630.

Blondin P., Coenen K., Sirard M.A. 1997. The impact of reactive oxygen species on bovine sperm fertilizing ability and oocyte maturation. *J Androl,* **18**(4): 454-460.

Bolet, G., Brun, J.M., Monnerot, M., Abeni, F., Arnal, C., Arnold, J., et al. 2000. Evaluation and conservation of European rabbit (Oryctolagus cuniculus) genetic resources. First results and inferences. *World Rabbit Sci*, **8**(A): 281–315.

Bond, T.E., Kelly, C.F. 1955. The globe thermometer in agricultural research. *Agr Eng,* **36**: 251-255.

Borg, K. E., Lunstra, D.D., Christenson, R.K. 1993. Semen characteristics, testicular size, and reproductive hormone concentrations in mature Duroc, Meishan, Fengjing, and Minzhu boars. *Biol Reprod,* **49**: 515-521.

Bridges, P.J., Brusie, M.A., Fortune, J.E. 2005. Elevated temperature (heat stress) in vitro reduces androstenedione and estradiol and increases progesterone secretion by follicular cells from bovine dominant follicles. *Dom Anim Endocrinol,* **29**: 508-522.

Brigelius-Flohe, R., Aumann, K.D., Blocker, H., Gross, G., Kiess, M., Kloppel, K.D., Maiorino, M., Roveri, A., Schuckelt, R., Ursini, F., Wingender, E., Flohé, L. 1994. Phospholipid-hydroperoxide glutathione peroxidase. Genomic DNA, cDNA, and deduced amino acid sequence. *J Biol Chem,* **269**: 7342–7348.

Brito, L.F.C., Silva, A.E.D.F., Marbosa, R.T., Unanian, M.M., Kastelic, J.P. 2003. Effects of scrotal insulation on sperm production, semen quality and testicular echotexture in Bos indicus and Bos indicus x Bos Taurus bulls. *Anim Reprod Sci*, **79**: 1-15.

Britt, J. H. 1975. Early postpartum breeding in dairy cows. A review. *J Dairy Sci,* **58**: 266-271.

Brodsky, J.L. & Chiosis, G. 2006. Hsp70 molecular chaperones: emerging roles in human disease and identification of small molecule modulators. *Curr Top Med Chem,* **6**: 1215–1225.

Bucak, M.N., Ateşşahin, A., Varişli, O., Yüce, A., Tekin, N., Akçay, A. 2007. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen Microscopic and oxidative stress parameters after freeze-thawing process. *Theriogenology*, **67**(5): 1060-1067.

Bucak, M.N., Ateşşahin, A., Yüce, A. 2008. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Rumin Res*, **75**(2): 128-134.

Bucak, M.N., Sariözkan, S., Tuncer, P.B. et al. 2010a. The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. *Small Rumin Res,* **89**(1): 24-30.

Bucak, M.N., Tuncer, P.B., Sanözkan, S., Baspinar, N., Taspinar, M., Coyan, K., Bilgili, A., Akalin, P.P., Büyükleblebici, S., Aydos, S., Ilgaz, S., Sunguroglu, A., Oztuna, D. 2010b. Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters. *Cryobiology,* **61**(3): 248-253.

Buccione, R., Schroeder, A.C., Eppig, J.J. 1990. Interactions between somatic cells and germ cells throughout mammalian oogenesis. *Biol Reprod,* **43**: 543–547.

Bucholtz, D.C., Vidwans, N.M., Herbosa, C.G., Schillo, K.K., Foster, D.L. 1996. Metabolic interfaces between growth and reproduction: pulsatile luteinizing hormone secretion is dependent on glucose availability. *Endocrinology,* **137**: 601–607.

Bucklin, R.A., Turner, L.W., Beede, D.K., Bray, D.R., Hemken, R.W. 1991. Methods to relieve heat stress for dairy cows in hot, humid climates. *Appl Eng Agric*, **7**: 241–247.

Burfening, P.J., Elliott, D.S., Eisen, E.J. & Ulberg, L.C. 1970. Survival of embryos resulting from spermatozoa produced by mice exposed to elevated ambient temperature. *Anim Sci,* **30**: 578-582.

Burfening, P.J., Ulberg, L.C. 1968. Embryonic survival subsequent to culture of rabbit spermatozoa at 38° and 40ºC. *J Reprod Fert,* **15:** 87-92.

Butler, W.R. 2000. Nutritional interactions with reproductive performance in dairy cattle. *Anim Reprod Sci*, **60-61**: 449-457.

Butler, W.R. 2001. Nutritional effects on resumption of ovarian cyclicity and conception rate in postpartum dairy cows. In Diskin MG, editor. Fertility in the high-producing dairy cow, vol. 26. BSAS Edinburgh, Occasional Publication; 133–145.

Calamari, L., Maianti, M.G., Amendola, F., Lombardi, G. 1999. On some aspects of the oxidative status and on antioxidants in blood of dairy cows during summer. *Proceedings of 13th Associazione Scientifica Produzioni Animali Congress*, Piacenza, Italy, 449-451.

Calus, M.P., Windig, J.J., Veerkamp, R.F. 2005. Associations among descriptors of herd management and phenotypic and genetic levels of health and fertility. *J Dairy Sci,* **88**: 2178–2189.

Calvin, H.I., Grosshan, K., Blake, E.J. 1986. Estimation and manipulation of glutathione levels in prepubertal mouse ovaries and ova: relevance to sperm nucleus transformation in the fertilized egg. *Gamete Res*, **14**: 265-275.

Camargo, L.S., Viana, J.H., Ramos, A.A, Serapiao, R.V., de Sa, W.F., Ferreira, A.M., Guimaraes, M.F., do Vale Filho, V.R. 2007. Developmental competence and expression of the Hsp 70.1 gene in oocytes obtained from *Bos indicus* and *Bos taurus* dairy cows in a tropical environment. *Theriogenology,* **68**: 626–632.

Can, A., Semiz, O., Cinar, O. 2005. Bisphenol-A induces cell cycle delay and alters centrosome and spindle microtubular organization in oocytes during meiosis. *Mol Hum Reprod,* **11**: 389–396.

Cao, W., Huang, P., Zhang, L., Wu, H.Z., Zhang, J., Shi, F.X. 2009. Acute heat stress increases HSP70 expression in the testis, epididymis and vas deferens of adult male mice. *Zhonghua Nan Ke Xue.* **15**(3): 200-206.

Carlson, J.C., Sawada, M., Boone, D.L., Stauffer, J.M. 1995. Stimulation of progesterone secretion in dispersed cells of rat corpora lutea by antioxidants. *Steroids,* **60**: 272–276.

Cartwright, T.C. 1955. Responses of beef cattle to high ambient temperatures. *J Anim Sci,* **14**: 350–362.

Casady, R.B., Myers, R.M., Legates, J.E. 1953. The effect of exposure to high ambient temperature on spermatogenesis in the dairy bull. *J Dairy Sci,* **36**: 14-20.

Castellini, C., Lattaioli, P., Bernardini, M., Dal Bosco, A. 2000. Effect of dietary α-tocopheryl acetate and ascorbic acid on rabbit semen stored at 5ºC. *Theriogenology*, **54**: 523-533.

Cavestany, D., E1-Wishy, A.B. and Foote, R.H. 1985. Effect of season and high environmental temperature on fertility of Holstein cattle. *J Dairy Sci*, **68**: 1471-1478.

Celeghini, E.C.C., Arruda, R.P.; Andrade, A.F.C.; Nascimento, J., Raphael, C.F. 2007. Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes. *Reprod Dom Anim*, **42**: 479-488.

Cetica, P.D., Pintos, L.N., Dalvit, G.C., Beconi, M.T. 2001. Antioxidant enzyme activity and oxidative stress in bovine oocyte in vitro maturation. *IUBMB Life*. **51**(1): 57-64.

Chandolia, R.K., Peltier, M.R., Tian, W., Hansen, P.J. 1999. Transcriptional control of development, protein synthesis and heat-induced heat shock protein 70 synthesis in 2-cell bovine embryos. *Biol Reprod,* **61**: 1644–1648.

Chaudiere, J., Ferrari-Illiou, R. 1999. Intracellular antioxidant from chemical to biochemical mechanism. *Food Chem Toxicol*, **37**: 949–962.

Chen, L., Wert, S.E., Hendrix, E.M., Russell, P.T., Cannon, M., Larsen, W.J. 1990. Hyaluronic acid synthesis and gap junction endocytosis are necessary for normal expansion of the cumulus mass. *Mol Reprod Dev,* **26**: 236-247.

Cheon, Y.M., Kim, H.K., Yang, C.B., Yi, Y.J. and Park, C.S. 2002. Effect of Season Influencing Semen Characteristics, Frozen-Thawed Sperm Viability and Testosterone Concentration in Duroc Boars. *Asian-Aust J Anim Sci,* **15**(4): 500-503.

Chew, B.P., Holpuch, D.M., O'Fallon, J.V. 1984. Vitamin A and beta-carotene in bovine and porcine plasma, liver, corpora lutea, and follicular fluid. *J Dairy Sci*, **67**: 1316-1322.

Chiamenti, A., Aguiar Filho, C.R., Freitas Neto, L.M., Chaves, R.M., Paula-Lopes, F.F., Lima, P.F., *et al.* 2010. Effects of retinoids on the in vitro development of *Capra hircus* embryos to blastocysts in two different culture systems. *Reprod Domest Anim,* **45**: e68–72.

Chian, R.C., Sirard, M.A. 1995. Effects of cumulus cells and follicle stimulating hormone during in vitro maturation on parthenogenetic activation of bovine oocytes. *Mol Reprod Dev*, **42**: 425-431.

Chou, J.P., Yi-Chuan, L., Chen-Chao, C. 1974. Effects of heating on rabbit spermatogenesis. *Chinese Medical Journal.* **6**: 365-367.

Chow, C.K. 1979. Nutritional influence on cellular antioxidant defense systems. *Am J Clin Nutr,* **32**: 1066-1081.

Clagett-Dame, M., Knutson, D. 2011. Vitamin A in reproduction and development. *Nutrients*, **3**: 385-428.

Clermont, Y. 1972. Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev*, **52**: 198-236.

Collier, R.I., Beede, D.K., Thatcher, W.W., Israel, L.A., Wilcox, C.J. 1992. Influences of environment and its modification on dairy animal health and production. *J Dairy Sci,* **65**: 2213-2227.

Collier, R.J., Dahl, G.E., VanBaale, M.J. 2006. Major advances associated with environmental effects on dairy cattle. *J Dairy Sci,* **89**: 1244-1253.

Collier, R.J., Eley, R.M., Sharma, A.K., Pereira, R.M., Buffington, D.E. 1981. Shade management in subtropical environment for milk yield and composition in Holstein and Jersey cows. *J Dairy Sci*, **64**(5): 844-849.

Collins, P. & Lacy, D. 1969. Studies on the structure and function of the mammalian testis. II. Cytological and histochemical observations on the testis of the rat after a single exposure to heat applied for different lengths of time. *Proc R Soc Lond B Biol Sci,* **172**: 17–38.

Comporti, M. 1989. Three models of free radical induced cell injury. *Chem Biol Interact,* **72**: 1-56.

Conner, S., Leaf, D., Wessel, G. 1997. Members of the SNARE hypothesis are associated with cortical granule exocytosis in the sea urchin egg. *Mol Reprod Dev,* **48**: 106–118.

Córdova, B., Morató, R., Izquierdo, D., Paramio, T., Mogas, T. 2010. Effect of the addition of insulin transferrin-selenium and/or L-ascorbic acid to the in vitro maturation of prepubertal bovine oocytes on cytoplasmic maturation and embryo development. *Theriogenology*, **74**(8): 1341–1348.

Coss, R.A., Dewey, W.C. & Bamburg, J.R. 1982. Effects of hyperthermia on dividing Chinese hamster ovary cells and microtubules in vitro. *Cancer Res,* **42:** 1059–1071.

Coss, R.A. & Linnemans, W.A.M. 1996. The effects of hyperthermia on the cytoskeleton. *Int J Hyperthermia,* **12**: 173–196.

Cozzi, J., Monier-Gavelle, F., Lievre, N., Bomsel, M., Wolf, J.P. 2001. Mouse offspring after microinjection of heated spermatozoa. *Biol Reprod*, **65**: 1518-1521.

Craig, E.A. & Gross, C.A. 1991. Is hsp70 the cellular thermometer? *Trend Biochem Sci,* **16**: 135–140.

Cummins, J.M. 2004. The role of mitochondria in the establishment of oocyte functional competence. *Eur J Obstet Gynecol Reprod Biol,* **115**: S23–29.

Cummins, J.M. & Glover, T.D. 1970. Artificial cryptorchidism and fertility in the rabbit. *J Reprod Feri,* **23**: 423-434.

Curci, A., Bevilacqua, A., Mangia, F. 1987. Lack of heatshock response in preovulatory mouse oocytes. *Dev Biol*, **123**: 154-160.

Daader, A.H., Seleem, T.S.T. 1999. Recent trends in rabbit production. In: Proceedings of 1st International Conference on Indigenous Versus Acclimatized Rabbits, El-Arish, North Sinai, Egypt, 23–50.

Daader, A.H., Gabr, H.A., Bahgat, L.B., Zeidan, A.E.B., Selem, T.S.T. 1997. Effect of intramuscular injection of gonadotropin releasing hormone on semen characteristics of buck rabbits, under different seasons of the year. In: Proceedings of International Conference of Animal Production and Health, Dokki, Egypt, 587–592.

Darley-Usmar, V., Wiseman, H. and Halliwell, B. 1995. Nitric oxide and oxygen radicals: a question of balance. *FEBS Letters,* **369**: 131-135.

De Alba, J. & Riera, S. 1966. Sexual maturity and spermatogenesis under heat stress in the bovine. *Anim Prod,* **8**: 137-144 (Abstract).

Deb, G.K., Dev, S.R., Bang, J.I., Lee, J.G., Kong, I.K. 2012. 9-cis Retinoic acid inhibits cumulus cell apoptosis during the maturation of bovine cumulus-oocyte-complexes. *J Anim Sci*, **90**(6): 1798-1806.

De Castro, L.A., Hansen, P.J. 2007. Interactions between oxygen tension and glucose concentration that modulate actions of heat shock on bovine oocytes during in vitro maturation. *Theriogenology*, **68**: 763–770.

De Lamirande, E., Gagnon, C. 1992. Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. *J Androl*, **13**: 379-386.

De Lamirande, E., Gagnon, C. 1992. Reactive oxygen species and human spermatozoa I Effects on the motility of intact spermatozoa and on sperm anoxemes. *J Androl*, **13**(5): 379-386.

De Lamirande, E., Gagnon, C. 1995. Impact of reactive oxigen species on spermatozoa: a balancing act between beneficial and detrimental effects. *Hum Reprod,* **10**(Suppl 1): 15-21.

De Lamirande, E., Jiang, H., Zini, A., Kodama, H., Gagnon, C. 1997a. Reactive oxygen species and sperm physiology. *Rev Reprod,* **2**: 48–54.

De Lamirande, E., Leclerc, P., Gagnon, C. 1997b. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol Hum Reprod,* **3**: 175–194.

De la Sota, R.L., Burke, J.M., Risco, C.A., Moreira, F., De Lorenzo, M.A., Thatcher, W.W. 1998. Evaluation of timed insemination during summer heat stress in lactating dairy cattle. *Theriogenology*. **49**: 761–770.

Delasota, R.L., Lucy, M.C., Staples, C.R., Thatcher, W.W. 1993. Effects of Recombinant Bovine Somatotropin (Sometribove) on Ovarian-Function in Lactating and Nonlactating Dairy-Cows. *J Dairy Sci,* **76**(4): 1002-1013.

De Matos, D.G., Furnus, C.C., Moses, D.F., Martinez, A.G., Matkovic, M. 1996. Stimulation of glutathione synthesis of in vitro matured bovine oocytes and its effect on embryo development and freezability. *Mol Reprod Dev*, **45**: 451-457.

De Matos, D.G., Furnus, C.C. 2000. The Importance of having high glutathione (GSH) level after nbovine in vitro maturation on embryo development effect of beta-mercaptoethanol, cysteine and cystine. *Theriogenology*, **53**: 761-771.

Dercole, A.J., Applewhite, G.T., and Underwood, L.E. 1980. Evidence That Somatomedin Is Synthesized by Multiple Tissues in the Fetus. *Dev Biol,* **75**(2): 315-328.

De Rensis, F., Marconi, P., Capelli, T., Gatti, F., Facciolongo, F., Franzini, S., et al. 2002. Fertility in post-partum dairy cows in winteror summer following estrus synchronization and fixed time A.I. after the induction of an LH surge with gonadotropin releasing hormone (GnRH) or human chorionic gonadotropin (hCG). *Theriogenology,* **58**: 1675–1687.

De Rensis, F., Scaramuzzi, R.J. 2003. Heat stress and seasonal effects on reproduction in the dairy cow – a review. *Theriogenology*, **60**: 1139-1151.

Desai, N., Sharma, R., Makker, K., Sabanegh, E. and Agarwal, A. 2009. Physiologic and pathologic levels of reactive oxygen species in neat semen of infertile men. *Fertil Steril,* **92**(5): 1626-1631.

De S. Torres-Júnior, J.R., de F.A. Pires, M., de Sá, W.F., de M. Ferreira, A., Viana, J.H.M., Camargo, L.S.A., Ramos, A.A., Folhadella, I.M., Polisseni, J., de Freitas, C., Clemente, C.A.A., de Sá Filho, M.F., Paula-Lopes, F.F., Baruselli, P.S. 2008. Effect of maternal heat-stress on follicular growth and oocyte competence in Bos indicus cattle. *Theriogenology,* **69**: 155-166.

De Vita, R., Calugi, A., Chiarantano, C., Forte, D., Mauro, F. and Uccelli, R. 1990. Effects of heat on mouse spermatogenesis monitored by flow cytometry. *Int J Hyperthermia,* **6**: 543-551.

Devkota, B., Sadaki, M., Matsui, M., Takahashi, K.I., Matsuzaki, S., Koseki, T. And Miyake, Y.I. 2010. Effects of scrotal insulation and pathological lesions on α-smooth muscle actin (SMA) and vimentin in the bull testes. *J Reprod Develop,* **56**(2): 187-190.

Di Mascio, P., Kaiser, S., Sies, H. 1989. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch Biochem Biophys*, **274**: 532-538.

Dobao, M., Rodriganez, J. and Silio, S. 1983. Seasonal influence on fecundity and litter performance in Iberian pigs. *Livest Prod Sci,* **10**: 601-610.

Dong, J., Albertini, D.F., Nishimori, K., Kumar, T.R., Lu, N., Matzuk, M.M. 1996. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature,* **383**: 531-535.

Ducibella, T., Buetow, J. 1994. Competence to undergo normal, fertilization-induced cortical activation develops after metaphase I of meiosis in mouse oocytes. *Dev Biol,* **165**: 95-104.

Ducibella, T., Duffy, P., Buetow, J. 1994. Quantification and localization of cortical granules during oogenesis in the mouse. *Biol Reprod,* **50**: 467–473.

Duncan, R.F. & Hershey, J.W.B. 1989. Protein synthesis and protein phos-phorylation during heat stress, recovery, and adaptation. *J Cell Biol,* **109**: 1467–1481.

Dunlap, S.E. & Vincent, C.K. 1973. Influence of postbreeding thermal stress on conception rate in beef cattle. *J Anim Sci,* **32**(6): 1216-1218.

Duque, P., Diez, C., Royo, L., Lorenzo, P.L., Carneiro, G., Hidalgo, C.O., *et al.* 2002. Enhancement of developmental capacity of meiotically inhibited bovine oocytes by retinoic acid. *Hum Reprod,* **17**: 2706–2714.

Dutt, R.H. 1961. Temperature and light as factors in reproduction among farm animals. *J Dairy Sci*, **43**: 123-144.

Dutt, R.H. 1963. Critical period for early embryo mortality in ewes exposed to high ambient temperature. *J Anim Sci,* **22**: 713–719.

Dutt, R.H. & Simpson, E.C. 1957. Environmental temperature and fertility of Southdown rams early in the breeding season. *J Anim Sci,* **16**: 136-143.

Ealy, A.D., Arechiga, C.F., Bray, D.R., Risco, C.A., Hansen, P.J. 1994. Effectiveness of short-term cooling and vitamin E for alleviation of infertility induced by heat stress in dairy cows. *J Dairy Sci,* **77**: 3601–3607.

Ealy, A.D., Drost, M. and Hansen, P.J. 1993. Developmental changes in embryonic resistance to adverse effects of maternal heat stress in cows. *J Dairy Sci,* **76**: 2899-2905.

Ealy, A.D. & Hansen, P.J. 1994. Induced thermotolerance during early development of murine and bovine embryos. *J Cell Physiol*, **160**: 463-468.

Ealy, A.D., Howell, J.L., Monterroso, V.H., Arechiga, C.F., Hansen, P.J. 1995. Developmental changes in sensitivity of bovine embryos to heat shock and use of antioxidants as thermoprotectants. *J Anim Sci*, **73**: 1401–1407.

Eberhardt, D.M., Will, W.A., Godkin, J.D. 1999. Retinol administration to superovulated ewes improves in vitro embryonic viability. *Biol Reprod*, **60**: 1483–1487.

Edwards, J.L. & Hansen, P.J. 1996. Elevated temperature increases heat shock protein 70 synthesis in bovine two-cell embryos and compromises function of maturing oocyte. *Biol Reprod,* **55**: 340–346.

Edwards, J.L. & Hansen, P.J. 1997. Differential responses of bovine oocytes and preimplantation embryos to heat shock. *Molec Reprod Dev,* **46**: 138-145.

Edwards, J.L., Ealy, A.D., Hansen, P.J. 1995. Regulation of heat shock protein 70 synthesis by heat shock in the preimplantation murine embryo. *Theriogenology*, **44**: 329–337.

Edwards, J.L., Ealy, A.D., Monterroso, V.H., Hansen, P.J. 1997. Ontogeny of temperature-regulated heat shock protein 70 synthesis in pre-implantation bovine embryos. *Mol Reprod Dev,* **48**: 25–33.

Edwards, J.L., King, W.A., Kawarsky, S.J., Ealy, A.D. 2001. Responsiveness of early embryos to environmental insults: potential protective roles of HSP70 and glutathione. *Theriogenology*. **55**(1): 209-223.

Edwards, J.L., Saxton, A.M., Lawrence, J.L., Payton, R.R. & Dunlap, J.R. 2005. Exposure to a physiologically relevant elevated temperature hastens in vitro maturation in bovine oocytes. *J Dairy Sci,* **88**: 4326-4333.

Edwards, R.L., Omtvedt, I.T., Turman, E.J., Stephens, D.F., Mahoney, G.W.A. 1968. Reproductive performance of gilts following heat stress prior to breeding and in early gestation. *J Reprod Fertil,* **52**(Suppl): 47-61.

Egbunike, G.N. & Dede, T.I. 1979. The influence of short-term exposure to tropical sunlight on boar seminal characteristics. *Biol Reprod,* **20**: 235-241.

Eitel, K., Staiger, H., Mathias, D.B., Daniel, B., Bretzel, R.G., Haring, H.U., Kellerer, M. 2002. Different role of saturated and unsaturated fatty acid in cell apoptosis. *Biochem Biophys Res Commun*, **299**: 853–856.

Elnagar, S.A. 2010. Royal jelly counteracts bucks' "summer infertility". *Anim Reprod Sci,* **121**(1-2): 174-180.

El-Sayiad, G.H.A., Habeeb, A.A.M., El Maghawry, A.M. 1994. A note on the effects of breed, stage of lactation and pregnancy status on milk composition of rabbits. *Anim Prod*, **58**: 153-157.

El-Sherbiny, A.M. 1987. Seasonal variations in seminal characteristics of rabbits. M.Sc. Thesis, Faculty of Agriculture, Ain-Shams University, Cairo, Egypt.

El-Sherry, M.I., El-Naggar, M.A., Nassar, S.M. 1980. Experimental study of summer stress in rabbits. 2. The quantitative and qualitative pathogenesis of spermatogenic cell cycle in rabbits. *Assiut Vet Med J,* **7**(13/14): 17-31.

El-Sheshtawy, R.I., El-Sisy, G.A., El-Nattat, W.S. 2008. Use of Selected Amino Acids to Improve Buffalo Bull Semen Cryopreservation. *Global Veterinaria*, **2**(4): 146-150.

Elvin, J.A., Clark, A.T., Wang, P., Wolfman, N.M., Matzuk, M.M. 1999. Paracrine actions of growth differentiation factor-9 in the mammalian ovary. *Mol Endocrinol*, **13**: 1035-1048.

Eppig, J.J. 1982. The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation and cumulus expansion. *Dev Biol,* **89**: 268–272.

Eppig, J.J. 1991. Intercommunication between mammalian oocytes and companion somatic cells. *BioEssays*, **13**: 569-574.

Eppig, J.J. 2001. Oocyte control of ovarian follicular development and function in mammals. *Reproduction*. **122**: 829-838.

Eppig, J.J. & Downs, S.M. 1984. Chemical signals that regulate mammalian oocyte maturation. *Biol Reprod,* **30**: 1-11.

Eppig, J.J., O'Brien, M., Wigglesworth, K. 1996. Mammalian oocyte growth and development in vitro. *Mol Reprod Devel*, **44**: 260–273.

Eppig, J.J., Wigglesworth, K., Pendola, F., Hirao, Y. 1997. Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. *Biol Reprod,* **56**: 976-984.

Fahey, J., O'Sullivan, K., Crilly, J., Mee, J.F. 2002. The effect of feeding and management practices on calving rate in dairy herds. *Anim Reprod Sci*, **74**: 133-150.

Fair, T., Hyttel, P., Lonergan, P., Boland, M.P. 2001. Immunolocalization of nucleolar proteins during bovine oocyte growth, meiotic maturation and fertilization. *Biol Reprod,* **64**: 1516–1525.

Fan, H.Y. & Sun, Q.Y. 2004. Involvement of Mitogen-Activated Protein Kinase cascade during oocyte maturation and fertilization in mammals. *Biol Reprod,* **70**: 535–547.

FAO. 2001. FAO recognizes the increasingly important role of rabbit breeding. Press release 01/57. http://www.Fao.org/waicent/ois/press-ne/presseng/2001/pren0157.htm.

FAO. 2009. Livestock in the balance. State of Food and Agriculture 2009. FAO Rome. http://www.fao.org/docrep/012/i0680e/i0680e00.htm

FAO. 2011. World Livestock 2011. Livestock in food security. http://www.fao.org/docrep/014/i2373e/i2372e00.htm

Fatma, B.A., Nozha, C.F., Ines, D., Hamadi, A., Basma, H., Leila, A.K. 2009. Sperm quality improvement after date seed oil in vitro supplementation in spontaneous and induced oxidative stress. *Asian J Androl*, **11**(3): 393-398.

Faust, M.A., Mcdaniel, B.T., Robison, O.W., and Britt, J.H. 1988. Environmental and Yield Effects on Reproduction in Primiparous Holsteins. *J Dairy Sci*, **71**(11): 3092-3099.

Fernández-Santos, M.R., Domínguez-Rebolledo, A.E., Esteso, M.C., Garde, J.J., Martínez-Pastor, F. 2009. Refrigerated Storage of Red Deer Epididymal Spermatozoa in the Epididymis, Diluted and with Vitamin C Supplementation. *Reprod Dom Anim,* **44**: 212–220.

Ferreira, E.M., Vireque, A.A., Adona, P.R., Meirelles, F.V., Ferriani, R.A., Navarro, P.A.A.S. 2009. Cytoplasmic maturation of bovine oocytes: Structural and biochemical modifications and acquisition of developmental competence. *Theriogenology*, **71**(5): 836-848.

Finch, V.A. 1986. Body-Temperature in Beef-Cattle - Its Control and Relevance to Production in the Tropics. *J Anim Sci,* **62**(2): 531-542.

Finch, V.A., Bennett, I.L. and Holmes, C.R. 1982. Sweating Response in Cattle and Its Relation to Rectal Temperature, Tolerance of Sun and Metabolic-Rate. *J Agric Sci,* **99**(Dec): 479-487.

Findlay, J.K. 1993. An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis. *Biol Reprod,* **48**: 15–23.

Finzi, A., Morera, P., Kuzminsky, G., 1988. Acclimation and repeatability of thermotolerance parameters in rabbit. In: *Proc. 4th World Rabbit Congr*, **2**: 419-426.

Finzi, A., Morera, P., Kuzminsky, G. 1995. Sperm abnormalities as possible indicators of rabbits chronic heat stress. *J World Rabbit Sci,* **3**(4): 157–161.

Finzi, A., Morera, P., Kuzminsky, G. 2010. Sperm abnormalities as possible indicators of rabbit chronic heat stress. *World Rabbit Sci*, **3**(4): 157-161.

Fisher, H.M. & Aitken, R.J. 1997. Comparative analysis of the ability of precursor germ cells and epididymal spermatozoa to generate reactive oxygen metabolites. *J Exp Zoolog,* **277**: 390–400.

Fissore, R.A., Kurokawa, M., Knott, J., Zhang, M., Smyth, J. 2002. Mechanisms underlying oocyte activation and postovulatory aging. *Reproduction*, **124**: 745–754.

Flesch, F.M., Gadella, B.M. 2000. Dynamics of the mammalian sperm membrane in the process of fertilization. *Biochim Bioph Acta*, **1469**: 197-235.

Foote, R.H.F. 1996. Review: Dairy cattle reproductive physiology research and management-past progress and future prospects. *J Dairy Sci*, **79**: 980-990.

Foote, R.H.F. 1967. Factors prolonging survival of unfrozen bovine spermatozoa. *J Dairy Sci,* **50**: 1338–1340.

Foote, R.H.F., Brockett, C.C., Kaproth, M.T. 2002. Motility and fertility of bull sperm in whole milk exgtender containing antioxidants. *Anim Reprod Sci*, **71**(1-2): 13-23.

Fouladi-Nashta, A.A., Gutierrez, C.G., Gong, J.G., Garnsworthy, P.C., Webb, R. 2007. Impact of dietary fatty acids on oocyte quality and development in lactating dairy cows. *Biol Reprod,* **77**: 9–17.

Fouladi-Nashta, A.A., Gutierrez, C.G., Russell, H.F., Garnsworthy, P.C.,Webb, R. 2004. Effects of dietary fatty acids on oocyte quality and development in lactating dairy cows. *Biol Reprod* (Special Issue), Abstract 107.

Fowler, D.G. & Dun, R.B. 1966. Skin folds and merino breeding. The susceptibility of rams selected for a high degree of skin wrinkle to heat induced infertility. *Aust J exp Agrie Anim Husband,* **6**: 121-127.

Franci, O., Amici, A., Margarit, R., Merendino, N., Piccolella, E. 1996. Influence of Thermal and Dietary Stress on Immune Response of Rabbits. *J Anim Sci,* **74**: 1523-1529.

Fuquay, J.W. 1981. Heat-Stress as It Affects Animal Production. *J Anim Sci*, **52**(1): 164-174.

Gallicano, G.I. 2001. Composition, regulation and function of the cytoskeleton in mammalian eggs and embryos. *Frontiers in Bioscience*, **6**: 1089–1108.

Galloway, S.M., McNatty, K.P., Cambridge, L.M., Laitinen, M.P., Juengel, J.L., Jokiranta, T.S., McLaren, R.J., Luiro, K., Dodds, K.G., Montgomery, G.W. et al. 2000. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosagesensitive manner. *Nature Genet,* **25**: 279-283.

Gambhir, K.K., Ahluwalia, B.S. 1975. Vitamin A in bovine sperm acrosomes*. J Reprod Fertil*, **43**(1): 129-132.

Gandini, L., Lombardo, F., Paoli, D., Caponecchia, L., Familiari, G., Verlengia, C., et al. 2000. Study of apoptotic DNA fragmentation in human spermatozoa. *Hum Reprod*, **15**: 830–839.

Gangwar, P.C., Branton, C. and Evans, D.L. 1965. Reproductive and physiological response of Holstein heifers to controlled and natural climatic conditions. *J Dairy Sci,* **48**: 222-227.

García-Ispierto, I., López-Gatius, F., Bech-Sabat, G., Santolaria, P., Yaniz, J.L., Nogareda, C., De Rensis, F. and López-Bejar, M. 2007. Climate factors affecting conception rate of high-producing dairy cows in northeastern Spain. *Theriogenology,* **67**: 1379-1385.

García-Ispierto, I., López-Gatius, F., Santolaria, P., Yaniz, J.L., Nogareda, C., López-Bejar, M., and De Rensis, F. 2006. Relationship between heat stress during the periimplantation period and early fetal loss in dairy cattle. *Theriogenology,* **65**(4): 799-807.

García-Tomás, M., Tusell, Ll., López-Béjar, M., Ramon, J., Rafel, O., Piles, M. 2008. Influence of environmental temperature and relative humidity on quantitative and qualitative semen traits of rabbits. (Abstract). 9th World Rabbit Congress, June 10-13, Verona, Italy, 359-364.

Gasinska, A. & Hill, S. 1990. The effect of hyperthermia on the mouse testis. *Neoplasma,* **37**: 357-366.

Gatzuli, E., Aten, R.F., Behrman, H.R. 1991. Inhibition of gonadotropic action and progesterone synthesis by xanthine oxidase in rat luteal cells. *Endocrinology,* **128**: 2253–2258.

Gaughan, J.B., Mader, T.L., Holt, S., Josey, M.J., Rowan, K.J. 1999. Heat tolerance of Boran and Tuli crossbred steers. *J Anim Sci*, **77**: 2398-2405.

Georgopoulos, C. & Welch, W.J. 1993. Role of themajor heat shock proteins as molecular chaperones. *Annu Rev Cell Biol,* **9**: 601–634.

Geva, E., Bartoov, B., Zabludovsky, N., Lessing, J.B., Lerner-Geva, L., Amit, A. 1996. The effect of antioxidant treatment on human spermatozoa and fertilization rate in an in vitro fertilization program. *Fertil Steril*, **66**(3): 430-434.

Gilad, E., Meidan, R., Berman, A., Graber, Y., Wolfenson, D. 1993. Effect of heat stress on tonic and GnRH-induced gonadotropin secretion in relation to concentration of estradiol in plasma of cyclic cows. *J Reprod Fertil,* **99**: 315-321.

Gómez, E., Caamaño, J.N., Rodríguez, A., De Frutos, C., Facal, N., Díez, C. 2006. Bovine Early Embryonic Development and Vitamin A. *Reprod Dom Anim*, **41**(Suppl. 2): 63–71.

Gomez, E., Rodriguez, A., Goyache, F., Diez, C., Jose Royo, L., Moreira, P.N., *et al.* 2004. Retinoid-dependent mRNA expression and poly-(A) contents in bovine oocytes meiotically arrested and/or matured *in vitro*. *Mol Reprod Dev,* **69**: 101–108.

Gomez, E., Royo, L.J., Duque, P., Carneiro, G., Hidalgo, C., Goyache, F., *et al.* 2003. 9-*cis*-Retinoic acid during *in vitro* maturation improves development of the bovine oocyte and increases midkine but not IGF-I expression in cumulus–granulosa cells. *Mol Reprod Dev,* **66**: 247–255.

Gómez, E., Rodríguez, A., Muñoz, A., Caamaño, J.N., Carrocera, S., Martín, D., Facal, N., Díez, C. 2008. Development and quality of bovine morulae cultured in serum-free medium with specific retinoid receptor agonists. *Repro Fertil Develop*, **20**(8): 884–891.

Gonçalves, F.S., Barretto, L.S.S., Arruda, R.P., Perri S.H.V. and Mingoti G.Z. 2010. Effect of antioxidants during bovine in vitro fertilization procedures on spermatozoa and embryo development. *Reprod Domest Anim,* **45**: 129-135.

Gong, J.G. 2002. Influence of metabolic hormones and nutrition on ovarian follicle development in cattle: practical implications. *Dom Anim Endocrin,* **23**(1-2): 229-241.

Gosden, R., Nagano, M. 2002. Preservation of fertility in nature and ART. *Reproduction,* **123**(1): 3-11.

Grinsted, J., Kjer, J.J., Blendstrup, K., Pedersen, J.F. 1985. Is low temperature of the follicular fluid prior to ovulation necessary for normal oocyte development? *Fertil Steril,* **43**: 34-39.

GrØndahl, C., Greve, T., Schmidt, M., Hunter, R.H.F. 1996. Bovine preovulatory follicles are cooler than ovarian stroma and deep rectal temperature. *Theriogenology*. **45**: 289.

Grossman, A.D., Erickson, J.W. and Gross, C.A. 1984. The htpR gene product of E. coli is a sigma factor for heat-shock promoters. *Cell,* **38**(2): 383-390.

Guérin, P., El Mouatassim, S., Menezo, Y. 2001. Oxidative stress and protection against reactive oxygen species in the preimplantation embryo and its sorroundings. *Hum Reprod Update,* **7**: 175-189.

Guille, J.J.P. & Joenje, H. 1991. Biological significance of oxygen toxicity: an introduction. In Membrane Lipid Oxidation (Vigo-Pelfrey, C., ed). pp. 1–32, CRC Press, Inc., Boca Raton, FL.

Guillomot, M., Fléchon, J.E., Leroy, F. 1993. Blastocyst development and implantation. Thibault C, Levasseur MC, Hunter RHF, editors. Reproduction in mammals and man. Paris: Ellipses; 387–410.

Guthrie, H.D., Welch, G.R. 2006. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in percoll-treated viable boar sperm using fluorescence-activated flow cytometry. *J Anim Sci*, **84**: 2089‑2100.

Guzeloglu, A., Ambrose, J.D., Kassa, T., Diaz, T., Thatcher, M.J., Thatcher, W.W. 2001. Long term follicular dynamics and biochemical characteristics of dominant follicles in dairy cows subjected to acute heat stress. *Anim Reprod Sci,* **66**: 15–34.

Gwazdauskas, F.C. 1984. Effects of climate on reproduction in cattle. *J Dairy Sci,* **68**: 1568-1578.

Gwazdauskas, F.C., Thatcher, W.W., Kiddy, C.A., Pape, M.J., Wilcox, C.J. 1981. Hormonal pattern during heat stress following PGF2alpha-tham salt induced luteal regression in heifers. *Theriogenology,* **16**: 271–285.

Gwazdauskas, F.C., Thatcher, W.W. and Wilcox, C.J. 1973. Physiological, Environmental, and Hormonal Factors at Insemination Which May Affect Conception. *J Dairy Sci,* **56**(7): 873-877.

Gwazdauskas, F.C., Wilcox, C.J., Tatcher, W.W. 1975. Environmental and managemental factors affecting conception rate in a subtropical climate. *J Dairy Sci,* **58**(1): 88-92.

Habeeb, A.A.M., Aboul-Naga, A.I., Khadr, A.F. 1999. Deterioration effect of summer hot climate on bunnies of acclimatized rabbits during suckling period. In: Proceedings of 1st International Conference on Indigenous Versus Acclimatized Rabbits, El-Arish, North Sinai, Egypt, 253–263.

Hahn, G.L. 1969. Predicted vs measured production differences using summer air conditioning for lactating cows. *J Dairy Sci,* **52**(6): 800-802.

Hahn, G.L. 1976. Rational environmental planning for efficient livestock production. *Biometeorology,* **6**(part II): 106–108.

Hahn, G.L. 1981. Housing and management to reduce climatic impacts on livestock. *J Anim Sci,* **52**(1): 175-186.

Hajializadeh, N., Babaei, H., Nematollahi-Mahani, S.N., Azizollahi, S. 2008. The development of mouse early embryos *in vitro* in fibroblasts and cumulus cells cocultures supplemented with retinoic acid. *Iranian J Vet Res,* **9**(1,22): 1-8.

Haliloglu, S., Baspinar, N., Serpek, B., Erdem, H., Bulut, Z. 2002. Vitamin A and beta-carotene levels in plasma, corpus luteum and follicular fluid of cyclic and pregnant cattle. *Reprod Domest Anim*, **37**: 96-99.

Halliwell, B. & Gutteridge, J.M. 1988. Free radicals and antioxidant protection: mechanisms and signi. cance in toxicology and disease. *Hum Toxicol,* **7**: 7–13.

Halliwell, B., Gutteridge, J.M.C., Cross, C.E. 1992. Review article: free radicals, antioxidants and human disease: where are we now? *J Lab Clin Med*, **119**: 598–620.

Hand, J.W., Walker, H., Hornsey, S., Field, S.B. 1979. Effects of hyperthermia on the mouse testis and its response to X-rays, as assayed by weight loss. *Int J Radiat Biol,* **35**: 521–528.

Hansen, P.J., 1997a. Effects of environment of bovine reproduction. In: Youngquist, R.S. Ed., Current Therapy in Large Animal Theriogenology. W.B. Saunders, Philadelphia, PA, 403–415.

Hansen, P.J. 1997b. Strategies for enhancing reproduction of lactating dairy cows exposed to heat stress. In: Proceedings of the 16th Annual Convention American Embryo Transfer Association, Madison, 62–72.

Hansen, P.J. 2002. Embryonic mortality in cattle from the embryo's perspective. *J Anim Sci*, **80**(E Suppl 2): E33–E44.

Hansen, P.J. 2005. Managing the Heat-Stressed Cow to Improve Reproduction. *Proceedings of the 7th Western Dairy Management Conference*, Reno, NV, 63-75.

Hansen, P.J. 2007a. Improving dairy cow fertility through genetics. *44th Florida Dairy Production Conference, Proceedings*, 23-30.

Hansen, P.J. 2007b. To be or not to be - Determinants of embryonic survival following heat shock. *Theriogenology*, **68S**: S40–S48.

Hansen, P.J. 2009. Effects of heat stress on mammalian reproduction. *Phil Trans R Soc B,* **364**: 3341–3350.

Hansen, P.J. & Arechiga, C.F. 1999. Strategies for managing reproduction in the heat stressed dairy cow. *J Dairy Sci,* **82**(Suppl. 2): 36-50.

Hansen, P.J., Drost, M., Rivera, R.M., Paula-Lopes, F.F., Al-Katanani, Y.M., Krininger, C.E. and Chase, C.C. 2001. Adverse impact of heat stress on embryo production: Causes and strategies for mitigation. *Theriogenology*, **55**(1), 91-103.

Harmon, R.J., Lu, M., Trammell, D.S., Smith, B.A., Spain, J.N., Spiers, D. 1997. Influence of heat stress and calving on antioxidant activity in bovine blood. *J Dairy Sci,* **80**: 264 (Abstract).

Harrison, R. & Weiner, J. 1948. Abdomino-testicular temperature gradients. *J Physiol,* **18**: 256–262.

Heitman, H., Cockrell, J.R. 1984. Cycling ambient temperature effect on boar semen. *Anim Prod*, **38**: 129-132.

Hendin, B.N., Kolettis, P.N., Sharma, R.K., Thomas, A.J. Jr., Agarwal, A. 1999. Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. *J Urol*, **161**(6): 1831-1834.

Hendrey, J.J. & Kola, I. 1991. Thermolability of mouse oocytes is due tothe lack of expression and/or inducibility of Hsp70. *Mol Reprod Dev,* **25**: 1–8.

Hendricks, K.E., Martins, L., Hansen, P.J. 2009. Consequences for the bovine embryo of being derived from a spermatozoon subjected to post-ejaculatory aging and heat shock: development to the blastocyst stage and sex ratio. *J Reprod Develop,* **55**(1): 69-74.

Herrler, A., Krusche, C.A., Beier, H.M. 1998. Insulin and insulin-like growth factor-1 promote rabbit blastocyst development and prevent apoptosis. *Biol Reprod,* **59**: 1302–1310.

Hidalgo, C.O., Diez, C., Duque, P., Facal, N., Gomez, E. 2003. Pregnancies and improved early embryonic development with bovine oocytes matured *in vitro* with 9-*cis*-retinoic acid. *Reproduction,* **125**: 409–416.

Hidalgo, C., Diez, C., Duque, P., Prendes, J.M., Rodriguez, A., Goyache, F., *et al.* 2005. Oocytes recovered from cows treated with retinol become unviable as blastocysts produced in vitro. *Reproduction,* **129**: 411–421.

Hirao, Y., Yanagimachi, R. 1978. Temperature-dependence of sperm-egg fusion and post-fusion events in hamster fertilisation. *J Exp Zool,* **205**: 433–438.

Ho, Y.S., Dey, M.S. and Crapo, J.D. 1996. Antioxidant enzyme expression in rat lungs during hypoxia. *Am J Physiol,* **270**: 810–818.

Hogarth, C.A., Griswold, M.D. 2010. The key role of vitamin A in spermatogenesis. *J Clin Invest,* **120**(4): 956-962.

Holter, J.B., West, J.W., and McGilliard, M.L. 1997. Predicting ad libitum dry matter intake and yield of Holstein cows. *J Dairy Sci,* **80**(9): 2188-2199.

Holter, J.B., West, J.W., McGilliard, M.L., and Pell, A.N. 1996. Predicting ad libitum dry matter intake and yields of Jersey cows. *J Dairy Sci,* **79**(5): 912-921.

Hoodbhoy, T. & Talbot, P. 1994. Mammalian cortical granules: contents, fate, and function. *Mol Reprod Dev,* **39**: 439–448.

Hosoe, M., Shioya, Y. 1997. Distribution of cortical granules in bovine oocytes classified by cumulus complex. *Zygote,* **5**: 371–376.

Hourcade, J.D., Pérez-Crespo, M., Fernández-González, R., Pintado, B., Gutiérrez-Adán, A. 2010. Selection against spermatozoa with fragmented DNA after postovulatory mating depends on the type of damage. *Reprod Biol Endocrinol*, **8**: 9.

Howarth, B. 1969. Fertility in the ram following exposure to elevated ambient temperature and humidity. *Reprod Fert,* **19**: 179-183.

Howarth, B., Alliston, C.W. & Ulberg, L.C. 1965. Importance of uterine environment on rabbit sperm prior to fertilization. *J Anim Sci,* **24**: 1027-1032.

Howell, J.L., Fuquay, J.W., Smith, A.E. 1994. Corpus luteum growth and function in lactating Holstein cows during spring and summer. *J Dairy Sci,* **77**: 735-739.

Hsu, P.C., Liu, M.Y., Hsu, C.C., Chen, L.Y., Guo, Y.L. 1998. Effects of vitamin E and/or C on reactive oxygen species-related lead toxicity in the rat sperm. *Toxicology*, **128**(3): 169-179.

Hu, J.H., Tian, W.Q., Zhao, X.L., Zan, L.S., Xin, Y.P., Li, Q.W. 2011. The cryoprotective effects of vitamin B12 supplementation on bovine semen quality. *Reprod Domest Anim*, **46**(1): 66-73.

Huber, J.T. 1996. Amelioration of heat stress in dairy cattle. In: Phillips, C.J.C. Ed., Progress in Dairy Science. CAB Int. Oxon, UK, 211–243.

Hunter, R.H., Bogh, I.B., Einer-Jensen, N., Muller, S., Greve, T. 2000. Pre-ovulatory Graafian follicles are cooler than neighbouring stroma in pig ovaries. *Hum Reprod,* **15**: 273-283.

Hunter, R.H., Grondahl, C., Greve, T., Schmidt, M. 1997. Graafian follicles are cooler than neighbouring ovarian tissues and deep rectal temperatures. *Hum Reprod,* **12**: 95-100.

Hyttel, P., Fair, T., Callesen, H. and Greve, T. 1997. Oocyte growth, capacitation and final maturation in cattle *Theriogenology,* **47**: 23–32.

Hyttel, P., Viuff, D., Fair, T., Laurincik, J., Thomsen, P.D., Callesen, H., *et al.* 2001. Ribosomal RNA gene expression and chromosome aberrations in bovine oocytes and preimplantation embryos. *Reproduction*, **122**: 21–30.

Hyttel, P., Xu, K.P., Smith, S., Greve, T. 1986. Ultrastructure of in vitro oocyte maturation in cattle. *J Reprod Fertil,* **78**: 615–625.

Igono, M.O., Bjotvedt, G., Sanford-Crane, H.T. 1992. Environmental profile and critical temperature effects on milk production of Holstein cows in desert climate. *Int J Biometeorol,* **36**: 77–87.

Igono, M.O. & Johnson, H.D. 1990. Physiological Stress Index of Lactating Dairy-Cows Based on Diurnal Pattern of Rectal Temperature. *J Interdiscipl Cycle,* **21**(4): 303-320.

Igono, M.O., Johnson, H.D., Stevens, B.J., Krause, G.F., Shanklin, M.D. 1987. Physiological, productive, and economic benefits of shade, spray, and fan system versus shade for Holstein cows during summer heat. *J Dairy Sci,* **70**: 1069–1079.

Ikeda, S., Kitagawa, M., Imai, H., Yamada, M. 2005. The roles of vitamin A for cytoplasmic maturation of bovine oocytes. *J Reprod Dev,* **51**: 23–35.

Ikeda, M., Kodama, H., Fukuda, J., Shimizu, Y., Murata, M., Kumagai, J. and Tanaka, T. 1999. Role of radical oxygen species in rat testicular germ cell apoptosis induced by heat stress. *Biol Reprod*, **61**: 393-399.

Ingraham, R.H.G.D., Wager, W.D. 1971. Relationship of temperature and humidity to conception rate of Holstein cows in subtropical climate. *J Dairy Sci,* **57**: 476-481.

Ingraham, R.H., Stanley, R.W., Wagner, W.C. 1976. Relationship of temperature and humidity to conception rate of Holstein cows in Hawaii. *J Dairy Sci*, **59**: 2086–2090.

Ingraham, R.H., Stanley, R.W. and Wagner, W.C. 1979. Seasonal effects of tropical climate on shaded and nonshaded cows as measured by rectal temperature, adrenal cortex hormones, thyroid hormone, and milk production. *Am J Vet Res,* **40**: 1792–1797.

Irvine, D.S. 1996. Glutathione as a treatment for male infertility. *Rev Reprod*, **1**: 6-12.

Ivell, R. 2007. Lifestyle impact and the biology of the human scrotum. *Reprod Biol Endocrinol,* **5**: 15-22.

James, W.H. 1998. Hypotheses on mammalian sex ratio variation at birth. *J Theor Biol,* **192**: 113-116.

James, L.F., Panter, K.E., Nielsen, D.B., Molyneux, R.J. 1992. The effect of natural toxins on reproduction in livestock. *J Anim Sci,* **70**: 1573–1579.

Jannes, P., Spiessens, C., Van der Auwera, I., D'Hooghe, T., Verhoeven, G. And Vanderschueren, D. 1998. Male subfertility induced by acute scrotal heating affects embryo quality in normal female mice. *Hum Reprod,* **13**: 372-375.

Jeong, W.J., Cho, S.J., Lee, H.S., Deb, G.K., Lee, Y.S., Kwon, T.H., Kong, I.K. 2009. Effect of cytoplasmic lipid content on in vitro developmental efficiency of bovine IVP embryos. *Theriogenology*, **72**: 584-589.

Johnson, H.D. 1965. Environmental temperature and lactation (with special reference to cattle). *Int J Biom,* **9**: 103–116.

Johnson, H.D. Bioclimates and livestock. 1987. H.D. Johnson, (Ed.) Bioclimatology and adaptation of livestock. Elsevier, Amsterdam, 127.

Johnson, H.D., Ragasdale, A.C., Eheng, C.S. 1957. Influence of constant environmental temperatures on growth responses and physiological reactions of rabbits and cattle. *Mo Agric Exp Sta Res Bull,* **648**.

Johnson, H.D., Ragsdale, A.C., Berry, I.L., Shanklin, M.D. 1962. Effect of various temperature–humidity combinations on milk production of Holstein cattle. *Missour Agric Exp Stn Res Bull,* **791**: 1–39.

Johnson, H.D., Ragsdale, A.C., Berry, I.L., Shanklin, M.D. 1963. Temperature-humidity effects including influence of acclimation in feed and water consumption of Holstein cattle. *Missour Agric Exp Stn Res Bull,* **846**.

Johnson, J. E., Branton, C. 1953. Effects of seasonal climatic changes on certain physiological reactions, semen production and fertility of dairy bulls. *J Dairy Sci,* **36**: 934.

Johnson, M.H., Nasr-Esfahani, M.H. 1994. Radical solutions and cultural problems: could free oxygen radicals be responsible for the impaired development of mammalian embryos *in vitro*? *Bioessays,* **16**: 31–38.

Jolly, P.D., McDougall, S., Fitzpatrick, L.A., Macmillan, K.L., Entwhitsle, K. 1995. Physiological effects of under nutrition on postpartum anoestrous in cows. *J Reprod Fertil Suppl,* **49**: 477–492.

Jonsson, N.N., McGowan, M.R., McGuigan, K., Davison, T.M., Hussain, A.M., Matschoss, M. 1997. Relationships among calving season, heat load, energy balance and postpartum ovulation of dairy cows in a subtropical environment. *Anim Reprod Sci,* **47**: 315–326.

Jorritsma, R., Cesar, M.L., Hermans, J.T., Kruitwagen, C.L., Vos, P.L., Kruip, T.A. 2004. Effects of non-esterified fatty acids on bovine granulosa cells and developmental potential of oocytes in vitro. *Anim Reprod Sci,* **81**(3–4): 225–235.

Jousan, F.D. & Hansen, P.J. 2004. Insulin-like Growth Factor-I as a Survival Factor for the Bovine Preimplantation Embryo Exposed to Heat Shock. *Biol Reprod,* **71**(5): 1665-1670.

Joyce, I.M., Clark, A.T., Pendola, F.L., Eppig, J.J. 2000. Comparison of recombinant growth differentiation factor-9 and oocyte regulation of KIT ligand messenger ribonucleic acid expression in mouse ovarian follicles. *Biol Reprod*, **63**: 1669-1675.

Ju, J.C., Jiang, S., Tseng, J.K., Parks, J.E., Yang, X. 2005. Heat shock reduces developmental competence and alters spindle configuration of bovine oocytes *Theriogenology*, **64**: 1677–1689.

Ju, J.C., Parks, J.E., Yang, X. 1999. Thermotolerance of IVM-derived bovine oocytes and embryos after short-term heat shock. *Mol Reprod Dev,* **53**: 336–340.

Ju, J.C., Tseng, J.K. 2004. Nuclear and cytoskeletal alterations of in vitro matured porcine oocytes under hyperthermia. *Mol Reprod Dev*, **68**: 125–133.

Kadokawa, H., Sakatani, M., Hansen, P.J. 2012. Perspectives on improvement of reproduction in cattle during heat stress in a future Japan. *Anim Sci J,* **83**(6): 439-445.

Kadzere, C.T., Murphy, M.R., Silanikove, N., and Maltz, E. 2002. Heat stress in lactating dairy cows: a review. *Lives Prod Sci,* **77**(1): 59-91.

Kamaruddin, M.B. 1998. Heat shock protein 70 in bovine spermatogenesis and fertilization. Ph.D. thesis University of Guelph, Ontario, Canada.

Kamwanja, L.A., Chase Jr, C.C., Gutierrez, J.A., Guerriero, V.J., Olson, T.A., Hammond, A.C., et al. 1994. Responses of bovine lymphocytes to heat shock as modified by breed and antioxidant status. *J Anim Sci,* **72**: 438–44.

Kaneko, H., Nakanishi, Y., Akagi, S., Arai, K., Taya, K., Watanabe, G., Sasamoto, S. and Hasegawa, Y. 1995. Immunoneutralization of inhibin and oestradiol during the follicular phase of the oestrous cycle in cows. *Biol Reprod,* **53**: 931–939.

Kaneko, H., Terada, T., Taya, K., Watanabe, G., Sasamoto, S., Hasegawa, Y. and Igarashi, M. 1991. Ovarian follicular dynamics and concentration of oestradiol 17β, progesterone, luteinizing hormone and follicle stimulating hormone during the preovulatory phase of the oestrous cycle in the cow. *Reprod, Fertil Develop,* **3**: 529–535.

Kao, S.H., Chao, H.T., Chen, H.W., Hwang, T.I., Liao, T.L., Wei, Y.H. 2008. Increase of oxidative stress in human sperm with lower motility. *Fertil Steril*, **89**(5): 1183-1190.

Karabinus, D.S., Evenson, D.R., Jost, L.K., Baer, R.K., Kaproth, M.T. 1990. Comparison of semen quality in young and mature Holstein bulls measured by light microscopy and flow cytometry. *J Dairy Sci*, **73**: 2367-2371.

Karabinus, D.S., Vogler, C.J., Saacke, R.G., Evenson, D.P. 1997. Chromatin structural changes in sperm after scrotal insulation of Holstein bulls. *J Androl,* **18**: 549-555.

Kiang, J.G. & Tsokos, G.C. 1998. Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol Ther,* **80**: 183–201.

Kim, H.J., Kang, B.S., and Park, J.W. 2005. Cellular defense against heat shock-induced oxidative damage by mitochondrial NADP(+)-dependent isocitrate dehydrogenase. *Free Radical Res,* **39**(4): 441-448.

Kim, J.G. & Parthasarathy, S. 1998. Oxidation and the spermatozoa. *Semin Reprod Endocrinol,* **16**: 235–239.

Kim, N., Chung, K., Day, B. 1997. The distribution and requirements of microtubles and microfilaments during fertilization and parthenogenesis in pig oocytes. *J Reprod Fert,* **111**: 143–149.

Kim, N.H., Cho, S.K., Choi, S.H., Kim, E.Y., Park, S.P., Lim, J.H. 2000. The distribution and requirements of microtubule and microfilaments in bovine oocytes during in vitro maturation. *Zygote*, **8**: 25–32.

Kothari, S., Thompson, A., Agarwal, A., du Plessis, S.S. 2010. Free radicals: their beneficial and detrimental effects on sperm function. *Indian J Exp Biol*, **48**(5): 425-435.

Kregel, K.C. 2002. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J Appl Physiol,* **92**: 2177–2186.

Krisher, R.L. & Bavister, B.D. 1998. Responses of oocytes and embryos to the culture environment. *Theriogenology.* **59**: 103–114.

Kurzawa, R., Glabowski, W., Baczkowski, T., Brelik, P. 2002. Evaluation of mouse preimplantation embryos exposed to oxidative stress cultured with insulin-like growth factor I and II, epidermal growth factor, insulin, transferrin and selenium. *Reprod Biol,* **2**: 143–162.

Kusakabe, H., Tateno, H. 2011. Characterization of chromosomal damage accumulated in freeze-dried mouse spermatozoa preserved under ambient and heat stress conditions. *Mutagenesis*, **26**(3): 447–453.

Labèrnia, J., López-Gatius, F., Santolaria, P., Hanzen, C., Laurent, Y., Hountain, Y. 1998. Influence of calving season on the interactions among reproductive disorders of dairy cows. *Anim Sci,* **67**: 387–393.

Lane, M. 2001. Mechanisms for managing cellular and homeostatic stress in vitro. *Theriogenology*, **55**: 225–236.

Lane, M.A., Chen, A.C., Roman, S.D., Derguini, F., Gudas, L.J. 1999. Removal of LIF (leukemia inhibitory factor) results in increased vitamin A (retinol) metabolism to 4-oxoretinol in embryonic stem cells. *Proc Nat Acad Sci*, **96**: 13524–13529.

Lapointe, J. & Bilodeau, J.F. 2003. Antioxidant Defenses Are Modulated in the Cow Oviduct During the Estrous Cycle. *Biol Reprod,* **68**, 1157–1164.

Larsson, K., Einarsson, S. 1984. Seminal changes in boars after heat stress. *Acta Vet Scand,* **25**: 57–66.

Lasso, J.L., Noiles, E.E., Alvarez, J.G., Storey, B.T. 1994. Mechanism of superoxide dismutase loss from human sperm cells during cryopreservation. *J Androl*, **15**(3): 255-265.

Lawrence, J.L., Payton, R.R., Godkin, J.D., Saxton, A.M., Schrick, F.N., Edwards, J.L. 2004. Retinol improves development of bovine oocytes compromised by heat stress during maturation. *J Dairy Sci,* **87**(8): 2449-2454.

Lecha, L.B. 2007. IPCC (2007). Fourth Assessment Report. Cambridge, Cambridge University Press.

Lee, D.H. 1965. Climatic stress indices for domestic animals. *Int J Biometeorol,* **9** (1): 29-35.

Lenz, R.W., Ball, G. D., Leibfried, M. L., Ax, R. L., First, N. L. 1983. In vitro maturation and fertilization of bovine oocytes are temperature-dependent processes. *Biol Reprod,* **29**: 173–179.

Lenzi, A., Gandini, L., Lombardo, F., Picardo, M., Maresca, V., Panfili, E. 2002. Polyunsaturated fatty acids of germ cell membranes, glutathione and glutathione-dependent enzyme-PHGPx: from basic to clinic. *Contraception,* **65**: 301–304.

Lenzi, A., Lombardo, F., Gandini, L., Alfano, P., Dondero, F. 1993. Computer assisted sperm motility analysis at the moment of induced pregnancy during gonadotropin treatment for hypogonadotropic hypogonadism. *J Endocrinol Invest*, **16**: 683-686.

Leroy, J.L.M.R., Vanholder, T., Mateusen, B., Christophe, A., Opsomer, G., de Kruif, A., Genicot, G., Van Soom, A. 2005. Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes in vitro. *Reproduction,* **130**: 485–495.

Leroy, J.L.M.R., Vanholder, T., Opsomer, G., Van Soom, A., Bols, P.E.J., Bossaert, P., de Kruif, A. 2006. Typical metabolic changes in high producing dairy cows early postpartum and their consequences on oocyte and embryo quality. *Vlaam Diergeneeskd Tijdschr,* **75**: 94–104.

Leroy, J.L.M.R., Van Soom, A., Opsomer, G., Bols, P.E.J. 2008. The consequences of metabolic changes in high-yielding dairy cows on oocyte and embryo quality. *Animal,* **2**:8: 1120–1127.

Lewis, S.E.M., Sterling, E.S.L., Young, I.S., Thompson, W. 1997. Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. *Fertil Steril*, **67**: 142-147.

Li, G.C., Mivechi, N.F., Weitzel, G. 1995. Heat shock proteins, thermotolerance, and their relevance to clinical hyperthermia. *Int J Hyperthermia,* **11**: 459–488.

Li, G.C. & Werb, Z. 1982. Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. *Proc Natl Acad Sci USA,* **79**: 3218-3222.

Li, G.P., Liu, Y., Bunch, T.D., White, K.L., Aston, K.I. 2005. Asymmetric division of spindle microtubules and microfilaments during bovine meiosis from metaphase I to metaphase III. *Mol Reprod Dev,* **71**: 220–226.

Li, R., Norman, R.J., Armstrong, D.T., Gilchrist, R.B. 2000. Oocytesecreted factor(s) determine functional differences between bovine mural granulosa cells and cumulus cells. *Biol Reprod,* **63**: 839-845.

Liang, S., Kang, J., Xin Liu, H.J., Li, J., Y. 2012. The influence of 9-cis-retinoic acid on nuclear and cytoplasmic maturation and gene expression in canine oocytes during in vitro maturation. *Theriogenology*, **77**(6): 1198-1205.

Lima, P.F., Oliveira, M.A., Goncalves, P.B., Montagner, M.M., Reichenbach, H.D., Weppert, M., et al. 2004. Effects of retinol on the in vitro development of Bos indicus embryos to blastocysts in two different culture systems. *Reprod Domest Anim*, **39**(5): 356-360.

Lindquist, S. 1986. The Heat-Shock Response. *Annu Rev Biochem,* **55**: 1151-1191.

Line, C. 1986. Intensification of dairying. In. Cole, DJA, DC. Brander (eds). Bioindustrial ecosystem. Ed. Esevier. Amsterdam, 161-164.

Liu, E., Kitajima, S., Wiese, E., Reifenberg, K., Morimoto, M., Watanabe, T., Fan, J. 2007. Re-establishment of complement C6-deficient rabbit colony by cryopreserved sperm transported from abroad. *Exp Anim*, **56**: 167–171.

Liu, Z., Stocco, D.M. 1997. Heat shock-induced inhibition of acute steroidogenesis in MA-10 cells is associated with inhibition of the synthesis of the steroidogenic acute regulatory protein. *Endocrinology,* **138**(7): 2722-2728.

Livingston, T., Eberhardt, D., Edwards, J.L., Godkin, J. 2004. Retinol improves bovine embryonic development *in vitro*. *Reprod Biol Endocrinol,* **2**: 83.

López-Gatius, F. 2003. Is fertility declining in dairy cattle? A retrospective study in northeastern Spain. *Theriogenology*, **60**: 89-99.

López-Gatius, F., García-Ispierto, I., Santolaria, P., Yániz, J., Nogareda, C., López-Béjar, M. 2006. Screening for high fertility in high-producing dairy cows. *Theriogenology,* **65**: 1678-1689.

López-Gatius, F., López-Béjarb, M., Fenechb, M., Huntera, R.H.F. 2005. Ovulation failure and double ovulation in dairy cattle: risk factors and effects. *Theriogenology,* **63**: 1298-1307.

López-Gatius, F., Santolaria, P., Yániz, J.L., Garbayo, J.M., Hunter, R.H.F. 2004a. Timing of early foetal loss for single and twin pregnancies in dairy cattle. *Reprod Dom Anim,* **39**: 429–433.

López-Gatius, F., Santolaria, P., Yániz, J.L., Hunter, R.H.F. 2004b. Progesterone supplementation during the early fetal period reduces pregnancy loss in high-yielding dairy cattle. *Theriogenology,* **62**: 1529–1535.

López-Gatius, F., Santolaria, P., Yániz, J., Rutllant, J., López-Béjar, M. 2002. Factors affecting pregnancy loss from gestation Day 38 to 90 in lactating dairy cows from a single herd. *Theriogenology,* **57**: 1251–1261.

Loven, D.P. 1988. A role for reduced oxygen species in heat induced cell killing and induction of thermotolerance. *Med Hypotheses,* **26**: 39–50.

Lu, Z.H., Mu, Y.M.,Wang, B.A., Li, X.L., Lu, J.M., Li, J.Y., Pan, C.Y., Yanase, T., Nawata, H. 2003. Saturated fatty acids, palmitic and stearic acid, induce apoptosis by stimulation of ceramide generation in rat testicular Leydig cell. *Biochem Biophys Res Commun*, **303**: 1002–1007.

Lucy, M.C. 2001. Reproductive loss in high-producing dairy cattle: where will it end? *J Dairy Sci*, **84**: 1277-1293.

Lucy, M.C., Savio, J.D., Badinga, L., de la Sota, R.L., Thatcher, W.W. 1992. Factors that affect ovarian follicular dynamics in cattle. *J Anim Sci,* **70**: 3615–3626.

Lue, Y.H., Hikim, A.P., Swerdloff, R.S., Im, P., Taing, K.S., Bui, T., Leung, A., Wang, C. 1999. Single exposure to heat induces stage-specific germ cell apoptosis in rats: role of intratesticular testosterone on stage specificity. *Endocrinology,* **140**: 1709–1717.

Lue, Y., Hikim, A.P., Wang, C., Im, M., Leung, A., Swerdloff, R.S. 2000. Testicular heat exposure enhances the suppression of spermatogenesis by testosterone in rats: The ‘‘two-hit’’ approach to male contraceptive development. *Endocrinology,* **141**: 1414–1424.

Lussier, J.G., Matton, P. and Dufour, J.J. 1987. Growth rates of follicles in the ovary of the cow. *J Reprod Fertil,* **81**: 301–307.

Maatje, K., Loeffler, S.H., Engel, B. 1997. Predicting optimal time of insemination in cows that show visual signs of estrus by estimating onset of estrus with pedometers. *J Dairy Sci*, **80**(6): 1098-1105.

MacCracken, M.C. 2008. The 2008 Critical Review—Prospects for Future Climate Change and the Reasons for Early Action. *J Air & Waste Manage Assoc*, **58**(6): 735-786.

Macirone, C., Walton, A. 1983. Fecundity of male rabbits as determined by ‘Dummy mating’. *J Ag Sci,* **28**: 122-134.

Macmillan, K.L., Taufa, V.K., Barnes, D.R., Day, A.M., Henry, R. 1988. Detecting estrus in synchronized heifers-using tailpaint and an aerosol raddle. *Theriogenology*. **30**(6): 1099-1114.

Madan, M.L. & Johnson, H.D. 1973. Environmental heat effects on bovine luteinizing hormone. *J Dairy Sci,* **56**: 1420–1423.

Mader, T.L., Davis M.S., and Brown-Brandl, T. 2006. Environmental factors influencing heat stress in feedlot cattle. *J Anim Sci*, **84**: 712-719.

Maia, A.S.C., DaSilva, R.G. and Loureiro, C.M.B. 2005. Respiratory heat loss of Holstein cows in a tropical environment. *Int J Biometeorol,* **49**(5): 332-336.

Maia, Mda.S., Bicudo, S.D., Sicherle, C.C., Rodello, L., Gallego, I.C. 2010. Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in extenders with antioxidants. *Anim Reprod Sci*, **122**(1-2): 118-123.

Makarevich, A.V., Olexikova, L., Chrenek, P., Kubovicova, E., Freharova, K., Pivko, J. 2007. The effect of hyperthermia in vitro on vitality of rabbit preimplantation embryos. *Physiol Res*, **56**: 789-796.

Malayer, J.R., Hansen, P.J. 1990. Differences between Brahman and Holstein cows in heat-shock induced alterations of protein synthesis and secretion by oviducts and uterine endometrium. *J Anim Sci,* **68**: 266–280.

Malayer, J.R., Hansen, P.J., Gross, T.S., Thatcher, W.W. 1990. Regulation of heat shock-induced alterations in release of prostaglandins by the uterine endometrium of cows. *Theriogenology,* **34**: 219–230.

Malmgren, L. 1989. Experimentally induced testicular alterations in boars: sperm morphology changes in mature and peripubertal boars. *Zbl Vet Med* A, **36**: 411-420.

Malmgren, L., Larsson, K. 1984. Semen quality and fertility after heat stress in boars. *Acta Vet Scandinavica*, **25**: 425-435.

Mammoto, A., Masumoto, N., Tahara, M., Ikebuchi, Y., Ohmichi, M., Tasaka, K., Miyake, A. 1996. Reactive oxygen species block sperm-egg fusion via oxydation of sperm sulfhydryl proteins in mice. *Biol Reprod*, **55**: 1063–1068.

Mann, G.E., Lamming, G.E., Robinson, R.S., Wathes, D.C. 1999. The regulation of interferon-tau production and uterine hormone receptors during early pregnancy. *J Reprod Fertil,* **54**: 317–328.

Marai, I.F.M., Abdel-Samee, A.M., El-Gafaary, M.N. 1991. Criteria of response and adaptation to high temperature for reproductive and growth traits in rabbits. *Options Mediterraneennes Ser,* **A 17**: 127–134.

Marai, I.F.M., Ayyat, M.S., Abd el-Monem, U.M. 2001. Growth performance and reproductive traits at first parity of New Zealand white female rabbits as affected by heat stress and its alleviation under Egyptian conditions. *Trop Anim Health Prod,* **33**(6): 451-462.

Marai, I.F.M., Habeeb, A.A. 1994. Thermoregulation in rabbits. *Options Méditerranéennes*, **8**: 33-41.

Marai, I.F.M., Habeeb, A.A.M., Gad, A.E. 2002. Rabbits' productive, reproductive and physiological performance traits as affected by heat stress: a review. *Lives Prod Sci,* **78**(2): 71-90.

Marei, W.F., Wathes, C., Fouladi-Nashta, A.A. 2009. The Effect of Linolenic Acid on Bovine Oocyte Maturation and Development. *Biol Reprod*, **81**: 1064–1072.

Marei, W.F., Wathes, C., Fouladi-Nashta, A.A. 2010. Impact of linoleic acid on bovine oocyte maturation and embryo development. *Reproduction*, **139**: 979–988.

Marei, W.F., Wathes, C., Fouladi-Nashta, A.A. 2011. Differential effects of linoleic and alpha-linolenic fatty acids on spatial and temporal mitochondrial distribution and activity in bovine oocytes. *Reprod Fertil Develop*, **24**(5): 679-690.

Maresca, B. & Lindquist, S. 1991. Heat shock response. In: Luts Nover, editor. Boca Raton, FL: CRC Press, Inc, 5–7.

Marques, A., Santos, P., Antunes, G., Chaveiro, A., Moreira da Silva, F. 2010. Effect of a-Tocopherol on Bovine In Vitro Fertilization. *Reprod Dom Anim*, **45**: 81–85.

Mathias, S., Pena, L.A., Kolesnick, R.N. 1998. Signal transduction of stress via ceramide. Ceramide also plays a pivotal role in stress-induced apoptosis. *Biochem J*, **335**: 465–480.

Matsuoka, T., Imai, H., Kohno, H., Fukui, Y. 2006. Effects of bovine serum albumine and trehalose in semen diluents for improvement of frozen-thawed ram spermatozoa. *J Reprod Develop*, **52**: 675-683.

Matsuzuka, T., Ozawa, M., Nakamura, A., Ushitani, A., Hirabayashi, M. and Kanai, Y. 2005. Effects of heat stress on the redox status in the oviduct and early embryonic development in mice. *J Reprod Dev*, **51**: 281-287.

Matwee, C., Kamaruddin, M., Betts, D.H., Basrur, P.K. and King, W.A. 2001. The effects of antibodies to heat shock protein 70 in fertilization and embryo development. *Mol Hum Reprod,* **7**: 829-837.

Mauro, L. & Surmacz, E. 2004. IGF-I receptor, cell–cell adhesion, tumour development and progression. *J Mol Histol,* **35**: 247–253.

McDowell, D., Hooven, N., Cameron, K. 1979. Effects of climate on performance of Holsteins in first lactation. *J Dairy Sci,* **68**: 2418–2435.

McEvoy, T.G., Coull, G.D., Broadbent, P.J., Hutchinson, J.S.M., Speake, B.K. 2000. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucid. *J Reprod Fertil,* **118**: 163–170.

McLaren, T.T., Foster, P.M., Sharpe, R.M. 1994. Identification of stage-specific changes in protein secretion by isolated seminiferous tubules from rats following exposure to short-term local testicular heating. *J Reprod Fertil,* **102**: 293–300.

Mclean, J.A. 1963. Measurement of Cutaneous Moisture Vaporization from Cattle by Ventilated Capsules. *J Physiol-London,* **167**(3): 417-426.

McNitt, J. I., First, N.L. 1970. Effects of 72-hour heat stress on semen quality in boars. *Int J Biometeor,* **14**: 373-380.

McNitt, J.I., Moody, Jr. G.L. 1991. Gestation length of four medium breeds of rabbits in Lousiana. *J Appl Rabbit Res,* **14:** 80–82.

Medeiros, C.M., Forell, F., Oliveira, A.T., Rodrigues, J.L. 2002. Current status of sperm cryopreservation: why isn't it better? *Theriogenology*, **57**: 327-344.

Meirelles, F.V., Caetano, A.R., Watanabe, Y.F., Ripamonte, P., Carambula, S.F., Merighe, G.K., et al. 2004. Genome activation and developmental block in bovine embryos. *Anim Reprod Sci,* **82–83**: 13–20.

Meirelles, F.V., Rosa, A.J.M., Lobo, B.R. 1999. Is the American Zebu really *Bos indicus*? *Genet Mol Biol,* **22**: 543–547.

Meister, A. 1983. Selective modification of glutathione metabolism. *Science*, **220**: 472-477.

Meister, A., Anderson, M.E. 1983. Glutathione. *Annu Rev Biochem*, **52**: 11–60.

Memili, E. & First, N.L. 2000. Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. *Zygote,* **8**: 87–96.

Mermillod, P., Oussaid, B., Cognie, Y. 1999. Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. *J Reprod Fertil Suppl,* **54:** 449–460.

Meyerhoeffer, D.C., Wettemann, R.P., Coleman, S.W. and Wells, M.E. 1985. Reproductive Criteria of Beef Bulls during and after Exposure to Increased Ambient Temperature. *J Anim Sci,* **60**: 352-357.

Mieusset, R., Bengoudifa, B. and Bujan, L. 2007. Effect of posture and clothing on scrotal temperature in fertile men. *J Androl,* **28**: 170-175.

Mieusset, R. & Bujan, L. 1995. Testicular heating and its possible contributions to male infertility: a review. *Int J Androl,* **18**: 169–184.

Mieusset, R., Bujan, L., Mansat, A. and Pontonnier, F. 1992a. Scrotal hyperthermia and male infertility. *Prog Urol,* **2**: 31-36.

Mieusset, R., Quintana Casares, P., Sanchez Partida, L.G., Sowerbutts, S.F., Zupp, J.L., Setchell, B.P. 1992b. Effects of heating the testes and epididymides of rams by scrotal insulation on fertility and embryonic mortality in ewes inseminated with frozen semen. *J Reprod Fertil,* **94**: 337–343.

Mieusset, R., Bujan, L., Mondinat, C., Mansat, A., Pontonnier, F. and Grandjean, H. 1987. Association of scrotal hyperthermia with impaired spermatogenesis in infertile men. *Fertil Steril,***48**: 1006-1011.

Mieusset, R., Quintana Casares, P., Sanchez Partida, L.G., Sowerbutts, S.F., Zupp, J.L., Setchell, B.P. 1991. Effects of heating the testes and epididymides of rams by scrotal insulation on fertility and embryonic mortality in ewes inseminated with frozen semen. *J Reprod Fertil,* **94**: 337-343.

Mihm, M., Baguisi, A., Boland, M.P., Roche, J.F. 1994. Association between the duration of dominance of the ovulatory follicle and pregnancy rate in beef heifers. *J Reprod Fertil,* **102**: 123-130.

Mihm, M., Curran, N., Hyttel, P., Knight, P.G., Boland, M.P., Roche, J.F. 1999. Effect of dominant follicle persistence on follicular fluid oestradiol and inhibin and on oocyte maturation in heifers. *J Reprod Fertil,* **116**(2): 293-304.

Miller, D., Brough, S. and Al-Harbi, O. 1992. Characterization and cellular distribution of human spermatozoa heat shock proteins. *Hum Reprod,* **7**: 637-645.

Miller, J.G.O. & Shultz, G.A. 1987. Amino acids content of preimplantation embryos and fluids of the reproductive tract. *Biol Reprod,* **36**: 125–129.

Miller, J.K., Brezinska-slebodzinskai, E. 1993. Oxidative stress, antioxidants and animal function. *J Dairy Sci,* **76**: 2812-2823.

Minton, J.E., Wettemann, R.P., Meyerhoeffer, D.C., Hintz, R.L. and Turman, E.J. 1981. Serum luteinizing hormone and testosterone in bulls during exposure to elevated ambient temperature. *J Anita Sci,* **53**: 1551-1558.

Mitchell, J.B., Russo, A., Kinsella, T.J., Glatstein, E. 1983. Glutathione Elevation during Thermotolerance Induction and Thermosensitization by Glutathione Depletion. *Cancer Res*, **43**(3): 987-991.

Mohan, M., Malayer, J.R., Geisert, R.D., Morgan, G.L. 2001. Expression of retinol-binding protein messengerRNAand retinoic acid receptors in preattachment bovine embryos. *Mol Reprod Dev*, **60**: 289–296.

Mohan, M., Malayer, J.R., Geisert, R.D., Morgan, G.L. 2002. Expression patterns of retinoid X receptors, retinaldehyde dehydrogenase, and peroxisome proliferator activated receptor gamma in bovine preattachment embryos. *Biol Reprod*, **66**: 692–700.

Monteiro, J.C., Gonçalves, J.S., Rodrigues, J.A., Lúcio, C.F., Silva, L.C., Assumpção, M.E., Vannucchi, C.I. 2009. Influence of ascorbic acid and glutathione antioxidants on frozen-thawed canine semen. *Reprod Domest Anim*, **44**(Suppl2): 359-362.

Monterroso, V.H., Drury, K.C., Ealy, A.D., Edwards, J.L., Hansen, P.J. 1995. Effect of heat shock on function of frozen/thawed bull spermatozoa. *Theriogenology,* **44**: 947-961.

Monty, D.E. Jr. & Racowsky, C. 1987. In vitro evaluation of early embryo viability and development in summer heat-stressed, superovulated dairy cows. *Theriogenology,* **28**: 451–465.

Moor, R.M., Smith, M.W., Dawson, M.C. 1980. Measurement of intercellular coupling between oocytes and cumulus cells using intracellular markers. *Exp Cell Res,* **126**: 15–29.

Moore, C.E., Kay, J.K., Collier, R.J., VanBaale, M.J., Baumgard, L.H. 2005. Effect of supplemental conjugated linoleic acids on heat-stressed Brown Swiss and Holstein cows. *J Dairy Sci,* **88**: 1732-1740.

Morera, P., Kuzminsky, G., Finzi, A. 1995. Estrés térmico crónico: Variaciones de algunos caracteres del semen y de la libido del conejo macho. *Boletín Cunicultura*, **80**: 42 (Abstract).

Morris-Kay, G.M., Ward, S.J. 1999. Retinoids and mammalian development. *Int Rev Cytol,* **188**: 73–131.

Morrow, D.A. (Ed). 1980. Current Therapy in Theriogenology. W.B. Saunders Co., Philadelphia, USA.

Moule, G.R., Waites, G.M.H. 1963. Seminal degeneration in the ram and its relation to the temperature of the scrotum. *J Reprod Fertil*, **5**: 433-446.

Muller, W.U., Li, G.C., Goldstein, L.S. 1985. Heat does not induce synthesis of heat shock proteins or thermotolerance in the earliest stage of mouse embryo development. *Int J Hyperthermia,* **1**: 97–102.

Murphy, B.D., Lalli, E., Walsh, L.P., Liu, Z., Soh, J., Stocco, D.M., Sassone-Corsi, P. 2001. Heat shock interferes with steroidogenesis by reducing transcription of the steroidogenic acute regulatory protein gene. *Mol Endocrinol*, **15**(8): 1255-1263.

Nabenishi, H., Ohta, H., Nishimoto, T., Morita, T., Ashizawa, K., Tsuzuki, Y. 2011. The effects of cysteine addition during in vitro maturation on the developmental competence, ROS, GSH and apoptosis level of bovine oocytes exposed to heat stress. *Zygote*, **18**: 1-11.

Nakanishi, T., Isotani, A., Yamaguchi, R., Ikawa, M., Baba, T., Suarez, S.S., Okabe, M. 2004. Selective passage through the uterotubal junction of sperm from a mixed population produced by chimeras of calmegin-knockout and wild-type male mice. *Biol Reprod*, **71**(3): 959-965.

Narendranath, R. & Kiracofe, G.H. 1975. Effects of heat--stress in rats. II. Factor(s) responsible for reduced embroynic and/or foetal survival percentage. *Indian J Physiol Pharmacol*, **19**: 140-145.

Nasiri, E., Mahmoudi, R., Bahadori, M.H., Amiri, I. 2011. The Effect of Retinoic Acid on In vitro Maturation and Fertilization Rate of Mouse Germinal Vesicle Stage Oocytes. *Cell Journal*, **13**(1): 19-24.

Neagu, V.R., García, B.M., Sandoval, C.S., Rodríguez, A.M., Ferrusola, C.O., Fernández, L.G., Tapia, J.A., Peña, F.J. 2010. Freezing dog semen in presence of the antioxidant butylated hydroxytoluene improves postthaw sperm membrane integrity. *Theriogenology*, **73**(5): 645-650.

Nilsson, A., Isgaard, J., Lindahl, A., Dahlstrom, A., Skottner, A. and Isaksson, O.G.P. 1986. Regulation by Growth-Hormone of Number of Chondrocytes Containing Igf-I in Rat Growth Plate. *Science,* **233**(4763): 571-574.

Nobel, R.L., Jobst, S.M., Dransfield, M.B.G., Pandolfi, S.M., Balley, T.L. 1997. Use of radio frequency data communication system, HeatWatch, to describe behavioural estrus in dairy cattle. *J Dairy Sci,* **179** (Abstract).

Nonogaki, T., Nado, Y., Narimoto, K., Umaoka, Y., Mori, T. 1992. Effects of superoxide dismutase on mouse in vitro fertilization and embryo culture system. *J Assist Reprod Genet*, **9**: 274-280.

O'Callagan, D. & Boland, M.P. 1999. Nutritional effects on ovulation, embryo development and the establishment of pregnancy in ruminants. *Anim Sci,* **68**: 299–314.

O'Flaherty, C., Beconi, M., Beorlegui, N. 2009. Effect of natural antioxidants, superoxide dismutase and hydrogen peroxide on capacitation of frozen-thawed bull spermatozoa. *Andrologia*, **29**(5): 269-275.

Okada, A., Inomata, K., Nagae, T. 1993. Spontaneous cortical granule release and alteration of zona pellucida properties during and after meiotic maturation of mouse oocytes. *Anat Rec,* **237**: 518–526.

Okada, A, Yanagimachi, R., Yanagimachi, H. 1986. Development of a cortical granule-free area of cortex and the perivitelline space in the hamster oocyte during maturation and following ovulation. *J Submicrosc Cytol*, **18**: 233–247.

Olson, S.E., Seidel, G.E. Jr. 2000. Culture of in vitro-produced bovine embryos with Vitamin E improves development in vitro and after transfer to recipients. *Biol Reprod*, **62**: 248-252.

Ominski, K.H., Kennedy, A.D., Wittenberg, K.M., Nia, S.A.M. 2002. Physiological and production responses to feeding schedule in lactating dairy cows exposed to shortterm, moderate heat stress. *J Dairy Sci,* **85**(4): 730-737.

Omtvedt, I.T., Nelson, R.E., Edwards, R.L., Stephens, D.F., Turman, E.J. 1971. Influence of heat stress during early, mid, and late pregnancy of gilts. *J Anim Sci,* **32**: 312–317.

Otsuka, F., Yamamoto, S., Erickson, G.F., Shimasaki, S. 2001. Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. *J Biol Chem,* **276**: 11387-11392.

Õura, C., Toshimori, K. 1990. Ultrastructural studies on the fertilization of mammalian gametes. *Int Rev Cytol*, **122**: 105-151.

Ozawa, M., Hirabayashi, M. and Kanai, Y. 2002. Developmental competence and oxidative state of mouse zygotes heat-stressed maternally or in vitro. *Reproduction,* **124**: 683-689.

Parks, J.E., Lynch, D.V. (1992). Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. *Cryobiology*, **29**: 255–266.

Patial, K.K., Manja, N.K., Gupta, K., Sanjeet, K. 1991. The effects of season on litter size of rabbits in Himachal Pradesh (India). *J Appl Rabbit Res,* **14**: 257–259.

Patriarca, E.J., Maresca, B. 1990. Acquired Thermotolerance Following Heat-Shock Protein-Synthesis Prevents Impairment of Mitochondrial Atpase Activity at Elevated-Temperatures in Saccharomyces-Cerevisiae. *Exp Cell Res*, **190**(1): 57-64.

Paul, C., Murray, A.A., Spears, N. and Saunders, P.T. 2008. A single, mild, transient scrotal heat stress causes DNA damage, subfertility and impairs formation of blastocysts in mice. *Reproduction,* **136**: 73-84.

Paula-Lopes, F.F., Chase, C.C.J., Al-Katanani, Y.M., Krininger, C.E., Rivera, R.M., Tekin, S., et al. 2003. Genetic divergence in cellular resistance to heat shock in cattle: differences between breeds developed in temperate versus hot climates in responses of preimplantation embryos, reproductive tract tissues and lymphocytes to increased culture temperatures. *Reproduction.* **125**: 285–294.

Paula-Lopes, F.F., Hansen, P.J. 2002. Heat-shock induced apoptosis in preimplantation bovine embryos is a developmentally-regulated phenomenon. *Biol Reprod,* **66**: 1169–1177.

Paulenz, H., Taugbøl, O., Kommisrud, E., Grevle, I.S. 1999. Effect of dietary supplementation with cod liver oil on cold shock and freezability of boar semen. *Reprod Dom Anim*, **34**: 431–435.

Paumen, M.B., Ishida,Y., Muramatsu, M.,Yamamoto, M., Honjo, T. 1997. Inhibition of carnitine palmitoyltransferase I augments sphingolipid synthesis and palmitate-induced apoptosis. *J Biol Chem,* **272**: 3324–3329.

Payton, R.R., Romar, R., Coy, P., Saxton, A.M., Lawrence, J.L., Edwards, J.L. 2004. Susceptibility of bovine germinal vesicle-stage oocytes from antral follicles to direct effects of heat stress in vitro. *Biol Reprod,* **71**: 1303–1308.

Payton, R. R., Lawrence, J.L., Saxton, A.M., Dunlap, J.R., Edwards, J.L. 2003. Cortical Granule Types and Nuclear Stage of Bovine Oocytes after Exposure to Elevated Temperature During Maturation. *Theriogenology*, **59**: 496.

Pennington, J.A., Albright, J.L., Diekman, M.A., Callahan, C.J. 1985. Sexual activity of Holstein cows: seasonal effects. *J Dairy Sci,* **68**: 3023–3030.

Pérez-Crespo, M., Pintado, B., Gutiérrez-Adán, A. 2008. Scrotal heat stress effects on sperm viability, sperm DNA integrity, and the offspring sex ratio in mice. *Mol Reprod Dev*, **75**: 40–47.

Perreault, S.D., Aitken, R.J., Baker, H.W., Evenson, D.P., Huszar, G., Irvine, D.S., Morris, I.D., Morris, R.A., Robbins, W.A., Sakkas, D., Spano, M., Wyrobek, A.J. 2003. Integrating new tests of sperm genetic integrity into semen analysis: breakout group discussion. *Adv Exp Med Biol*, **518**: 253-268.

Perreault, S.D., Barbee, R.R., Slott, V.I. 1988. Importance of glutathione in the acquisition and maintenance of sperm nuclear decondensing activity in maturing hamster oocytes. *Dev Biol*, **125**: 181-186.

Piper, P.W., Millson, S.H., Mollapour, M., Panaretou, B., Siligardi, G., Pearl, L.H., Prodromou, C. 2003. Sensitivity to Hsp90-targeting drugs can arise with mutation to the Hsp90 chaperone, cochaperones and plasma membrane ATP binding cassette transporters of yeast. *Eur J Biochem,* **270**(23): 4689-4695.

Pribenszky, C., Vajta, G., Molnar, M., Du, Y., Lin, L., Bolund, L., et al. 2010. Stress for stress tolerance? A fundamentally new approach in mammalian embryology. *Biol Reprod*, **83**(5): 690-697.

Pryce, J.E., Esslemont, R.J., Thompson, R., Veerkamp, R.F., Kossaibati, M.A., Simm, G. 1998. Estimation of genetic parameters using health, fertility and production data from a management recording system for dairy cattle. *Anim Sci*, **66**: 577-584.

Pursley, J.R., Mee, M.O., Wiltbank, M.C. 1995. Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. *Theriogenology,* **44**: 915–923.

Pursley, J.R., Wiltbank, M.C., Stevenson, J.S., Ottobre, J.S., Gaverick, H.A., Anderson, L.L. 1997. Pregnancy rates per artificial insemination for cows and heifers inseminated at synchronized ovulation or synchronized estrus. *J Dairy Sci*, **80**: 295–300.

Putney, D.J., Drost, M., Thatcher, W.W. 1988a. Embryonic development in superovulated dairy cattle exposed to elevated ambient temperatures between days 1 to 7 post insemination. *Theriogenology*, **30**: 195–209.

Putney, D.J., Drost, M., Thatcher, W.W. 1989a. Influence of summer heat stress on pregnancy rates of lactating dairy cattle following embryo transfer or artificial insemination *Theriogenology,* **31**: 765–778.

Putney, D.J., Malayer, J.R., Gross, T.S., Thatcher, W.W., Hansen, P.J., Drost, M. 1988b. Heat stress-induced alterations in the synthesis and secretion of proteins and prostaglandins by cultured bovine conceptuses and uterine endometrium. *Biol Reprod,* **39**: 717–728.

Putney, D.J., Mullins, S., Thatcher, W.W., Drost, M., Gross, T.S. 1989b. Embryonic development in superovulated dairy cattle exposed to elevated ambient temperatures between the onset of estrus and insemination. *Anim Reprod Sci,* **19**: 37–51.

Quintero-Moreno, A., Rigau, T., Rodríguez-Gil, J.E. 2007. Multivariate cluster analysis regression procedures as tolos to identify motile sperm subpopulations in rabbit semen and to predict semen fertility and litter size. *Reprod Dom Anim*, **42**: 312-319.

Rabiee, A.R., Lean, I.J., Gooden, J.M., Miller, B.J., Scaramuzzi, R.J. 1997. An evaluation of transovarian uptake of metabolites using arterio-venous difference methods in dairy cattle. *Anim Reprod Sci,* **48**: 9–25.

Racowsky, C. 1985. Effect of forskolin on maintenance of meiotic arrest and stimulation of cumulus expansion, progesterone and cyclic AMP production by pig oocyte±cumulus complexes. *J Reprod Fertil,* **74**: 9-21.

Rafai, P., Papp, Z. 1984. Temperature requirement of rabbit does for optimal performance. *Arch Exp Vet Med*, **38**: 450–457.

Rajesh, N., Shankar, M.B., Deecaraman, M. 2010. Effect of vitamin A supplementation at different gaseous environments on *in vitro* development of pre-implantation sheep embryos to the blastocyst stage. *Animal,* **4**(11): 1884–1890.

Rao, B., Soufir, J.C., Martin, M., David, G. 1989. Lipid peroxidation in human spermatozoa as related to midpiece abnormalities and motility. *Gamete Res,* **24**(2): 127-134.

Rathore, A.K. 1968. Effects of high temperature on sperm morphology and subsequent fertility in Merino sheep. *Proc Austr Soc anim Prod,* **7**: 270-274.

Rathore, A.K. 1970. Fertility of rams heated for 1, 2, 3 and 4 days, mated to superovulated ewes. *Aust J agrie Res,* **21:** 355-358.

Rauw, W.M., Kannis, E., Noordhuizen-Stassen, E.N., Grommers, F.J. 1998. Undesirable side effects of selection for high production efficiency in farm animals: a review. *Livest Prod Sci,* **56**: 15-33.

Ravagnolo, O., Misztal, I., Hoogenboom, G. 2000. Genetic component of heat stress n dairy cattle development of heat index function. *J Dairy Sci,* **83**: 2120–2125.

Reddy, N.S.S., Mohanarao G.J., Atreja, S.K. 2010. Effects of adding taurine and trehalose to a tris-based egg yolk extender on buffalo (Bubalua bubalis) sperm quality following cryopreservation. *Anim Reprod Sci*, **119**(3-4): 183-190.

Ren, L., Medan, M.S., Ozu, M., Li, C., Watanabe, G., Taya, K. 2006. Effects of experimental cryptorchidism on sperm motility and testicular endocrinology in adult male rats. *J Repro Develop*, **52**(2): 219-228.

Rhynes, W. E. & Ewing, L.L. 1973. Testicular endocrine function in Hereford bulls exposed to high ambient temperature. *Endocrinology,* **92**: 509-515.

Ribarov, S.R. & Benov, L.C. 1981. Relationship between the Hemolytic Action of Heavy-Metals and Lipid-Peroxidation. *Biochim Biophys Acta,* **640**(3): 721-726.

Richter, K., Haslbeck, M. and Buchner, J. 2010. The Heat Shock Response: Life on the Verge of Death. *Mol Cell,* **40**(2): 253-266.

Rivera, R.M. & Hansen, P.J. 2001. Development of cultured bovine embryos after exposure to high temperatures in the physiological range. *Reproduction,* **121**: 107–115.

Rivera, R.M., Kelley, K.L., Erdos, G.W., Hansen, P.J. 2004. Reorganization of microfilaments and microtubules by thermal stress in two-cell bovine embryos. *Biol Reprod,* **70**: 1852–1862.

Robaire, B., Hales, B.F. 2003. Mechanisms of action of cyclophosphamide as a male-mediated developmental toxicant. *Adv Exp Med Biol*, **518**: 169-180.

Robinson, J.J., Ashworth, C.J., Rooke, J.A., Mitchell, L.M., McEvoy, T.G. 2006. Nutrition and fertility in ruminant livestock. *Anim Feed Sci Technol*, **126**: 259–276.

Roca, J., Martínez, S., Orengo, J., Parrilla, I., Vázquez, J.M., Martínez, E.A. 2005. Influence of constant long days on ejaculate parameters of rabbits reared under natural environment conditions of Mediterranean area. *Lives Prod Sci,* **94**: 169-177.

Rocha, A., Randel, R.D., Broussard, J.R., Lim, J.M., Blair, R.M., Roussel, J.D., Godke, R.A., Hansel, W. 1998. High environmental temperature and humidity decrease oocyte quality in *Bos taurus* but not in *Bos indicus* cows. *Theriogenology,* **49**: 657–665.

Rockett, J.C., Mapp, F.L., Garges, J.B., Luft, J.C., Mori, C. and Dix, D.J. 2001. Effects of Hyperthermia on Spermatogenesis, Apoptosis, Gene Expression, and Fertility in Adult Male Mice. *Biol Reprod,* **65**(1): 229-239.

Rodriguez-Martinez, H., Barth, A.D. 2007. In vitro evaluation of sperm quality related to in vivo function and fertility. *Soc Reprod Fertil Suppl*, **64**: 39-54.

Rodriquez, L.A., Mekonnen, G., Wilcox, C.J., Martin, F.G., and Krienke, W.A. 1985. Effects of Relative-Humidity, Maximum and Minimum Temperature, Pregnancy, and Stage of Lactation on Milk-Composition and Yield. *J Dairy Sci,* **68**(4): 973-978.

Roels, O.A.,Anderson, O.R., Luis, N.S., Shah, D.O., Trout, M.E. 1969. Vitamin A and membranes. *Am J Clin Nutr*, **22**(8): 1020-1032.

Roman-Ponce, H., Thatcher, W.W., Buffington, D.E., Wilcox, C.J., Van Horn, H.H. 1977. Physiological and production responses of dairy cattle to a shade structure in a subtropical environment. *J Dairy Sci*, **60**(3): 424-430.

Roman-Ponce, H., Tatcher, W.W., Caton, D., Barron, D.H., Wilcox, C.J. 1978. Thermal stress effects on uterine blood flow in dairy cows. *J Anim Sci,* **46**(1): 175-180.

Roman-Ponce, H., Thatcher, W.W. and Wilcox, C.J. 1981. Hormonal interelationships and physiological responses of lactating dairy cows to a shade management system in a subtropical environment. *Theriogenology,* **16**(2): 139-154.

Ron, M., Bar-Anan, R. and Wiggans, G.R. 1984. Factors affecting conception rate of Israeli Holstein cows. *J Dairy Sci,* **67**: 854–860.

Ronchi, B., Stradaioli, G., Verini Supplizi, A., Bernabuci, U., Lacetera, N., Accorsi, P.A. et al. 2001. Influence of heat stress or feed restriction on plasma progesterone, oestradiol-17beta, LH, FSH, prolactin and cortisol in Holstein heifers. *Lives Prod Sci,* **68**: 231–241.

Rooke, J.A., Shao, C.C., Speake, B.K. 2001. Effects of feeding tuna oil on the lipid composition of pig spermatozoa and in vitro characteristics of semen. *Reproduction*, **121**: 315–322.

Rosenberg, M., Folman, Y., Herz, Z., Flamenbaum, I., Berman, A., Kaim, M. 1982. Effect of climatic conditions on peripheral concentrations of LH, progesterone and estradiol-17b in high milk-yielding cows. *J Reprod Fertil,* **66**: 139–146.

Ross, S.A., McCaffery, P.J., Drager, U.C., De Luca, L.M. 2000. Retinoids in embryonal development. *Physiol Rev*, **80**: 1021-1054.

Roth, Z. 2008. Heat stress, the follicle, and its enclosed oocyte: mechanism and potential strategies to improve fertility in dairy cows. *Reprod Domest Anim,* **43**(Suppl. 2): 238–244.

Roth, Z., Arav, A., Bor, A., Zeron, Y., Braw-Tal, R. and Wolfenson, D. 2001a. Improvement of quality of oocytes collected in the autumn by enhanced removal of impaired follicles from previously heat-stressed cows. *Reproduction,* **122**: 737–744.

Roth, Z., Arav, A., Bor, A., Zeron, Y., Ocheretny, A., Wolfenson, D. 1999. Enhanced removal of impaired follicles improves the quality of oocytes collected in the autumn from summer heat-stressed cows. Ann. Meeting, Soc. for the Study of Fertility, Aberystwyth, Abstract 78.

Roth, Z., Arav, A., Braw-Tal, R., Bor, A., Wolfenson, D. 2002. Effect of treatment with follicle-stimulating hormone or bovine somatotropin on the quality of oocytes aspirated in the autumn from previously heat-stressed cows. *J Dairy Sci,* **85**: 1398–1405.

Roth, Z., Hansen, P.J. 2004. Involvement of apoptosis in disruption of developmental competence of bovine oocytes by heat shock during maturation. *Biol Reprod,* **71**: 1898–1906.

Roth, Z. & Hansen, P.J. 2005. Disruption of nuclear maturation and rearrangement of cytoskeletal elements in bovine oocytes exposed to heat shock during maturation. *Reproduction*, **129**: 235–244.

Roth, Z., Meidan, R., Braw-Tal, R. and Wolfenson, D. 2000. Immediate and delayed effects of heat stress on follicular development and its association with plasma FSH and inhibin concentration in cows. *J Reprod Fertil,* **120**: 83–90.

Roth, Z., Meidan, R., Shaham-Albalancy, A., Braw-Tal, R., Wolfenson, D. 2001b. Delayed effect of heat stress on steroid production in medium-sized and preovulatory bovine follicles. *Anim Reprod Sci,* **121**: 745-751.

Roti, J.L. 2008. Cellular responses to hyperthermia (40–46ºC): cell killing and molecular events. *Int J Hyperthermia*, **24**: 3–15.

Rougoor, C.V., Hanekamp, W.J.A., Dijkhuizen, A.A., Nielen, M., Wilmink, J.B.M. 1999. Relationships between dairy cow mastitis and fertility management and farm performance. *Prev Vet Med,* **27**(39): 247–64.

Royal, M.D., Darwash, A.O., Flint, A.P.F., Webb, R., Woolliams, J.A., Lamming, G.E. 2000. Declining fertility in dairy cattle: changes in traditional and endocrine parameters of fertility. *Anim Sci,* **70**: 487-501.

Russell, L.D., Ettlin, R.A., Sinha Hikim, A.D., Clegg, E.P. 1990. Histological and histopathological evaluation of the testis. Clearwater, FL: Cache River Press; 1990.

Ryan, D.P., Blakewood, E.G., Lynn, J.W., Munyakazi, L., Godke, R.A. 1992. Effect of heat-stress in bovine embryo development in vitro. *J Anim Sci,* **70**: 3490–3497.

Safaa, H.M., Emarah, M.E., Saleh, N.F.A. 2008. Seasonal effects on semen quality in Black Baladi and white New Zealand rabbit bucks. *World Rabbit Sci*, **16**: 13-20.

Sailer, B.L., Sarkar, L.J., Bjordahl, J.A., Jost, L.K. and Evenson, D.P. 1997. Effects of heat stress on mouse testicular cells and sperm chromatin structure. *J Androl,* **18**: 294-301.

Sakatani, M., Kobayashi, S., Takahashi, M. 2004. Effects of heat shock on in vitro development and intracellular oxidative state of bovine preimplantation embryos. *Mol Reprod Dev,* **67**: 77–82.

Sakkas, D., Urner, F., Bizzaro, D., Manicardi, G.C., Bianchi, P.G., Shoukir, Y., Campana, A. 1998. Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. *Hum Reprod,* **13**(4): 11-19.

Salah, M.S., El-Nouty, F.D., Al-Hajri, M.R. 1992. Effect of wáter sprinkling during the hot-dry summer season on semen quality of Holstein bulls in Saudi Arabia. *Anim Prod*, **55**: 59-63.

Salustri, A. & Siracusa, G. 1983. Metabolic coupling, cumulus expansion and meiotic resumption in mouse cumuli oophori cultured in vitro in the presence of FSH or dcAMP, or stimulated in vivo by hCG. *J Reprod Fertil,* **68**: 335-341.

Sanocka, D., Kurpisz, M. 2004. Reactive oxygen species and sperm cells. *Reprod Biol Endocrinol*, **2**: 12-19

Sartori, R., Sartor-Bergfelt, R., Mertens, S.A., Guenther, J.N., Parrish, J.J., Wiltbank, M.C. 2002. Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. *J Dairy Sci*, **85**: 2803-2812.

Sartori, R., Haughian, J.M., Shaver, R.D., Rosa, G.J.M., Wiltbank, M.C. 2004. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. *J Dairy Sci,* **87**(4): 905-920.

Sartorelli, E.S., Satrapa, R.A., Barcelos, A.C.Z., Potiens, J.R., Barros, C.M. 2006. Influencia do estresse térmico calórico na taxa de apoptose em embriones bovinos (indicus vs taurus) produzidos in vitro e na capacidade dos mesmos em originarem gestaçones. *Acta Scientiae Veterinariae,* **34**(Suplemento 1): s476.

Saunders, P.T.K. 2003. Germ cell-somatic cell interactions during spermatogenesis. *Reprod Suppl*, **61**: 91-101.

Schiller, J., Muller, K., Sus, R., Arnhold, J., Gey, C., Herrmann, A., Lesig, J., Klaus, Arnold, K., Muller, P. 2003. Analysis of the lipid composition of bull spermatozoa by MALDI-TOF mass spectrometry-a cautionary note. *Chem Phys Lipids,* **126**: 85–94.

Schmitt, E.J., Diaz, T., Barros, C.M., De la Sota, R.L., Drost, M., Fredriksson, E.W., Staples, C.R., Thorner, R., Thatcher, W.W. 1996a. Differential response of the luteal phase and fertility in cattle following ovulation of the first-wave follicle with human chorionic gonadotropin or an agonist of gonadotropin-releasing hormone. *J Anim Sci,* **74**: 1074–1083.

Schmitt, EJ-P., Diaz, T., Drost, M., Fredriksson, E.W., Thatcher, W.W. 1996b. Use of a gonadothropin releasing hormone or human chorionic gonadotrophin for timed insemination in cattle. *J Anim Sci,* **74**: 1084–1091.

Schrock, G.E., Saxton, A.M., Schrick, F.N., and Edwards, J.L. 2007. Early in vitro fertilization improves development of bovine ova heat stressed during in vitro maturation. *J Dairy Sci,,* **90**(9): 4297-4303.

Schuel, H. 1985. Function of egg cortical granules. In Metz CB and Monroy A (eds). Biology of Fertilization. Vol. 3. Academic Press, New York, USA, 1–43.

Schwaab, V., Faure, J., Dufaure, J.P., Drevet, J.R. 1998. GPx3: the plasma-type glutathione peroxidase is expressed under androgenic control in the mouse epididymis and vas deferens. *Mol Reprod Dev,* **51**: 362–372.

Seif, S.M., Johnson, H.D., Lippincott, A.C. 1979. The effects of heat exposure (31ºC) on Zebu and Scottish Highland cattle. *Int J Biome,* **23**: 9–14.

Setchell, BP. 1978. The Mammalian Testis. London, United Kingdom: Elek Books and Ithaca, NY: Cornell University Press.

Setchell, B.P. 1998. The Parkes Lecture. Heat and the testis. *J Reprod Fertil,* **114**: 179–194.

Setchell, B.P. & Breed, W.G. 2006. Anatomy, vasculature and innervation of the male reproductive tract. In: Neill JD (Ed.). Knobil and Neill's physiology of reproduction, San Diego, USA: Elsevier, 771-825.

Setchell, B.P., D'Occhio, M.J., Hall, M.J., Laurie, M.S., Tucker, M.J. and Zupp, J.L. 1988. Is embryonic mortality increased in normal females mated to subfertile males? *J Reprod Fert,* **82**: 567-574.

Setchell, B.P., Ekpe, G., Zupp, J.L. and Surani, M.A. 1998. Transient retardation in embryo growth in normal female mice made pregnant by males whose testes had been heated. *Hum Reprod,* **13**: 342-347.

Setchell, B.P. & Mieusset, R. 1996. Testis thermorégulation. In Male Gametes Production and Quality, pp 65-81. Eds S Hamamah and R Mieusset. INSERM, Paris.

Setchell, B.P. & Waites, G.M.H. 1972. The effects of local heating of the testis on the flow and composition of rete testis fluid in the rat, with some observations on the effects of age and unilateral castration. *J Reprod Fertil,* **30**: 225-233.

Setchell, B.P., Zupp, J.L., Ekpe, G., Maddocks, S. and Grigg, G. 1994. The effect of environmental temperature on scrotal temperature in rams at pasture, recorded by telemetry. *Proceedings of the Australian Society for Reproductive Biology,* **26**: 87.

Shafie, M.M., Kamar, G.A.R., Borady, A.M., Hassanein, A.M. 1982. Thermoregulation in rabbits under different environmental conditions. In: *Proceedings of 6th International Conference on Animal and Poultry Production*, Zagazig, Egypt, 21–23.

Shafie, M.M., Kamar, G.A.R., Borady, A.H.A., Hassanien, A.M. 1984. Reproductive performance of Giza rabbit does under different natural and artificial environmental conditions. *Egyptian J Anim Prod,* **24**(1-2): 167-174.

Shaham-Albalancy, A., Meidan, R., Rosenberg, M., Folman, Y., Wolfenson, D. 1996a. The effect of plasma progesterone on steroidogenic capacity of granulosa and theca cells from dominant follicles. *Biol Reprod,* **45**(Suppl. 1): 59.

Shaham-Albalancy, A., Rosenberg, M., Folman, Y., Nyska, A., Wolfenson, D. 1996b. The effect of progesterone concentration during the luteal phase of the estrous cycle on uterine endometrial morphology and function during the subsequent estrous cycle of dairy cows. The 13th Int. Cong. Anim. Reprod., 18.

Sharma, R.K. & Agarwal, A. 1996. Role of reactive oxygen species in male infertility. *Urology*, **48**(6): 835–850.

Sharpe, R.M., Maddocks, S., Millar, M., Kerr, J.B., Saunders P.T., McKinnell, C. 1992. Testosterone and spermatogenesis. Identification of stage-specific, androgen-regulated proteins secreted by adult rat seminiferous tubules. *J Androl,* **13**(2): 172-184.

Shaw, D.W., Farin, P.W., Washburn, S.P., Britt, J.H. 1995. Effect of retinol palmitate on ovulation rate and embryo quality in superovulated cattle. *Theriogenology*, **44**: 51–58.

Shearer, J.K. & Beede, D.K. 1990a. Heat-Stress.1. Thermoregulation and Physiological- Responses of Dairy-Cattle in Hot Weather. *Agri-Practice,* **11**(4): 5-8.

Shearer, J.K. & Beede, D.K. 1990b. Heat-Stress.2. Effects of High Environmental-Temperature on Production, Reproduction, and Health of Dairy-Cattle. *Agri-Practice,* **11**(5): 6-16.

Shehab-El-Deen, M.A.M.M., Leroy, J.L.M.R., Fadel, M., Saleh, S., Maes, D., Van Soom, A. 2010. Biochemical changes in the follicular fluid of the dominant follicle of high producing dairy cows exposed to heat stress early post partum. *Anim Reprod Sci,* **117**: 189-200.

Shehab-El-Deen, M.A.M.M. 2011. Effects of metabolic stressors and high temperature on oocyte and embryo quality in high yielding dairy cows. Ph.D. thesis. Faculty of Veterinary Medicine, Ghent University.

Shi, D.S., Avery, B., Greve, T. 1998. Effects of temperature gradients on in vitro maturation of bovine oocytes. *Theriogenology*, **50**: 667-674.

Shimizu, T., Ohshima, I., Ozawa, M., Takahashi, S., Tajima, A., Shiota, M. *et al*. 2005. Heat stress diminishes gonadotropin receptor expression and enhances susceptibility to apoptosis of rat granulose cells. *Reproduction*, **129**: 463–472.

Shin, M.R., Kim, N.H. 2003. Maternal gamma (g)-tubulin is involved in microtubule reorganization during bovine fertilization and parthenogenesis. *Mol Reprod Develop*, **64**: 438–445.

Shoubridge, E.A. & Wai, T. 2007. Mitochondrial DNA and the mammalian oocyte. *Curr Top Dev Biol,* **77**: 87–111.

Sikka, S.C. 1996. Oxidative stress and role of antioxidants in normal and abnormal sperm function. *Frontiers in Bioscience*, **1**: e78–e86.

Sikka, S.C. 2001. Relative impact of oxidative stress on male reproductive function. *Curr Med Chem,* **8**: 851–862.

Sikka, S.C. 2004. Role of oxidative stress and antioxidants in and urology and assisted reproductive technology. *J Androl,* **25**: 5–18.

Sikka, S.C., Rajasekaran, M., Hellstrom, W.J.G. 1995. Role of oxidative stress and antioxidants in male infertility. *J Androl,* **16**: 464-468.

Simon, A.M., Goodenough, D.A., Li, E., Paul, D.L. 1997. Female infertility in mice lacking connexin 37. *Nature*. **385**: 525-529.

Sirard, M. A., Richard, F., Blondin, P., Robert, C. 2006. Contribution of the oocyte to embryo quality. *Theriogenology*, **65**: 126–136.

Skibba, J.L., Powers, R.H., Stadnicka, A., Cullinane, D.W., Almagro, U.A., and Kalbfleisch, J.H. 1991. Oxidative Stress as a Precursor to the Irreversible Hepatocellular Injury Caused by Hyperthermia. *Int J Hyperther,* **7**(5): 749-761.

Skibba, J.L. & Stadnicka, A. 1986. Xanthine oxidase (XO) activity at hyperthermic temperatures as a source of free radicals. *Proc Am Assn Cancer Res,* **27**: 400.

Skinner, J.D., Louw, G.N. 1966. Heat stress and spermatogenesis in Bos indicus and Bos taurus cattle. *J Appl Physiol,* **21**: 1784-1790.

Smith, J.F. 1971. The effect of temperature on characteristics of semen in rams. *Australian J Agri Res*, **22**: 481-490.

Spano, M., Seli, E., Bizzaro, D., Manicardi, G.C., Sakkas, D. 2005. The significance of sperm nuclear DNA strand breaks on reproductive outcome. *Curr Opin Obstet Gynecol*, **17**: 255-260.

Soto, P., Smith, L.C. 2009. BH4 Peptide Derived From Bcl-xL and Bax-Inhibitor Peptide Suppresses Apoptotic Mitochondrial Changes in Heat Stressed Bovine Oocytes. *Mol Reprod Dev*, **76**(7): 637-646.

Stevens, D.G. 1981. A Model of Respiratory Vapor Loss in Holstein Dairy-Cattle. *T Asae,* **24**(1): 151-153.

Stock, A.E., Fortune, J.E. 1993. Ovarian follicular dominance in cattle: relationship between prolonged growth of the ovulatory follicle and endocrine parameters. *Endocrinology*, **132**: 1108-1114.

Stojkovic, M., Machado, S.A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Gonçalves, P.B., *et al.* 2001. Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biol Reprod,* **64**: 904–909.

Stone, B.A. 1981. Thermal characteristics of the testis and epididymis of the boar. *J Reprod Fertil,* **63**(2): 551-557.

Stone, B.A. 1982. Heat induced infertility of boars: The inter-relationship between depressed sperm output and fertility and an estimation of the critical air temperature above which sperm output is impaired. *Anim Reprod Sci,* **4**(4): 283–299.

Stone, B.A. & Seamark, R.F. 1984. Effects of acute and chronic testicular hyperthermia on levels of testosterone and corticosteroids in plasma of boars. *Anim Reprod Sci,* **7**: 391-403.

Stott, G.H. & Wiersma, F. 1976. Short-Term Thermal Relief for Improved Fertility in Dairy-Cattle during Hot Weather. *Int J Biometeorol*, **20**(4): 344-350.

Streffer, C. 1984. Mechanism of heat injury. In: Overgaard J, editor. Hyperthermic oncology. London: Taylor and Francis; 213–22.

Strzezek, J. 2002. Secretory activity of boar seminal vesicle glands. *Reprod Biol,* **2:** 243–266.

Strzezek, J., Fraser, L., Demianowicz, W., Kordan, W., Wysocki, P., Holody, D. 2000. Effect of depletion tests (DT) on the composition of boar semen. *Theriogenology*, **54**: 949–963.

Suckow, Mark A., Douglas, Fred, The Laboratory Rabbit, a volume in the Laboratory Animal Pocket Reference Series (Boca Raton: CRC Press, 1997).

Sugiyama, S., McGowan, M., Phillips, N., Kafi, M. and Young, M. 2007. Effects of Increased Ambient Temperature During IVM and/or IVF on the In Vitro Development of Bovine Zygotes. *Reprod Dom Anim,* **42**: 271-274.

Sun, Q.Y. & Schatten, H. 2006. Regulation of dynamic events by microfilaments during oocyte maturation and fertilization. *Reproduction*, **131**: 193–205.

Sutton, M.L., Gilchrist, R.B., Thompson, J.G. 2003. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Hum Reprod Update*, **9**(1): 35-48.

Swanson, P.E., Carroll, S.B., Zhang, X.F. & Mackey, M.A. 1995. Spontaneous premature chromosome condensation, micronucleus formation and non-apoptotic cell death in heated HeLa S3 cells. *Am J Pathol,* **146**: 969–971.

Swiestra, E.E., Foote, R.H. 1965. Duration of spermatogenesis and spermatozoon transport in the rabbit based on cytological changes, DNA synthesis and labelling with tritiated thymidine. *Am J Anat,* **116**(2): 401-412.

Szczesniak-Fabianczyk, B., Bochenek, M., Smorag, Z., Silvestre, M.A. 2006. Effect of antioxidants added to boar semen extender on the semen survival time and sperm chromatin structure. *Reprod Biol*, **3**: 81-87.

Szendro, Zs., Papp, Z., Kustos, K. 1998. Effect of environmental temperature and restricted feeding on production of rabbit does. *Cahiers Opt Med,* **41**: 11–17.

Szollosi, D. 1971. Morphological changes inmouse eggs due to aging in the fallopian tube. *Am J Anat,* **130**: 209–225.

Szollosi, D. 1974. The spindle structure in mammalian eggs: the effect of ageing. Colloque: Les accidents chromosomiques de la reproduction. 241–50.

Szollosi, D. 1975. Mammalian eggs aging in the fallopian tubes. In Aging Gametes, Their Biology and Pathology. Proceedings of the International Symposium on Aging Gametes, 13–16 June 1973. pp. 98–121. Seattle: Washington, USA.

Tahaei, L.S., Eimani, H., Yazdi, P.E., Ebrahimi, B., Fathi, R. 2011. Effects of retinoic acid on maturation of immature mouse oocytes in the presence and absence of a granulosa cell co-culture system. *J Assist Reprod Genet*, **28**(6): 553-558.

Takahashi, T., E. Takahashi, H. Igarashi, N. Tezuka, H. Kurachi. 2003. Impact of oxidative stress in aged mouse oocytes on calcium oscillations at fertilization. *Mol Reprod Dev,* **66**: 143–152.

Takami, M., Preston, S.L., Toyloy, V.A. and Behrman, H.R. 1999. Antioxidants reversibly inhibit the spontaneous resumption of meiosis. *Am J Physiol,* **276:** E684–688.

Tarazona, A.M., Rodriguez, J.I., Restrepo, L.F., Olivera-Angel, M. 2006. Mitochondrial activity, distribution and segregation in bovine oocytes and in embryos produced in vitro. *Reprod Dom Anim,* **41**: 5–11.

Tarín, J.J., Brines, J., Cano, A. 1998. Is antioxidant therapy a promising strategy to improve human reproduction? Antioxidants may protect against infertility. *Hum Reprod*, **13**(6): 1415–1424.

Tarín, J.J., Vendrell, F.J., Ten, J., Blanes, R., van Blerkom, J., Cano, A. 1996. The oxidizing agent tertiary butyl hydroperoxide induces disturbances in spindle organization, c-meiosis, and aneuploidy in mouse oocytes. *Mol Hum Reprod,* **2**(12): 895-901.

Tatemoto, H., Sakuri, N., Muto, N. 2000. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during in vitro maturation: role of cumulus cells. *Biol Reprod,* **63**: 805–810.

Tanghe, S., Van Soom, A., Nauwynck, H., Coryn, M., de Kruif, A. 2002. Minireview: functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev*, **61**: 414–424.

Thatcher, W.W. & Hansen, P.J. 1993. Environment and reproduction. In GJ King (ed): "Reproduction in Domesticated Animals." Elsevier World Animal Science Series B, vol. 9. Amsterdam: Elsevier, 433–457.

Thatcher, W.W. 1974. Effects of season, climate, and temperature on reproduction and lactation. *J Dairy Sci,* **57**(3): 360-368.

Theau-Clément, M., Michel, N., Esparbié, J., Bolet G. 1995. Effects of artificial photoperiods on sexual behaviour and sperm output in the rabbit. *Anim Sci,* **60:** 143-149.

Thom, E.C. 1958. Cooling degree days. *Air Conditioning, Heating and Ventilating,* **55**: 65-69.

Thom, E.C. 1959. The discomfort index. *Weatherwise*, **12**(1): 57-60.

Toivola, D.M., Strnad, P., Habtezion, A., and Omary, M.B. 2010. Intermediate filaments take the heat as stress proteins. *Trends Cell Biol*, **20**(2): 79-91.

Tomek, W., Torner, H., Kanitz, W. 2002. Comparative analysis of proteinsynthesis, transcription and cytoplasmic polyadenylation of mRNA during maturation of bovine oocytes in vitro. *Reprod Dom Anim,* **37**: 86–91.

Tompkins, E.C., Heidenreich, C.J., Stob, M. 1967. Effect of after-breeding thermal stress on embryonic mortality of swine. *J Anim Sci*, **26**: 377–380.

Torres-Junior, J.R.D., Pires, M.D.A., De Sa, W.F., Feffeira, A.D., Viana, J.H.M., Camargo, L.S.A., Ramos, A.A., Folhadella, I.M., Polisseni, J., de Freitas, C., Clemente, C.A.A., De Sa, M.F., Paula-Lopes, F.F., and Baruselli, P.S. 2008. Effect of maternal heat stress on follicular growth and oocyte competence in Bos indicus cattle. *Theriogenology,* **69**(2): 155-166.

Tremellen, K. 2008. Oxidative stress and male infertility—A clinical perspective. *Hum Reprod Update,* **14**: 243-258.

Trimarchi, J.R., Liu, L., Porterfield, D.M., Smith, P.J., Keefe, D.L. 2000. Oxidative phosphorylation-dependent and -independent oxygen consumption by individual preimplantation mouse embryos. *Biol Reprod.* **62**: 1866-1874.

Trounson, A., Anderiesz, C., Jones, G. 2001. Maturation of human oocytes in vitro and their developmental competence. *Reproduction*, **121**: 51–75.

Trout, J.P., McDowell, L.R., Hansen, P.J. 1998. Characteristics of the oestrous cycle and antioxidant status of lactating Holstein cows exposed to stress. *J Dairy Sci,* **81**: 1244–1250.

Trudeau, V. & Sanford, L.M. 1986. Effect of Season and Social Environment on Testis Size and Semen Quality of the Adult Landrace Boar V. *J Anim Sci,* **63**: 1211-1219.

Tseng, J.K. 2004. Nuclear and cytoskeletal alterations of in vitro matured porcine oocytes under hyperthermia. *Mol Reprod Dev,* **68**: 125–133.

Tseng, J.K., Chen, C.H., Chou, P.C., Yeh, S.P. and Ju, J.C. 2004. Influences of Follicular Size on Parthenogenetic Activation and in Vitro Heat Shock on the Cytoskeleton in Cattle Oocytes. *Reprod Dom Anim,* **39**: 146–153.

Tseng, J.K., Tang, P.C., Ju, J.C. 2006. In vitro thermal stress induces apoptosis and reduces development of porcine parthenotes. *Theriogenology*, **66**: 1073–1082.

Tuncer, P.B., Bucak, M.N., Büyükleblebici, S., Sarıözkan, S., Yeni, D., Eken, A., Akalın, P.P., Kinet, H., Avdatek, F., Fidan, A.F., Gündoğan, M. 2010. The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen. *Cryobiology*, **61**(3): 303-307.

Turner, H.G. 1982. Genetic variation of rectal temperature in cows and its relationship to fertility. *Anim Prod*, **35**(3): 401-412.

Tusell, L., David, I., Bodin, L., Legarra, A., Rafel, O., López-Bejar, M. et al. 2011. Using the product threshold model for estimating separately the effect of temperature on male and female fertility. *J Anim Sci*, **89**: 3983-3995.

Ulberg, L.D., Burfening, P.J. 1967. Embryo death resulting from adverse environment on spermatozoa or ova. *J Anim Sci*, **26**: 571-577.

Ullah, G., Fuquay, J.W., Keawkhong, T., Clark, B.L., Pogue, D.E., Murphey, E.J. 1996. Effect of gonadotropin-releasing hormone at estrus on subsequent luteal function and fertility in lactating Holsteins during heat stress. *J Dairy Sci,* **79**: 1950–1953.

Uysal, O., Bucak, M.N. 2007. Effects of Oxidized Glutathione, Bovine Serum Albumin, Cysteine and Lycopene on the Quality of Frozen-Thawed Ram Semen. *Acta vet Brno*, **76**: 383-390.

Uysal, O., Korkmaz, T., Tosun, H. 2005. Effect of bovine serum albumine on freezing of canine semen. *Indian Vet J*, **82**: 97-98.

Vahedi, V., Zeinoaldini, S., Kohram, H., Farahavar, A. 2009. Retinoic acid effects on nuclear maturation of bovine oocytes in vitro. *African J Biotechnol,* **8**: 3974-3978.

Vallorani, C., Spinaci, M., Bucci, D., Tamanini, C., Galeati, G. 2010. Effects of antioxidants on boar spermatozoa during sorting and storage. *Anim Reprod Sci*, **122**: 58–65.

Van Blerkom, J., Antczak, M., Schrader, R. 1997. The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. *Hum Reprod,* **12**: 1047-1055.

Vanholder, T., Leroy, J.L., Van Soom, A., Maes, D., Coryn, M., Fiers, T., de Kruif, A., Opsomera, G. 2006. Effect of non-esterified fatty acids on bovine theca cell steroidogenesis and proliferation in vitro. *Anim Reprod Sci*, **92**(1-2): 51-63.

Vanholder, T., Leroy, J.L.M.R., Van Soom, A., Opsomer, G., Maes, D., Coryn, M., and de Kruif, A. 2005. Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation in vitro. *Anim Reprod Sci,* **87**(1-2): 33-44.

Vasconcelos, J.L.M., Silcox, R.W., Lacerda, J.A., Pursley, G.R., Wiltbank, M.C. 1998. Pregnancy rate, pregnancy loss, and response to heat stress after AI at 2 different times from ovulation in dairy cows. *Biol Reprod,* **56**(Suppl 1): 140 (Abstract).

Vaught, L.W., Monty, D.W., Foote, W.C. 1977. Effect of summer heat stress on serum LH and progesterone values in Holstein-Frisian cows in Arizona. *Am J Vet Res,* **38**: 1027–1032.

Vega, M., Carrasca, I., Castillo, T., Troncoso, J.L., Videla, I.A., Devoto, L. 1995. Functional luteolysis in response to hydrogen peroxide in human luteal cells. *J Endo,* **147**: 177–182.

Velazquez, A., Rosado, A. Jr., Rosado, A. 1975. Participation of vitamin A in the maturation of rabbit spermatozoa. *Int J Fertil*, **20**(3): 151-155.

Verga, M., Luzi, F., Carenzi, C. 2007. Effects of husbandry and management systems on physiology and behaviour of farmed and laboratory rabbits. *Horm Behav,* **52**: 122-129.

Vidair, C.A., Doxsey, S.J. & Dewey, W.C. 1993. Heat shock alters centrosome organization leading to mitotic dysfunction and cell death. *J Cell Physiol,* **154**: 443–445.

Vigh, L., Nakamoto, H., Landry, J., Gomez-Munoz, A., Harwood, J.L., Horvath, I. 2007. Membrane regulation of the stress response from prokaryotic models to mammalian cells. *Ann Ny Acad Sci*, **1113**: 40-51.

Vigodner, M., Lewin, L.M., Schochat, L., Oschry, I., Lotan, G., Kleen, B., Golan, R. 2003. Evaluation of damage to the testicular cells of golden hamsters caused by experimental cryptorchidism using flow cytometry and confocal microscopy. *Int J Androl,* **26**: 84-90.

Vilakazi, D.M., Webb, E.C. 2004. Effect of age and season on sperm morphology of Friesland bulls at an artificial insemination centre in South Africa. *SA J Anim Sci*, **34**: 62-69.

Vincent, A.M. & Feldman, E.L. 2002. Control of cell survival by IGF signaling pathways. *Growth Horm IGF Res,* **12**: 193-197.

Virro, M.R., Larson-Cook, K.L., Evenson, D.P. 2004. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in *in vitro* fertilization and intracytoplasmic sperm injection cycles. *Fertil Steril,* **81**: 1289-1295.

Waberski, D., Schmid, S., Henning, H. 2010. Summer heat stress on boar sperm membranes and extender influences. http://www.minitube.de/DE_eng/Media/Images/SUMMER-HEAT-STRESS-ON-BOAR-SPERM-MEMBRANES-AND-EXTENDER-INFLUENCES

Waites, G.M.H. & Setchell, B.P. 1990. Physiology of the mammalian testis. In Marshall's Physiology of Reproduction, 4th Edn, Vol 2, pp 1-105 Ed. GE Lamming, Churchill Livingstone, London.

Walker, W.L., Nebel, R.L., McGilliard, M.L. 1996. Time of ovulation relative to mounting activity in dairy cattle. *J Dairy Sci*, **79**(9): 1555-1561.

Walters, A.H., Eyestone, W.E., Saacke, R.G., Pearson, R.E., Gwazdauskas, F.C. 2004. Sperm morphology and preparation method affect bovine embryonic development. *J Androl,* **25**: 554-563.

Walters, A.H., Eyestone, W.E., Saacke, R.G., Pearson, R.E., Gwazdauskas, F.C. 2005. Bovine embryo development after IVF with spermatozoa having abnormal morphology. *Theriogenology,* **63**: 1925-1937.

Walters, A.H., Saacke, R.G., Pearson, R.E. and Gwazdauskas, F.C. 2006. Assessment of pronuclear formation following in vitro fertilization with bovine spermatozoa obtained after thermal insulation of the testis. *Theriogenology,* **65**: 1016-1028.

Wang, A.W., Zhang, H., Ikemoto, I., Anderson, D.J., Loughlin, K.R. 1997. Reactive oxygen species generation by seminal cells during cryopreservation. *Urology,* **49**: 921–925.

Wang J.Z., Sui, H.S., Miao, D.Q., Liu, N., Zhou, P., Ge, L., Tan, J.H. 2008. Effects of heat stress during in vitro maturation on cytoplasmic versus nuclear components of mouse oocytes *Reproduction,* **137**: 1–10.

Wang, X., Sharma, R.K., Sikka, S.C., Thomas, A.J., Falcone, T. Jr. and Agarwal, A. 2003. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. *Fertil Steril,* **80**: 531-535.

Warnes, G.M., Moor, R.M., Johnson, M.H. 1977. Changes in protein synthesis during maturation of sheep oocytes in vivo and in vitro. *J Reprod Fertil,* **49**: 331–335.

Welch, W.J. 1992. Mammalian stress response: cell physiology, structure/function of stress proteins and implications for medicine and disease. *Physiol Rev,* **72**: 1063-1081.

Welch, W.J. 1993. Come le cellule reagiscono allo stress. *Le Scienze*, **51**(299): 50-57.

Welch, W. & Suhuan, J. 1985. Morphological study of the mammalian stress response characterization of changes in cytoplasmic organelles, cytoskeleton, and nucleoli, and appearance of intranuclear actin filaments in rat fibroblasts after heat shock treatment. *J Cell Biol,* **101**: 1198–1211.

Wells, P.G., Kim, P.M., Laposa, R.R., Nicol, C.J., Parman, T., Winn, L.M. 1997. Oxidative damage in chemical teratogenesis. *Mutat Res,* **396**: 65–78.

Wessel, G.M., Brooks, J.M., Green, E., Haley, S., Voronina, E., Wong, J. et al. 2001. The biology of cortical granules. *Int Rev Cytol,* **209**: 117–206.

Wessel, G.M., Conner, S.D., Berg, L. 2002. Cortical granule translocation is microfilament mediated and linked to meiotic maturation in the sea urchin oocyte. *Development*, **129**: 4315–4325.

West, J.W. 2003. Effects of heat stress on production in dairy cattle. *J Dairy Sci,* **86**: 2131-2144.

Wettemann, R.P. & Bazer, F.W. 1985. Influence of environmental temperature on prolificacy of pigs. *J Reprod Fert Suppl,* **33**: 199-208.

Wettemann, R.P. & Desjardins, C. 1979. Testicular function in boars exposed to elevated ambient temperature. *Biol Reprod,* **20**: 235-241.

Wettemann, R.P., Wells, M.E. and Johnson, R.K. 1979. Reproductive characteristics of boars during and after exposure to increased ambient temperature. *J Anim Sci*, **49**(6): 1501-1505.

Wettemann, R.P., Wells, M.E., Omtvedt, I.T., Pope, C.E., Turman, E.J. 1976. Influence of elevated ambient temperature on reproductive performance of boars. *J Anim Sci,* **42**: 664–669.

Whaley, S.L., Hedgpeth, V.S., Farin, C.E., Martus, N.S., Jayes, F.C.L., Britt, J.H. 2000. Influence of vitamin A injection before mating on oocyte development, follicular hormones, and ovulation in gilts fed high-energy diets. *J Anim Sci,* **78**: 1598–1607.

Wildeus, S., Entwistle, K.W. 1983. Spermiogram and sperm reserves in hybrid Bos indicus X Bos taurus bulls after scrotal insulation. *J Reprod Fertil*, **69**: 711-716.

Wildt, D.E., Riegle, G.D. and Dukelow, W.R. 1975. Physiological temperature response and embryonic mortality in stressed swine. *Am J Physiol*, **229**: 1471-1475.

Wilson, S.J., Kirby, C.J., Koenigsfeld, A.T., Keisler, D.H. and Lucy, M.C. 1998a. Effects of controlled heat stress on ovarian function of dairy cattle: II. Heifers. *J Dairy Sci,* **81**: 2132–2138.

Wilson, S.J., Marion, R.S., Spain, J.N., Spiers, D.E., Keisler, D.H. and Lucy, M.C. 1998b. Effects of controlled heat stress on ovarian function of dairy cattle: I. Lactating cows. *J Dairy Sci,* **81**: 2124–2131.

Windig, J.J., Calus, M.P.L. and Veerkamp, R.F. 2005. Influence of herd environment on health and fertility and their relationship with milk production. *J Dairy Sci*, **88**: 335–347.

Winfield, C.G., Hemsworth, P.H., Galloway, D.B. and Makin, A.W. 1981. Sexual behaviour and semen characteristics of boars: effects of high temperature. *Aust J Exp Agric Anim Husbandry,* **21**(108): 39-45.

Wise, M.E., Rodreguez, R.E., Armstrong, D.V., Huber, J.T., Weirsma, F., Hunter, R. 1988. Fertility and hormonal responses to temporary relief of heat stress in lactating dairy cows. *Theriogenology,* **29**: 1027–1035.

Wolbach, S.B., Howe, P.R. 1925. Tissue changes following deprivation of fat-soluble A vitamin. *J Exp Med*, **42**(6): 753-777.

Wolfenson, D., Barton, F.F., Badinga, L., Barros, C.M., Marple, D.N., Cummins, K., Wolfe, D., Lucy, M.C., Spencer, T.E., Thatcher, W.W. 1993a. Secretion of PGF2α and oxytocin during hyperthermia in cyclic and pregnant heifers. *Theriogenology*, **39**: 1129–1141.

Wolfenson, D., Blüm, O. 1988. Embryonic-development, conception rate, ovarian-function and structure in pregnant rabbits heat-stressed before or during implantation. *Anim Reprod Sci*, **17**: 259–270.

Wolfenson, D., Flamenbaum, I., Berman, A. 1988. Hyperthermia and body energy store effects on estrous behavior, conception rate, and corpus luteum function in dairy cows. *J Dairy Sci,* **71**: 3497–3504.

Wolfenson, D., Lew, B.J., Thatcher, W.W., Graber, Y. and Meidan, R. 1997. Seasonal and acute heat stress effects on steroid production by dominant follicles in cows. *Anim Reprod Sci,* **47**: 9–19.

Wolfenson, D., Luft, O., Berman, A., Meidan, R. 1993b. Effects of season, incubation temperature and cell age on progesterone and prostaglandin F2α production in bovine luteal cells. *Anim Reprod Sci,* **32**: 27–40.

Wolfenson, D., Roth, Z., and Meidan, R. 2000. Impaired reproduction in heat-stressed cattle: basic and applied aspects. *Anim Reprod Sci,* **60/61**: 535-547.

Wolfenson, D., Thatcher, W.W., Badinga, L., Savio, J.D., Meidan, R., Lew, B.J. et al. 1995. Effect of heat stress on follicular development during the estrous cycle in lactating dairy cattle. *Biol Reprod,* **52**: 1106–1113.

Wong, W.Y., Thomas, C.M., Merkus, J.M., Zielhuis, G.A., Steegers-Theunissen, R.P. 2000. Male factor subfertility: possible causes and the impact of nutritional factors. *Fertil Steril*, **73**: 435–442.

Woody, C.O. & Ulberg, L.C. 1964. Viability of One-Cell Sheep Ova as Affected by High Environmental Temperature. *J Reprod Fertil,* **7**: 275-80.

Yaeram, J., Setchell, B.P. and Maddocks, S. 2006. Effect of heat stress on the fertility of male mice in vivo and in vitro. *Reprod Fertil Dev*, **18**: 647-653.

Yanagimachi, R. 1994. Mammalian fertilization. In Knobil E and Neill JD (eds). The Physiology of Reproduction. 2nd edn. Raven Press, New York, USA, 189–317.

Yin, Y., Hawkins, K.L., DeWolf, W.C. and Morgentaler, A. 1997. Heat stress causes testicular germ cell apoptosis in adult mice. *J Androl,* **18**(2): 159-165.

Yoshida, M. 1993. Role of glutathione in the maturation and fertilization of pig oocytes in vitro. *Mol Reprod Dev*, **35**: 76-81.

Yost, H.J., Lindquist, S. 1986. Rna Splicing Is Interrupted by Heat-Shock and Is Rescued by Heat-Shock Protein-Synthesis. *Cell,* **45**(2): 185-193.

Younas, M., Fuquay, J.W., Smith, A.E., Moore, A.B. 1993. Estrus and endocrine responses of lactating Holsteins to forced ventilation during summer. *J Dairy Sci,* **76**: 430–434.

Young, B.A. 1981. Cold Stress as It Affects Animal Production. *J Anim Sci*, **52**(1): 154-163.

Young, W.C. 1927. The influence of high temperature on the guinea pig testis. Histological changes and effects on reproduction. *J Exp Zool,* **49**: 459-499.

Yousef, M.I., Abdallah, G.A., Kamel, K.I. 2003. Effect of ascorbic acid and Vitamin E supplementation on semen quality and biochemical parameters of male rabbits. *Anim Reprod Sci,* **76**: 99–111.

Yousef, M.K. 1984. Stress physiology: definition and terminology. In: Yousef, M.K. (Ed.) Stress Physiology in Livestock. CRC Press, Boca Raton, FL, 3-7.

Yousef, M.K. 1985. Stress physiology in livestock. CRC Press, Boca Raton, FL (USA).

Yu, B.P. 1994. Cellular defenses against damage from reactive oxygen species. *Physiol Rev,* **74**: 139–162.

Yuan, Y., Hao, Z.D., Liu, J., Wu, Y., Yang, L., Liu, G.S., Tian, J.H., Zhu, S.E., Zeng, S.M. 2008. Heat shock at the germinal vesicle breakdown stage induces apoptosis in surrounding cumulus cells and reduces maturation rates of porcine oocytes in vitro. *Theriogenology*. **70**(2): 168-178.

Zeron, Y., Ocheretny, A., Kedar, O., Borochov, A., Sklan, D. and Arav, A. 2001. Seasonal changes in bovine fertility: relation to oocyte developmental competence, membrane properties and follicle fatty acid composition. *Reproduction,* **121**: 447–454.

Zeron, Y., Sklan, D., Arav, A. 2002. Effect of polyunsaturated fatty acid supplementation on biophysical parameters and chilling sensitivity of ewe oocytes. *Mol Reprod Dev*, **61**(2): 271-278.

Zeidan, A.E.B., Marai, I.F.M., Abd El-Kariem, Z.A. 1997. Effects of intratesticular injection of gonadotropin-releasing hormone on reproductive performance of low fertile male rabbits under Egyptian summer conditions. In: Proceedings of 1st International Conference on Animal Production and Health, Dokki, Egypt, 557–566.

Zhang, L., Jiang, S., Wozniak, P.J., Yang, X., Godke, R.A. 1995. Cumulus cell function during bovine oocyte maturation, fertilization, and embryo development in vitro. *Mol Reprod Dev,* **40**(3): 338-344.

Zhang, R.D., Wen, X.H., Jong, L.S., Deng, X.Z., Peng, B., Huang, A.P., Wan, Y., Yang, Z.W. 2002. A quantitative (stereological) study of the effects of experimental unilateral cryptorchidism and subsequent orchidopexy on spermatogenesis in adult rabbit testis. *Reproduction,* **124**: 95-105.

Zhu, B.K. & Setchell, B.P. 2004. Effects of paternal heat stress on the in vivo development of preimplantation embryos in the mouse. *Reprod Nutr Dev,* **44**: 617–629.

Zhu, B., Walker, S.K., Oakey, H., Setchell, B.P. and Maddocks, S. 2004. Effect of paternal heat stress on the development in vitro of preimplantation embryos in the mouse. *Andrologia,* **36**: 384–394.

Zimbelman, R.B., Rhoads, R.P., Rhoads, M.L., Duff, G.C., Baumgard, L.H., and Collier, R.J. 2009. A Re-Evaluation of the Impact of Temperature Humidity Index (THI) and Black Globe Humidity Index (BGHI) on Milk Production in High Producing Dairy Cows. *The American Registry of Professional Animal Scientists, Southwest Nutrition & Management Conference,* Proceedings 26–27.

APPENDIX

Author information

Academic degrees

2008–2012 **Ph.D. in Veterinary Science.**

Fellowship from UAB (PIF), Universitat Autònoma de Barcelona.

PhD thesis: Heat stress and antioxidant agents: Effects on gamete development. Expected date (viva defense): November 2012.

2010 **Postgraduated course: Research staff user of animals for experimental research and other aims.**

Personal investigador usuario de animales para experimentación y otras finalidades (FELASA), Universitat Autònoma de Barcelona.

2008–2009 **Master in Veterinary Research**

Special field: Animal medicine and surgery, Universitat Autònoma de Barcelona

Research dissertation (awarded with honours): Effect of antioxidant agents on oocyte maturation under heat stress conditions.

2003–2008 **Degree in Veterinary Science**

Universitat Autònoma de Barcelona.

Academic experience

2008-2012 **Teaching and research staff.**

Department of Animal Health and Anatomy, Universitat Autònoma de Barcelona.

Member of the domestic and wild animal's reproductive diagnosis laboratory.

05-06/2012 **Short stay at the *In vitro* fertilization laboratory, UAB.**

Supervisors: Dr. Maite Paramio, Dr. Maria Catalá.

10/2011 **Research stay at the European Xenopus Resource Centre.**

School of Biological Science, University of Portsmouth, Portsmouth, UK.

Awarded scholarship stay ESTPIF 2011-30 from UAB.

Amphibian cellular and embryo cryopreservation for Gene-banks.

Protein crystallography (X-ray crystallography).

Supervisors: Dr. Rhiannon Lloyd, Dr. Matt Guille.

05/2011 **Short stay at the Department of Genetics from Biology Faculty, Universidad Autónoma de Madrid.**

Nuclear DNA fragmentation using the chromatin dispersion test.

Supervisor: Dr. Jaime Gosálvez.

12/2010-02/2011 **Research stay at the Institute of Zoology (ZSL).**

Institute of Zoology, London, UK.

Awarded scholarship stay ESTPIF 2010-39 from UAB.

Development of molecular barcoding techniques in endangered amphibian tissues from the Amphibian Ark (genebank).

Cellular cryopreservation for genebanks.

Supervisors: Dr. William V. Holt, Dr. Rhiannon Lloyd.

05/2010 **Workshop: Ultrasonography of marine mammals (by Thomas Hildebrandt).**

International conference on diseases of zoo and wild animals (EAZWV), Madrid.

Ultrasonography techniques (conventional and 3D) developed on dolphins, gray seals, sea lions and bamboo sharks.

10/2008 **Volunteer at the 43 European Veterinary Conference SEVC 2008, Barcelona.**

10/2006-10/2008 **Professional practices at ARS Veterinary Hospital, Barcelona.**

Services in: Intensive care unit, Orthopedics, Neurology, Intern medicine, Anaesthesia, Ophthalmology.

10/2007 **Workshop: Reconstructive surgery in small animals (by Dick White).**

42 AVEPA-SEVC, Barcelona.

07/2007 **Practices at the Neurology service, UAB Veterinary Hospital.**

Grants

2009-2012

Heat stress on reproduction and survival. Determination of environmental and genetic variable factors which influences on their tolerance. Study of their effects on gamete and embryo development (MICINN, Ref. RTA2008–00070-C02–02).

Publications

Bovine oocytes show a higher tolerance to heat shock in the warm compared to the cold season of the year.

M.J. Maya-Soriano, F. López-Gatius, C. Andreu-Vázquez, M. López-Béjar

Theriogenology (2012). Accepted for publication (THE_12268).

Retinol improves in vitro oocyte nuclear maturation under heat stress in heifers.

MJ Maya-Soriano, E Taberner, M López-Béjar

Zygote (2012), Epub 11 julio. DOI : 10.1017/S0967199412000135.

Biobanked amphibian samples confirmed to species level using 16S rRNA DNA barcodes.

MJ Maya-Soriano, R Lloyd, WV Holt.

Biopreservation & Biobanking (2012), 10(1): pp 22-28.

Does heat stress provoke the loss of a continuous layer of cortical granules beneath the plasma membrane?

Andreu-Vázquez C., Maria José Maya Soriano, López-Gatius F., García-Ispierto I., Hunter RHF., López-Béjar M.

Zygote (2010), 18(4): pp 293-299.

Papers under review

Absence of beneficial effects on rabbit sperm cell cryopreservation by several antioxidant agents.

Maya-Soriano M.J., Taberner E., Sabés-Alsina M, Piles M, López-Béjar M.

Zygote (2012).

Daily exposure to summer temperatures affects epididymal sperm cells in an in vivo rabbit model.

Maya-Soriano M.J., Taberner E., Sabés-Alsina M., Ramon J., Rafel O., Tussell Ll., Piles M., López-Béjar M.

Reproduction (2012).

Retinol stabilizes acrosome membrane in heat-shocked bull spermatozoa.

Maya-Soriano MJ, Taberner E, Sabés-Alsina M, López-Béjar M.

Theriogenology (2012).

Published communications

***In vivo* heat stress affects spermatogenesis in rabbits.**

M Sabes, N Planell, E Torres-Mejía, E Taberner, M Maya-Soriano, L Tusell, J Ramon, M Piles, M Lopez-Bejar.

Reproduction in Domestic animals (2012), 47(Suppl 5): pp 94. Poster at 16th ESDAR conference.

***In vivo* heat stress affects development of preimplantational rabbit embryos.**

E Torres, M Sabés-Alsina, E Taberner, MJ Maya-Soriano, J Ramon, O Rafel, M Piles, M López-Béjar

Reproduction in Domestic Animals (2012), 47(Suppl 4) : pp 419. Poster at 17th ICAR conference.

Cryopreservation of giraffe (*Giraffa camelopardalis*) epididymal sperm cells.

MJ Maya-Soriano, H Fernández-Bellón, E Taberner, P Mayor, V Almagro, C Enseñat, M López-Béjar.

Reproduction in Domestic Animals (2012), 47(Suppl 3): pp 122-123. Poster at 11th AERA conference.

***In vivo* heat stress affects potential oocyte maturation in rabbits.**

E Torres, M Sabés-Alsina, E Taberner, MJ Maya-Soriano, LL Tusell, O Rafel, J Ramon, M Piles, M López-Béjar.

Reproduction in Domestic Animals (2012), 47(Suppl 3): pp 120. Poster at 11th AERA conference.

Daily exposure to summer temperatures affects epididymal sperm cells in an *in vivo* rabbit model.

MJ Maya-Soriano, E Taberner, M Sabés-Alsina, LL Tusell, J Ramon, O Rafel, M Piles, M López-Béjar.
Reproduction in Domestic Animals (2012), 47(Suppl 3): pp 120. Poster at 11th AERA conference.

Biobanked amphibian samples confirmed to species level using 16S rRNA DNA barcodes.
MJ Maya-Soriano, R Lloyd, WV Holt.
Biopreservation & Biobanking (2011), 9(3): pp 303. Oral communication at ESBB conference.

Retinol improves in vitro oocyte nuclear maturation under heat stress in dairy cows.
MJ Maya-Soriano, C Andreu-Vazquez, F López-Gatius, M López-Béjar.
Reproduction in Domestic Animals (2010), 45(Suppl 3): pp 63-113. Poster at 14th ESDAR conference.

Cryopreservation of epididymal sperm cells from zoo ruminants.
M López-Béjar, MJ Maya-Soriano, E Taberner, C Andreu-Vazquez, C Enseñat, H Fernández-Bellon.
Reproduction in Domestic Animals (2010), 45(Suppl 3): pp 63-113. Poster at 14th ESDAR conference.

Successful refrigeration storage of epididymal sperm cells from a black-faced impala (*Aepyceros melampus petersi*).
MJ Maya-Soriano, H Fernández-Bellon, C Andreu-Vazquez, C Enseñat, M López-Béjar.
Reproduction in Domestic Animals (2010), 45(Suppl 2): pp 100. Poster at 10th AERA conference.

Role of antioxidant agents on steroidogenic activity of cumulus cells during oocyte *in vitro* culture.
MJ Maya-Soriano, C Andreu-Vazquez, M López-Béjar.
Reproduction in Domestic Animals (2010), 45(Suppl 2): pp 85. Poster at 10th AERA conference.

Other communications

Successful electroejaculation and long-term refrigeration of epididymal sperm cells from a lowland tapir (*Tapirus terrestris*).
MJ Maya-Soriano, E Taberner, H Fernández-Bellon, P Mayor, V Almagro, M López-Béjar.
Poster at International Conference on Disease of Zoo and Wild Animals, EAZWV, Lisbon (2011).

Successful refrigeration storage of epididymal sperm cells from an african hunting dog (*Lycaon pictus pictus*) for 72 hours.
MJ Maya-Soriano, E Taberner, H Fernández-Bellon, V Almagro, M López-Béjar.
Poster at International Conference on Disease of Zoo and Wild Animals, EAZWV, Lisbon (2011).

Effect of antioxidant agents on oocyte maturation under heat stress conditions.
Maria José Maya Soriano.
Oral communication for the Animal Health and Anatomy Department, Veterinary Faculty, UAB (2010).

Preliminary results of long-term refrigeration and cryopreservation of sperm cells from a drill (*Mandrillus leucopaheus*) after electroejaculation.

MJ Maya-Soriano, MT Abelló, H Fernández-Bellon, J Vidal, C Salvador, M López-Béjar.
Poster at International Conference on Disease of Zoo and Wild Animals, EAZWV, Madrid (2010).

Successful long-term refrigeration and cryopreservation of epididymal sperm cells from a patas monkey (*Erythrocebus patas*).
MJ Maya-Soriano, MT Abelló, H Fernández-Bellon, M López-Béjar.
Poster at International Conference on Disease of Zoo and Wild Animals, EAZWV, Madrid (2010).

Successful cryopreservation of epididymal sperm cells from a black-faced impala (*Aepyceros melampus petersi*).
MJ Maya-Soriano, H Fernández-Bellon, C Andreu-Vazquez, C Enseñat, M López-Béjar.
Oral communication at VIIe Congrès international francophone sur les animaoux sauvages et exotiques, Paris (2010).

Adenocarcinoma de glándulas hepatoides : tres casos clínicos.
C Díaz-Bertrana, MJ Maya-Soriano, I Durall, A Burballa, A Martínez, J Franch.
Poster at 43 Congreso Europeo Veterinario, SEVC, Barcelona (2008).

Tratamiento de una otitis media mediante ablación del conducto auditivo externo y trepanación de la bulla timpánica.
C Díaz-Bertrana, I Durall, A Burballa, A Martínez, E Rapado, MJ Maya-Soriano.
Poster at 43 Congreso Europeo Veterinario, SEVC, Barcelona (2008).

Attended courses

So long, and thanks for all the frogs!
Dr. Phil Bishop, Chief Scientist, IUCN Amphibian Survival Alliance, New Zeland.
European Xenopus Resource Center, University of Portsmouth (2011).

From endangered species to tracking cars; Reproductive biology, Wildlife and Modern biotechnology. A Symposium to celebrate contributions in comparative reproductive biology at ZSL.
Institute of Zoology (ZSL), Londres (2011).

The contribution of the international zoo community to the conservation of great apes and their habitats.
Consorci Universitat Internacional Menéndez Pelayo de Barcelona-Centre Ernest Lluch and Zoo de Barcelona (2011).

The current mission of zoos: new allies of biodiversity.
Consorci Universitat Internacional Menéndez Pelayo de Barcelona-Centre Ernest Lluch and Zoo de Barcelona (2009).

Creative teaching in the use of educational resources.
Unitat d'Innovació Docent en Educació Superior (IDES), UAB, Bellaterra, Barcelona (2009).

Felid intern medicine.

IVSA-Hill's, Veterinary Faculty, UAB, Bellaterra, Barcelona (2008).

Primate conservation and medicine.

AVAFES, Veterinary Faculty, UAB, Bellaterra, Barcelona (2006-2007).

Clinical cases in wild animals.

AVAFES, Veterinary Faculty, UAB, Bellaterra, Barcelona (2006-2007).

Felid conservation and medicine.

AVAFES, Veterinary Faculty, UAB, Bellaterra, Barcelona (2005-2006).