Universitat Autònoma de Barcelona

Climate change and male animal reproduction: effects of heat stress and season

Maria Sabés Alsina

Doctoral Thesis

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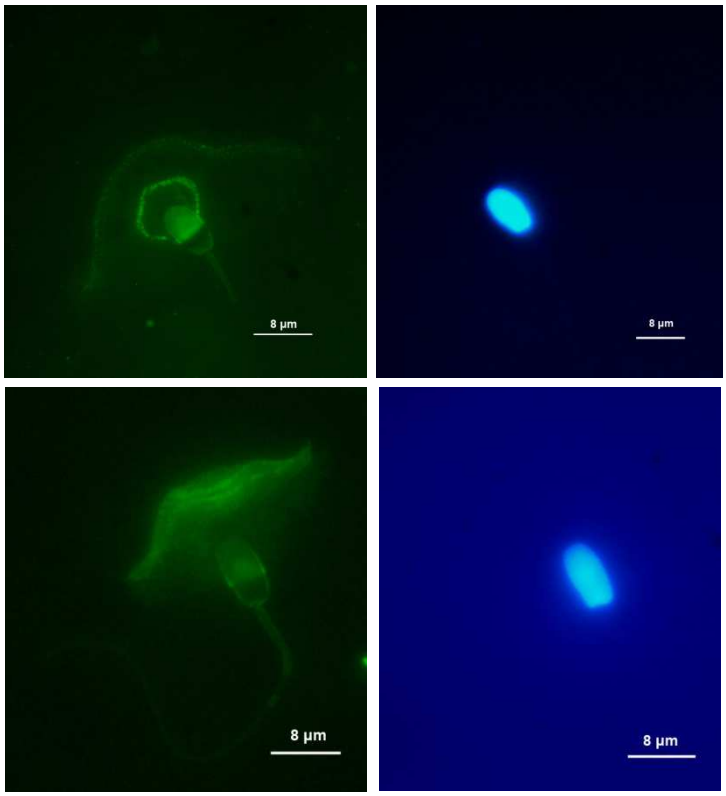

Under the direction of:

Dr. Manel López Béjar

PhD program: **Animal Medicine and Health**

Dpt. of Animal Health and Anatomy

Faculty of Veterinary

Universitat Autònoma de Barcelona

July, 2016

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Dissertation to obtain the Degree of Doctor by:

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CERTIFICA

Que la Tesi Doctoral titulada "**Climate change and male animal reproduction: effects of heat stress and season**" presentada per Maria Sabés Alsina per optar al grau de Doctor en Veterinària s'ha realitzat sota la meva direcció i, considerant-la finalitzada, autoritzo la seva presentació perquè sigui jutjada pel tribunal corresponent.

I, perquè així consti, firmo el present certificat.

Manel López Béjar

Maria Sabés Alsina
(Doctoranda)

Bellaterra, juliol 2016.

Als meus pares

i al Sergi.

"La verdadera ciencia enseña, por encima de todo, a dudar y a ser ignorante".

Miguel de Unamuno.

AGRAÏMENTS

M'agradaria donar les gràcies a totes aquelles persones que han fet que aquesta tesi sigui possible. A tots ells i elles, MOLTES GRÀCIES.

En primer lloc m'agradaria agrair al meu director de tesi, el Doctor **Manel López Béjar**, que m'hagi donat la possibilitat de realitzar aquest treball i també per tot l'ajut i consells rebuts durant aquests anys. Moltes gràcies.

Agrair als Doctors **Carlos López** i **Pedro Mayor** la dedicació i paciència que han tingut durant tots aquests anys escoltant-me, ajudant-me, animant-me i guiant-me en tots els dubtes i entrebancs que m'han anat sorgint. Les classes pràctiques al vostre costat han valgut molt la pena.

Al grup de treballadors de l'IRTA, sobretot a la **Míriam**, la **Llibertat** I l'**Oscar**, donar-los les gràcies per la paciència i les facilitats que m'han donat sempre que ho he necessitat.

I am very thankful to Professor **Jane M. Morrell** for giving me the possibility of doing part of my thesis at the Division of Reproduction of the Swedish University of Agricultural Sciences (SLU). I really appreciate all the time, dedication and resources that were invested in order to make my stay at SLU as profitable as possible. This thesis would not be the same without all your help and support. Also, I would like to thank **Anders**, **Nils**, **Ylva**, **Celina**, **Essraa**, **Tiger**, **Inês**, **Ziyad** and **Thong** for the unconditional help and support.

Dar las gracias al Doctor **Ramiro Alberio** por haberme mostrado lo interesante que puede ser el mundo de la reproducción animal y hacer que me apasione durante el tiempo que estuve en el departamento de *Animal Sciences* de la Facultad de Nottingham.

Gràcies a tots els que han estat i/o són (encara que alguns per poc temps) companys de laboratori i despatx. Fent un especial èmfasi en l'**Uri**, l'**Annaïs,** la **Laura**, la **Meritxell**, l'**Encarna**, l'**Elen**, l'**Ester** i la **Mari**. Moltes gràcies a tots vosaltres per fer que les hores de despatx i laboratori siguin molt més amenes i divertides. També voldria donar les gràcies

a la **Carme,** la **Tamara**, la **Blanca** i l'**Aida** per tot el seu suport tècnic i pels moments de desconnexió. Agrair també al Doctor **Quim Caselles** pel suport logístic ofert aquests últims mesos de tesi.

Moltes gràcies a les meves amigues de Sant Quirze, però sobretot a la **Núria Planell** per encoratjar-me i per les hores dedicades a ajudar-me en els meus inicis amb l'estadística. També donar les gràcies a l'**Anna Albert**, al **Dani Guerrero**, la **Neus Torres**, el **Jordi Aguilar** i el **Dani Costa** per interessar-se en el meu dia a dia, per la paciència en les meves converses sobre la tesi i pels milions de missatges donant-me ànims.

M'agradaria donar les gràcies a tota la meva **família**, però sobretot als meus **pares** i **germà**. Encara que no sempre he estat convençuda d'haver triat correctament aquest camí, m'agradaria donar-vos les gràcies per introduir-me en aquest món i fer que no tirés la tovallola encara que les coses no anessin tal com esperava. És més que obvi que sense vosaltres no hagués arribat fins aquí.

Finalment, però no per això menys important, m'agradaria donar les gràcies d'una manera molt especial al **Sergi**. Tot i estar atabalat amb la tesi i/o la feina, has sigut capaç de recolzar-me i donar-me tot el suport necessari en tot moment (fins i tot estan en països diferents). Moltissimes gràcies per ser com ets, per fer-me sempre costat, per la teva paciència pràcticament il·limitada, però sobretot pel teu suport incondicional.

I a la **Gumi**, perquè ella no en té cap culpa.

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ABSTRACT

The general purpose of this doctoral thesis has been to study the impact of climate changes, such as heat stress, and season on sperm quality using an experimental model (rabbit) and a livestock model (bovine).

The aim of the first chapter was to evaluate the effect of an *in vivo* heat stress model of continuous summer circadian cycles with periods of recovery on sperm quality, fertility, prolificacy and concentrations of stress hormones in rabbits. The reliability of the model was demonstrated by the higher values of faecal cortisol metabolites of the animals exposed to heat stress. The exposure to the *in vivo* heat stress decreased the percentage of viable spermatozoa, and increased the proportion of acrosome abnormalities and tailless spermatozoa. Although negative effects were observed in some sperm quality parameters, fertility and prolificacy were not adversely affected. A hypothesis of development of stress tolerance or species adaptation to stress is suggested.

In Chapter 2a, a vital fluorometric method (resazurin reduction test; RRT) for evaluating metabolic activity of rabbit sperm cells was developed. A positive correlation between metabolic activity through RRT and the proportion of viable spermatozoa, and a negative correlation between metabolic activity and sperm cells with acrosomic abnormalities were observed. RRT is a promising method for a dual indirect assessment of viability and morphological parameters.

Chapter 2b evaluated the impact of an *in vitro* heat stress model on rabbit sperm quality and to determine possible seasonal effects on sperm heat tolerance. Sperm samples collected both in the warm and the cold seasons were exposed to scrotal (32.5ºC), body (37ºC) or hyperthermic (42ºC) temperatures for 3 hours. Only the exposure to hyperthermic conditions decreased the average values of total motility, metabolic activity, and curvilinear and mean velocity. Metabolic activity was also influenced by the interaction of heat stress and season. Thus, extrapolating to *in vivo* conditions, the presence of sperm cells in a hyperthermic uterus or oviduct could compromise fertility.

In Chapter 3, the possible effects of climate factors on sperm quality of bulls housed in northern Spain were evaluated. Samples collected in spring showed higher average kinematic values (total and progressive motility, average path velocity and straight line velocity), proportion of viable spermatozoa and proportion of viable spermatozoa with non-reacted acrosome, and proportion of live spermatozoa not producing superoxide or hydrogen peroxide when compared to ejaculates from the same bull obtained in winter and summer. These results may be indicative of better sperm quality in samples collected in spring than samples collected in other seasons.

The aim of chapter 4 was to study the effects of season on the fertilizing ability of bovine spermatozoa and subsequent embryo development *in vitro*. Bovine oocytes were matured and fertilized *in vitro* with sperm cells from ejaculates collected in different seasons. From the studied parameters, only embryo development rates showed a trend towards significance among seasons. Semen samples collected in spring results in embryos that developed at a faster rate. Further studies should be performed in order to verify these preliminary interesting results.

Climate change can be the cause of several physiological changes in animals and human beings to enable them to cope with the demands for species survival. A phenomenon of adaptation or tolerance to persistent stressful factors, such as heat stress, is needed for maintaining reproductive function. Our results support this theory and indicate the necessity of developing strategies to alleviate the demands of climate change, which will be needed in the future to maintain efficiency in animal reproduction.

RESUM

L'objectiu general d'aquesta tesi doctoral ha estat estudiar l'impacte que pot tenir el canvi climàtic, provocant situacions d'estrès tèrmic, i l'estacionalitat climàtica sobre la reproducció animal, en concret sobre la qualitat espermàtica, utilitzant un model experimental (conill) i un model d'una espècie de gran interès ramader (boví).

L'objectiu del primer capítol va ser avaluar l'efecte d'un model d'estrès tèrmic *in vivo* de cicles circadians d'estiu continus amb períodes de recuperació sobre la qualitat espermàtica, la fertilitat, la prolificitat i en la concentració d'hormones d'estrès utilitzant el conill com model. La fiabilitat del model es va demostrar mitjançant l'increment de metabòlits de cortisol fecal en animals sotmesos a estrès tèrmic. L'exposició d'animals a l'estrès tèrmic *in vivo* va comportar la disminució del percentatge d'espermatozoides viables, i l'augment de la proporció d'alteracions acrosòmiques i d'espermatozoides sense cua. Tot i que es van observar efectes negatius en alguns paràmetres de qualitat espermàtica, la fertilitat i la prolificitat no es van veure negativament afectades. Per tant, es suggerí la hipòtesi d'un possible desenvolupament de tolerància o d'adaptació dels animals a l'estrès tèrmic.

En el capítol 2a es va desenvolupar un mètode fluoromètric vital (resazurin reduction test, RRT) per tal d'avaluar l'activitat metabòlica dels espermatozoides de conill. Els resultats van mostrar una correlació positiva entre l'activitat metabòlica a través del RRT i la proporció d'espermatozoides viables, i una correlació negativa entre l'activitat metabòlica i la presència d'espermatozoides amb anomalies acrosòmiques. El RRT és per tant un mètode prometedor per a l'avaluació indirecta de la viabilitat i els paràmetres morfològics.

En el capítol 2b es va avaluar l'impacte d'un model d'estrès tèrmic *in vitro* en la qualitat espermàtica del conill. A més, es va determinar els possibles efectes d'estacionalitat climàtica en la tolerància tèrmica de l'esperma. Les mostres espermàtiques recollides en estacions càlides i fredes van ser exposades a temperatures escrotals (32,5ºC), corporals (37ºC) o d'hipertèrmia (42ºC). Només l'exposició de les mostres espermàtiques a temperatures d'hipertèrmia va comportar la reducció dels valors mitjans de motilitat total, activitat metabòlica, i velocitat curvilínia i mitjana. L'activitat metabòlica també va

ser influenciada per la interacció entre l'estrès tèrmica i l'estació climàtica. Per tant, es pot extrapolar que en condicions *in vivo*, la presència d'espermatozoides en úters i oviductes de femelles en estat d'hipertèrmia podria comprometre la posterior fertilitat.

En el capítol 3 es van avaluar els possibles efectes climàtics en la qualitat espermàtica de toros estabulats al nord d'Espanya. Les mostres seminals recollides a la primavera van mostrar valors cinemàtics (motilitat total i progressiva, velocitat mitjana i velocitat rectilínia), percentatges d'espermatozoides viables, proporció d'espermatozoides viables amb acrosoma no reaccionat, i proporció d'espermatozoides vius no productors de superòxid o peròxid d'hidrogen més elevats que les mostres recollides en les altres estacions climàtiques estudiades. Aquests resultats poden ser indicatius d'una millor qualitat espermàtica en les mostres recollides a la primavera comparat amb les mostres recollides en altres estacions climàtiques.

L'objectiu del capítol 4 va ser estudiar l'efecte de l'estacionalitat en la capacitat fecundant dels espermatozoides bovins i el seu posterior desenvolupament embrionari *in vitro*. Oòcits bovins van ser madurats i fecundats *in vitro* amb espermatozoides de toro recollits en diferents estacions climàtiques. Dels paràmetres estudiats, només la taxa de divisió embrionària mostrava una tendència cap a la significació entre estacions. Les mostres espermàtiques recollides a la primavera van mostrar desenvolupaments embrionaris més ràpids. S'hauran de realitzar més estudis per tal de verificar aquest resultats preliminars.

El canvi climàtic pot ser la causa de diversos canvis fisiològics en animals i éssers humans per tal de fer front a les demandes de supervivència de les espècies. Fenòmens d'adaptació o tolerància als factors estressants persistents, com és l'estrès tèrmic, són necessaris per mantenir la funció reproductiva. Els nostres resultats donen suport a aquesta teoria i ens indiquen la necessitat de desenvolupar estratègies per alleujar les demandes del canvi climàtic per tal de mantenir l'eficiència en la reproducció animal en un futur proper.

RESUMEN

El objetivo general de esta tesis doctoral ha sido estudiar el impacto que puede tener el cambio climático, provocando situaciones de estrés térmico, y la estacionalidad climática en la reproducción animal, concretamente sobre la calidad espermática, utilizando un modelo experimental (conejo) y un modelo de una especie de gran interés ganadero (bovino).

El objetivo del primer capítulo fue evaluar el efecto de un modelo de estrés térmico *in vivo* de ciclos circadianos de verano continuos con períodos de recuperación sobre la calidad espermática, la fertilidad, la prolificidad y en la concentración de hormonas de estrés utilizando el conejo como modelo. La fiabilidad el modelo se demostró mediante el incremento de metabolitos de cortisol fecal en los animales sometidos a estrés térmico. La exposición de animales a estrés térmico *in vivo* conllevó a la disminución del porcentaje de espermatozoides viables, y el aumento de la proporción de alteraciones acrosómicas y de espermatozoides sin cola. Aunque se observaron efectos negativos en algunos parámetros de calidad espermática, la fertilidad y la prolificidad no se vieron negativamente afectadas. Por tanto, se sugirió la hipótesis de un posible desarrollo de tolerancia o adaptación de los animales al estrés térmico.

En el capítulo 2a se desarrolló un método fluorométrico vital (resazurin reduction test, RRT) para evaluar la actividad metabólica de los espermatozoides de conejo. Los resultados mostraron una correlación positiva entre la actividad metabólica a través del RRT y la proporción de espermatozoides viables, y una correlación negativa entre la actividad metabólica ya la presencia de espermatozoides con anomalías acrosómicas. El RRT es por tanto un método prometedor para la evaluación indirecta de la viabilidad y los parámetros mofológicos en espermatozoides de conejo.

En el capítulo 2b se evaluó el impacto de un modelo de estrés térmico *in vitro* en la calidad espermática del conejo. Además, se determinó los posibles efectos de estacionalidad climática en la tolerancia térmica del esperma. Las muestras espermáticas recogidas en estaciones cálidas y frías fueron expuestas a temperaturas escrotales

(32,5ºC), corporales (37ºC) o de hipertermia (42ºC). Solo la exposición de las muestras espermáticas a temperaturas de hipertermia comportó la reducción de los valores medios de motilidad total, actividad metabólica, y velocidad curvilínea y media. La actividad metabólica fue influenciada por la interacción entre el estrés térmico y la estación climática. Por lo tanto se puede extrapolar que en condiciones *in vivo,* la presencia de espermatozoides en úteros y oviductos de hembras en estado de hipertermia podría comprometer la posterior fertilidad.

En el capítulo 3 se evaluaron los posibles efectos climáticos en la calidad espermática de toros estabulados en el norte de España. Las muestras seminales recogidas en primavera mostraron valores cinemáticos (motilidad total y progresiva, velocidad media y velocidad rectilínea), porcentajes de espermatozoides viables, proporción de espermatozoides viables con acrosoma no reaccionado, y proporción de espermatozoides vivos no productores de superóxido o peróxido de hidrógeno más elevados que las muestras recogidas en las ostras estaciones climáticas estudiadas. Estos resultados pueden ser indicativos de una mejor calidad espermática en las muestras recogidas en primavera comparado con las muestras recogidas en otras estaciones climáticas.

El objetivo del capítulo 4 fue estudiar el efecto de la estacionalidad en la capacidad fecundante de los espermatozoides bovinos y su posterior desarrollo embrionario *in vitro*. Ovocitos bovinos fueron madurados y fecundados *in vitro* con espermatozoides de toro recogidos en diferentes estaciones climáticas. De los parámetros estudiados, sólo la tasa de división embrionaria mostraba una tendencia hacia la significación entre estaciones. Las muestras espermáticas recogidas en primavera mostraron desarrollos embrionarios más rápidos. Se deberán realizar más estudios para verificar estos resultados preliminares.

El cambio climático puede ser la causa de varios cambios fisiológicos en animales y seres humanos para hacer frente a las demandas que requiere la supervivencia de las especies. Fenómenos de adaptación o tolerancia a los factores estresantes persistentes, como es el estrés térmico, son necesarios para mantener la función reproductiva. Nuestros resultados apoyan esta teoría y nos indican la necesidad de desarrollar estrategias para

aliviar las demandas del cambio climático a fin de mantener la eficiencia en la reproducción animal en un futuro cercano.

Introduction

1. Global climate change

There is evidence that climate change is causing several negative effects on the planet. Since the seventies, a substantial decrease of mountain glaciers and snow cover in both hemispheres has been observed producing a rise in sea level (19 cm from 1901 to 2010). It is predicted that average sea level is going to rise between 24 – 30 cm by 2065 and 40 - 63 cm by 2100. Patterns of precipitations have significantly changed since the Industrial Revolution. Nowadays, there are more areas affected by drought, but also there are regions affected by heavy rainfall events: floods, tropical storms and hurricanes. Seasons are also affected by climate change producing earlier spring events, and long periods of heat waves in summer followed by severe winters (Kuczynski et al., 2011; UNFCC, 2016). In the Paris Agreement in 2015, Parties to the United Nations Framework Convention on Climate Change (UNFCCC) committed to combat climate change by limiting a global temperature increase to below 2ºC and to pursue efforts to limit the temperature increase even further to 1.5ºC (Figure 1) (UNFCCC, 2016). The UNFCCC promotes research and supports international programmes, networks and organizations. Also, the European Union and the Spanish Government have prioritized some research lines on fighting the effect of global climate changes (Horizon 2020, 2016).

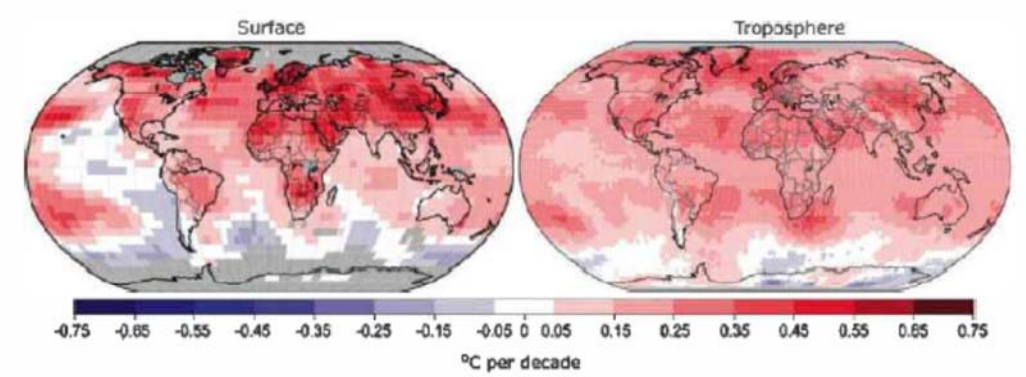

Figure 1. Patterns of linear global temperature trends from 1979 to 2005 estimated at the surface and the troposphere from satellite records (Kuczynski et al., 2011).

Climate changes and global warming have a considerable impact on plants, animals and human populations. The effect of the global temperature increase and stress factors such as the destruction of habitats by drought or floods could lead to change in the population density of species, changes in the timing of events such as migration or egg laying can occur,

changes in morphology (body size) and behaviour may occur, and genetic frequencies may shift. Moreover, the warming of the planet can induce numerous species extirpations and possible extinctions (Root et al., 2003).

2. Importance of domestic animals

Livestock have an important role in feeding the world's human population providing a source of high-quality protein to consumers and incomes to producers. In order to potentiate their sustainability they must be managed with care, and efficiency, under appropriate welfare conditions (FAO, 2011). In the last three decades (from 1983 to 2013), the world milk production has increased more than a 50% (from 500 million tonnes to 769 million tonnes). Cattle produce 83% of this milk, followed by buffalo (13%), goats (2%), sheep (1%) and camels (0.4%). In developed countries, almost all milk production originates from cattle. Europe is one of the world regions with high consumption of milk per capita (>150 kg/capita/year) (FAO, 2016). The increase in milk production of the dairy cows is due to an improvement in genetics, feeding and management. However, this genetic gain is accompanied by, and correlated with, a decline in reproductive performance such as fertility (Rodriguez-Martinez et al., 2008). Any adverse effects of climate on fertility can have a marked negative effect on reproductive efficiency.

Rabbits have a higher efficiency system converting fodder to food than other farmed species. These herbivores can turn 20% of the proteins they consume into edible meat compared with 16-18% in the pig and 8-12% in the beef (Rochambeau, 1986). From 1961 to 2014, Europe has produced the 30% of the rabbit meat production in the world. Spain is one of the leading countries in Europe for rabbit meat production (FAOSTAT, 2016). In 2001, the Food and Agriculture Organization of the United Nations (FAO) recognized the increasingly importance of the role of rabbit breeding (FAO, 2001). Apart from being important as a livestock animal in the Mediterranean countries, rabbits are also commonly used as laboratory mammals (Cummins and Glover, 1970).

3. Effect of hot environment in domestic animals

Exposure of domestic animals such as cattle and rabbits to hot environments can cause negative effects on its nutrition, physiology, growth rate, health (higher susceptibility to disease because of modifications of the immune system), production and reproductive functions (Das et al., 2016; Kuczynski et al., 2011; Takahashi, 2012). The body temperature of most mammals is between 35ºC and 39ºC (Takahashi, 2012). The thermal comfort zone (range of environmental temperature which minimal physiological cost and maximum productivity are achieved for the animal [Kadzere et al., 2002]) in the dairy cow has been established as being from 5ºC to 25ºC (De Rensis et al., 2015)**,** while in rabbits it is 15ºC to 21ºC (Marai et al., 2002)**.** Environmental temperatures above these ranges can cause heat stress to these animals.

4. Heat stress definition

Heat stress is a term that has been used widely and rather loosely by physiologist to denote an excessive demand on the animal for heat dissipation under high ambient temperature (Kuczynski et al., 2011). Lee (1965) defined heat stress as a magnitude of external forces to the bodily system that displace and modify the body temperature from its resting or ground state.

4.1 Indices of heat stress

Heat stress can be expressed by a number of indices such as the temperature-humidity index (THI; combination of ambient temperature and the relative humidity), the temperature-humidity-velocity index (combination of ambient temperature, relative humidity and air velocity over the animals), the effective temperature (combination of solar radiation and ambient temperature) and the black globe-humidity index (combination of solar radiation, ambient temperature, wind speed and relative humidity)(Kuczynski et al., 2011). Among these indices, the THI is one of the stress indices widely used for detecting the impact of heat stress on livestock production (Bouraoui et al., 2002; De Rensis et al., 2015; Safaa et al., 2008). Maximum THI values (corresponding to maximum temperature and minimum relative humidity) below 70 units (68 units for dairy cows) are considered comfortable conditions

and generally do not cause safety problems. Between values of 70 and 74 units animals are exposed to mild discomfort. Maximum THI values above 75 units are considered stressful and animals can show noticeable decreases in performance. In dairy cows, maximum THI about 80 units at the time of artificial insemination cause severe stress and was observed to decrease fertility and conception rates (De Rensis et al., 2015; Kuczynski et al., 2011). In rabbits, the THI was modified for small animals in 2001. Values obtained from this equation are lower than those observed in large animals. These values are classified as follows: lower than 27.8 units = absence of heat stress; between 27.8 and 28.9 units = moderate heat stress; between 28.9 and 30 units = severe heat stress; and above 30 units = very severe heat stress (Marai et al., 2002).

4.2 Consequences of heat stress on the male

In most mammals, testis are located outside the body cavity in the scrotum allowing a testis temperature between 2ºC and 8ºC below the core body temperature (Alvarez and Storey, 1985; Kim et al., 2013; Takahashi, 2012). Different mechanisms exist for dissipating or increasing heat from the testes such as the contraction or relaxation of the external cremaster muscle and tunica dartos, and the extensive vascularization next to the spermatic cord (pampiniform plexus) (Chenoweth and Lorton, 2014; Hansen, 2009). The testes are susceptible to damage by exposure to the temperature normally found in the abdomen. An increase of the testicular temperature can induce testicular degeneration and a reduction of sexual desire (libido) resulting in sub- or infertility (Abdul Niyas et al., 2015; Marai et al., 2002). One of the possible effects of acute or chronic heat stress on the testis is a reduction in testis weight, and a decrease of the volume and sperm concentration of the ejaculates (Marai et al., 2002; Setchell, 1998). Also, heat stress can cause adverse effects on sperm quality producing a decrease in the sperm motility, and an increase in the proportion of dead sperm cells or sperm cells with morphological abnormalities in the ejaculate (Abdul Niyas et al., 2015; Marai et al., 2002; Setchell, 1998). Moreover, the presence of spermatozoa with abnormalities of chromatin structure in the ejaculates maybe increased (Hansen, 2009; Takahashi, 2012).

4.3 Consequences of heat stress on the female

High environmental temperatures can affect different components of the female reproductive system such as the ovarian follicle with the oocyte and granulosa and theca cells, the corpus luteum, and the developing embryos and uterine endometrium. Moreover, the estrus cycle itself may be affected (Abdul Niyas et al., 2015). In dairy animals, heat stress reduces the intensity and length of estrus and, increases anestrus and silent ovulations. These effects are due to an increase of ACTH and cortisol production that blocks the secretion of estradiol (inducer of sexual behaviour) (Das et al., 2016; De Rensis et al., 2015; Takahashi, 2012). Follicular growth, oocyte development, steroid secretion, gene expression, conception rates, and embryonic growth and development can also be affected by high temperatures (Das et al., 2016; García-Ispierto et al., 2007; Hansen, 2009). In the rabbit, heat stress can delay age at puberty, decrease the conception rate, alter the gestation length, and affect the litter size and weight (Marai et al., 2002).

The severity of the damage on the male and female reproductive system varies depending on the duration, frequency and intensity of heat stress exposure (Das et al., 2016; Durairajanayagam et al., 2014; Setchell, 1998).

5. Seasonal effects on reproduction

Not only heat stress but also global climate change can lead alterations in reproduction, seasonality (variations in atmospheric temperature and photoperiods length) can also induce adverse effects on reproduction (De Rensis and Scaramuzzi, 2003; Erb et al., 1942; Safaa et al., 2008). Seasonality can produce alterations in sperm quality such as motility, metabolic activity, acrosome status and sperm chromatin structure in livestock animals (Erb et al., 1942; Nakabayashi and Salisbury, 1959; Valeanu et al., 2015; Wells et al., 1972).

In dairy cows, a clear pattern of low fertility and poor oocyte quality exists associated with the warm months (from June to September in the Northern hemisphere). However these adverse effects remain in autumn (October and November) even when animals are no longer exposed to heat stress (De Rensis and Scaramuzzi, 2003; De Rensis et al., 2015).

6. Adaptive process against heat stress

Despite all these possible changes on the reproductive function produced by the impact of heat stress, livestock possess a wide range of adaptive mechanisms for modulating the response to environmental challenges, which allow the animals to develop heat tolerance. These mechanisms to maintain homeothermy can be physiological (increased respiratory rate, pulse rate, rectal temperature and sweating rate), morphological (skin colour and concentration of sweat glands), behavioural (decreased feed intake and increased water intake), biochemical, endocrinological, cellular and molecular (increasing expression of heat shock proteins and decreasing synthesis of other proteins). However, not all animals have the ability of using all these mechanisms, and species and individual responses differ (Abdul Niyas et al., 2015).

7. Sperm quality

The concept of "sperm quality" is an attempt to describe the ability of the spermatozoa to function i.e. to move from the site of semen deposition to the site of fertilization, to penetrate and oocyte and activate it to develop an embryo.

In an *in vivo* situation, the female reproductive tract is considered to function as a filtering mechanism providing various physical barriers (the cervix in some species and the uterotubal junction) for selecting spermatozoa. Only sperm cells with desirable functional parameters such as progressive motility, normal morphology, functional membranes, being capable of capacitating and undergoing acrosome reaction would be able to cross these barriers and survive in the female reproductive tract, reach the oocyte, bind to the zona pellucida, penetrate the oolema and activate the oocyte (Morrell and Rodriguez-Martinez, 2009; Saacke, 2008).

In contrast, in an *in vitro* fertilization situation the natural selection that occurs in the reproductive tract is avoided and some of the sperm quality parameters become less important because sperm cells are co-cultivated close to the oocytes instead of being required to navigate through the female reproductive tract to find them (Morrell and Rodriguez-Martinez, 2009).

Sperm quality can be described in terms of motility, morphology, plasma membrane integrity, metabolic activity and sperm acrosome reaction in order to determine the fertilizing potential of a semen sample (Mocé and Graham, 2008; Morrell and Rodriguez-Martinez, 2009). It can be evaluated in several ways.

7.1 Sperm motility

Estimation of the proportion of motile sperm cells in ejaculates is one of the parameters more commonly used for analysing sperm quality. For artificial insemination and *in vitro* fertilization, it is customary to evaluate sperm motility of each ejaculate (Chenoweth and Lorton, 2014). Initially, the assessment of sperm motility was performed using visual microscopic evaluation. However, this evaluation is subjective (and therefore varies among evaluators, human bias) and few motility parameters such as total and progressive motility could be evaluated. The use of Computer-Assisted Sperm Analysis (CASA) systems has allowed the possibility of increasing the objectivity and repeatability of the motility analysis, and the number of kinematic analysed (Chenoweth and Lorton, 2014). The CASA system reconstructs the sperm trajectory by taking consecutive images of each sperm head and digitizing with a computer (Mortimer, 2000).

7.2 Sperm morphology

For many years, the presence of a high proportion of abnormal spermatozoa in the ejaculate has been related with reduced fertility or sterility in males (Mocé and Graham, 2008; Saacke, 2008). Normal sperm morphology is necessary for cell functionality. The production of normal sperm cells can be negatively affected by external influences such as reactive oxygen species, temperature, hormonal changes and external chemicals (Chenoweth and Lorton, 2014). Sperm morphology can be assessed visually using microscopy or automated morphometry estimations (Saacke, 2008).

7.3 Sperm plasma membrane integrity

Sperm viability is often linked to the integrity of the plasma membrane that surrounds the entire sperm cell. The plasmalemma is essential for sperm interaction with other cells and

their environment, such as the epithelium lining the female genital tract, and with the oocyte-cumulus cell complex. The plasma membrane has the capability of maintaining the homeostasis of the sperm cell. Sperm cells with no functional intact plasma membrane are considered dead or dying, and not capable of fertilizing *in vivo.* The assessment of the sperm plasma membrane integrity is usually performed by staining with membrane impermeable-dyes. Sperm cells capable of excluding the dye can be considered to be alive (Figure 2) (Hossain et al., 2011; Rodríguez-Martínez, 2007; Silva and Gadella, 2006).

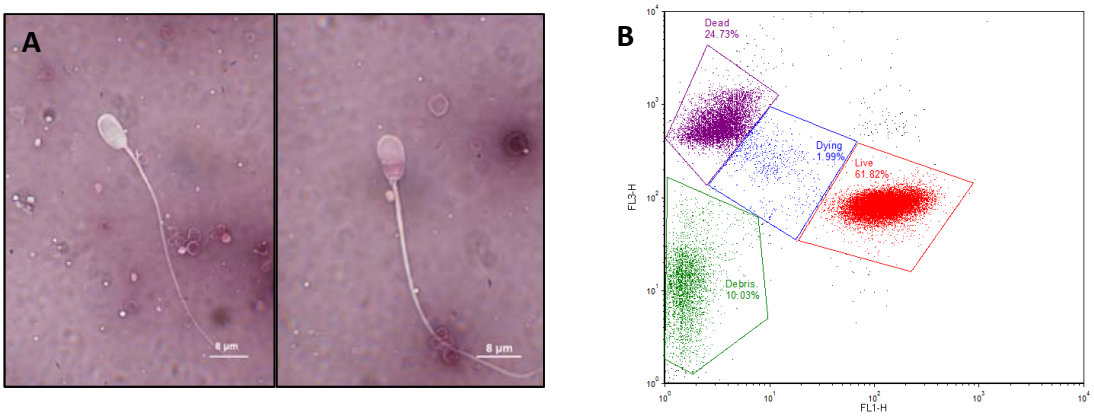

Figure 2. Two classical stainings for assessing plasma membrane integrity in sperm cells using light microscope (a) and flow cytometer (b). A: Viability analysis using eosin-nigrosin staining of rabbit sperm cells at 1000 x. Viable sperm cell remain white or unstained (left) and non-viable sperm cell stained pink (right). B: Viability evaluation by SYBR 14/propidium iodide (PI) labelling in bull sperm cells. Four regions show the proportion of viable (red), dying (blue) and dead (purple) bull sperm cells, and cell debris (green).

7.4 Sperm metabolic activity

Reproductive performance depends on metabolic processes. The sperm mid-piece has a battery of mitochondria (less than 100) that are wound around the flagellum and elongates further down the tail; it is the site where aerobic metabolism occurs. These organelles generate the majority of the ATP necessary for sperm metabolism, membrane function and motility. Also, mitochondria are implicated in sperm maturation, coordinate the apoptosis mechanism, and protects against cryopreservation damage (Chenoweth and Lorton, 2014; Hossain et al., 2011).

7.5 Sperm acrosome reaction

The acrosome is a membrane-enclosed structure located at the apical edge of the sperm head covering its nucleus. This cap-shaped organelle consisting of two membranes (inner and outer acrosomal membrane) is filled with hydrolytic, glycosidic and proteolytic enzymes. During the acrosome reaction, these enzymes are released producing a fusion and vesiculation of the plasma membrane and outer acrosomal membrane. These changes in the sperm structure allow the sperm cell to bind to the zona pellucida, penetrate the oocytes and fuse with the oocyte's plasma membrane. Therefore, the ability of the acrosome to react at the correct time is a prerequisite for fertilization (Aitken, 2006; Chenoweth and Lorton, 2014; Hossain et al., 2011). Acrosome integrity can be assessed *in vitro* by microscopy (fluorescent or light) and flow cytometry (Figure 3) (Hossain et al., 2011). The dyes most commonly used for measuring acrosome integrity are fluorescent conjugated plant lectins (*Pisum sativum* [pea] agglutinin: PSA; and *Arachis hypogaea* [peanut] agglutinin: PNA). Lectins bind to specific carbohydrate moieties of glycoproteins exclusive of acrosome localization (Silva and Gadella, 2006).

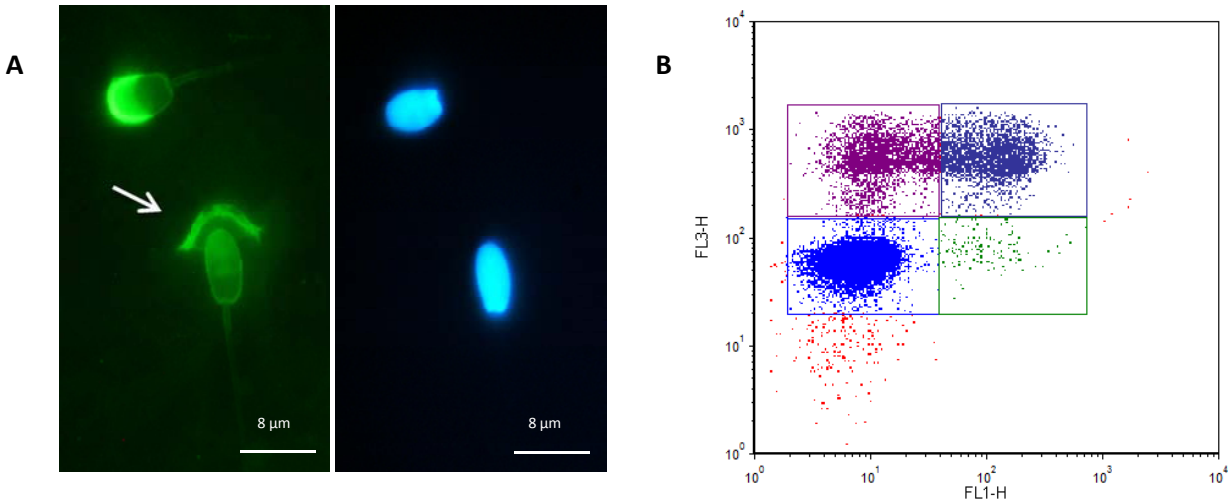

Figure 3. Acrosome evaluation methods. A: Rabbit sperm cells with altered (arrow) and intact acrosome membrane after fluorescein isothiocyanate-labeled PSA (FITC-PSA) staining (green fluorescence) and DAPI nuclear staining (blue fluorescence) evaluated with fluorescent microscopy at 1000x. B: Simultaneous evaluation of viability and acrosome integrity in bull sperm cells using PNA conjugated with FITC/PI labelling. Colour dot plots on the lower left region show viable spermatozoa with intact acrosome; lower right region show viable spermatozoa with reacted acrosome; upper left region show dead sperm cells with intact acrosome; upper right region show dead spermatozoa with reacted acrosome.

7.6 Comparison of assessment methods

The assessment of some of the sperm quality parameters can be performed using fluorescent microscopy or flow cytometry. These instruments are compared in Table 1 (Gillan et al., 2005; Graham, 2001; Hossain et al., 2011; Martínez-Pastor et al., 2010).

Table 1. Comparison of fluorescent microscopy and flow cytometry for assessment of sperm quality.

	Fluorescent microscopy	**Flow cytometry**
Advantages	- Analysis of spermatozoa shapes - Fluorescent localization on the spermatozoa - Analysis of multiple sperm characteristics and fluorescent stains simultaneously - Useful for small sperm samples or samples with low number of spermatozoa	- Objective - High sensitivity and repeatability - Analysis of small and large sperm samples - Analysis of large number of spermatozoa in short periods of time - Analysis of multiple sperm characteristics and fluorescent stains simultaneously - Samples can be recovered after analysis - Measures the fluorescent intensity of the sperm sample
Disadvantages	- Subjective (human bias) - Tedious and time-consuming - Needs immobilized sperm samples (spermatozoa cannot be recovered after analysis)	- Impossible locating the fluorescence on the spermatozoa - Needs expertise for operation and data interpretation - Expensive

7.7 Relation of sperm quality with fertility

Several sperm quality parameters have been correlated with fertility (Gillan et al., 2005; Simonik et al., 2015). However, currently there is no one sperm quality parameter that can predict the fertility capability of a sperm sample (Mocé and Graham, 2008). A possible

explanation for this fact is that ejaculates are composed of a heterogeneous suspension of pluricompartmental cell population produced in different spermatogenic waves that matured along the ducti epididymides and were stored in the tail of the epididymis or the ampulla as different cell cohorts. This heterogenic population should retain their integrity and fertility over time, to sustain selection steps and to response to exogenous stimuli and stress environmental conditions on the female genital tract (Mocé and Graham, 2008; Rodríguez-Martínez, 2007). Therefore, the assessment of various sperm quality parameters may give us more complete information about this heterogenic population and their fertility capability.

8. Specific aspects of heat stress and seasonality in male reproduction

The effect of global climate changes on reproduction is a complex and multifactorial topic that cannot be explained, approached and solved easily. Nowadays, animal reproduction is fundamental for human food production and extremely important for our daily lives.

Under adverse situations, reproduction is one of the first physiological parameters to be compromised. For example, dairy cows gene-selected for high milk production are more sensitive to heat stress due to the increase in metabolic heat output. Also, these animals have fertility problems (Das et al., 2016). The assessment of sperm quality parameters is a convenient way of detecting alterations in male reproduction caused by external factors. Rabbits and bulls are good reproductive animal models because they are not seasonal breeders and thus their reproduction can be assessed all year.

We aim to assess the impact that a situation of heat stress would have on animal reproduction. In this case, two models of animals would be bioindicators of the physiological modifications that the climate change could cause with the loss of proper seasons and increase of the temperatures.

Aims

The main objective of this doctoral thesis is to analyse the impact of climate changes, such as heat stress, and season on sperm quality using an experimental model (rabbit) and a livestock model (bovine).

The specific objectives are:

1- Evaluate the effect of continuous summer circadian cycles in an *in vivo* heat stress model on semen production and quality, stress, fertility and prolificacy of rabbits.

2- Develop a fluorometric method for evaluating metabolic activity of rabbit sperm cells.

3- Assess the impact on sperm parameters of an *in vitro* HS model that simulated the conditions to which spermatozoa may be exposed in the testis (32.5ºC) and in the reproductive tract of rabbit females subjected to normal (37ºC) or hyperthermic conditions (42º). Also, determine if there were seasonal effects on sperm heat tolerance.

4- Study the possible effects of climate factors on the sperm quality of Holstein dairy bulls housed in northern Spain.

5- Evaluate the effects of season on the fertilizing ability of bovine spermatozoa and subsequent embryo development *in vitro*.

Chapters

I

Daily exposure to summer circadian cycles affects spermatogenesis, but not fertility in an *in vivo* rabbit model

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Received 10 January 2014; received in revised form 9 September 2014**;**
accepted 11 September 2014

Theriogenology 83 (2015) 246 - 252

Abstract

Heat stress (HS) in mammals is a determining factor in the deterioration of spermatogenesis and can cause infertility. The aim of this study was to evaluate the effect of continuous summer circadian cycles on semen production, sperm cell features, fertility, prolificacy, and faecal cortisol metabolites from rabbits kept under an *in vivo* HS model. We split randomly 60 New Zealand White rabbits into two temperature-controlled rooms: the control group was maintained at comfort temperature (18ºC – 22ºC) and for the HS group, where the environmental temperature was programmed to increase from 22ºC to 31ºC and maintained for 3 hours to this temperature at the central part of the day. Faecal cortisol metabolites were assessed to evaluate the stress conditions. Seminal parameters were analysed.

Although animals exposed to HS showed higher values of faecal cortisol metabolites (P = 0.0003), no differences were detected in fertility or prolificacy. Semen samples from HS males showed a significant decrease ($P < 0.05$) with respect to the controls in the percentage of viable spermatozoa (80.71 vs. 74.21%), and a significant increase ($P \leq 0.01$) in the percentage of acrosomic abnormalities (22.57 vs. 36.96%) and tailless spermatozoa (7.91 vs. 12.83). Among motility parameters, no significant differences were found. This study describes a model of HS simulating a continuous summer daily cycle that allows periods of time to recover as it occurs under natural conditions. Although negative effects have been detected in several sperm parameters, fertility and prolificacy were not affected, suggesting a recovery of the reproductive function when normal conditions are re-established.

Keywords: heat stress, fertility, prolificacy, sperm viability, rabbit.

1. Introduction

Global warming is having many ramifications on the planet weather conditions. Effects of this phenomenon are an increase in air temperature, the shortening of winters, loss of the temperate seasons, an increase in periods of drought and flooding, and as a result, a decrease in periods of bioclimatic comfort. Forecasts indicate that there will be a rise in the maximum and mean temperatures around the year 2020 of 1.5ºC to 2.1ºC, which will significantly increase the heat stress (HS) on the world (MacCracken, 2008)

One of the biggest problems in most domestic animals during summer is the period of infertility or subfertility caused by HS (Setchell, 1998; Yaeram et al., 2006). HS has negative effects on a large number of physiological functions because all tissues are susceptible to its effects. The reproductive function is the first physiological feature impaired in adverse situations. Thus, the study of the reproductive function could be a direct indicator of conditions affecting animal welfare such as maintenance under HS conditions (Tilbrook et al., 2002). Testes are particularly sensitive to HS because they can even be influenced by the physiological abdominal temperature (Bedford et al., 1982; Setchell, 1998). The scrotal temperature of male mammals should be about 2ºC to 8ºC below the body temperature to achieve a normal spermatogenesis (Alvarez and Storey, 1985; Pérez-Crespo et al., 2008a). A high testicular temperature, either acute or chronic, produces an impairment of spermatogenesis that results in a reduction of the spermatozoa number, associated with a transitional period of partial or complete infertility in several species (Ikeda et al., 1999; Marai et al., 2002; Pérez-Crespo et al., 2008a; Setchell, 1998; Yaeram et al., 2006). Some studies have detected that the most important effects of HS are changes in ejaculate volume, sperm concentration and motility. HS also produces an increase in morphological abnormalities and dead spermatozoa (Finzi et al., 1995; Hansen, 2009; Pérez-Crespo et al., 2008a; Setchell, 1998; Yaeram et al., 2006; Zhu and Setchell, 2004). HS applied on the day of artificial insemination (AI) cause a 6% of decrease in male fertility rate respect thermoneutrality (Tusell et al., 2011a). However, many of these experiments consisted in exposing the animals to very high temperatures, in some instances above physiological range, that is, temperatures higher than 40ºC, usually for long periods of time. To better represent real weather conditions, we aimed to analyse the effects of a continuous exposure to a

summer circadian cycle on sperm cell characteristics and fertility. No previous studies in the rabbit have analysed the effects of a continuous summer circadian cycle with exposure to high temperatures in the central part of the day, followed by a recovery period to comfort temperatures. In addition, to the best of our knowledge, no studies have performed crossed AI between rabbits submitted to HS and control conditions. These cross inseminations could assess if the effect of HS in fertility and prolificacy is caused by one of the two genders or by the interaction of both.

Rabbits are very sensitive to HS owing to their difficulty in eliminating the excess body heat; rabbits have few functional sweat glands. Exposure to high levels of humidity and temperature causes a negative effect on their growth and reproduction, and reduces their resistance against diseases (Marai et al., 2002). However, rabbits can adapt to adverse situations after exposure to HS conditions, as we have reported recently (de Lima et al., 2013). In addition, the impact of HS on prolificacy could be alleviated if the temperature decreases during the day (Piles et al., 2013).

Stress has been defined as a state that occurs when an animal is required to make abnormal or extreme adjustments to cope with adverse aspects of its environment and management (Fraser et al., 1974). At high temperatures, rabbits react showing physiological and behavioural changes (Baêta, 1998). Physiologically, stress agents promote the activation of the hypothalamic-pituitary-adrenal axis, which significantly increases corticosteroids levels (Ludwig et al., 2010). A plasmatic cortisol secretion peak provokes an increase in cortisol metabolites, which are detectable in faeces some hours later (Möstl and Palme, 2002). Faecal cortisol assessment has the advantage of providing an integrated measure of cortisol secretion over one or two previous days (Monfort et al., 1998; Palme et al., 1999) and the time of day does not affect its measurement.

The aim of the present study was to evaluate the effect of continuous summer circadian cycles on semen production and sperm cell features such as viability, morphology and motility. Additionally, faecal cortisol metabolites, fertility and prolificacy of rabbits kept under an *in vivo* HS model were also assessed.

2. Material and methods

All experiments were approved by the Institutional Animal Care and Use Committee of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA).

2.1 Animals and experimental conditions

The study was performed at the experimental farm of the IRTA (Torre Marimon) from February 2010 through July 2011 using New Zealand White breed (71 males and 161 females). The animals belonged to the Caldes line selected for growth rate during the fattening period (Gómez et al., 2002).

At 2 months of age, bucks and does were randomly split and housed in 2 identical, closed rooms (46.5m^2) with ventilation and cooling/heating systems. Rooms only differed in the environmental conditions as described. Daily climate variables of the rooms (temperature and relative humidity) were automatically recorded each 4 minutes using a data logger (Tinytag, Gemini Data Loggers, Chichester, UK) located inside the chambers during the experiment.

Animals located in the HS room were continuously exposed to a summer temperature daily cycle, with a period of high temperature at the central part of the day for 3 hours. The resultant environmental conditions were as follows: The temperature in the HS room at 9 am was programmed to start to increase from 22ºC to reach a maximum temperature of 30ºC at 12 pm. This temperature was maintained for 3 hours and then started to decrease to a temperature of 22ºC until 9 am of the following day. Animals located in the control room were exposed to a daily constant temperature of the thermo neutral zone (from 18ºC to 22ºC).

Temperature Humidity Index (THI) was estimated from the following equation described by Marai *et al.* (2002) for rabbits:

$$THI: db^{o}C - [(0.31 - 0.31\ (RH))\ (db^{o}C - 14.4)]$$

Where $db^{o}C$ is dry bulb temperature and RH is relative humidity percentage /100.

Figure 1 shows the THI values in the HS and control rooms. The THI of the HS room from 16 to 9 hours ranged from 18.3 to 20.2. Thereafter, the THI was gradually increased from 9 to 16 hours from 23.6 to 28.2. Animals located in the control room were exposed to a daily constant THI between 16.5 and 19.3. The values for the temperatures established at

the present study were chosen based on the average minimum and maximum temperature values registered during the three previous summers on the farm where the current experiment was performed.

All animals had *ad libitum* access to water and all-mash pellet. The photoperiod followed a 24 hours rhythm and included an uninterrupted light period of 16 hours and 8 hours of darkness.

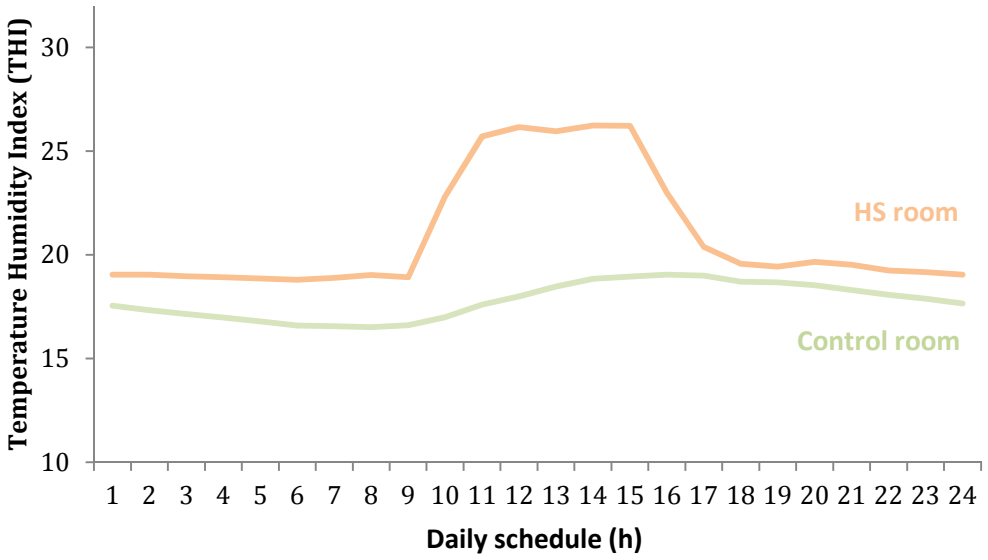

Figure 1. THI values at the control and HS rooms following a daily schedule.

2.2 Faecal cortisol metabolites analysis

A total of 42 samples of fresh faeces (18 from males and 24 from females) to assess faecal cortisol metabolites were taken per room over the course of 1 year (from February 2010 to February 2011) and analysed following the methodology described in our previous study (de Lima et al., 2013). Briefly, the samples were frozen after collection to – 21ºC until analysis. Samples were dried at 60°C for at least 24 hours and crushed to obtain a homogenous powder. We placed 0.3 g of powdered faeces was placed into a 15-mL Pyrex glass tube with screw-cap and 2.5 mL distilled water and 3.0 mL of methanol were added to extract the cortisol metabolites. Samples were vortexed for 30 minutes and then centrifuged at 3000 rpm for 15 minutes. We transferred 1-mL aliquots of the methanol fraction into minivials and stored at -20°C until assaying. Extracts were thawed at room temperature and mixed thoroughly before use. Cortisol metabolites were measured using an ELISA kit (Rabbit Cortisol ELISA Kit; USCN Life Science Inc., Wuhan, China) according to

the manufacturer instructions. The assay was validated by determination of assay specificity (dilutional parallelism), accuracy from spike recovery, precision from intra-assay and inter-assay variabilities, and sensitivity (Reimers and Lamb, 1991). Extracts from 10 animals were pooled for assay validation. Parallelism was tested using serial dilutions of extracts and assayed to test for a parallel response to the cortisol standards. Immunoreactivity of serial dilutions was parallel to assay standards, confirming samples interacted with the assay antibody in a dose-dependent manner. Accuracy was tested by calculation of recoveries from samples spiked with known amounts of cortisol (three increments of the cortisol standard solution provided with the kit). Hormone standards spiked with faecal extracts produced accurate results of hormone recovery (98.5 ± 7.6% in 12 assays, r^2 = 0.97). Inter-assay and intra-assay coefficients of variation were 12.1% and 6.8%, respectively. Sensitivity of the assay was determined to be 17.7 ng cortisol metabolites per 100 mg of dry faeces. Cortisol metabolites concentrations are expressed as nanograms per 100 mg of dry faeces.

2.3 Semen collection

Before to the semen collection, all bucks were trained for ejaculate extraction with an artificial vagina at 4.5 month of age. A homemade polyvinyl chloride artificial vagina containing water at a temperature of 50ºC was used. One ejaculate was collected per male each week for the first two weeks. At 5 months of age - when bucks are considered to be adults - two ejaculates per male were collected in the same day of each week, with an interval of 30 minutes between collections. Analysed semen samples were obtained after the seventh month of age.

Ejaculates that contained urine and calcium carbonate deposits to eye inspection were discarded, and gel plugs removed. After this initial pre-evaluation, ejaculate volume was assessed using a micropipette. Sperm concentration was measured using a sperm cell counter (Nucleocounter SP-100, Chemometec A/S, Allerød, Denmark) and the total sperm number was determined multiplying ejaculate volume by sperm concentration. Ejaculates were stored in an isothermal chamber at 37ºC until insemination or evaluation for no more than 1 hour after collection.

In this study, 375 seminal samples (193 from 29 control and 182 from 42 HS bucks) were analysed. One aliquot of the ejaculate (20µL) was resuspended in a commercial extender

(Galap, IVM Technologies, Saint Ouen sur Iton, France) and used for individual evaluation. The remainder of the ejaculate was used for AI.

2.4 Evaluation of sperm viability and morphology

Galap diluent was used to adjust the sperm concentrations to 60 x 10^6 cells/mL. Eosin-nigrosin staining was performed for each sample (Bamba, 1988). We mixed 10 µL of the sperm sample and 10 µL of the dye, and smeared the mixture onto the center of the glass slide and allowed air dry. Then, the slide was covered with mounting medium and a cover glass.

Slides were analysed using an optical microscope at 1000 x under immersion oil. As many as 200 cells were counted on each slide and the following ratios were calculated: Sperm viability, as the percentage of spermatozoa that remained white or unstained versus non-viable sperm cells stained pink, because the integrity of their plasma membranes had been compromised causing an increase in membrane permeability that led to uptake of the dye (Bamba, 1988), and percentages of sperm with acrosome abnormalities, sperm with proximal and distal cytoplasmic droplets, sperm with morphological abnormalities of the head, sperm with morphological abnormalities of the tail, and sperm without tails.

2.5 Sperm motility parameters

Motility characteristics were determined using a computer-assisted sperm analysis system (CASA system, Proiser SL, Valencia, Spain). The CASA system is based on the analysis of 25 consecutive digital images taken from a single field at a magnification of 100 x in a dark ground in a time lapse of 1s. Sample aliquots (5µL) were placed on a prewarmed slide and viewed in a phase contrast microscope equipped with a warmer stage at 37ºC. Four to five separate fields were taken for each sample and a minimum of 200 cells per sample were analysed. The motility descriptors obtained after CASA were: Curvilinear velocity (VCL), as the mean path velocity of the sperm head along its actual trajectory (units: µm/s); Linear velocity (VSL), as the mean path velocity of the sperm head along its average from its first to its last position (units: µm/s); Mean velocity (VAP), as the mean velocity of the sperm head along its average trajectory (units: µm/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), as the mean value of the extreme side-to-side movement of the sperm head in each beat cycle (units: µm); and Frequency of head displacement (BCF), as the frequency with which the actual sperm trajectory crosses the average path trajectory (units: Hz). Total motility was defined as the percentage of spermatozoa that showed a VAP above 10 µm/s, and progressive motility as the percentage of spermatozoa which showed a VAP above 50 µm/s and 70% of STR, as defined by the CASA manufacturer.

2.6 Artificial Insemination

Ejaculates from all the animals (control and HS) were individually diluted to a final concentration of 20 x 10^6 spermatozoa/mL in Galap diluent to prepare monospermic insemination doses. The AI doses of 0.5 mL were applied within one hour after ejaculate collection. Ejaculates from control bucks were used to inseminate either control or HS does. The same procedure was done with the ejaculates from HS bucks. Thus we obtained four different types of cross-inseminations: Control male with control female (n=75), control male with HS female (n=56), HS male with control female (n=72), and HS male with HS female (n=80).

Females followed a semi-intensive reproductive rhythm: First AI was done at approximately 4.5 months of age, with subsequent 42-day reproductive cycles. At 48 hours before AI, all females were treated with 15 IU eCG subcutaneously (Foligon, Intervet International B.V., Booxmeer, Holland). Ovulation was induced immediately after AI with 0.02 mg of gonadorelin administered intramuscularly (Fertagyl, Intervet Internacional B.V.). The fertility score was assigned at birth (1 if the female gave birth and 0 otherwise). Prolificacy was defined as the total number of kits born per parity.

2.7 Statistical analysis

All results are expressed as mean ± standard error. Faecal cortisol metabolite concentrations were analysed with PROC GENMOD of the Statistical Analysis System (SAS 9.2; software SAS Institute Inc. 2002-2008) by means of a negative binomial distribution, in accordance with the deviance (Cameron and Trivedi, 1998). The independent variable considered was group (control or HS). The evaluation of differences between the control and HS groups for ejaculate volume, sperm concentration, sperm cell viability, sperm

normal morphology, motility and velocity were studied fitting a linear mixed-effects model in order to take into account individual effect.

Logistic regression analyses were performed on data from each insemination using parturition as the dependent variable (0 or 1) inseminating rabbit buck and inseminated rabbit doe as random effects. The room of the animal (control versus HS) and the type of crossed AI (male control with a female control, male control with a female HS, male HS with a female control and male HS with a female HS) were fitted as class-fixed effects. A logistic regression with the same model was performed for the analysis of prolificacy as the dependent variable (0 = < 6 born rabbits, 1 = ≥ 6 born rabbits).

All the statistical analyses described above were performed using the R program (version 2.10.0; R development Core Team, 2009). In all cases, differences between groups with $P < 0.05$ were considered significant.

3. Results

The effects of HS on the levels of faecal cortisol metabolites are shown in Figure 2. Animals housed in the HS room showed higher values of faecal cortisol metabolites than the controls ones (Chi-square = 12.86; df = 1; $P = 0.0003$). Significant effects between males and females were not detected in faecal cortisol metabolites (Chi-square = 0.27; df = 1; $P = 0.6038$).

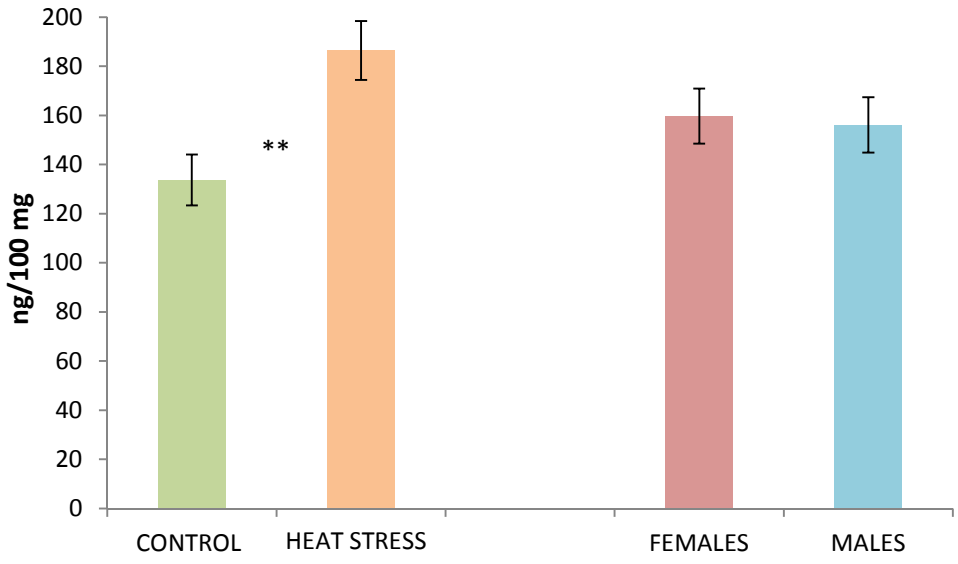

Figure 2. Concentration of cortisol in dried faeces (ng/100 mg). ** $P = 0.003$.

No significant differences were detected in ejaculate volume, sperm cell concentration and total sperm cell number between control and HS males (Table 1).

Variations in viability and normal sperm morphology attributable to HS are shown in Table 1. Animals from HS treatment showed a significant decrease in the percentages of viable sperm cells (*P* = 0.01*)*, and an increase in the percentage of acrosome abnormalities (*P* = 0.00253) and tailless spermatozoa (*P* = 0.01). No significant differences were found for the rest of the morphology and motility parameters analysed between the control and HS group.

Table 1. Effects of HS on rabbit semen volume, sperm cell concentration, total number of spermatozoa, sperm cell viability, morphology and sperm motility parameters. Evaluation of 375 seminal samples (193 from 29 control bucks and 182 from 42 HS bucks).

Parameters	**Control group**	**Heat stress group**
Volume of ejaculate (mL)	0.90 ± 0.07	0.84 ± 0.10
Spermatozoa concentration (x10^6 spz/mL)	246.46 ± 23.43	208.95 ± 33.08
Total number of spermatozoa (x10^6 spz)	189.42 ± 19.20	152.82 ± 26.99
Viable (%)	80.71 ± 1.62	74.21 ± 2.28*
Acrosome abnormalities (%)	22.57 ± 3.09	36.96 ± 4.32*
Proximal cytoplasmic droplet (%)	5.71 ± 1.39	5.88 ± 1.94
Distal cytoplasmic droplet (%)	1.48 ± 0.21	1.34 ± 0.29
Head abnormalities (%)	0.26 ± 0.04	0.31 ± 0.06
Bent tails (%)	4.62 ± 0.60	4.98 ± 0.85
Coiled tails (%)	2.55 ± 0.45	2.61 ± 0.63
Tailless spermatozoa (%)	7.91 ± 1.21	12.83 ± 1.71*
MP (%)	26.06 ± 1.70	28.52 ± 2.43
MT (%)	64.61 ± 3.33	59.91 ± 4.72
VCL (µm/s)	101.54 ± 2.66	99.51 ± 3.92
VSL (µm/s)	40.67 ± 1.90	42.50 ± 2.80
VAP (µm/s)	66.70 ± 2.75	65.86 ± 4.04
LIN (%)	41.60 ± 1.70	43.99 ± 2.51
STR (%)	61.07 ± 1.45	64.36 ± 2.14
WOB (%)	65.11 ± 1.72	65.63 ± 2.53
ALH (µm)	3.69 ± 0.09	3.61 ± 0.13
BCF (Hz)	9.41 ± 0.24	9.43 ± 0.35

Values are mean ± standard error. * Significance $P \leq 0.01$

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity coefficient; MP, progressive motility; MT, total motility; spz, spermatozoa; VAP, average path velocity; VCL; curvilinear velocity; VSL; straight linear velocity; WOB, wobble coefficient.

Fertility and prolificacy rates after AI are shown in Figure 3 and 4, respectively. No differences were detected in fertility or prolificacy among AI groups. Logistic regression analysis showed that the analysed variables had no significant effects on fertility and prolificacy.

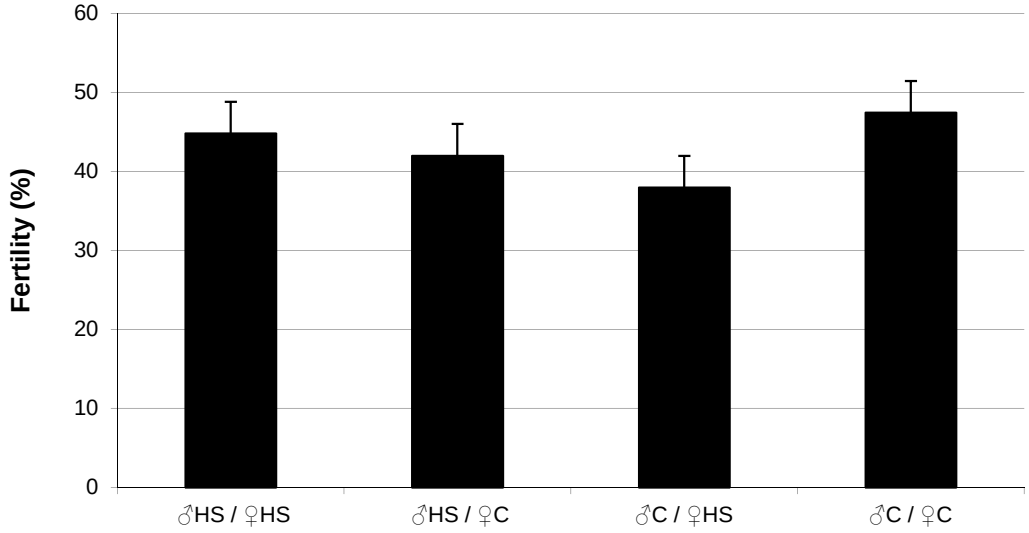

Figure 3. Effects of HS on fertility after AI.

♂HS / ♀HS = HS male with HS female; ♂HS / ♀C = HS male with control female;
♂C / ♀HS = control male with HS female; ♂C / ♀C = control male with control female.

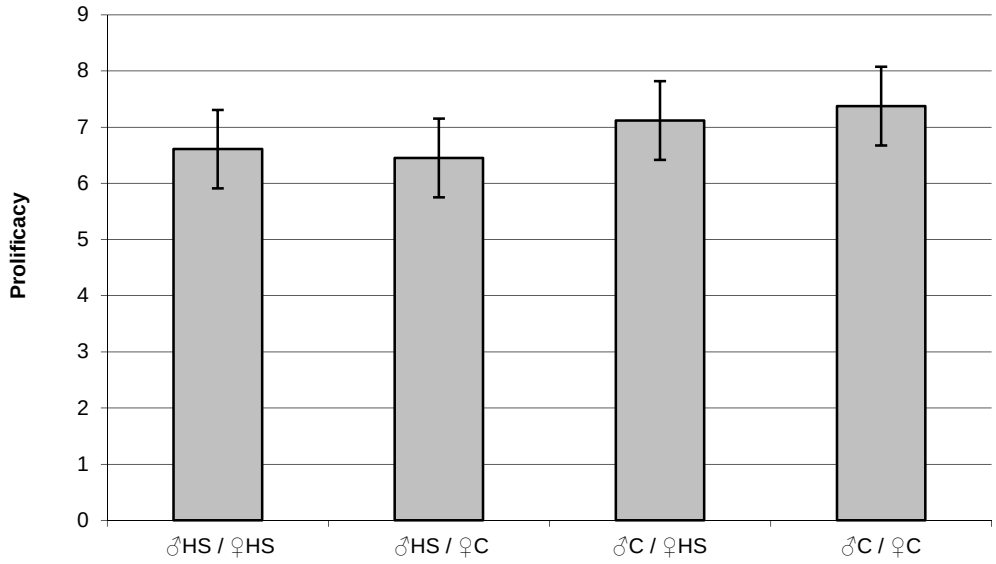

Figure 4. Effects of HS on prolificacy after AI.

♂HS / ♀HS = HS male with HS female; ♂HS / ♀C = HS male with control female;
♂C / ♀HS = control male with HS female; ♂C / ♀C = control male with control female.

4. Discussion

This study confirms that an *in vivo* experimental model of continuous daily summer cycles has moderate effects on sperm quality in rabbits, especially on sperm viability and acrosome integrity. However, these anomalies do not significantly affect fertility and prolificacy suggesting that animals are able to maintain the reproductive function by a process of adaptation or, alternatively, that the proportion of potential fertile sperm cells in an ejaculate is greater enough to preserve fertility and thus preservation of the species, as an example of compensable seminal deficiencies (Saacke, 2008). These seminal deficiencies would not be associated with fertilizing sperm that are incompetent to maintain the fertilization process or subsequent embryogenesis (Saacke, 2008).

Some studies previously performed in rabbits (Burfening and Ulberg, 1968; El-Sheikh and Casida, 1955) and mice (Burfening et al., 1970; Pérez-Crespo et al., 2008b; Yaeram et al., 2006; Zhu and Setchell, 2004; Zhu et al., 2004) detected significant decreases in fertilization rates after exposing animals or sperm cells to high temperatures for short periods (minutes to hours) (Burfening et al., 1970; Pérez-Crespo et al., 2008b; Yaeram et al., 2006) or long periods (24h) (Burfening et al., 1970; Zhu and Setchell, 2004; Zhu et al., 2004). However, in the majority of these studies, animals were exposed to a whole body hot environment with high temperatures (36ºC) for 24h (Zhu and Setchell, 2004) or local heating of the testes by inducing cryptorchidism (Bedford, 1978), or scrotal insulation (Setchell, 1998) or short-term heating by immersion in a hot water bath (Pérez-Crespo et al., 2008b). In our study, we simulated a summer daily cycle with a period of HS at the central part of the day followed by a period of coming back to comfort condition, following the typical cycle of a summer day. This period of recovery could allow the recovery of physiological functions and alleviate the HS negative effects, allowing an efficient reproduction function.

Our study shows that continuous summer HS cycles do not affect sperm cell production, or morphological, or sperm motility parameters. However, viability and mainly acrosome integrity are significantly affected by HS. This is in accordance with results reported by Marai *et al.* (2002) and Finzi *et al.* (1995). However, these authors detected negative effects on sperm motility features and fertility. Again, the recovery periods used in our

study to simulate the conditions of a summer's day may alleviate the negative effects of HS and allow recovery to normal values of motility and also fertility.

The experimental model designed in this study provoked stress in the animals, demonstrated by higher values of cortisol metabolites in faeces of the animals exposed to HS. Although the experimental conditions induce a moderate HS condition, as showed by THI values from 27.8 to 28.9, this is enough to cause an increase in the activity of the hypothalamic-pituitary-adrenal axis. Therefore, animals from the HS group were under metabolic stress, but this stress did not negatively affect the reproductive function. It is likely that the daily recovery periods were enough to maintain the normal reproductive function demonstrating the animal tolerance to this specific stress factor. It is interesting to note that in this study animals were submitted to the same experimental conditions at 2 months of age (when bucks are considered to be maturing sexually). This could explain the possible adaptation of the animals to the HS environment.

The current fertility using monospermic inseminations was similar to that described by our group with this rabbit line (Tusell et al., 2011b) and both lower than had been observed previously in the nucleus of selection of this paternal line over purebred females when using either natural mating (86.2%) (Piles et al., 2005) or AI (71.7%) (Tusell et al., 2010). The AI conditions of this experiment, with monospermic inseminations and smaller sperm dosage, could be more unfavourable than natural mating and commercial AI conditions, respectively. After strong sperm quality selection, Brun *et al.* (2002) obtained similar fertility rates after AI using monospermic doses in 2 purebred lines and their reciprocal crosses, but current fertility was lower than that described by Vicente *et al.* (2004) with similar sperm dosage and 3 different rabbit lines.

In conclusion, this study describes a model of HS simulating a continuous summer daily cycle that allows periods of time to recover as would occurs under natural conditions. Although negative effects of HS have been detected in sperm viability and acrosome integrity, fertility and prolificacy were not affected, suggesting a fast recovery of the reproductive function when normal conditions are re-established. This could be considered a key strategy in the management of rabbits in warm areas during the summer. Hence, to ensure a good reproductive status of the animals under HS conditions

a certain period of refreshment in relation to the environmental temperatures must be provided.

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Metabolic activity of sperm cells: correlation with sperm cell concentration, viability and motility in the rabbit

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Received 15 July 2015; received in revised form 6 November 2015**;**
accepted 29 November 2015

Zygote (2016). DOI: 10.1017/S0967199415000726

Abstract

Resazurin reduction test (RRT) is a useful technique to assess the metabolic rate of sperm cells. RRT depends on the ability of metabolically active cells to reduce the non-fluorescent dye resazurin to the fluorescent resorufin. The aim of this study was to develop a vital fluorometric method to evaluate metabolic activity of rabbit sperm cells. Twenty-five rabbit males were included in the study. Viability and morphology, motility and metabolic activity were evaluated using an eosin-nigrosin staining, a computer-assisted semen analysis (CASA) and the RRT, respectively. Spearman rank correlation analysis was used to determine the correlation between RRT and semen parameters. After evaluation, a concentration of 10 x 10^6 sperm cells/mL was selected for further experiments with RRT. No significant correlation was found between the RRT results and the motility parameters. However, after RRT a significant positive correlation between relative fluorescence units and the percentage of alive spermatozoa ($r = 0.62$; $P = 0.001$) and a negative one with the percentage of sperm cells with acrosomic abnormalities ($r = -0.45$; $P < 0.05$) were detected. The vital assessment of metabolic rate of sperm cells by RRT could provide more information about semen quality than other routine semen analysis, correlating with sperm viability and acrosome status information.

Keywords: Resazurin reduction test; metabolic activity; sperm cell; acrosome; rabbit.

1. Introduction

Several features are necessary for sperm cells in order to fertilize an oocyte, such as motility, normal morphology, sufficient metabolism for energy production, and membrane integrity (Zrimsek et al., 2005). Although several analytical techniques have been developed to evaluate these features, there is no a single method that provides a complete picture of semen quality or complete information for predicting fertility (Rodríguez- Martínez, 2007).

Metabolism is a basic process of cellular function and biological activities. Reproductive performance depends on several metabolic processes since a fine metabolic activity is necessary for fertilization to occur (Zrimsek et al., 2004). The assessment of metabolic rates of sperm cells could provide pertinent information for evaluating sperm cell quality (Wang et al., 1998b). The resazurin reduction seems to be a useful technique to assess the metabolic rate of sperm cells. The analyses of resazurin reduction have been used in human and ovine sperm cells after adaptation of the test previously described to asses the bacterial content of milk, and the quality control of pasteurized and ultra-high temperature-treated food (Rahman and Kula, 1997; Martin et al., 1999). Resazurin, as a nontoxic redox dye, allows to follow-up *in vivo* cellular activity whereas other dyes such as 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) are non-vital (O'Brien et al., 2000; Aziz, 2006; Aziz et al., 2005; Iqbal et al., 2010). Mahmoud *et al.* (1994) found that the resazurin is a better and more accurate metabolic assay than ATP measurements thanks its higher sensitivity as indicator of cellular dehydrogenase activity.

Inside the cell, resazurin undergoes enzymatic reduction in mitochondria (Czekanska, 2011). This reaction is manifested by a visual colour change from blue (resazurin) to a spectrum ranging from dark purple to pink (resorufin) and further to a colourless compound (dihydroresorufin) (Glass et al*.,* 1991; Erb and Ehlers, 1950; Zalata et al., 1998). Initially, the colour change of resazurin was usually graded by using colour charts but the measurements were subjective and variable among evaluators (Glass et al*.,* 1991; Dart et al., 1994). Later, Mahmoud *et al*. (1994), Rahman *et al.* (1997) and Wang *et al.* (1998b) developed a spectrophotometric evaluation of the resazurin that provided a quantitative and objective method for measuring metabolic activity.

Resazurin reduction has been used successfully by spectrophotometry or colorimetrical methods in several studies to evaluate the quality and fertility potential of semen from humans (Mahmoud et al., 1994; Rahman and Kula, 1997; Glass et al., 1991; Zalata et al., 1998) and from animals such as stallions (Carter et al., 1998), bulls (Dart et al., 1994), rams (Wang et al., 1998b; Martin et al., 1999) and boars (Zrimsek et al., 2004; Zrimsek et al., 2006). However, some of these studies have described the use of resazurin without standardizing for sperm cell concentration, which could saturate the reaction and give variable results (Erb and Ehlers, 1950; Dart et al., 1994).

Recent studies have shown that resazurin is a non-fluorescent dye while resorufin is a strong fluorescent redox indicator. Both dyes can be detected by fluorometric methods using a lower concentration of resazurin and fluorimetry, which has a greater sensitivity and a lower detection limit than spectrophotometry (O'Brien et al., 2000; Wang et al., 1998a; Gil et al., 2011; Hamoutene et al., 2000). Besides being able to provide a reliable analysis of metabolic activity of cells, Kuisma *et al*. (2006) described the capacity of resazurin for also evaluating the plasma membrane integrity of cells.

The aim of this study was to develop a new fluorometric method for evaluating metabolic activity of rabbit sperm cells. In this study, we assessed the effect of sperm cell number on the reaction of resazurin reduction, which allows quantitative measurement of sperm cells metabolic activity in rabbit sperm cells. It also enables to study the correlation between metabolic activity and sperm cells features, such as viability, acrosome status, morphology and motility.

2. Material and Methods

2.1 Semen samples

Twenty-five New Zealand White adult rabbit bucks (older than seven months) from the nucleus of selection of the farm of the Institut de Recerca i Tecnologia Agroalimentaries (IRTA, Torre Marimon, Spain) were used in this study and maintained for commercial purposes. Each animal was housed in a single cage (85 x 40 x 30 cm) equipped with a plastic footrests, a feeder and nipple drinker. Animals were given an all-mash pellet and offered water, both of them *ad libitum*.

All males began the training to an artificial vagina at 4.5 month of age. A homemade polyvinyl chloride artificial vagina containing water at a temperature of 50ºC was used. One ejaculate was collected per male. Ejaculates which contained urine and calcium carbonate deposits to eye inspection were discarded, and gel plugs were removed. After this initial pre-selection, each sperm sample was diluted 1:3 (vol/vol) in a commercial liquid diluent for rabbit semen (Galap, IVM Technologies, Saint Ouen sur Iton, France). Ejaculates were stored in an isothermal chamber and evaluated within 30 minutes after collection.

Individual fertility data was not available because pooling ejaculates from several males for heterospermic insemination is a common practice in rabbit AI centers (Tusell et al., 2011). Collective fertility with heterospermic inseminations was 85% on average.

2.2 Semen analysis perform

Following the determination of the ejaculate volume, concentration and total sperm number with a haemocytometer chamber, sperm morphology and viability were determined by an eosin-nigrosin staining. The eosin-nigrosin staining was performed by mixing 10 µL of sperm sample and 10 µL of the dye solution, smeared onto a glass slide and allowed air-drying. Then, the slide was covered with mounting medium and a cover glass. Slides were analysed using an optical microscope (Motic BA210, Spain) at 1000 x magnification under immersion oil. As many as 200 cells were counted on each slide and the following ratios were calculated: percentage of sperm viability (percentage of sperm cells that remained white or unstained while non-viable sperm cells stained pink or stained, since the integrity of their plasma membranes had been compromised causing an increase in membrane permeability that led to uptake of the dye (Bamba, 1988)), percentage of sperm with acrosome abnormalities (percentage of sperm cells with irregularities in the acrosome membrane), percentage of sperm with morphological abnormalities of head (abnormalities in shape and dimensions), percentage of sperm morphological tail abnormalities (bent tails and coiled tails), percentage of proximal and distal cytoplasmic droplets, and percentage of sperm without tails. The eosin-nigrosin staining provides similar measurements of sperm membrane integrity to those observed using flow cytometry (Foster et al., 2011), and also allows the sperm morphology analysis.

Motility characteristics were determined using a computer-assisted sperm analysis system (CASA system; Integrated Sperm Analysis System V.1.2.; Proiser SL, Valencia, Spain). The CASA system is based on the analysis of 25 consecutive digital images taken from a single field at a magnification of 100X in a dark ground in a time lapse of 1s.

Sample aliquots (5 µL) were previously adjusted to 60 x 10^6 cells/mL with Galap extender. Aliquots were placed on a pre-warmed slide for 1 minute and viewed in a phase contrast microscope equipped with a warmer stage of 37ºC for CASA evaluation.

Four to five separate fields were taken for each sample and a minimum of 200 cells/sample were analysed. The motility descriptors obtained after CASA analyses were: Curvilinear velocity (VCL), as the mean path velocity of the sperm head along its actual trajectory (units: µm/s); linear velocity (VSL), as the mean path velocity of the sperm head along its average from its first to its last position (units: µm/s); mean velocity (VAP), as the mean velocity of the sperm head along its average trajectory (units: µm/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), as the mean value of the extreme side-to-side movement of the sperm head in each beat cycle (units: µm); and frequency of head displacement (BCF), as the frequency with which the actual sperm trajectory crosses the average path trajectory (units: Hz). Finally, total motility was defined as the percentage of sperm cells which showed a VAP above 10 µm/s, and progressive as the percentage of sperm cells which showed a VAP above 50 µm/s and 70% of STR.

A sperm index (SI) was calculated by multiplying sperm concentration by the square root of the percentage of sperm motility (% total motility) multiplied by the percentage of morphological normal sperm (Mahmoud et al., 1994):

Sperm index = sperm concentration x [root](% total motility x % normal forms).

2.3 Resazurin reduction test (RRT)

The sperm cell metabolic activity was evaluated by the RRT using the QBlue cell viability test (QBlue Cell Assay kits, Biochain, USA).

In order to determine which concentration of sperm cells was the most appropriate before saturating the reaction of resazurin reduction, four sperm concentrations were

assessed at 5 x 10^6, 10 x 10^6, 15 x 10^6 and 20 x 10^6 sperm cells/mL. Three replicates were performed for each concentration of each sample in this assay.

Concentrations were adjusted to one of the four evaluated sperm concentrations with phosphate-buffered saline (PBS; Sigma, Madrid, Spain). Each sample was placed into a 1.5 mL eppendorf and centrifuged at 4,442.7 x g for 10 minutes. The supernatant was discarded and samples were gently resuspended with 100 µL QBlue solution (10% QBlue diluted in PBS).

Blanks were made by taking an equal amount of QBlue solution without sperm cells. All eppendorfs were incubated at 37ºC in a humidified air atmosphere with 5% CO_2 for 25 minutes. This time of incubation was standardized previously in our laboratory for rabbit sperm cells, and it corresponds to the time required to observe a change of colour in the culture medium from purple to pink. After incubation, samples were immediately centrifuged at 4,846.6 x g for 10 minutes. Part of the supernatant (90 µL) of each sample was taken and measured with a Wallac Fluorometry plate reader (VICTOR^3 V 1420 multilabel counter, PerkinElmer precisely, Waltham, USA) (excitation wavelength at 550 nm and emission at 615 nm). All procedures were made in the dark to avoid light effects in the wavelength emission. The results of RRT were quantified in relative fluorescence units (RFU).

2.4 Statistical analysis

The normal distribution of all parameters was assessed using a Shapiro-Wilk test. Statistical comparison of differences in RRT among the different sperm concentrations was carried out by means of the non-parametric test of Kruskal-Wallis and Mann-Whitney-Wilcoxon. Spearman rank correlation analysis was calculated to determine the correlation between the RRT and semen parameters such as total sperm number, viability, sperm morphology, motility parameters and SI.

All statistical analyses described were performed using the R program (version 2.10.0 ISBN 3-900051-07-0). P values < 0.05 were considered statistically significant.

3. Results

Average semen characteristics and range of the samples were as follows [mean ± SD (Min - Max)]: total number of sperm cells: 1020.5 x 10^6 ± 722.4 x 10^6 (108 – 2684.5 x 10^6), viable sperm cells: 80.3% ± 11.3% (46 – 93.5 %), normal morphology: 75.5% ± 13.4% (37 – 91.2 %), total motility: 74.4% ± 15.5% (33.8 – 92.9 %), and progressive motility 34.4% ± 12.8% (12.8 – 62.8 %).

3.1 Relationship between RRT and sperm concentration

Figure 1 shows that there were significant differences ($P < 0.05$) between the RFU of the concentration of 5 x 10^6 sperm cells/mL and the other three evaluated concentrations. No significant differences ($P > 0.05$) were found among the RFU of 10 x 10^6, 15 x 10^6 and 20 x 10^6 sperm cells/mL concentrations. Taking into account these results, the concentration of 10 x 10^6 sperm cells/mL was used for further experiments.

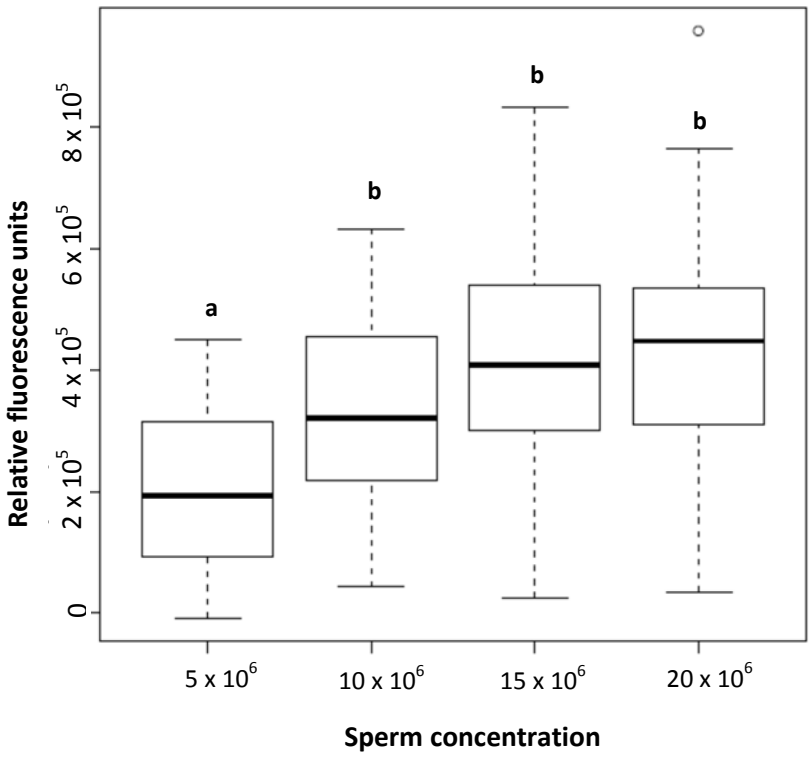

Figure 1. Values of Resazurin Reduction Test in Relative Fluorescence Units (RFU) based on the sperm concentrations evaluated.

Note: a - b = Different scripts between box plots indicate significant differences.

3.2 Correlation between RRT and sperm viability, acrosome status and morphology

A significant positive correlation between the results of RRT in RFU and the percentage of viable sperm cells was detected (r = 0.62; *P* = 0.001) (Fig. 2). Additionally, the results of RRT were inversely correlated with the percentage of acrosomic abnormalities (r = -0.45; *P* < 0.05) (Fig. 3). No significant correlation was found between the results of RRT and the following: abnormalities in shape and head dimensions, cytoplasmic proximal and distal droplets, bent tails, coiled tails, swollen tails and heads without tails (data not shown).

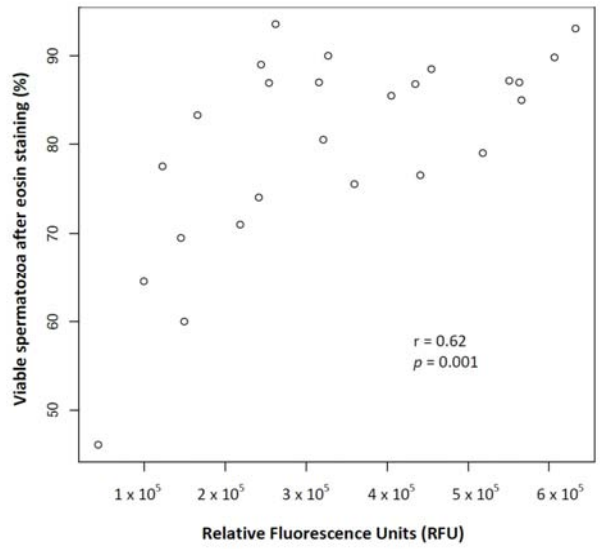

Figure 2. Correlation between the percentage of viable sperm cells and the results of the Resazurin Reduction Test in Relative Fluorescence Units (RFU).

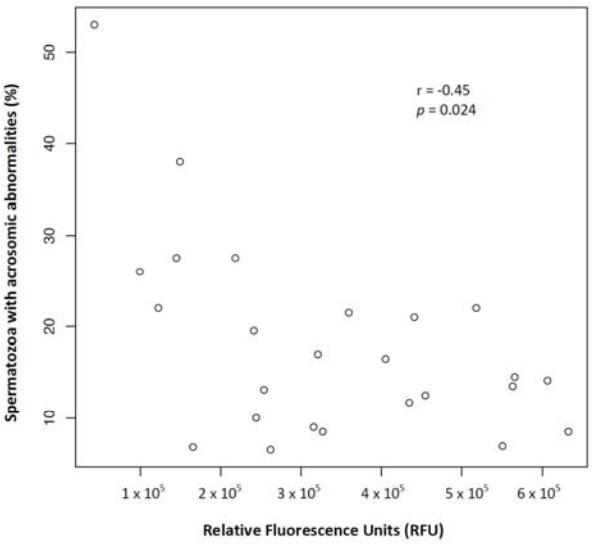

Figure 3. Correlation between the percentage of acrosomic abnormalities and the results of the Resazurin Reduction Test in Relative Fluorescence Units (RFU).

3.3 Correlation between RRT and sperm motility parameters

The correlation of the results of RRT with the motility and velocity parameters is shown in Table 1. No significant correlation was found between these parameters. Nonetheless, there was a positive tendency to being significant for the correlation between the results of RRT and total motility (r = 0.38; *P* = 0.059) (Fig. 4). There was no significant correlation between the results of the RRT and the SI (r = 0.23; *P* > 0.05).

Table 1. Correlation of RRT results with motility and velocity parameters (n = 25, Spearman rank correlation analysis).

Parameters	Correlation coefficient	Statistical significance	Parameters	Correlation coefficient	Statistical significance
MP (%)	-0.07	*P* = 0.747	LIN (%)	-0.31	*P* = 0.128
MT (%)	0.38	*P* = 0.059	STR (%)	-0.25	*P* = 0.235
VCL (µm/s)	-0.02	*P* = 0.934	WOB (%)	-0.32	*P* = 0.119
VSL (µm/s)	-0.31	*P* = 0.136	ALH (µm)	0.60	*P* = 0.078
VAP (µm/s)	-0.25	*P* = 0.235	BCF (Hz)	0.03	*P* = 0.093

Abbreviations: ALH, amplitude of lateral head displacement; BCF, beat cross frequency; LIN, linearity coefficient; MP, progressive motility; MT, total motility; VAP, average path velocity; VCL; curvilinear velocity; VSL; straight linear velocity; WOB, wobble coefficient.

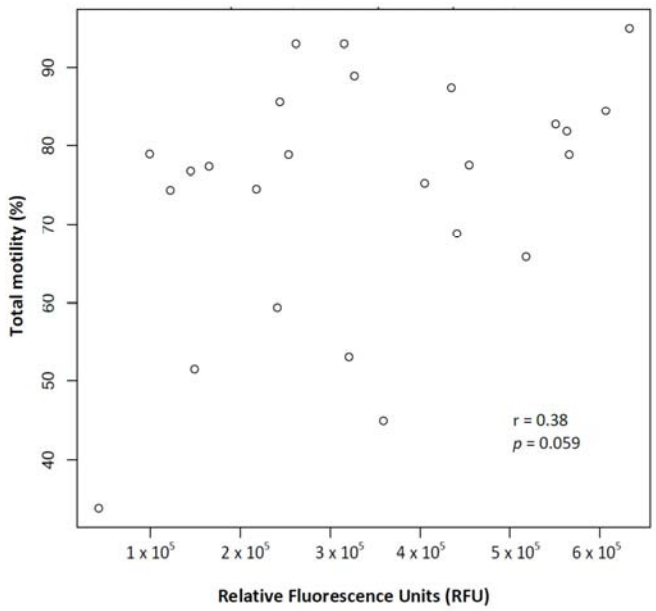

Figure 4. Correlation between the percentage of total motility using the CASA system and the results of the Resazurin Reduction Test in Relative Fluorescence Units (RFU).

4. Discussion

Our results confirm that the RRT determined by fluorimetry is a useful test to quantitatively evaluate the metabolic activity of sperm cells in the rabbit and it is in direct relation to the sperm cell concentration, viability and acrosome integrity.

Previous studies shown that the use of resazurin was cell concentration dependent and thus the efficient prediction of fertility could be related to the time of exposure (Wang et al., 1998b; Rahman and Kula, 1997; Glass et al., 1991; Erb and Ehlers, 1950; Dart et al., 1994; Carter et al., 1998; Erb et al., 1952; Zalata et al., 1995). For this reason, in this experiment a fluorometric method has been developed based on a fixed sperm cells number and a known reduction time of resazurin so to measure sperm cell metabolic activity quantitatively.

Taking into account that the values of RFU for the concentration of 5 x 10^6 sperm cells/mL was significantly lower than the other three concentrations and that no significant differences were detected among those other concentrations, we suggest the use of 10 x 10^6 sperm cells/mL for further experiments with the RRT.

A positive correlation of RRT and sperm viability has been demonstrated in this study. Also, it can be stated that the higher percentage of damage, the lower metabolic activity. These results agree with those from Rahman *et al.* (1997) and Fuse *et al.* (1993) demonstrating a significant correlation between acrosome abnormalities and viability of human sperm cells with the results of resazurin. By contrast, in our study the results of RRT were not related with other sperm morphological parameters.

To the authors' knowledge, prior to this study and according to the available literature, only one assay had used the reduction of resazurin to evaluate sperm quality in rabbits (El-Battawy, 2008). In contrast to our study, this assay used spectrophometry to evaluate the resazurin reaction, performed a trypan blue/Giemsa staining to determine the acrosomal integrity, evaluated the motility parameters by a subjective score by optical microscopy, and also, did not standardize the initial sperm concentration. Although the staining used to evaluate the acrosomal integrity was different, their results confirm our findings that resazurin reduction values inversely correlates with acrosomal

abnormalities. Despite the obvious fact that the metabolic activity is affected by the acrosomal abnormalities of sperm cells, it still represents a significant result and gives additional value to the RRT study.

El-Battawy (2008) observed a significant correlation between the resazurin results and sperm motility. These findings do not totally match with the results from our study. Our results suggest a tendency that total motility could be correlated with resazurin results, but not with other motility parameters such as progressive motility or sperm velocities. A possible explanation for this is that, in our study, motility characteristics were determined by a computer-assisted semen analysis in contrast to the previous study by El-Battawy (2008). CASA facilitates results comparison and enables the finding of subtle differences between treatments increasing accuracy and repeatability (Awad, 2011).

Moreover, in the study by El-Battawy (2008), initial sperm cell concentration was not defined. This might eventually saturate the resazurin reaction and consequently give variable or false positive results (Erb and Ehlers, 1950; Dart et al., 1994).

In other species, it has been reported that resazurin reduction results significantly correlate with sperm concentration and motile sperm cells concentration (Mahmoud et al., 1994; Rahman and Kula, 1997; Martin et al., 1999; Glass et al., 1991; Zalata et al., 1998; Dart et al., 1994; Zrimsek et al., 2004; Zalata et al., 1995, Eljarah, 2007). Only Eljarah (2007) in bulls, and Zalata *et al.* (1998; 1995) and Mahmoud *et al.* (1994) in humans used a semi-computer-assisted semen analysis to evaluate progressive motility, total motility and linear velocity. Those studies found a positive correlation between resazurin reduction ability and progressive and total motility which is consistent with the findings of the current study.

Further experiments are needed to correlate RRT with fertility after insemination. However, in the rabbit industry heterospermic insemination is a common practice in order to compensate for possible infertile ejaculates. This fact makes the evaluation of individual fertility more difficult when animals are kept for commercial purposes, as it was the case of our study.

In conclusion, the RRT is a vital, simple and reproducible test that can be performed by fluorimetry in the rabbit with a small volume of semen. We suggest that the most

appropriate concentration of sperm cells for performing the RRT in rabbit sperm cells seems to be 10 x 10^6 sperm cells/mL. The assessment of metabolic rates of sperm cells could provide better or more complete information about semen quality than other routine semen analysis. Although further research involving fertility trials using RTT in rabbits is required in order to confirm our findings, we believe this test has a great potential as an additional tool for evaluating semen quality in rabbits.

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Heat stress has an effect on motility and metabolic activity of rabbit sperm cells

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Submitted 23 May 2016.

Submitted for publication.

Abstract

In the warm months the function of the spermatozoa can be affected by the temperature of the reproductive tract of the female exposed to hyperthermic conditions. The aim of this study was to evaluate the impact of heat stress on sperm parameters in an *in vitro* model and to determine if there were seasonal effects on sperm heat tolerance. Sperm samples from 32 New Zealand White rabbits were collected in two seasons and incubated at scrotal (32.5ºC), body (37ºC) or hyperthermic (42ºC) temperatures for 3h. Sperm viability and morphology were evaluated using nigrosin-eosin staining. Motility and metabolic activity parameters were determined using computer-assisted sperm analysis and the QBlue cell viability test, respectively. The incubation of spermatozoa at 42ºC decreased ($P < 0.05$) the mean values of total motility, curvilinear (VCL) and mean velocity (VAP) as well as the metabolic activity with respect to the incubation at 32.5ºC and 37ºC. No seasonal effects were observed except for the highest percentages of bent and coiled tails in the cold season, and the highest mean values of VCL, linear velocity and VAP in the warm season ($P < 0.01$). The interaction between *in vitro* heat stress and season was significant for metabolic activity ($P = 0.02$). Our results suggest that rabbit spermatozoa parameters are largely modified by a short exposure to hyperthermic conditions, in terms of metabolic activity and motility parameters. Thus, a short exposure of spermatozoa to an environment of 42ºC in temperature for only 3h may compromise sperm functionality. Additionally, sperm metabolic activity was influenced by the interaction of heat stress and season.

Keywords: heat stress, hyperthermia, sperm, rabbit.

1. Introduction

Exposure of animals to high environmental temperatures has an adverse effect on physiological and reproductive functions (García-Ispierto et al., 2007; Hansen, 2009; Takahashi, 2012). High environmental temperatures during the warm months of the year or experimental exposure to heat stress (HS) can decrease conception rates, fertility and embryo development (De Rensis and Scaramuzzi, 2003; Marai et al., 2002; Yaeram et al., 2006). In several species, exposure of the testes to an acute or chronic increase of temperature during the spermatogenic cycle can reduce the sperm number in the ejaculate and affect sperm parameters, such as motility, morphology and plasma membrane integrity. These events may be followed by periods of partial or complete infertility (Ikeda et al., 1999; Pérez-Crespo et al., 2008; Rahman et al., 2014; Setchell, 1998; Yaeram et al., 2006).

After leaving the testis, sperm cells are vulnerable to environmental changes. The impact on sperm function can persist in the warm months of the year by the exposure of sperm cells to HS environmental conditions found in the reproductive tract of an hyperthermic female before fertilization.

High temperatures can be achieved in the uterus of domestic species, such as cow (De Rensis and Scaramuzzi, 2003; Ealy et al., 1993; West, 2003), when the environmental temperatures are also high. Although the effects of conditions of hyperthermia have been largely analysed for the oocyte (Maya-Soriano et al., 2012, 2013), few studies have analysed the changes in sperm cell physiology when the spermatozoa reach a female genital tract under HS conditions. In this study, we wanted to compare changes in sperm cell features when they leave the testis, at a temperature of 32.5ºC (Alvarez and Storey, 1985), and they reach a genital tract at a physiological temperature (37ºC; Alvarez and Storey, 1985) or under hyperthermia conditions (42ºC; Howarth et al., 1965). Thus, this study was designed to evaluate the impact on sperm parameters of an *in vitro* HS model where rabbit sperm cells were exposed to temperatures of 32.5ºC, 37ºC or 42ºC for three hours. An additional objective was to determine if there were seasonal effects on sperm heat tolerance.

2. Material and methods

2.1 Animals and semen collection

Thirty-two New Zealand White (NZW) adult rabbit bucks (older than seven months) from the nucleus colony at the farm of the Institut de Recerca i Tecnologia Agroalimentaries (IRTA, Torre Marimon, Spain) were used in this study. The animals belonged to the Caldes line selected for growth rate during the fattening period (Gómez et al., 2002). Each animal was housed in a single cage (85 x 40 x 30 cm) equipped with plastic footrests, a feeder and nipple drinker. Animal feed was restricted to 180 g/d of an all-mash pellet and fresh water was always available. Bucks were kept under a controlled photoperiod of 16h of light and 8h of darkness, and a range of temperature between 15-20ºC in winter and 20-26ºC in summer.

All males started to be trained with an artificial vagina at 4.5 month of age. A homemade polyvinyl chloride artificial vagina containing water at a temperature of 50ºC was used. One ejaculate was collected per male. Ejaculates which contained urine and calcium carbonate deposits on visual inspection were discarded. After this initial pre-selection, gel plugs were removed and each sperm sample was diluted 1:3 (vol/vol) in a commercial liquid diluent for rabbit semen (Galap, IVM Technologies, Saint Ouen sur Iton, France). Ejaculates were stored until evaluation in an isothermal chamber at 37ºC for no more than 30 minutes after collection.

2.2 Sperm preparation

Two millilitres of the diluted ejaculate were used as a non-incubated sperm control, the rest of the sample was split between three vials. For the control group, sperm cells were incubated at 32.5ºC for 3 hours, an optimal temperature for testicular germ cells**.** For HS conditions, sperm cells were incubated at abdominal temperature (37ºC), or at hyperthermic conditions (42ºC) for 3 hours. The incubation was done in a humidified air atmosphere with 5% of CO_2. After 3 hours of incubation, sperm viability, morphology, motility parameters and metabolic activity were evaluated for all the experimental groups (non-incubated control, and sperm cells incubated at 32.5ºC, 37ºC or 42ºC).

2.3 Evaluation of sperm viability and morphology

The nigrosin-eosin stain method was used to evaluate viability and morphology. Smears of the sperm suspensions were prepared by mixing 10 µL of sperm sample with 10 µL of stain, smearing the mixture over a glass slide and leaving to air-dry. Then the slide was covered with mounting medium and a cover glass. The spermatozoa were evaluated by counting at least 200 cells per slide under an optical microscope (Motic BA210, Spain) at X1000 magnification (oil immersion). Spermatozoa that remained white or unstained were considered to be viable whereas non-viable sperm cells stained pink, since the integrity of their plasma membranes had been compromised causing an increase in membrane permeability that led to uptake of the dye (Bamba, 1988). The following proportions were calculated (%): sperm viability, sperm with acrosome abnormalities, sperm with proximal and distal cytoplasmic droplets, sperm with morphological abnormalities of the head, sperm with morphological abnormalities of the tail, and sperm without tails.

2.4 Sperm motility parameters

Motility characteristics were determined using a computer-assisted sperm analysis system (CASA system, Proiser SL, Valencia, Spain). The CASA system is based on the analysis of 25 consecutive digital images taken from a single field at a magnification of 100X on a dark ground with a time lapse of 1 second.

Sample aliquots (5 µL) were placed on a pre-warmed slide and viewed in a phase contrast microscope equipped with a warm stage at 37ºC. Four to five separate fields were taken for each sample and a minimum of 200 cells/sample were analysed. The motility descriptors obtained after CASA were: Curvilinear velocity (VCL), as the mean path velocity of the sperm head along its actual trajectory (units: µm/s), Linear velocity (VSL), as the mean path velocity of the sperm head along its average from its first to its last position (units: µm/s), Mean velocity (VAP), as the mean velocity of the sperm head along its average trajectory (units: µm/s).

Finally, total motility (MT) was defined as the percentage of spermatozoa that showed a VAP above 10 µm/s, and progressive motility (MP) as the percentage of spermatozoa that showed a VAP above 50 µm/s and 70% of STR, as defined by the CASA manufacturer.

2.5 Sperm metabolic activity

In order to evaluate the metabolic activity by the Resazurin reduction test (RRT), we used the QBlue cell viability test (QBlue Cell Assay kits, Biochain, USA) as described previously (Sabés-Alsina et al., 2016). Briefly, samples were adjusted to an approximate concentration of 10 x 10^6 sperm cells/mL with phosphate-buffered saline (PBS; Sigma, Madrid, Spain). Each sample was centrifuged at 2,750 rpm for 10 minutes in a 1.5 mL eppendorf. The supernatant was discarded and samples were gently resuspended with 100 µL of QBlue solution (10% QBlue diluted in PBS).

Blanks were made by taking an equal amount of QBlue solution without sperm. All eppendorf tubes were incubated at 37ºC in a humidified air atmosphere with 5% of CO_2 for 25 minutes. After incubation, samples were immediately centrifuged at 3,000 rpm for 10 minutes in order to separate the sperm cells from the medium, thereby stopping the reaction. Ninety microliters of supernatant were taken from each sample and measured with a Wallac Fluorometry plate reader (VICTOR^3 V 1420 multilabel counter, PerkinElmer precisely, Waltham, USA) (excitation wavelength at 550 nm and emission at 615 nm). All procedures were made in the dark to avoid light effects on the wavelength emission. The results of RRT were quantified in relative fluorescence units (RFU).

2.6 Experimental design

In order to evaluate possible effects of season, sperm samples were collected in two periods of the year: warm season (September 2012; n=16) and cold season (February 2013; n=16).

2.7 Statistical analysis

To ascertain the effect of temperature and season of the year for each of the dependent variables ANOVA was conducted using Statistical Package for the Social Sciences (SPSS, Chicago, Ill., USA) version 15.0. According to the design of the experiment, temperature of incubation was a within-subject factor with four modalities (0, 32.5, 37, and 42 degrees) and month of the year was an inter-subject factor with two modalities (warm and cold season). Interaction between factors was also screened. The alpha level was set at 0.05. All results are expressed as mean values ± standard error.

3. Results

3.1 Effect of HS

3.1.1 Viability and morphology parameters

Sperm quality parameters following incubation at 32.5ºC, 37ºC or 42ºC for 3 hours are shown in Table 1. No significant differences ($P < 0.05$) were observed in the percentage of viability and acrosome abnormalities among treatment groups. The presence of distal cytoplasmic droplets was lower for the spermatozoa incubated at hyperthermic conditions (42ºC) compared with those incubated at optimal testicular temperature (32.5ºC) or abdominal temperature (37ºC) ($P = 0.01$). No significant differences ($P > 0.05$) were observed in the remaining morphological parameters.

3.1.2 Motility parameters

As shown in Table 1, the incubation of spermatozoa under hyperthermic conditions (42ºC) for 3 hours decreased significantly the percentage of MT and VCL compared with those incubated at 32.5ºC or 37ºC ($P < 0.05$). In contrast, the mean values of MP and VSL were not significantly affected by the incubation of the sperm cells at the different temperatures ($P > 0.05$). Mean values of VAP were significantly lower ($P < 0.05$) for spermatozoa incubated at 32.5ºC and 42ºC compared with those incubated at 37ºC.

3.1.3 Metabolic activity

Sperm metabolic activity was affected by HS (Table 1). After 3h of incubation at 42ºC, the sperm metabolic activity decreased significantly ($P < 0.001$) compared with those incubated at 32.5ºC or 37ºC. No significant differences ($P > 0.05$) in metabolic activity were found between the samples incubated at 32.5ºC and 37ºC.

3.2 Seasonal effects

The sperm quality parameters for the control groups at the different seasons are shown in Table 2. For the viability and morphology assessments, only sperm cells collected in the cold season showed significantly higher percentages of bent and coiled tails than the sperm cells collected in the warm one ($P < 0.01$). No significant differences ($P > 0.05$) were found in the percentage of viable cells and the rest of the sperm morphological parameters analysed.

Table 1. Viability, morphology, motility parameters and metabolic activity of sperm cells incubated at 32.5ºC, 37ºC or 42ºC for 3 hours.

Parameters	Incubated at 32.5ºC	Incubated at 37ºC	Incubated at 42ºC
Viable (%)	82.3 ± 1.6[a]	81.0 ± 1.6[a]	80.9 ± 1.8[a]
Acrosome abnormalities (%)	17.3 ± 1.8[a]	18.1 ± 1.6[a]	18.2 ± 1.8[a]
Proximal cytoplasmic droplet (%)	6.0 ± 0.9[a]	6.4 ± 1.0[a]	6.9 ± 1.2[a]
Distal cytoplasmic droplet (%)	6.0 ± 0.8[a]	3.4 ± 0.6[a]	2.5 ± 0.5[b]
Head abnormalities (%)	0.3 ± 0.1[a]	0.2 ± 0.04[a]	0.2 ± 0.1[a]
Bent and coiled tails (%)	5.2 ± 1.1[a]	5.8 ± 1.0[a]	6.7 ± 1.0[a]
Tailless spermatozoa (%)	4.7 ± 0.6[a]	4.3 ± 0.6[a]	4.5 ± 0.8[a]
MT (%)	72.3 ± 3.3[a]	72.9 ± 3.6[a]	63.5 ± 3.6[b]
MP (%)	33.6 ± 2.7[a]	29.4 ± 2.9[a]	35.7 ± 2.9[a]
VCL (μm/s)	106.4 ± 3.0[a]	110.2 ± 2.6[a]	99.3 ± 3.6[b]
VSL (μm/s)	38.4 ± 1.7[a]	36.7 ± 1.6[a]	38.3 ± 1.8[a]
VAP (μm/s)	61.3 ± 2.5[a,b]	63.9 ± 2.3[a]	57.4 ± 3.0[b]
Metabolic activity (RFU)	442063.1 ± 170510.6[a]	427768.5 ± 163158.1[a]	262293 ± 141871.8[b]

Values are mean ± standard error. Within rows, values with different superscript letters differ significantly ($p < 0.05$).

Abbreviations: MT, total motility; MP, progressive motility; VCL, curvilinear velocity; VSL, straight linear velocity; VAP; average path velocity

Table 2: Viability, morphology, motility parameters and metabolic activity of sperm cells collected in the warm or cold season.

Parameters	Average values at sperm cell recovery	Cold Season	Warm Season	P-*value*
Viable (%)	85.1 ± 1.4	85.8 ± 2.2	84.5 ± 2.0	NS
Acrosome abnormalities (%)	14.6 ± 1.6	14.2 ± 2.4	15.0 ± 2.0	NS
Proximal cytoplasmic droplet (%)	7.5 ± 1.2	8.1 ± 1.5	6.9 ± 1.9	NS
Distal cytoplasmic droplet (%)	5.0 ± 0.8	5.5 ± 1.4	4.5 ± 0.7	NS
Bent and coiled tails (%)	4.9 ± 0.7	6.6 ± 1.3	3.3 ± 0.6	< 0.01
Tailless spermatozoa (%)	4.0 ± 0.5	4.4 ± 0.8	3.6 ± 0.5	NS
MT (%)	76.0 ± 1.9	74.7 ± 2.6	77.4 ± 2.8	NS
MP (%)	38.1 ± 2.1	36.3 ± 3.9	40.1 ± 3.0	NS
VCL (μm/s)	106.6 ± 2.8	104.1 ± 0.6	108.5 ± 2.1	< 0.01
VSL (μm/s)	46.1 ± 1.9	43.4 ± 0.5	49.9 ± 2.3	< 0.01
VAP (μm/s)	70.3 ± 2.2	65.3 ± 0.5	75.7 ± 2.2	< 0.01
Metabolic activity (RFU)	512217.9 ± 32098.2	526523.7 ± 48037.5	499468.2 ± 46324.1	NS

Values are mean ± standard error. Abbreviations: MT, total motility; MP, progressive motility; VCL, curvilinear velocity; VSL, straight linear velocity; VAP; average path velocity.

Although the percentages of MT and MP were higher in the warm season, no significant differences were observed between seasons. Mean values of VCL, VSL and VAP were significantly ($P < 0.01$) higher in the warm season.

No significant differences were found in metabolic activity between seasons at control groups.

No significant interactions were found between the *in vitro* HS and season for viability, morphology and motility parameters. However, a significant interaction was found in the metabolic activity between the *in vitro* HS (32.5ºC, 37ºC or 42ºC) and season (warm vs. cold season) ($P = 0.02$) (Figure 1).

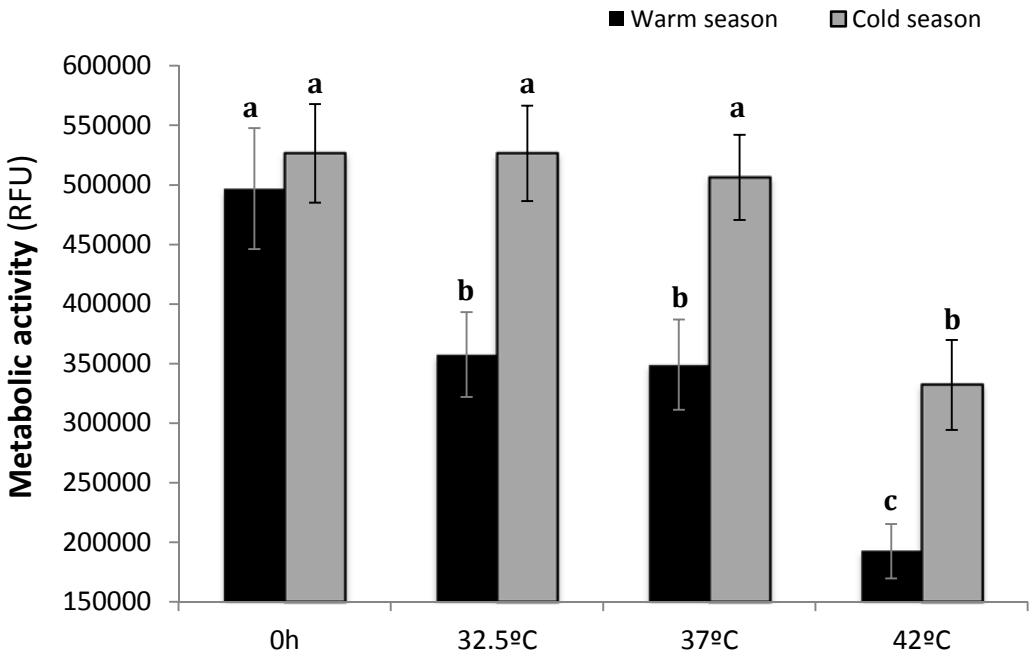

Figure 1. Metabolic activity parameters in different seasons (warm and cold) of non-incubated control spermatozoa and spermatozoa incubated for 3 hours at 32.5ºC, 37ºC or 42ºC.
Within columns, values with different superscript letters differ significantly ($P < 0.01$).

4. Discussion

Exposure of rabbit sperm cells to hyperthermic conditions (42ºC) for a short period of time significantly affected their metabolic activity and some morphologic and motility parameters when compared to the exposure to the theoretical temperature of the testis

(32.5ºC) and a physiological temperature of the uterus (37ºC). Regarding seasonally-mediated effects, the metabolic activity of sperm cells was found to be higher in the cold season than in the warm season of the year.

Artificial cryptorchidism induced by surgery has been used to study the influence of abdominal temperature on spermatogenesis (Bedford, 1978; Setchell, 1998). In contrast, *in vitro* HS has been identified as a good model for studying the mechanisms of HS without the possible adverse effects of surgery (Ikeda et al., 1999). The results of the present study demonstrated that the exposure of sperm cells for 3 hours to body temperature does not affect sperm quality parameters suggesting that they may have a good chance of fertilizing an oocyte. These findings are in agreement with those of Cummins *et* al. (1970) who reported that the appearance of some morphological abnormalities is not observed until after 2 days of exposure of rabbit testis to body temperature and they were not related with loss of fertility. Male sterility did not appear until 7 days of exposure to abdominal temperature.

Previous studies have noticed the impact of HS on the testis (Hansen, 2009; Setchell, 1998), however the effects on the sperm cells themselves vary depending on the kind of exposure and the time. The exposure of rabbits to an *in vivo* HS model resulted in a decrease of the viable sperm cell number and an increase of the percentage of acrosome abnormalities but without affecting fertility and prolificacy (Sabés-Alsina et al., 2015). It can thus be suggested that although some parameters of sperm quality are altered by high temperatures, there are sufficient sperm cells available for retaining the male fertility.

Exposure to hyperthermic conditions (42ºC) for 3 hours in the present study was associated with a reduction in the percentage of distal cytoplasmic droplets, as well as the mean values of MT, VCL, VAP and metabolic activity. These findings are in agreement with Hendricks *et* al. (2009) who demonstrated that the aging of bovine sperm cells by incubation to normothermic (38.5ºC) and heat shock temperatures (40ºC) for 4 hours significantly decrease their kinematics. However, neither the ability to fertilize an oocyte nor the competence of the zygote to develop to the blastocyst stage was affected (Hendricks et al., 2009).

A possible explanation for the reduction of the distal cytoplasmic droplets might be that spermatozoa are maturing during the incubation time, as suggested by Cummis and Glover (1970).

Rabbit sperm production and quality can be affected for several factors such as breed, age and season (Alvariño, 2000). Seasonal effects on sperm quality have been attributed to a variation of the atmospheric temperature and length of the photoperiod (Marai et al., 2002). The results of the present study demonstrated a seasonal effect only on sperm velocities and on tail abnormalities. Safaa *et* al. (2008) analysed the seasonal effects on sperm parameters of Black Baladi and NZW rabbit bucks housed in Egypt. These authors reported that quality parameters (viability and acrosome abnormalities) of NZW semen ejaculates collected in winter were better than those collected in summer, although no differences were found in motility parameters between seasons. The differences in viability and acrosome abnormalities found in that study in relation to the present one may be due to the fact that the NZW bucks were exposed to higher temperatures during summer (23ºC – 34.4ºC) and different photoperiods during the year (winter: 10-11 hours light per day, summer: 13-14 hours light per day). Despite the differences in the climate factors, the results of the present study corroborate that exposure to different seasons does not affect sperm motility parameters.

Sperm cells collected in the warm or cold season were similarly affected by the exposure to HS in the study presented here. Only one interaction in metabolic activity was found between *in vitro* HS and season. This interaction might reflect the observation that *in vitro* HS tended to maintain lower values of RFU in the warm season than in the cold season. Metabolic processes are essential for cellular functions and reproductive performances, thus the evaluation of the metabolic status on the spermatozoa could provide relevant information about the sperm fertilizing capacity (Zrimsek et al., 2004).
Rabbit sperm show a higher metabolic activity in the winter than in the summer. They respond to HS *in vitro* by decreasing metabolic activity, with the result that heat stressed sperm have a lower metabolic activity in summer than in winter. The higher sperm metabolic activity in winter than in summer could be associated with an increase in metabolic by-products and reduced longevity, in accordance with the observations of

Gibb *et* al. (2014), who observed that spermatozoa from fertile stallions with a high metabolic activity showed decreased longevity compared to spermatozoa with lower metabolic activity. This mechanism could help to maintain fertility under heat stress conditions. These results support the previous study of Maya-Soriano *et al.* (2013) who reported that the exposure of bovine oocytes during maturation in an *in vitro* HS model in different seasons produce an increased rate of premature oocytes in the cold season as a response to the heat shock. Their study demonstrated that bovine oocytes collected in the cold season might be more sensitive to high temperatures than bovine oocytes collected in the warm one.

In conclusion, our results suggest that the quality of rabbit sperm cells appears to be altered by a short exposure to HS temperatures (42ºC), in terms of metabolic activity and motility parameters, such as MT and VCL. Thus, on exposure for a short period of time to the reproductive tract of a hyperthermic female, their functionality may well be compromised. Only metabolic activity was influenced by the interaction of heat stress and season.

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III

Effects of season on bull sperm quality in thawed samples in northern Spain

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Submitted 3 May 2016

Submitted for publication.

Abstract

The aim of the present study was to evaluate the possible effects of climate factors on sperm quality of Holstein dairy bulls housed in northern Spain. Semen samples from 11 Holstein dairy bulls were collected and cryopreserved in winter, spring and summer. Sperm quality parameters such as motility, morphology, plasma membrane integrity, acrosome status, mitochondrial membrane potential, DNA fragmentation index and reactive oxygen species were assessed. Samples collected in spring showed higher mean values of total and progressive motility when compared with samples collected in winter. Mean values of average path velocity and straight line velocity were higher in spring than in summer. The proportion of viable spermatozoa was higher in spring than in winter as was the proportion of viable spermatozoa with non-reacted acrosome. The proportion of live cells that were not producing superoxide or hydrogen peroxide was higher in samples collected in spring than in winter. No differences were found in sperm morphology or the DNA fragmentation index among seasons. In conclusion, our results suggest that sperm quality of bulls housed in the northern Spain is affected by season. Samples collected in spring appear to have better sperm quality than samples collected in other seasons.

Keywords: heat stress, reactive oxygen species, chromatin integrity, CASA.

1. Introduction

Globally temperatures have risen about 0.7ºC during the last 3 centuries provoking summer heat waves and heavy rains or drought (Takahashi, 2012). The heat stress caused by global warming has a substantial impact on human and animal physiology and reproduction (Takahashi, 2012). High ambient temperatures can cause adverse effects on fertility due to alterations of heat stress on germ cells (Hansen, 2009; Setchell, 1998; Takahashi, 2012).

In dairy cows, high ambient temperatures negatively affect milk composition and production (Bouraoui et al., 2002). Moreover, oestrus signs are weaker in summer months, and conception rates and fertility are lower (De Rensis and Scaramuzzi, 2003) due to changes in male and female physiology. Males exposed to heat stress show an increase of respiratory rate and rectal temperature (Kadzere et al., 2002), reduction in testis weight, alteration in testosterone production, and abnormalities in sperm quality (Setchell, 1998).

The effect of heat stress on bull sperm quality has been studied by scrotal insulating (Karabinus et al., 1997; Setchell, 1998). However, this technique does not accurately reflect changes in sperm quality that might occur in warmer climates because it overrides the normal physiological mechanisms of testicular temperature regulation. In most mammals, the testes are outside the body cavity to reduce exposure to core body temperature. Also, the arterio-venous pampiniform plexus allows heat exchange between warm blood entering, and cool blood draining, the testis (Hansen, 2009).

Although Koonjaenak *et al.* (2007) and Nichi *et al.* (2006) studied the effect of hot and humid weather on sperm quality of bulls and related species, few studies have investigated the effects of climate on sperm quality in Europe. There is one study in Sweden (Valeanu et al., 2015), a country with a relatively temperate climate, reporting seasonal differences in bull sperm quality. The aim of the present study was to evaluate the possible effects of climate on the sperm quality of Holstein dairy bulls housed in northern Spain, evaluated post-thawing.

2. Material and methods

2.1 Animals and semen collection

Semen from 11 Holstein dairy bulls, 1 to 6 years old, kept in the outdoor facilities of the ASCOL cooperative (Genetics selection center, Gijón, Spain, latitude 43.466218620724 and longitude -5.752450247406), were available. Semen was collected by artificial vagina; samples with a good mass motility (>70%) were cryopreserved according to the company's routine practice. The semen was extended with BioXcell® (IMV Technologies, L'Aigle, France), loaded into 0.25 mL plastic straws at ambient temperature (18 to 20ºC) and cooled to 5ºC before freezing using a programmable freezing machine (IMV Technologies – Digitcool, L'Aigle, France). After thawing, only straws with post-thaw motility ≥ 50% were retained by the bull center and thus were included in the study. Straws were available from semen collected in winter (December - January), spring (March - April) and summer (July - August) for eight of the bulls, and in winter and summer for three bulls. Samples from these months were chosen to match the previous study by Valeanu *et al.* (2015) with semen from Swedish bulls.

2.2 Climate factors

Temperature (ºC), humidity (%), atmospheric pressure (hPa) and visible daylight length (minutes per day) were obtained from the Agencia Estatal de Meteorología (AEMET, www.aemet.es) and the Instituto Geográfico Nacional (www.ign.es) for the period January 2013 to May 2015. The daily and monthly averages, and the mean and maximum temperature-humidity indices (THI) (García-Ispierto et al., 2006) were calculated and correlated with sperm quality parameters.

2.3 Sperm thawing and concentration determination

Straws were thawed in a water bath at 37ºC for 12 seconds. Sperm concentration was determined using the NucleoCounter® SP-100™, (ChemoMetec A/S, Allerød, Denmark), using an aliquot (5 µL) of sample mixed thoroughly with 500 µL of reagent S100. After loading a disposable SP-1 cassette containing propidium iodide (PI) with the mixture, the fluorescence of the stained DNA was measured.

2.4 Computer-assisted sperm analysis of sperm motility

Sperm kinematics were determined by computer-assisted sperm analysis (CASA) using the SpermVision™ (Minitüb, Tiefenbach, Germany) connected to an Olympus BX 51 microscope (Olympus. Japan). A 5 µL aliquot of semen was pipetted on to a pre-warmed microscope slide (38ºC), covered with an 18 x 18 mm coverslip and analysed using the manufacturer's settings for bull spermatozoa. A minimum of 200 cells per field was analysed in 8 fields. Data were collected for total motility (MT, %), progressive motility (MP, %), distance average path (DAP, µm), distance curved line (DCL, µm), distance straight line (DSL, µm), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), straightness (STR = VSL/VAP, %), linearity (LIN = VSL/VCL, %). wobble (WOB = VAP/VCL, %), amplitude of lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz).

2.5 Sperm morphology evaluation

Sperm morphology was evaluated as described previously (Morrell et al., 2008). Briefly, air-dried smears were stained using William's staining for assessing sperm head shape; 500 sperm were evaluated at 1,000 x magnification by light microscopy (Leitz, Wetzlar, Germany). In addition, aliquots (5 µL) were fixed with 45 µL of formol saline solution for preparation of wet smears. Slides were analysed using a phase-contrast microscope (Leica Microsystems, Wetzlar, Germany) at 1,000 x magnification, evaluating at least 200 sperm per sample. The percentage of spermatozoa with normal morphology was estimated by deducting the spermatozoa with morphological abnormalities from the total. Morphology evaluation was carried out by skilled personnel in the Swedish Sperm Reference Laboratory at SLU.

2.6 Plasma membrane integrity assessment

Plasma membrane integrity was assessed by flow cytometry using SYBR-14 and PI staining (Live-Dead® Sperm Viability KIT LIT L-7011; Invitrogen, Eugene, OR, USA). Briefly, aliquots from thawed semen samples were diluted with buffer B (patent applied for: JM Morrell and H Rodriguez-Martinez) to approximately 2 x 10^6 spermatozoa/mL. An aliquot of 300 µL of the diluted sample was stained with 1.2 µL of SYBR-14 (final stain concentration

0.04 µM) and 3 µL of PI (final concentration 12 µM) and incubated in a 37ºC water bath in the dark for 10 minutes before cytometric analysis.

Fluorescence was measured using a BD LSR flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with standard optics. The SYBR-14 and PI dyes were excited by an argon ion laser (488 nm); green fluorescence was detected with an FL 1 band-pass filter (530/30 nm), and red fluorescence was measured using an FL 3 long-pass filter (>670 nm). A total of 30,000 sperm specific-events was evaluated per sample and quantified according to the integrity of the plasma membrane (Johannisson et al., 2009): live spermatozoa with intact plasma membrane (SYBR-14 positive/PI negative), moribund spermatozoa (SYBR-14 negative/PI positive) and dead spermatozoa with disaggregated membrane (SYBR-14 negative/PI negative).

2.7 Assessment of acrosome status and the ability to undergo acrosome reaction when exposed to calcium ionophore

Sperm acrosomal changes and viability were evaluated with the acrosome-specific peanut agglutinin conjugated with fluorescein isothiocyanate (FITC-PNA, Sigma-Aldrich, Stockholm, Sweden) combined with Calcium ionophore A23187 (Sigma-Aldrich, Stockholm, Sweden) and PI. Aliquots from thawed semen samples were diluted with buffer B to a concentration of 2 x 10^6 spermatozoa/mL. A 300 µL aliquot was mixed with 3 µL of Calcium ionophore (final concentration 10 µM), 3 µL of FITC-PNA solution (FITC-PNA diluted with buffer B enriched with 10 mM Calcium and Magnesium) and 3 µL of PI (final concentration 24 µM). Samples were gently mixed and incubated at 37ºC in a water bath in the dark for 10 minutes before the cytometric analysis. Semen samples were analysed using the LSR flow cytometer described above with the argon-ion laser (488 nm). Green fluorescence was detected with an FL1 band pass filter (515/545 nm), and red fluorescence with an FL3 long-pass filter (>670 nm). Forward scatter (FSC, indicating size) and side scatter (SSC, measuring granularity) were collected for each event. An analysis gate was applied in the FSC/SSC two-dimensional dot-plot, restricting the analysis to spermatozoa by eliminating small debris and other particles from further analysis.

A total of 30,000 sperm specific-events was evaluated and quantified as percentages. Four sperm subpopulations were identified: live-reacted (PI negative/FITC-PNA positive),

dead-reacted (PI positive/FITC-PNA positive), live-non reacted (PI negative/FITC-PNA negative) and dead-non reacted (PI positive/FITC-PNA negative).

2.8 Mitochondrial membrane potential analysis

The lipophilic cation JC-1 (Molecular Probes, Eugene, OR, USA) was used to assess sperm mitochondrial membrane potential. Aliquots of semen were diluted with buffer B to approximately 2.5 x 10^6 spermatozoa/mL. An aliquot of 300 µL of the diluted semen was stained with 1.2 µL JC-1 (final stain concentration 12 µM), gently mixed and incubated at 37ºC in a water bath in the dark for 40 minutes. Fluorescence was measured the BD LSR flow cytometer as previously mentioned Green fluorescence was detected using an FL1 filter (530/30 nm), while orange fluorescence was measured using a FL2 bandpass filter (575/26 nm). Both FL1-FL2 and FL2-FL1 were compensated. A total of 30,000 sperm cells was evaluated and classified as percentages in two groups: spermatozoa with high respiratory activity (JC- 1 positive/orange fluorescence) and spermatozoa with low respiratory activity (JC-1 negative/green fluorescence).

2.9 Sperm chromatin structure assay

This assay (Evenson et al., 2002) uses the metachromatic fluorochrome acridine orange (AO) to assess sperm chromatin integrity. The metachromatic shift from green fluorescence (stable, double stranded DNA) to red fluorescence (denatured, single-stranded DNA) in damaged chromatin was quantified by flow cytometry. The DNA fragmentation index (%DFI) was calculated as the ratio of sperm cells with denatured single stranded DNA to total DNA (single stranded DNA + double stranded DNA). Briefly, samples of thawed semen (20 µL) were mixed with an equal volume of buffer containing Tris, sodium chloride and ethylenediamine tetraacetic acid (TNE buffer; 0.15 M NaCl, 0.01 M Tris-HCl, 1 mM EDTA; pH 7.4), and were immediately submerged in liquid nitrogen. These samples were stored at -80 ºC until analysis. They were thawed on crushed ice and were further diluted with TNE buffer (1:10, v/v). The spermatozoa were mixed with 200 µL of an acid-detergent solution (0.17% Triton X-100, 0.15 mol/L NaCl and 0.08 mol/L HCl; pH 1.2). Thirty seconds later, the spermatozoa were stained with 600 µL of AO (final concentration 4 µg/mL) and were analyzed within 3 to 5 minutes using the previously mentioned LSR flow cytometer, equipped with an argon-ion laser (488 nm), running at

200mW. From each sample 30,000 events were measured. For calculation of %DFI, the collected data-files were further processed using FCS Express version 2 (De Novo Software, Thornhill, ON, Canada).

2.10 Measurement of reactive oxygen species

Hydroethidine (HE; Molecular Probes Inc., Eugene, OR, USA) and 2',7'-dichlorodihydrofluorescein diacetate (DCFDA; Sigma-Aldrich, Stockholm, Sweden) were used to detect superoxide and hydrogen peroxide (H_2O_2), respectively. Hoechst 33258 (HO; Sigma-Aldrich, Stockholm, Sweden) was added to permit the simultaneous differentiation of living and dead cells. The sperm concentration was adjusted to 2 x 10^6 spermatozoa/mL with Buffer B and 300 µL of diluted sample was stained with 9 µL HE (final stain concentration 1.2 µM), 9 µL DCFDA (final stain concentration 60 µM) and 9 µL HO (final stain concentration 1.2 µM). Samples were gently mixed and incubated at 37ºC in a water bath in the dark for 30 minutes before analysis using the LSR flow cytometer (Becton Dickinson). The method is modified from Guthrie and Welch (2006), by the use of HO instead of Yo-Pro-1 as an independent analysis of living spermatozoa. Excitation was achieved with an argon-ion laser (488 nm) and a HeCd laser (325 nm). Detection of green fluorescence was measured with an FL1 pass filter (530/30 nm), red fluorescence was measured using an FL3 long pass filter (>670 nm) and blue fluorescence was detected in FL4 with a band pass filter (510/20 nm). An analysis gate in the FSC/SSC two-dimensional dot-plot restricted the analysis to spermatozoa, by eliminating small debris and other particles. In total, 30,000 specific-events were evaluated and quantified as percentages in the following categories: live (HO negative), superoxide negative; live (HO negative), superoxide positive; dead (HO positive), superoxide positive; live (HO negative), H_2O_2 negative; live (HO negative), H_2O_2 positive; dead (HO positive), H_2O_2 negative; and dead (HO positive), H_2O_2 positive.

2.11 Statistical analysis

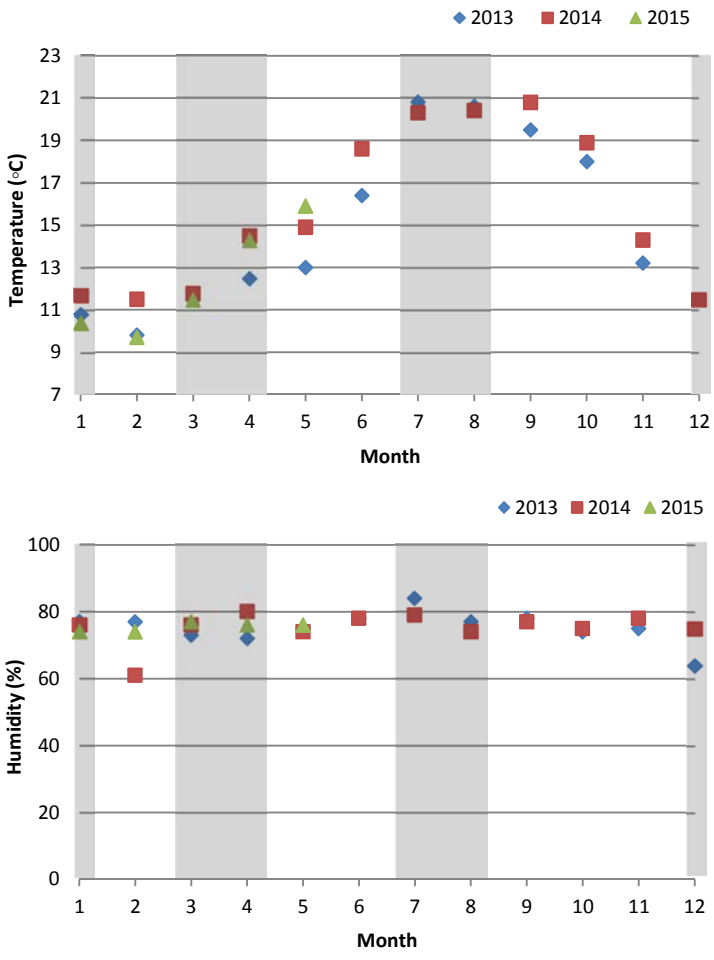
Analysis of variance was performed using the Statistical Analysis System software (SAS Institute Inc., version 9.3, Cary, NC, USA). The statistical model (PROC MIXED) included the fixed effect of season, and the random effect of bull. Pairwise tests of significance (between seasons) were performed using t-test. Two variables (%DFI and dead (HO

positive) with H_2O_2 positive), with a distribution deviating from normality, were log-transformed before the analyses. P values < 0.05 were considered statistically significant. All results are expressed as uncorrected Least-square means values ± standard deviation.

3. Results

3.1 Climate factors

The mean values of climate parameters are shown in Figure 1. Mean temperatures, humidity and atmospheric pressure for winter, spring and summer were 11.3ºC, 12.7ºC and 20.5ºC, 72.3%, 75.7% and 78.5%, and 1020 hPa, 1015.4 hPa and 1017.2 hPa, respectively. Mean visible daylight length increased from winter to summer (551.8 minutes, 760 minutes and 868.3 minutes, respectively). The mean values of mean THI and maximum THI increased from winter to summer (mean THI: 53.0, 55.3 and 67.6; maximum THI: 63.2, 66.8 and 72.5; respectively).



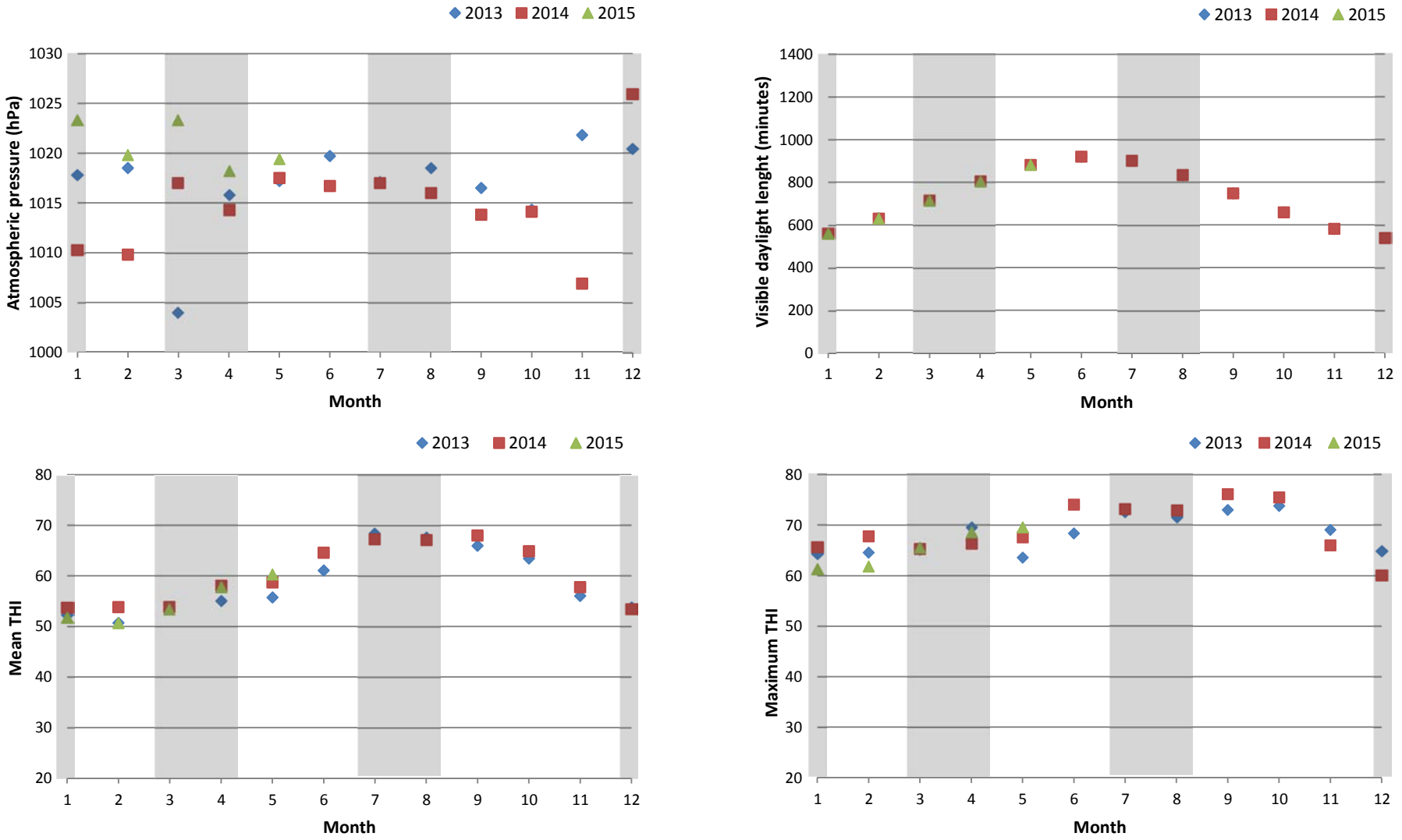


Figure 1. Monthly mean temperatures (ºC), humidity (%), atmospheric pressure (hPa), daylight length (minutes), mean and maximum temperature-humidity indices during January 2013 – May 2015 in Gijón, Spain; Agencia Estatal de Meteorología (AEMET) and Instituto Geográfico Nacional.

Footnote: Months (January – December) are numbered from 1 to 12. Shaded areas indicate the months when the semen samples were collected.

3.2 Computer-assisted sperm analysis of sperm motility

Sperm kinematics are shown in Table 1. Sperm samples collected in spring showed significantly higher ($P < 0.05$) mean values of MT and MP compared with samples collected in winter. No significant differences ($P > 0.05$) were observed for MT and MP between samples collected in spring and in summer. Mean values of DAP, DSL, VAP, VSL and BCF were significantly higher ($P < 0.05$) in spring than in winter and summer. Mean values of DCL and VCL were significantly higher ($P < 0.05$) in spring than in summer. However, no significant differences ($P > 0.05$) were observed in mean values of DCL and VCL between spring and winter. There were no significant differences ($P > 0.05$) between seasons in the parameters STR, LIN, WOB and ALH.

Table 1. Bull sperm kinematics in different seasons.

Parameters	Winter	Spring	Summer
MT (%)	51.00 ± 13.51^{b}	65.04 ± 14.26^{a}	58.17 ± 19.29^{a,b}
MP (%)	46.45 ± 13.12^{b}	60.74 ± 14.25^{a}	54.01 ± 18.89^{a,b}
DAP (µm)	28.01 ± 3.46^{b}	29.93 ± 3.51^{a}	27.04 ± 3.92^{b}
DCL (µm)	48.66 ± 6.80^{a,b}	51.93 ± 8.21^{a}	47.00 ± 6.94^{b}
DSL (µm)	22.50 ± 3.33^{b}	24.44 ± 2.56^{a}	21.66 ± 3.50^{b}
VAP (µm/s)	62.81 ± 7.82^{b}	67.37 ± 8.58^{a}	60.53 ± 9.20^{b}
VCL (µm/s)	108.86 ± 15.25^{a,b}	116.79 ± 19.52^{a}	104.95 ± 16.50^{b}
VSL (µm/s)	50.49 ± 7.36^{b}	55.04 ± 6.35^{a}	48.47 ± 8.05^{b}
STR (%)	0.80 ± 0.03	0.81 ± 0.02	0.79 ± 0.03
LIN (%)	0.46 ± 0.03	0.47 ± 0.03	0.46 ± 0.04
WOB (%)	0.57 ± 0.02	0.57 ± 0.03	0.57 ± 0.03
ALH (µm)	3.93 ± 0.51	3.92 ± 0.67	3.78 ± 0.63
BCF (Hz)	26.42 ± 2.12^{b}	28.02 ± 1.99^{a}	26.37 ± 1.81^{b}

Values are least-square means ± standard deviation. Means within row, with one letter in common are not significantly different ($P > 0.05$).

Abbreviations: MT, total motility; MP, progressive motility; DAP, distance average path; DCL, distance curved line; DSL, distance straight line; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; STR, straightness; LIN, linearity; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

3.3 Morphology, plasma membrane integrity, acrosome status, mitochondrial membrane potential and sperm chromatin structure evaluation

Sperm morphology, plasma membrane integrity, acrosome status, mitochondrial membrane potential and sperm chromatin structure parameters are presented in Table 2. No significant differences ($P > 0.05$) were found in the proportion of spermatozoa with normal morphology among seasons.

The proportion of viable spermatozoa was significantly higher ($P < 0.05$) in spring than in winter although no significant differences ($P > 0.05$) were found between spring and summer. A higher proportion of moribund spermatozoa was observed in winter ($P < 0.05$) than in the other seasons. The proportion of viable spermatozoa with non-reacted acrosome was significantly higher ($P < 0.05$) in spring than in winter. No significant differences ($P > 0.05$) were seen among seasons in the proportion of viable spermatozoa with reacted acrosome. A significantly higher ($P < 0.05$) proportion of dead spermatozoa with reacted acrosome was observed in samples collected in winter compared with samples collected in spring. The percentage of spermatozoa with high respiratory activity was significantly higher ($P < 0.05$) in samples collected in spring and summer than in samples collected in winter. No significant differences ($P > 0.05$) were observed in %DFI among seasons.

3.4 Assessment of reactive oxygen species

Mean values of ROS are shown in Table 3. The proportions of viable non-superoxide-containing spermatozoa and viable non-hydrogen peroxide-containing spermatozoa were significantly higher ($P < 0.05$) in semen samples collected in spring than in winter. The proportion of viable superoxide-containing spermatozoa was significantly higher ($P < 0.05$) in samples collected in winter than in the other seasons. Although no significant differences ($P > 0.05$) were observed among seasons in the proportion of dead superoxide-containing spermatozoa and dead non-hydrogen peroxide-containing spermatozoa, a trend towards significance ($P < 0.06$) was observed for these parameters between winter and spring (superoxide: 35.93 ± 8.23% versus 30.33 ± 8.65%; hydrogen peroxide: 35.74 ± 8.67% versus 30.09 ± 9.17%, respectively). No significant differences ($P > 0.05$) were observed in the proportion of viable and dead hydrogen peroxide-containing spermatozoa among seasons.

Table 2. Morphology, plasma membrane integrity, acrosome status, mitochondrial membrane potential and chromatin structure of bull spermatozoa in different seasons.

Parameters	Winter	Spring	Summer
Morphology (%)			
Normal morphology	78.14 ± 16.08	79.46 ± 24.84	73.05 ± 25.80
Plasma membrane integrity (%)			
Live with intact membrane	48.32 ± 8.30^{b}	59.33 ± 8.83^{a}	55.19 ± 14.86^{a,b}
Moribund	4.36 ± 1.55^{a}	3.08 ± 0.91^{b}	2.75 ± 1.16^{b}
Dead with disrupted membrane	47.32 ± 7.92^{a}	37.32 ± 8.89^{b}	42.05 ± 15.22^{a,b}
Acrosome status (%)			
Live - non reacted	46.84 ± 7.37^{b}	55.98 ± 8.07^{a}	51.78 ± 11.88^{a,b}
Dead - non reacted	32.54 ± 4.31^{a}	26.16 ± 6.14^{b}	28.07 ± 11.26^{a,b}
Live - reacted	0.78 ± 0.40	0.93 ± 0.18	0.93 ± 0.47
Dead - reacted	19.73 ± 4.65^{a}	16.84 ± 4.22^{b}	19.12 ± 4.16^{a,b}
Mitochondrial membrane potential (%)			
High respiratory activity	23.58 ± 10.85^{b}	30.23 ± 13.31^{a}	29.98 ± 12.74^{a}
Low respiratory activity	74.23 ± 10.30^{a}	67.19 ± 13.27^{b}	67.40 ± 12.31^{b}
Sperm chromatin structure (%)			
DFI	4.62 ± 4.72	3.86 ± 1.93	5.06 ± 6.51

Values are least-square mean ± standard deviation. Means within row, with one letter in common are not significantly different ($P > 0.05$).

Table 3. Reactive oxygen species in bull spermatozoa in different seasons.

Parameters	Winter	Spring	Summer
Live, Superoxide anion negative	39.83 ± 7.67^{b}	49.29 ± 5.54^{a}	44.88 ± 12.07^{a,b}
Live, Superoxide anion positive	24.48 ± 8.13^{a}	19.90 ± 8.39^{b}	19.73 ± 6.90^{b}
Dead, Superoxide anion	35.93 ± 8.23	30.33 ± 8.65	35.63 ± 12.89
Live, Hydrogen peroxide negative	63.22 ± 9.23^{b}	69.47 ± 9.57^{a}	63.42 ± 14.18^{a,b}
Live, Hydrogen peroxide positive	0.92 ± 0.70	0.52 ± 0.30	1.14 ± 1.24
Dead, Hydrogen peroxide negative	35.74 ± 8.67	30.09 ± 9.17	35.34 ± 13.09
Dead, Hydrogen peroxide positive	0.11 ± 0.32	0.04 ± 0.10	0.04 ± 0.07

Values are least-square mean ± standard deviation. Means within row, with one letter in common are not significantly different ($P > 0.05$).

4. Discussion

The present study investigated the impact of season on sperm quality of Holstein bulls housed in northern Spain. There were differences in sperm quality among seasons that are possibly attributable to climate factors, with bull sperm quality apparently being better in samples collected in spring than in winter and summer.

The THI incorporates the combined effects of temperature and relative humidity and has been used as a measure of heat stress (Bouraoui et al., 2002; Gholami et al., 2011). In summer, higher values of mean THI (78) or maximum THI (>69) have been related to alterations in milk production and composition (Bouraoui et al., 2002), and increasing pregnancy loss over the first 21-30 days of gestation in dairy cows (García-Ispierto et al., 2006). In the present study, bulls were exposed to mean THI values between 67.2 and 68.4 in summer, and maximum THI values of 71.5 and 74.1. Therefore it is likely that they experienced moderate heat stress which might have an effect on sperm quality.

CASA has been used as an objective and accurate tool to analyse sperm kinematics (Amann and Waberski, 2014). Different authors have suggested the possibility of correlating sperm kinematics with *in vivo* bull fertility. However, there is controversy about which particular sperm kinematic could best predict bull fertility (Pepper-Yowell, 2011; Simonik et al., 2015). Cseh *et al.* (2004) found that velocity parameters such as VAP, VCL and VSL from frozen-thawed bull sperm samples had high correlations with non-return rates (fertility rates), VAP being the one with highest correlation, whereas Nagy *et al.* (2015) reported that VAP values could be a potential predictor of bull fertility. However, Gillan *et al.* (2008) demonstrated that only VSL in post-thaw samples was correlated with fertility rates. In the present study, kinematic parameters such as MP and MT were higher in spring and summer than in winter. Velocity parameters such as VAP, VSL and BCF were higher in spring than in the other seasons, while VCL values were higher in winter and spring than in summer. These results suggest that sperm samples collected in spring may have a greater ability to fertilize oocytes than sperm samples collected in other seasons.

The controversy among authors about CASA kinematics could be due to extraneous factors affecting the analysis, such as different kinds of samples (fresh versus frozen-thawed samples), extenders, sample volume (from 4 to 10 µL) and sperm concentrations, temperature treatments, CASA instruments, settings (Simonik et al., 2015).

No seasonal effects were observed in any sperm morphology parameters. However, the proportion of spermatozoa with an intact plasma membrane was negatively affected by cold temperatures. Some controversies exists between the correlation of morphology and/or plasma membrane integrity with bull fertility (Gillan et al., 2008; Padrik et al., 2012; Zhang et al., 1998). Gillan *et al.* (2008) found that bull sperm morphology of post-thaw samples was correlated with bull fertility. However, Zhang *et al.* (1998) reported no significant correlation for either morphology or plasma membrane integrity with non-return rate at bull level. Although no differences were found in morphology between seasons in the present study, the higher proportion of live spermatozoa observed in spring suggest that these samples could be more fertile.

Acrosome status was seen to differ depending on the season. The percentage of viable spermatozoa with non-reacted acrosome was significantly higher in samples collected in spring than in winter. Moreover, the proportion of dead sperm cells with reacted or non-reacted acrosome was significantly higher in winter than in spring. These findings are in agreement with those of Wells *et al.* (1972) who found that the acrosome status of bull spermatozoa varies throughout the year, with early spring through early summer being the more favourable months.

Recently, Birck *et al.* (2010) suggested that the percentage of live, acrosome reacted spermatozoa could be a predictor of bull sperm fertility. Although, no significant differences were observed in the present study for proportion of live reacted spermatozoa between seasons, the percentage of non-reacted spermatozoa was higher in the samples collected in spring and summer than in winter. Thus according to Birck *et al.*, the samples collected in spring in the present study would be expected to have better fertilization capacity.

Different cytometer methods for assessing the mitochondrial function in bull spermatozoa exist, such as rhodamine 123, JC-1 and MitoTracker Green FM. However, JC-1 presents some advantages over the other methods due to minimal non-specific staining and the ability to differentiate between high and low membrane potential (Garner et al., 1997). In the present study, seasonal effects were observed in mitochondrial membrane potential. Samples collected in spring and summer showed higher proportions of spermatozoa with high respiratory activity compared with samples collected in winter. Moreover, the proportion of spermatozoa with low respiratory activity was significantly higher in samples collected in winter than in the other seasons. These results are in agreement with the findings of Nakabayashi and Salisbury (1959) who reported that respiratory activity of bull sperm samples collected in summer were higher than samples collected in winter.

A correlation between %DFI evaluated by SCSA and fertility in several species, including bulls, has been reported (Bochenek et al., 2001; Gillan et al., 2005). In the present study, no seasonal effects were observed in the %DFI. However, Karabinus *et al.* (1997) found that sperm chromatin structure of bull sperm cells was adversely affected by exposure to heat stress induced by scrotal insolation. The differences between studies may be due to the fact that normal physiological temperature regulation could occur in the animals in the present study, probably resulting in a lower testis temperature in summer than in the study by Karabinus *et al.* (1997).

Oxidative stress has been defined as an imbalance condition between ROS and antioxidant defence mechanisms, inducing an increased rate of cellular damage (Sanocka and Kurpisz, 2004; Sikka et al., 1995). In spermatozoa, oxidative stress can induce capacitation, DNA damage, and loss of sperm motility and fertilizing potential (Sanocka and Kurpisz, 2004; Sikka et al., 1995). In the present study, samples collected in spring had a higher proportion of living spermatozoa not producing ROS (superoxide and hydrogen peroxide) than samples collected in winter. Moreover, higher superoxide production was observed in living spermatozoa in winter compared with other seasons. The generation of ROS in samples collected in winter corresponded with lower progressive sperm motility and viability. These findings are in contrast to the Nichi *et al.*

(2006) who found an increase of ROS production in Simmental and Nelore bulls in Brazil during the summer months.

Erb *et al.* (1942) reported that seasonal factors such as temperature, relative humidity, and quantity and quality of light produced alterations in bull sperm quality (motility, pH, concentration, survival period and abnormal sperm forms). They observed that sperm quality of Holstein, Jersey, Guernsey and Ayrshire bulls housed in the United States was significantly superior in samples collected during spring and inferior in samples collected in summer. Similarly, Valeanu *et al.* (2015) observed small seasonal effects on sperm quality of Swedish Red and White dairy bulls housed in Sweden. There are similarities between the previous results and the findings of the present study. Samples collected in spring showed superior sperm quality to those collected in winter. It can therefore be assumed that samples collected in spring will have more possibilities to reach the oocyte and fertilize it. However, although bulls in summer were exposed to conditions likely to cause moderate heat stress, no differences were observed in some of the sperm quality parameters between samples collected in spring and summer.

Previous studies (Graham and Mocé, 2005; Sellem et al., 2015) suggested that sperm fertility potential should be predicted by the combination of several attributes instead of relying on a single parameter. Thus we suggest that samples collected in spring may have higher fertility potential than in the other seasons. However, a fertility trial would be needed to confirm whether the differences seen *in vitro* are reflecting changes in fertility *in vivo.*

In conclusion, our results suggest that sperm quality of bulls housed in northern Spain is affected by season. Samples collected in spring appear to have better sperm quality than samples collected in other seasons.

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IV

Preliminary study of the effect of season on the *in vitro* fertilizing ability of bovine spermatozoa

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In preparation.

Abstract

The purpose of the present study was to evaluate the effects of season on the fertilizing ability of bovine spermatozoa and subsequent embryo development *in vitro*. Bovine oocytes were matured and fertilized *in vitro* with Holstein dairy bull sperm cells collected and frozen in different seasons (winter, spring and summer). On Day 2 and 8 after *in vitro* fertilization, cleavage and blastocyst rates, respectively, were recorded; and the blastocysts were graded for quality. The number of sperm cells binding to the zona pellucida of oocytes, together with the number of nuclei in the developing blastocysts, was assessed after staining with Hoechst. No significant differences ($P > 0.05$) were observed among seasons in cleavage and blastocysts rate, number of bound sperm cells and number of nuclei in developed blastocysts. However, a trend towards significance of faster development rate was observed in embryos produced with sperm samples collected in spring. Also, semen samples collected in summer produced embryos that developed faster than those collected in winter. In conclusion, it appears that seasonality in sperm samples could affect the rate of embryo development. However, further studies should be performed in order to verify these interesting preliminary data.

Keywords: Season, *in vitro* fertilization (IVF), Bovine, heat stress.

1. Introduction

Reproduction of livestock animals is clearly affected by season. In warm months, detection of estrus, oocyte quality, conception rates and fertility of dairy cows are lower than in the cold months (De Rensis and Scaramuzzi, 2003; De Rensis et al., 2015). Males are also affected by warm months. High temperatures and changes in photoperiod can produce alterations in sperm quality such as motility, metabolic activity, acrosome status and sperm chromatin structure (Erb et al., 1942; Nakabayashi and Salisbury, 1959; Valeanu et al., 2015; Wells et al., 1972).

In a previous study, the presumptive seasonal effects on sperm quality from Holstein dairy bulls housed in northern Spain were investigated. The results showed that sperm quality parameters such as kinematics, viability, acrosome integrity and reactive oxygen species were affected by season. Sperm samples collected in spring appear to have better quality than samples collected in winter and summer (Sabés-Alsina et al., 2016a, 2016b).

In vitro fertilization is a useful model for assessing fertility in cows as it is less expensive and time consuming than an artificial insemination trial (Giritharan et al., 2005; Rodríguez-Martínez, 2007)**.** The *in vitro* production of bovine embryos has focused on improving and optimizing the production of large numbers of blastocysts of high quality by modifying the culture media (Holm et al., 1999; Lim et al., 2007). Usually a surplus of spermatozoa are added to the oocytes to maximize fertilization (Ward et al., 2003). Such a surplus of spermatozoa tends to mask small differences in fertility between treatments. Therefore, in the present study, half of the conventional sperm dose was used in order to detect any differences in fertilizing ability arising from seasonal differences in sperm cells and their effect on embryo development. This dose is considered to be the optimum for differentiating among bulls of differing fertility (Ward et al., 2003).

The techniques used for sperm preparation before *in vitro* fertilization, usually discontinuous gradients and swim-up, separate spermatozoa from seminal plasma and extenders, but also tend to select spermatozoa with good motility (Parrish et al., 1995) or good chromatin integrity (Abraham et al., 2016). A low-density colloid was used in this study, to separate the spermatozoa from the cryopreservation medium and seminal

plasma remnants, in order to avoid selecting only good quality sperm cells. In this way we hoped to detect any seasonal differences in fertility among groups.

The aim of this study was to evaluate the effects of season on the fertilizing ability of bovine spermatozoa and subsequent embryo development *in vitro*.

2. Material and methods

2.1 Experimental design

Bovine oocytes were matured and fertilized *in vitro* with bull sperm cells collected in different seasons (winter, spring and summer). A system control was included in all batches, using sperm samples of a bull of known fertility in *in vitro* fertilization. After fertilization, presumptive embryos were visually evaluated at 44h (first cleavage division) and on Day 8 of development. In total, 398 oocytes were included in the experiment.

2.2 Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (Stockholm, Sweden) unless otherwise stated.

2.3 Oocytes collection and in vitro maturation

Ovaries from heifers were collected from a local abattoir, placed in a thermos with 0.9% (w/v) sodium chloride solution at 30ºC and transported to the laboratory within 3-4h of collection. At the laboratory, ovaries were briefly washed twice with sterile 0.9% (w/v) sodium chloride solution at 35-38ºC. Subsequently, follicles from 3 to 8 mm were aspirated using an 18-gauge hypodermic sterile needle and a 5 mL syringe. Cumulus oocytes complexes (COCs) were collected into search medium consisting of HEPES-buffered TCM-199 supplemented with 50 µg/mL gentamicin (gentamicin sulphate) and 0.2% (w/v) bovine serum albumin (BSA) fraction V. Only unexpanded COCs with homogeneous cytoplasm and surrounded by five or more cumulus cell layers were selected for *in vitro* maturation. Selected COCs were washed twice with search medium and twice with maturation medium. The maturation medium contained TCM-199 supplemented with 0.68 mM L-glutamine, 0.5 µg/mL FSH and 0.1 µg/mL LH (Stimufol;

Partnar Animal Health, Port Huron, Canada), 50 µg/mL gentamicin and 0.4% (w/v) BSA. Then, COCs were randomly gathered in groups of 40-60 COCs, placed in 500 µL of maturation medium in four-well culture dish and cultured at 38.5ºC in a 5% CO_2 humidified air atmosphere for 24h.

2.4 Sperm collection and preparation

Semen samples from 11 Holstein dairy bulls, aged between 1 and 6 years old, kept in the outdoor facilities of the ASCOL cooperative (Genetics selection center, Gijón, Spain, latitude 43.466218620724 and longitude -5.752450247406**)** were used in this study. Semen was collected from the bulls by artificial vagina, extended with BioXcell® (IMV Technologies, L'Aigle, France), loaded into 0.25 mL plastic straws at ambient temperature (18 to 20ºC) and cooled to 5ºC. Freezing was done using a programmable freezing machine (IMV Technologies – Digitcool, L'Aigle, France). Semen samples were collected in winter (December - January), spring (March - April) and summer (July - August) for eight of the bulls; and in winter and summer for the remaining three bulls from January 2013 to May 2015.

Straws were thawed at 35ºC for 12 seconds and prepared by single layer centrifugation (SLC). The SLC was modified from a previous experiment (Abraham et al., 2016), to avoid selecting a sperm sub-population. Briefly, extended semen was carefully layered on top of 1 mL diluted Bovicoll (1:1 v/v Bovicoll and Buffer B; patent applied for) in a 15 mL tube. The tube was centrifuged at 300 x *g* for 20 min at room temperature. Then, the supernatant was removed and the pellet was transferred to pre-gassed fertilization medium (114 mM sodium chloride, 3.19mM potassium chloride, 25.88 mM sodium bicarbonate, 0.29 mM sodium phosphate, 0.49 mM magnesium chloride, 2 mM calcium chloride, 10 mM HEPES, 1 µL/mL phenol red, 0.5 µM sodium pyruvate, 0.03 µL sodium lactate, 50 µg/mL gentamicin, 0.6% (w/v) fatty acid-free BSA, with the addition of 3 µg/mL heparin and penicillamine, hypotaurine and adrenaline (PHE) giving a final concentration of 1.7 mM sodium chloride, 10 µM hypotaurine, 20 µM penicillamine, 1.5 µM adrenaline, 42 µM and sodium metabisulfite). Sperm motility was checked by microscopy and total sperm concentration was determined using a sperm cell counter (NucleoCounter® SP-100™, ChemoMetec A/S, Allerød, Denmark). Sperm concentration was adjusted with fertilization medium to a final concentration of 500,000 spz/mL.

2.5 In vitro fertilization and culture

After maturation, COCs were washed two times with washing medium (114 mM sodium chloride, 3.19 mM potassium chloride, 2 mM sodium bicarbonate, 0.29 mM sodium phosphate, 2 mM calcium chloride, 10 mM HEPES, 0.49 mM magnesium chloride, 1 µL/mL phenol red supplemented with 0.5 µM sodium pyruvate, 0.03 µM sodium lactate, 50 µg/mL gentamicin and 0.3% (w/v) BSA) and gentle pipetted, leaving 3-5 cumulus cells layers. The COCs were transferred to 50 µL drops of fertilization medium and were co-incubated with spermatozoa under mineral oil for 22h at 38.5ºC in a humidified 5% CO_2 incubator.

Fertilized oocytes were totally denuded of cumulus cells and spermatozoa by gentle pipetting and cultured in synthetic oviductal fluid (SOF) under mineral oil for 22h at 38.5ºC in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. SOF consisted of 0.11 M sodium chloride, 7 mM potassium chloride, 1.19 mM potassium phosphate monobasic, 25 mM sodium bicarbonate, 0.33 mM pyruvic acid sodium salt, 1 mM L-glutamine, 0.171 mM calcium chloride, 1.5 mM glucose, 110 mM sodium lactate and 0.49 mM magnesium chloride, with addition of minimum essential medium non-essential amino acids solution (100X), amino acids solution (50X), 0.4% (w/v) fatty acid-free BSA and 50 µg/mL gentamicin. Cleavage rate was checked at 44h after *in vitro* fertilization. On Day 8, blastocyst rate was recorded (early embryo, blastocyst, expanded blastocyst and hatching/ed blastocyst) and graded for quality (grade 1 -2; with grade 1 embryos having the best quality)(Laskowski et al., 2016).

2.6 Fixation and staining of embryos

Presumptive embryos were fixed in a solution of 2% paraformaldehyde in phosphate buffered saline solution (PBS) at 4ºC overnight. Embryos were then washed in PBS, stained with Hoechst 33342 (2.5 µg/mL) and mounted on a glass slides using Vectashield. The number of sperm cells binding to the zona pellucida of oocytes and the number of cell nuclei were assessed for each embryo using an epiflurescence microscopy (SM 510, Carl Zeiss AB, Jena, Germany) (figure 1).

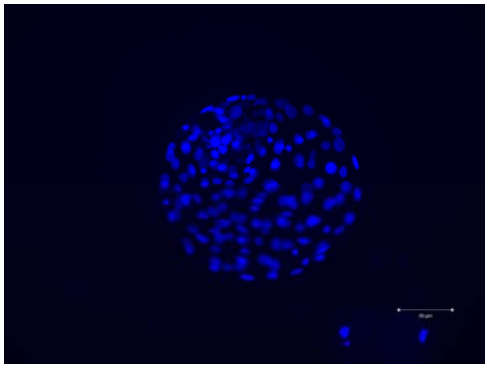

Figure 1. Epiflurescence microscopic image of a bovine blastocyst at 200 X with nucleus stained with Hoechst (blue).

2.7 Statistical analysis

The statistical analysis was performed using the Statistical Analysis System software (SAS Institute Inc., version 9.3, Cary, NC, USA).

Frequency analyses (incl. Fishers Exact test), was performed to analyse the effect of season on cleavage, embryo state and quality, and number of nuclei per blastocyst and number of spermatozoa binding to the zona. The statistical model included the random effect of bull, batch and drop. Rates of embryo development were compared using the Chi-Squared "Goodness of Fit" test (Campbell, 1974)**.**

P values < 0.05 were considered statistically significant. All results are expressed as uncorrected Least-square means values ± standard error.

3. Results

The production of embryos varied among animals. There were 3 bulls that did not produce any embryo. From the rests of the animals, 4 bulls achieved a lower embryo production than the control group (from 3.33% to 7.69%), 1 bull showed similar embryo production to the control group (11.25%) and 3 bulls produced more embryos than the control group (from 23.33% to 26.67%).

3.1 Evaluation of cleavage rates at 44h after in vitro fertilization

Cleavage rates are presented in Table 1. No significant differences ($P > 0.05$) were observed in cleavage rate and cleavage above the two cell stage among seasons.

Table 1. Oocyte cleavage rates in different seasons.

Treatment	No. Oocytes	Cleavage rate (%)	Cleavage above 2 cell stage (%)
Winter	117	49.98 ± 6.36	34.20 ± 8.79
Spring	85	50.02 ± 6.79	48.33 ± 10.11
Summer	117	51.75 ± 6.36	31.83 ± 8.79

Values are means ± error standard.

3.2. Embryo state and quality at Day 8.

The proportion of different stage embryos according to treatment is shown in Figure 2. The sum of total embryos (range from early embryo to hatching or hatched) according to treatment were [n (%)]: winter [13 (11.11%)], spring [9 (10.59%)] and summer [9 (7.69%)]. No significant differences ($P > 0.05$) were observed in embryo stages among seasons.

Although no significant differences ($P > 0.05$) were found in embryo development rate among seasons, a trend towards significance was observed for these parameters among seasons. Embryos produced with sperm samples obtained in the spring season had higher development rates than embryos produced with sperm samples obtained in other seasons. Moreover, embryos produced with sperm samples obtained in summer developed faster than embryos produced with winter sperm samples.

The quality of the embryo according treatment is presented in Figure 3. No significant differences ($P > 0.05$) were observed in quality among seasons.

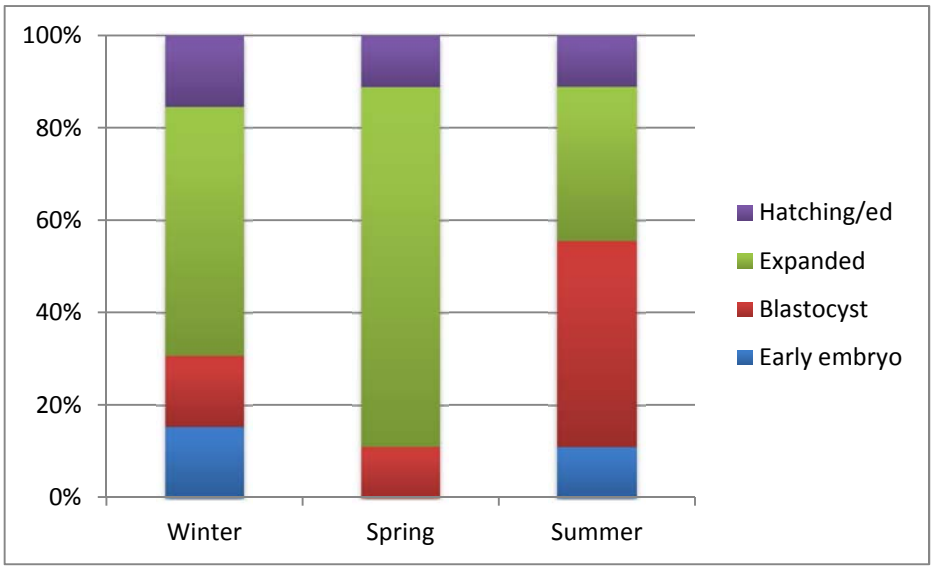

Figure 2. Bovine embryo development at Day 8.
Number of oocytes by seasons: winter n=117; spring n= 85; summer n=117.

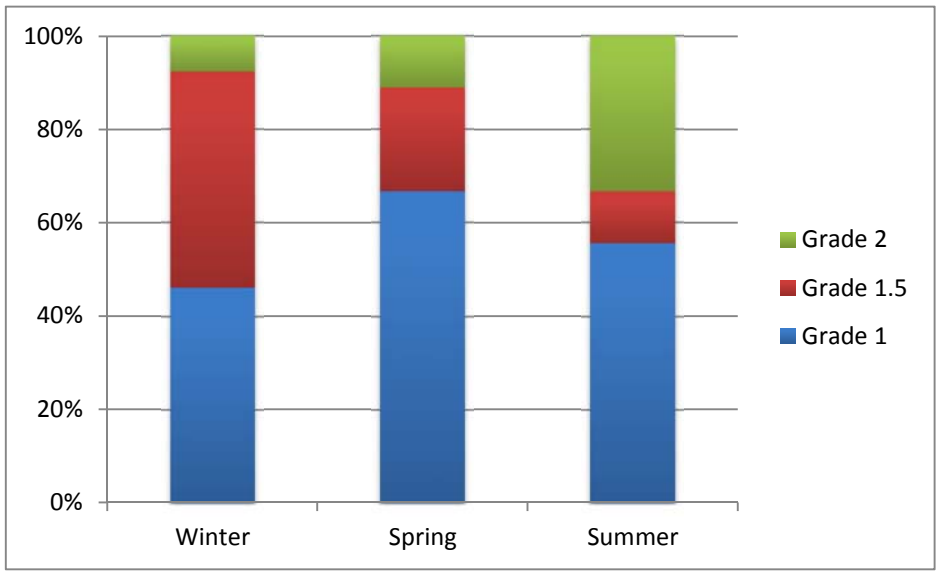

Figure 3. Blastocysts quality graded according to treatment at Day 8.

3.3 Evaluation of embryos stained with Hoechst

The number of nuclei per blastocyst and the number of sperm cells binding per oocyte in different treatments are shown in Table 2.

No significant differences ($P > 0.05$) among treatments were observed in the number of nuclei per blastocyst and spermatozoa binding to the zona pellucida of the oocytes.

Table 2. Number of nuclei and binding spermatozoa in different treatments.

Treatment	No. Oocytes	No. of nuclei per blastocyst	No. of spz binding per oocyte
Winter	117	47.62 ± 8.11	1.19 ± 0.10
Spring	85	57.63 ± 13.46	1.60 ± 0.21
Summer	117	42.89 ± 10.31	1.76 ± 0.29

Values are means ± error standard.
Abbreviations: spz=spermatozoa.

4. Discussion

This study investigated the possible effects of season on the *in vitro* fertilizing ability of frozen-thawed bovine spermatozoa collected in winter, spring and summer, and subsequent embryo development *in vitro*. From the studied parameters, only embryo development rate was affected by the season in which the ejaculates were obtained, being higher in bull sperm samples collected in spring.

Kinetics of early development of *in vitro* bovine embryos have been studied as a non-invasive indicator of bovine embryo viability and quality (Gutiérrez-Adán et al., 2004; Van Soom et al., 1992). Van Soom *et al.* (1997) demonstrated that embryos with faster rate of division (fast-cleaving) had greater possibilities of reaching advanced developmental stages than slower ones. Furthermore, timing of blastocyst formation was correlated with the number of cells in the inner cell mass and total cell number (blastocysts at day 6 and 7 had higher number of cells than blastocysts at day 8). In the present study, no significant differences were found in cleavage rates and the proportion of embryo stages at day 8 among seasons. However, the preliminary data appeared to indicate that the semen samples collected in spring resulted in embryos that developed at a faster rate than samples collected in other seasons (no presence of early embryos and a high proportion of expanded blastocysts); in addition, semen samples collected in summer produced embryos that developed faster than those collected in winter. This faster rate of division in embryos derived from semen samples collected in spring and summer suggests more competent and resistant embryos. There is no a specific reason for faster development of embryos; some possible causes that can influence the kinetics of embryo development are culture conditions, genetic influences, and paternal and maternal influences (intrinsic factors of the oocyte and/or sperm cell) (Gutiérrez-Adán et al., 2004; Van Soom et al., 1997). In the current study, the most likely cause was the paternal effect because oocytes were pooled and randomly distributed among treatments, and culture conditions were the same for all experimental groups.

Previously, the sperm quality of the semen samples used in this study was analysed among seasons (Sabés-Alsina et al., 2016a, 2016b). It was observed that sperm quality was affected by season and that sperm samples collected in spring and summer appeared to have better quality than samples collected in winter. These findings are compatible with the tendency for embryos to develop faster after fertilization with semen collected in spring or summer than in winter in the present *in vitro* fertilization study. A possible explanation for this tendency could be the exposure of bulls to heat stress during the spermatogenesis cycle in summer. Spermatogenesis in bulls takes approximately 60 days, being spermatocytes and spermatids cells easily damaged by heat stress (Amann and Almquist, 1962; Hansen, 2009; Setchell, 1998). Therefore, the presence of negative

effects in sperm samples and consequently in embryo development would appear from late autumn to an early winter. Another possible reason for this tendency could be explained by the decrease on testosterone concentration levels in bulls during the winter solstice (Stumpf et al., 1993).

Zona pellucida binding assays (sperm ability to bind to a homologous or heterologous zona pellucida) and sperm penetration assays have been used as a sperm diagnostic test in several species such as bulls (Larsson and Rodriguez-Martinez, 2000). In the present study, no differences were found among seasons in the number of sperm cells binding to oocytes, despite using a low sperm dose. However, there is some controversy between the correlation of the ability to bind to the zona pellucida *in vitro* and *in vivo* fertility results (Larsson and Rodriguez-Martinez, 2000).

Male fertility can be tested by mating or AI. However, these techniques are expensive and time-consuming, only allowing a limited number of male to be tested (Larsson and Rodriguez-Martinez, 2000). Fertilization is a complex process which is difficult to simulate under *in vitro* conditions (Larsson and Rodriguez-Martinez, 2000). Sperm cells are not exposed to the same challenges in reaching an oocyte in an *in vitro* fertilization system as in the female reproductive tract after insemination. Therefore it is likely that fertilization and development rates would be higher *in vitro* than *in vivo*. Moreover, development to hatching *in vitro* does not imply that the embryos would be capable of continued survival and implantation *in vivo* (Van Soom et al., 1997). Therefore, the results presented here can only be taken as an indication of the fertility of the sperm samples; nevertheless there was a suggestion that some aspects of fertility were different among samples collected in different seasons, with spring being the most favourable season for semen collection.

In conclusion, it appears that seasonality in sperm samples could have an affect on the rate of embryo development. However, further studies should be performed in order to verify these interesting preliminary data.

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General discussion

The current thesis confirmed that heat stress in bucks exposed to an *in vivo* experimental model of continuous daily summer cycles has moderate effects on sperm quality, especially on sperm viability and acrosome integrity. However, these sperm abnormalities do not significantly affect fertility and prolificacy. These results suggest that the proportion of rabbit sperm cells with fertility potential in the heterogeneous cell population of an ejaculate is enough to compensate for the increased proportion of abnormalities and thus maintain fertility. Alternatively, it is possible that animals had the capacity to adapt to environmental changes using behavioural, physiological and/or biochemical mechanisms, and therefore were able to preserve their reproductive function (Abdul Niyas et al., 2015).

While bucks were exposed to circadian heat cycles, they were also video-recorded in order to assess posture and behaviour as an indicator of heat stress in a behavioural study (Dalmau et al., 2015). Feeding and drinking behaviours were not affected by high temperatures, probably due to the fact that intake in rabbits is light dependent, taking place in late afternoon and at night when animals were not exposed to heat stress. However, it was observed that bucks and does exposed to circadian heat cycles were less active and tended to lie stretch in a prostrated position during the warmest parts of the day. This positioning would result in an increased body surface in contact with air in order to lose heat. Thus, it could be assumed that these behavioural variations observed in animals subjected to heat stress allowed adaptation to high environmental temperatures as well as counteracting its negative effects.

There are different ways of studying the impact of heat stress on sperm cells: whole body heating or local heating (cryptorchidism or scrotal isolation usually by water bath immersion). Whole body heating is the most physiological technique to study the effect of heat stress on the testis, however it involves some physiological, metabolic and endocrinological changes of the body to help coping with heat stress. Local heating involves the possible negative effects of anaesthesia and/or surgery (Setchell, 1998). In contrast, heat stress *in vitro* has been identified as a good model for studying the mechanisms of heat stress without possible body adaptation mechanisms and adverse effects of the techniques on the animal (Ikeda et al., 1999). In the present thesis, it was

observed that *in vitro* exposure of rabbit sperm cells to abdominal temperature did not affect sperm quality parameters suggesting that their fertilizing ability would be retained. However, the incubation of sperm cells for 3 hours to similar temperatures of a reproductive tract of a doe submitted to hyperthermic conditions (42ºC) affected metabolic activity, and some morphologic and motility parameters. Nonetheless, these alterations produced in the rabbit sperm quality may not have affected the fertilizing capacity of these heated samples. Our results are in agreement with Hendricks *et* al. (2009) who found that aging of bovine sperm cells by incubation to normothermic (38.5º) and heat shock temperatures (40ºC) for 4 hours significantly decreased their sperm kinematics. In their case, despite the changes in sperm quality, neither the ability to fertilize an oocyte nor the competence of the zygote to develop to the blastocyst stage was affected (Hendricks et al., 2009).

Sperm metabolic activity can be assessed using different stainings (rhodamine 123, mitoTracker, JC-1, etc.) and methods (microscope and flow cytometer) (Hossain et al., 2011). In the current thesis the use of the resazurin reduction test by fluorometry is described as a useful technique to quantitatively evaluate rabbit sperm metabolic activity. It was observed that measurements of this technique were directly related to rabbit sperm concentration, viability and acrosome integrity, suggesting a great potential of this test as a complementary tool for evaluating rabbit sperm quality.

After developing the resazurin reduction test for rabbit sperm cells, it was used for evaluating metabolic activity of rabbit sperm samples submitted to an *in vitro* heat stress model. Besides observing that metabolic activity was negatively affected by hyperthermic conditions (42ºC) as explained previously, an interaction was on metabolic activity in the heat stress model between the *in vitro* heat stress and season. This interaction indicated that sperm cells subjected to *in vitro* heat stress tended to exhibit lower values of metabolic activity measurements (relative fluorescent units; RFU) in the warm season than in the cold season. There could be two explanations for this. The first is that sperm cells respond to heat stress *in vitro* by decreasing metabolic activity, with the result that heat stressed sperm cells have a lower metabolic activity in summer than in winter. Therefore, fertility should be more likely to be affected by heat stress in summer than in

winter. The other is that higher rabbit sperm metabolic activity in winter could be associated with an increase in metabolic by-products and reduced longevity as Gibb *et al.* (2014) reported in stallions sperm cells. This mechanism could help to maintain fertility under heat stress conditions. This second hypothesis is in accordance with the previous study of Maya-Soriano *et al.* (2013) who observed that the incubation of bovine oocytes in an *in vitro* heat stress model during maturation in different seasons (warm and cold season) produced an increased rate of premature oocytes as a response to the *in vitro* heat stress in the cold season. These results suggested that bovine oocytes collected in the cold season might be more sensitive to high temperatures compared with oocytes collected in a warm season.

Seasonality can produce adverse effects on sperm quality in different species (Nakabayashi and Salisbury, 1959; Safaa et al., 2008). In the present thesis bull sperm quality of bulls housed in northern Spain was shown to vary among seasons, which may be attributable to climate factors. Sperm alterations produced by season effects could be related with the exposure of animals to high environmental temperatures (heat stress) during summer (Hansen, 2009; Marai et al., 2002)**.** In this thesis, animals were exposed to mean maximum THI values of 74.1 units (corresponding to mild discomfort) in summer. However, samples collected in spring and summer appear to have better quality than samples collected in winter. These observations can be explained by the fact that there is lack of information about the duration of the exposure of these animals to the THI and how animals coped with this THI (adaptation mechanisms). Another possible explanation is that during spermatogenesis, spermatocytes and spermatids are more susceptible to heat stress damage (Abdul Niyas et al., 2015; Hansen, 2009; Setchell, 1998). In bulls, spermatogenesis takes around 60 days (Amann and Almquist, 1962; Hansen, 2009). Therefore, the possible negative effects produced by heat stress during the spermatogenesis should appear in autumn or early winter, depending on the kind of heat stress exposure (intensity and duration). Material from this season was not available for the present study because this experiment was designed to match with a previous study performed with Swedish bulls (Valeanu et al., 2015). In view of the different results obtained in the two countries, it would be interesting to include some material from autumn in the study, if such material exists.

After determining the effects of season on bull sperm quality, a preliminary *in vitro* fertilization trial was performed in order to investigate this season effects on the fertilizing ability of bovine sperm cells and subsequent embryo development. *In vitro* fertilization is a useful model for assessing fertility as it is less expensive and time consuming than an artificial insemination trials (Giritharan et al., 2005; Rodríguez-Martínez, 2007)**.** However, in an *in vitro* fertilization system sperm cells are not exposed to the same challenges in reaching an oocyte as in the female reproductive tract after insemination (Larsson and Rodriguez-Martinez, 2000; Morrell and Rodriguez-Martinez, 2009). In the current thesis, no seasonal effects were observed on the *in vitro* fertilizing ability of frozen-thawed bovine spermatozoa collected in winter, spring and summer. Nonetheless, a tendency was found for semen samples collected in spring to have faster rates of embryo development than samples collected in other seasons. These preliminary findings are compatible with the results observed in sperm quality. A possible explanation for the lack of effect in most of the parameters studied could be explained by the small sample size. Only one drop replicate of 10 oocytes was run for each bull and season. Amann (2005) suggested that in order to not be flawed, *in vitro* fertilization studies should include at least four or five replicate droplets per male and treatment. Therefore, further replicates should be performed in order to increase the sample size and verify these preliminary interesting results.

Climate change can be the cause of several physiological changes in animals and human beings to enable them to cope with the demands for species survival. A phenomenon of adaptation or tolerance to persistent stressful factors, such as heat stress, is needed for maintaining reproductive function. Our results support this theory and indicate the necessity of developing strategies to alleviate the demands of climate change, which will be needed in the future to maintain efficiency in animal reproduction.

Conclusions

1- Continuous daily summer circadian cycles have moderate effects on sperm quality in the rabbit, especially on sperm viability and acrosome integrity. However, these anomalies did not affect fertility and prolificacy.

2- The vital assessment of metabolic rates of sperm cells by the resazurin reduction test could provide more complete information about semen quality than other routine semen analysis, correlating with sperm viability and acrosome status information. This test has a great potential as an additional tool for evaluating semen quality.

3- Short heat stress (42ºC) exposure appears to affect the quality of rabbit sperm cells in terms of metabolic activity and motility parameters. Thus, their functionality may well be compromised following exposure to the reproductive tract of a hyperthermic female for a short period.

4- Sperm quality of bulls housed in northern Spain is affected by season. Samples collected in spring appear to have better sperm quality than samples collected in other seasons.

5- Seasonality in sperm samples could affect the rate of embryo development. However, further studies should be performed in order to verify these preliminary data.

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