TESIS DE LA UNIVERSIDAD DE ZARAGOZA
2022
102

Melissa Carvajal Serna

Effect of the constant photoperiod and melatonin on the ram seminal characteristics of breeds reared in equatorial areas

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ISSN 2254-7606

Prensas de la Universidad
Universidad Zaragoza

ISSN 2254-7606

Universidad Zaragoza
1542

Tesis Doctoral

EFFECT OF THE CONSTANT PHOTOPERIOD AND MELATONIN ON THE RAM SEMINAL CHARACTERISTICS OF BREEDS REARED IN EQUATORIAL AREAS

Autor

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UNIVERSIDAD DE ZARAGOZA
Escuela de Doctorado

Programa de Doctorado en Bioquímica y Biología Molecular

2022

Facultad de Veterinaria
Universidad Zaragoza

Instituto Universitario de Investigación
en Ciencias Ambientales
de Aragón
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Tesis Doctoral

"Effect of the constant photoperiod and melatonin on the ram seminal characteristics of breeds reared in equatorial areas"

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2022

Las Doctoras **Rosaura Pérez Pe** y **Adriana Casao Gascón**, Profesoras Titulares de Zoología, del Departamento de Bioquímica y Biología Molecular y Celular de la Universidad de Zaragoza, y el Doctor **Jaime Antonio Cardozo Cerquera,** investigador PhD asociado de la Corporación colombiana de investigación agropecuaria (AGROSAVIA)

INFORMAN:

Que el trabajo realizado por la Graduada **Melissa Carvajal Serna** con título: "*"Effect of the constant photoperiod and melatonin on the ram seminal characteristics of breeds reared in equatorial areas*" ha sido realizado bajo su dirección en la Facultad de Veterinaria de Zaragoza y en las instalaciones de AGROSAVIA (Bogotá, Colombia) y reúne las condiciones exigidas para optar al grado de **Doctor con Mención Internacional**, por lo que consideran procedente su presentación. Asimismo, hacen constar su **correspondencia con el Proyecto de Tesis**, aprobado por el Departamento de Bioquímica y Biología Molecular y Celular. Los resultados obtenidos quedan recogidos en 4 artículos publicados en revistas SCI, todos ellos incluidos en el ejemplar de la Tesis Doctoral presentado.

Y para que conste a los efectos oportunos firman la presente en Zaragoza a 10 de diciembre de 2021.

Rosaura Pérez Pe **Adriana Casao Gascón** **Jaime A. Cardozo Cerquera**

UNIVERSIDAD AUTÓNOMA AGRARIA
“ANTONIO NARRO”

Centro de Investigación en Reproducción Caprina

Torreón, Coahuila, México, Diciembre 15 de 2021

A quien corresponda

Estimado Sr./Sra.

Reporte de la evaluación de la tesis doctoral de Melissa Carvajal-Serna

Les escribo en relación con la Tesis de Doctorado titulada “Efecto del fotoperiodo constante y de la melatonina en las características seminales de moruecos de razas criadas en zonas ecuatoriales“ y presentada por "Melissa Carvajal-Serna" en cumplimiento parcial de los requisitos para el título de la Universidad de Zaragoza, España, con Mención Internacional de Doctorado.

Ahora he tenido la oportunidad de leer con gran atención todo el documento y debo decir que me ha impresionado favorablemente. La tesis aborda un tema muy interesante y representa un paso adelante en nuestra comprensión de los mecanismos moleculares que subyacen a la capacitación de los espermatozoides en los carneros. El trabajo también brinda nuevos conocimientos sobre la función moduladora del plasma seminal. Vale la pena mencionar que, a pesar de que la presente disertación se centra principalmente en el esperma de carnero, todos los hallazgos también se colocan en el contexto más amplio de los espermatozoides de mamíferos.

En general, todo el trabajo está muy bien diseñado y concebido y el texto está muy bien escrito. En mi opinión, el candidato, que debe ser elogiado, merece justamente ser galardonado con un doctorado con mención internacional. También me gustaría mencionar que aprecio el papel de orientación de los tres supervisores, ya que esto definitivamente ha contribuido a la alta calidad de esta disertación.

La disertación está compuesta por una introducción general que cubre con gran detalle las vías de transducción involucradas en la capacitación del esperma de mamíferos y el papel de las proteínas plasmáticas seminales, así como de la melatonina. Esta sección también aborda el complicado concepto de apoptosis en espermatozoides de mamíferos y analiza críticamente el trabajo relevante realizado por otros grupos de investigación.

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La Introducción General es seguida por una sección detallada de Materiales y Métodos (completamente escrita en inglés) que claramente da una idea precisa de la gran cantidad de trabajo que el candidato ha realizado mientras realizaba la presente Tesis. La sección de resultados está compuesta por cuatro artículos que ya han sido publicados. Todas las publicaciones se encuentran en revistas indexadas en el Science Citation Index of Journal Citation Reports (SCI / JCR). Finalmente, hay una Discusión General que aborda adecuadamente todas las cuestiones planteadas en los Resultados. La redacción de esta sección también es impresionante y demuestra que el candidato ha alcanzado bastante madurez, el pensamiento independiente y la capacidad de discutir críticamente materiales de otros investigadores, tres cualidades / habilidades que cualquier doctorado debería tener. Las conclusiones encajan perfectamente con los hallazgos y la importancia de todo el trabajo.

Por tanto, me complace valorar positivamente la presente Tesis Doctoral de Melissa Carvajal-Serna y recomiendo a la Comisión de Doctorado de la Universidad de Zaragoza que la Tesis sea aprobada en el panel evaluador final (es decir, examen Viva).

A t e n t a m e n t e
"Alma Terra Mater"

Dr. osé Alberto Delgadillo Sánche
Profesor Investigador de tiempo Completo ersidad Autónoma Agraria Antonio Narro
Doctor en Fisiología de la Reproudcción Animal por la Université des Sciences et Tecniques du Languedoc, Montepellier, Francia
Miembro del Centro de Investigación en Reproducción Caprina (CIRCA), de la Academia Mexicana de Ciencias y del Sistema Nacional de Investigadores Nivel III

28 de diciembre de 2021, Balcarce, Argentina .-

Re: Informe de evaluación de Tesis doctoral

De mi mayor consideración:

Por medio de la presente, les hago llegar mi apreciación sobre la tesis doctoral titulada "Efecto del fotoperiodo constante y de la melatonina en las características seminales de moruecos de razas criadas en zonas ecuatoriales", presentada por Melissa Carvajal Serna, en cumplimiento parcial de los requisitos para optar el grado de Doctor en la Universidad de Zaragoza, España, con Mención Internacional de Doctorado.

Luego de leer detenidamente el manuscrito presentado por la doctoranda, debo decir que me ha impresionado grata y favorablemente. La Tesis aborda de un modo original, la función que desempeña la melatonina en la estacionalidad reproductiva de la especie ovina, cuando los animales se encuentran adaptados a ambientes con fotoperiodo constantes, con similares horas de luz y oscuridad. El trabajo se centra particularmente en el macho, y evalúa además la variabilidad estacional de la testosterona, y otros componentes en plasma seminal, como enzimas antioxidantes y proteínas. El trabajo de tesis ha permitido establecer diferencias marcadas entre individuos adaptados a latitudes ecuatoriales (fotoperiodos constantes) y aquellos de latitudes medias, las cuales han sido publicadas en revistas indexadas (Anim Reprod Science, Int. J. Mol. Sci., Trop Anim Health Prod, Animals) y componen la sección de resultados. A mi juicio, el aporte más significativo se refiere a la función de la melatonina sobre la calidad espermática, modulando la capacitación y los signos característicos de apoptosis. Si bien la tesis está enfocada en morruecos, estos hallazgos resultan de sumo interés para su estudio en especies consideradas con reproducción no estacional, y su potencial aplicación en técnicas de reproducción asistida y procesos de criopreservación de gametas.

El trabajo está muy bien escrito, presenta un diseño apropiado para alcanzar los objetivos planteados, haciendo un uso adecuado de figuras y gráficos que hace aún más fluida la lectura, y facilita la interpretación y comprensión de los resultados. En mi opinión, la doctoranda debe ser elogiada y merece justamente ser premiada con un doctorado con mención internacional. Es de destacar la labor llevada adelante por los orientadores, quienes sin lugar a duda han contribuido a la alta calidad del trabajo presentado. La Tesis está compuesta por una revisión bibliográfica exhaustiva y actualizada sobre las características particulares de los espermatozoides, plasma seminal, síntesis y mecanismos de acción de la melatonina. Seguidamente se plantean la hipótesis y los objetivos de manera clara y concisa. La sección de Materiales y métodos está escrita íntegramente en inglés, da una idea precisa de la gran cantidad de trabajo llevado adelante por la candidata durante la realización de la presente Tesis. La sección de resultados está compuesta por cuatro artículos que ya han sido publicados (2019, dos en el 2020 y uno en 2021) en revistas indexadas. Finalmente, hay una Discusión General que aborda

adecuadamente todas las cuestiones planteadas en los Resultados. La redacción de esta sección demuestra la capacidad de la candidata para discutir ideas y puntos de vista con pensamiento crítico. Las conclusiones se refieren a los objetivos planteados, y el significado de todo el trabajo. Por tanto, me complace valorar positivamente la presente Tesis Doctoral de Melissa Carvajal Serna y recomendar a la Comisión de Doctorado de la Universidad de Zaragoza que la Tesis sea aprobada, y premiada con un doctorado con mención internacional.

Dr. Federico Hozbor

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Este trabajo se realizó en el departamento de Bioquímica y Biología Molecular y Celular de la Universidad de Zaragoza, financiado por los proyectos CICYT-FEDER AGL2017-83799-R y DGA A07_20R por parte de España, en conjunto con el proyecto 110 157 635 854 del Ministerio de ciencias, tecnología e innovación de Colombia, y la beca predoctoral del Ministerio de ciencia e innovación del gobierno de España PRE2018-085198

Además de los artículos incluidos en el capítulo de Resultados, se han publicado otros artículos en los que he participado para su desarrollo durante el periodo predoctoral.

→ **NADPH Oxidase 5 and Melatonin: Involvement in Ram Sperm Capacitation**

Autores: Miguel-Jiménez S, Pina-Beltrán B, Gimeno-Martos S, Carvajal-Serna M, Casao A and Pérez-Pe R
Revista: Front. Cell Dev. Biol. 9:655794. (2021)
doi:10.3389/fcell.2021.655794

→ **Polymorphisms of the melatonin receptor 1A (MTNR1A) gene influence the age at first mating in autumn-born ram-lambs and sexual activity of adult rams in spring**

Autores: J.A. Abecia, M.C. Mura, M. Carvajal-Serna, L. Pulinas, A. Macías, A. Casao, R. Pérez-Pe, V. Carcangiu.
Revista: Theriogenology 157 (2020) 42-47.
https://doi.org/10.1016/j.theriogenology.2020.07.030

→ **The continuous presence of ewes in estrus in spring influences testicular volume, testicular echogenicity and testosterone concentration, but not LH pulsatility in rams**

J. A. Abecia, M. Carvajal-Serna, A. Casao, C. Palacios, L. Pulinas, M. Keller, P. Chemineau and J. A. Delgadillo. Animal, page 1 of 8 © 2020. Published by Cambridge University Press on behalf of The Animal Consortium
doi:10.1017/S1751731120001330

→ **Does Melatonin Exert Its Effect on Ram Sperm Capacitation Through Nitric Oxide Synthase Regulation?**

Autores: Sara Miguel-Jiménez, Melissa Carvajal-Serna, Silvia Calvo, Adriana Casao, José Alvaro Cebrián-Pérez, Teresa Muiño-Blanco and Rosaura Pérez-Pe.
Revista: Int. J. Mol. Sci. 2020, 21, 2093;
doi:10.3390/ijms21062093

→ **Presence of melatonin-catabolizing non-specific enzymes myeloperoxidase and indoleamine 2,3-dioxygenase in the ram reproductive tract.**

Autores: Paula Martínez-Marcos, Melissa Carvajal-Serna, Sofía Lázaro-Gaspar, Rosaura Pérez-Pé, Teresa Muiño-Blanco, José A. Cebrián-Pérez, Adriana Casao.
Revista: Reprod Dom Anim. 2019;54:1643–1650.
DOI: 10.1111/rda.13574

→ **Long days in winter or the presence of adult sexually active rams did not influence the timing of puberty of autumn-born Rasa Aragonesa ram lambs.**

Autores: José A. Abecia, Marianne Gave, Ana I. García, Adriana Casao, Melissa Carvajal-Serna, Carlos Palacios, Matthieu Keller, Philippe Chemineau & José A. Delgadillo
Revista: Biological Rhythms Research (2019)
doi.org/10.1080/09291016.2019.1613321

Los resultados obtenidos en esta tesis, al igual que otros correspondientes a otros proyectos en los que he participado, fueron también presentados como pósters en los siguientes congresos:

En modalidad de presentación oral:

→ **Evaluación preliminar del efecto de las dietas ricas en fitomelatonina sobre la reproducción del morueco**

Autores: Melissa Carvajal Serna, Sara Miguel-Jiménez; José Alfonso Abecia-Martìnez; Rosaura Pérez-Pe; Adriana Casao-Gascón.
Evento: VI Jornadas IUCA (Instituto Universitario de Investigación en Ciencias Ambientales de Aragón).
Lugar/año: Zaragoza. Spain. **2020**
Tercer premio mejor vídeo científico en Categoría no doctor

→ **Influencia de la melatonina en la funcionalidad espermática de distintas razas de moruecos en condiciones ecuatoriales**

Autores: Melissa Carvajal-Serna, Rosaura Peréz-Pe, Adriana Casao-Gscón.
Evento: 3ª Reunión Red de Excelencia PIVE.
Lugar/año: Madrid, Spain. **2019**

→ **Relación entre los datos obtenidos por ecografía testicular y la calidad seminal en sementales ovinos: resultados preliminares.**

Autores: Melissa Carvajal-Serna, María Santorromán, Juliano Barale, Ángel Macías, Francisco Quintín, Alfonso Abecia, Adrian Casao y Rosaura Pérez-Pe.
Evento: AIDA (2019), XVIII Jornadas sobre Producción Animal, 361-363.
Lugar/año: Zaragoza, Spain **2019**

Y en modalidad de póster

→ **Effect of melatonin on actin and α-tubulin distribution related to in vitro capacitation in ram sperm.**

Autores: Melissa Carvajal-Serna, Mónica Paesa, Juliano Barale, Silvia Gimeno-Martos, Victoria Peña-Delgado, Sara Miguel-Jiménez, Adriana Casao, Rosaura Pérez-Pe.
Evento:12th Biennial Meeting of the Association for Applied Animal Andrology Animal Reproduction Science 220 **(2020)** 106360
https://doi.org/10.1016/j.anireprosci.2020.106363

→ **Decapacitating effect of melatonin is not mediated by calcium-calmodulin kinase II in ram spermatozoa.**

Autores: Victoria Peña-Delgado, Sara Miguel-Jiménez, Melissa Carvajal-Serna, José Álvaro Cebrián-Pérez, Teresa Muiño-Blanco, Adriana Casao, Rosaura Pérez-Pe.
Evento: 12th Biennial Meeting of the Association for Applied Animal Andrology Animal Reproduction Science 220 **(2020)** 106360
https://doi.org/10.1016/j.anireprosci.2020.106407

→ **Monthly changes in testicular ultrasonography and their association with ram sperm quality**

Autroes: Melissa Carvajal-Serna; María Santorroman; Juliano Barale; Jaime Cardozo; José Cebrián-Pérez; Teresa Muiño- Blanco; Alfonso Abecia; Rosaura Pérez-Pe; Adriana Casao.
Evento: Proceedings of the 23rd Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR).
Lugar/año: St Petersburg, Russia. **2019**
DOI: 10.1111/rda.13462

→ **Changes in nitric oxide synthase isoforms localization in the presence of melatonin seem not to be related with changes in nitric oxide levels during ram spermatozoa capacitation**

Autores: Sara Miguel-Jimenez; Melissa Carvajal-Serna; Silvia Calvo; Adriana Casao; José Cebrián-Pérez; Teresa Muiño-Blanco; Rosaura Pérez-Pé.
Evento: Proceedings of the 23rd Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR).
Lugar/año: St Petersburg, Russia. **2019**
DOI: 10.1111/rda.13462

→ **Melatonin effect on sperm capacitation in sheep breeds under equatorial climates.**

Autores: Melissa Carvajal-Serna; Emma Conde-Silva; Esneider Rivera-Rincón; Jaime A Cardozo; José Alvaro Cebrián-Pérez; Teresa Muiño-Blanco; Rosaura Pérez-Pé; Adriana Casao.
Evento: Proceedings of the 22nd Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR).
Lugar/año: Cordoba, Spain. **2018**
DOI: 10.1111/rda.13300

→ **Comparative study of changes in intracellular calcium levels and localization in capacitated ram spermatozoa in presence of melatonin**

Autores: Sara Miguel Jiménez; Melissa Carvajal-Serna; Adriana Casao; José Álvaro Cebrián-Pérez; Teresa Muiño-Blanco; Rosaura Pérez-Pé.
Evento: Proceedings of the 22nd Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR).
Lugar/año: Cordoba, Spain. **2018**
DOI: 10.1111/rda.13300

→ **Identification of intracellular nitric oxide synthase isoforms in ram sperm.**

Autores: Silvia Calvo-García; Melissa Carvajal-Serna; Sara Miguel-Jiménez; Adriana Casao; José Álvaro Cebrián-Pérez; Teresa Muiño-Blanco; Rosaura Pérez-Pé.
Evento: Proceedings of the 22nd Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR).
Lugar/año: Cordoba, Spain. **2018**
DOI: 10.1111/rda.13300

→ **Presencia e inmunolocalización de los receptores de melatonina MT1 y MT2 en espermatozoides ovinos bajo fotoperiodo ecuatorial.**

Autores: Melissa Carvajal-Serna, Jaime Antonio Cardozo, Henry Grajales-Lombana, José Álvaro Cebrián-Pérez, Teresa Muiño-Blanco, Rosaura Pérez-Pe y Adriana Casao.
Congresos: XLIII congreso nacional y XIX congreso internacional de la sociedad española de ovinotecnia y caprinotecnia (SEOC 2018).
Lugar/año: Zaragoza, Spain. **2018**

→ **Effect of environmental factors on ram sperm quality under tropical conditions.**

Autores: Melissa Carvajal-Serna; Jaime Antonio Cardozo-Cerquera; Henry Alberto Gajales-Lombana; José Álvaro Cebrián-Pérez; Teresa Muiño-Blanco; Rosaura Pérez-Pé; Adriana Casao.

Evento: 14th International Congress of the Spanish Association for Animal Reproduction (AERA).
Lugar/año: Barcelona, Spain. **2017**

→ **Variation of melatonin, testosterone and antioxidant enzymes in seminal plasma of three ram breeds under tropical conditions.**

Autores: Melissa Carvajal-Serna, Jaime Antonio Cardozo, Henry Grajales-Lombana, José Álvaro. Cebrián-Pérez, Teresa Muiño-Blanco, Rosaura Pérez-Pé, and Adriana Casao.
Evento: 33rd scientific meeting Association of Embryo Technology in Europe.
Lugar/año: Bath, UK. **2017**

Abreviaturas

BP	Before present
AACD	L-aminoácido aromático descarboxilasa
AANAT	Arialkilamina N-acetiltransferasa
ADN	Ácido desoxirribonucleico
AFMK	1-N_2-formil-5-metoxiquinuramina
AMK	N_1-acetil-5-metoxiquinuramina
AMPC	Adenosín monofosfato cíclico
ASMT	Acetilserotonina O-meltiltransferasa
ATP	Adenosín -5- trifosfato o trifosfato de adenosina
BSA	Bovine serum albumin o albúmina de suero bovino
C	Capacitated o capacitado
CaM	Calmodulina
cAMP	Cyclic adenosine monophosphate
CFDA	Carboxyfluorescein diacetate odiacetato de carboxifluoresceina
CTC	Chlortetracycline o Clorotetraciclina
Cu/Zn-SOD	Superoxido dismutasa asociada al cobre o el zinc
DIGE	Difference gel electrophoresis
DMSO	Dimethyl sulphoxide o dimetilsulfóxido
EC-Cu/Zn-SOD	Superoxido dismutasa asociada al cobre o el zinc Extracelular
EDTA	Ácido etilenodiaminatetraacético
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid o ácido etilenglicol-bis (β-aminoetil éter) -N, N, N ′, N′-tetraacético
ELISA	Enzimoinmunoanálisis de adsorción
EPO	Eosinófilo peroxidasa
FSH	Hormona folículoestimulante
GnRH	Hormona liberadora de gonadotropinas
GPCRs	G-protein-coupled receptors o receptores acoplados a proteínas G
GPR50	G protein-coupled receptor 50
GPx	Glutatión peroxidasa
GRD	Glutatión reductasa
GSH	Glutatión reducido
GSSG	Glutatión oxidado
HRP	Horseradish peroxidase o peroxidasa de rábano picante
IDO	Indoleamina 2,3-dioxigenasa

IEF	Isoelectric focusing o isoelectroenfoque
IPCC	Intergovernmental Panel on Climate Change
IU	Unidades internacionales
kDa	Kilodalton
LH	Hormona luteinizante
Mn-SOD	Superoxido dismutasa asociada al magnesio
MPO	Mieloperoxidasa
MT1/MT1a	Receptor de melatonina 1
MT_2/MT1b	Receptor de melatonina 2
MT_3	Receptor de melatonina 3
MTNR1A	Melatonin Receptor 1a Gene
MTNR1B	Melatonin Receptor 1b Gene
NADPH	Nicotinamida adenina dinucleótido fosfato
NC	Non-capacitated o no capacitado
NOS	Especies reactivas de nitrógeno
PBS	Phosphate-buffered saline o buffer fosfato salino
PHS	Phosphate hepes sucrose
PKA	Proteína quinasa A
pM	Picomolar
PS	Phosphatidylserine o fosfatidilserina
PVDF	Polyvinylidene difluoride o fluoruro de polivinilideno
R	Acrosome-reacted o acrosoma reaccionado
$ROR_{\alpha 1}$, $ROR_{\alpha 2}$ y RZR_{β}	Receptores de acido retinoico
ROS	Especies reactivas de oxigeno
RT	Room temperature o Temperatura ambiente
SDS	Dodecilsulfato sódico
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis o electroforesis en gel de poliacrilamida con dodecilsulfato sódico
SM	Swim-up medium
SNC	Núcleos supraquiasmáticos
SOD	Superoxido dismutasa
TALP	Tyrode's Albumin Lactate Pyruvate o
TMB	Tetramethylbenzidine substrate o sustrato de tetrametilbencidina
TPH	Triptófano hidroxilasa
ZP	Zona pelúcida
µM	Micromolar

Contenido

1. Resumen y abstract

1.1 Resumen

La estacionalidad reproductiva de la especie ovina, ligada al fotoperiodo, es considerada como un factor limitante de la producción ovina en países cuya latitud determina la existencia de estaciones astronómicas bien diferenciadas. En estas zonas, los cambios en el fotoperiodo, a través de variaciones en la secreción de melatonina nocturna, regulan la actividad de la reproducción ovina tanto en hembras como en machos. En el caso del morueco, a pesar de que la producción de semen es continua a lo largo del año, se aprecian cambios significativos tanto en los componentes del plasma seminal, como en los parámetros de calidad y funcionalidad espermática entre la época reproductiva y la no reproductiva.

Durante los últimos años, nuestro grupo de investigación ha estudiado el efecto de la melatonina sobre la reproducción del morueco en condiciones de fotoperiodo variable o estacional. Estos trabajos han evidenciado variaciones en la concentración de melatonina en el plasma seminal de esta especie entre las distintas estaciones. Además, se ha demostrado la síntesis de melatonina extrapineal en el testículo, al igual que la presencia de receptores de melatonina (MT_1 y MT_2) tanto en este órgano como en la membrana plasmática de los espermatozoides, lo que sugiere que la melatonina estaría relacionada con la calidad seminal del morueco. El hecho de que la incubación de los espermatozoides ovinos con melatonina disminuya los marcadores apoptóticos y niveles de ROS apoya esta hipótesis. Además, esta hormona es capaz de modular la capacitación espermática en función de su concentración, posiblemente a través de su unión al receptor MT_2 en la membrana del espermatozoide.

Sin embargo, los ovinos se encuentran ampliamente distribuidos a nivel mundial, y algunas razas se hayan ubicadas en latitudes donde el fotoperiodo es constante, es decir, la variación de la luz a lo largo del año es mínima o casi nula. **En esta tesis se planteó como hipótesis que, aunque la melatonina no regule la estacionalidad reproductiva en los moruecos de la zona ecuatorial, esta hormona ejerce una función importante en la reproducción de estos animales.** Por ello, el objetivo de la presente tesis fue **evaluar el efecto del fotoperiodo constante y de la melatonina en las características seminales de moruecos ubicados en la zona ecuatorial.** Colombia es un país situado en la zona ecuatorial, donde el fotoperiodo es constante (12L:12D), con solo 32 minutos de diferencia entre el día más largo y el más corto del año. Sin embargo, los factores climáticos, como las precipitaciones, sí que pueden variar a lo largo del año, presentando un patrón bimodal de lluvias, alternadas con dos periodos de sequía. Este y otros factores podrían, de algún modo, modular la actividad reproductiva de los ovinos y otras especies ganaderas de esta zona. En Colombia, dependiendo de la altura sobre el nivel del mar, se crían ovinos de lana o de pelo. Tres de las principales razas ovinas de lana que se distribuyen en las zonas de altura son la raza Criolla de lana, autóctona y derivada de cruces indiscriminados de razas introducidas por los colonizadores desde el siglo XVI, y dos razas ovinas especializadas, Romney Marsh y Hampshire, de origen británico, que fueron introducidas en el país en el último siglo.

Para comprobar si en condiciones ecuatoriales de fotoperiodo constante existían variaciones en la concentración de melatonina en plasma seminal a lo largo del año, tal y como se había observado en moruecos situados en latitudes medias con fotoperiodo variable, **el primer objetivo de esta tesis fue evaluar la concentración de melatonina en el plasma seminal de los moruecos ubicados en la zona ecuatorial a lo largo del año.** El estudio se llevó a cabo en las tres razas anteriormente nombradas (Criolla de lana, Romney Marsh y Hampshire), y se analizaron también **los niveles de testosterona y la actividad de las enzimas antioxidantes,** ya que en razas de latitudes medias se había observado una correlación con los niveles de melatonina**.** Los resultados (Artículo 1) demostraron que, a pesar de estar en condiciones de fotoperiodo constante, **la concentración de melatonina en el plasma seminal de estos moruecos varía a lo largo del año** en todos los animales estudiados, independientemente de su raza. **El plasma seminal obtenido durante las épocas de sequía presentó mayores niveles de melatonina que el obtenido en las épocas de lluvia**. Debido a que la distribución de las lluvias afecta la disponibilidad y la calidad del alimento, es probable que los cambios en la concentración de melatonina en el plasma seminal se deban a cambios en la disponibilidad y concentración de fitomelatonina en el forraje, lo que explicaría la falta de diferencia entre razas (Artículo 1). Además, a diferencia de lo observado en moruecos de latitudes medias, en condiciones de fotoperiodo ecuatorial **la concentración de melatonina no correlaciona con la de testosterona o la actividad de las enzimas antioxidantes.** En relación a la concentración de testosterona sólo se observó variación anual en las razas introducidas (Romney Marsh y Hampshire), pero el incremento de testosterona ocurrió antes que el incremento en los niveles de melatonina, por lo que su variación no reflejaría una activación del eje hipotálamo-hipofisario-gonadal por la melatonina.

Con objeto de **dilucidar si el régimen fotoperiódico puede afectar a la composición del plasma seminal (objetivo 2),** se llevó a cabo un estudio comparativo de los componentes de este fluido entre moruecos ubicados en latitud ecuatorial (Colombia; razas Criolla de lana y Romney Marsh) y en una zona de latitud media (España; raza Rasa Aragonesa), durante el mismo periodo de tiempo (artículo 2)**.** El plasma seminal fue obtenido entre septiembre y noviembre, que coincide con el inicio de la época reproductiva en los moruecos de Rasa Aragonesa, y con la segunda época de lluvias en los moruecos de la zona ecuatorial. **La concentración de melatonina y de testosterona detectada en plasma seminal de los moruecos ubicados en la zona ecuatorial fue menor que la de moruecos de latitudes medias.** También se encontraron diferencias en la concentración de enzimas antioxidantes, pero no en la concentración total de proteína ni en la composición proteica.

Dadas las diferencias en la concentración de melatonina en plasma seminal entre moruecos ubicados en zonas de fotoperiodo constante y zonas de fotoperiodo variable y, puesto que la melatonina puede tener un origen pineal o testicular, nos planteamos como **tercer objetivo determinar el origen de la melatonina del plasma seminal de moruecos ubicados tanto en latitudes medias como en la zona ecuatorial.** Para ello, se analizó el plasma seminal de moruecos enteros y vasectomizados de las razas Criolla de la lana y

Romney Marsh (ubicadas en Colombia), y de la raza Rasa Aragonesa (ubicada en España), y se determinó, además de la concentración de melatonina, la de testosterona, la actividad de enzimas antioxidantes y la cantidad de proteínas (artículo 2). Los resultados mostraron que la concentración de melatonina era significativamente menor en moruecos vasectomizados, pero solo en aquellos situados en latitudes medias. Esto sugiere que, en machos enteros en estas latitudes, la melatonina del plasma seminal tiene un doble origen, testicular y pineal. Sin embargo, **en moruecos de la zona ecuatorial no se observaron diferencias en la concentración de melatonina en plasma seminal entre machos enteros y vasectomizados,** lo que podría sugerir que, o bien no hay síntesis de melatonina testicular, o bien esta no es secretada al plasma seminal**.** Por otro lado, la concentración de testosterona disminuyó tras la vasectomía en animales ubicados en ambas latitudes. También disminuyó la concentración de proteínas, sobre todo a nivel de las proteínas de alto peso molecular, que tendrían origen en el epidídimo y testículos. Finalmente, la actividad de las enzimas antioxidantes no se vio afectada por la vasectomía, lo que corrobora su origen en las glándulas accesorias.

Una vez identificada la presencia de melatonina en el plasma seminal de moruecos de la zona ecuatorial, y dado que esta hormona ejerce gran parte de sus funciones a través de sus receptores de membrana, el **cuarto objetivo de esta tesis fue identificar la presencia y la distribución de los receptores de melatonina MT_1 y MT_2, en la membrana de espermatozoides de moruecos de la zona ecuatorial y evaluar su posible variación entre las épocas de lluvia y de sequía** (artículo 3). Los resultados evidenciaron **la presencia de ambos receptores en los espermatozoides de las tres razas (Criolla de lana, Romney Marsh y Hampshire),** identificándose los mismos inmunotipos descritos para MT_1 en machos de latitudes medias, además de dos nuevos inmunotipos para el receptor MT_2. Se observaron diferencias en los porcentajes de inmunotipos, tanto para el receptor MT_1 como para el MT_2, con respecto a lo descrito previamente en moruecos de latitud media. También **se observaron diferencias entre razas y entre épocas para la distribución de ambos receptores**, a diferencia de lo que sucede en fotoperiodo variable, en los cuales sólo se habían observado diferencias estacionales para el receptor MT_2. Sin embargo, en cuanto a la densidad de receptores evidenciada por estudios densitométricos, únicamente la raza Criolla de lana presentó diferencias entre épocas en la cantidad del receptor MT_1, lo que sugiere que, salvo esta excepción, las diferencias en la distribución de este receptor no estarían acompañadas de cambios en la cantidad del mismo. Además, **la raza Criolla de lana también presentó una densidad del receptor MT_2 significativamente menor que las razas introducidas**, independientemente de la época climática, y que podría estar relacionada con su mayor adaptación al fotoperiodo ecuatorial. Los resultados también mostraron la tendencia de MT_2 a formar homodímeros o heterodímeros con otros receptores de la familia GPCR, pero no con MT_1, a diferencia de lo observado en moruecos de latitudes medias, donde se evidenciaron heterodímeros de MT_1 y MT_2.

Una vez detectada la presencia de melatonina en el plasma seminal y de los receptores para la misma en la membrana espermática, nos planteamos como quinto y último objetivo

evaluar si la melatonina podía regular la capacitación espermática en estas razas, al igual que lo hace en razas localizadas en zona de fotoperiodo variable. Previamente a la consecución de este objetivo, se hizo necesario **estudiar los requisitos y la respuesta a la capacitación *in vitro* de las razas Criolla de lana, Romney Marsh y Hampshire ubicadas en latitudes ecuatoriales,** ya que no se había estudiado hasta el momento. Este estudio se llevó a cabo tanto en época de lluvias como de sequía (artículo 4).

Nuestros resultados demostraron que **la adición al medio de capacitación de sustancias que elevan el AMPc intracelular (medio *cocktail*) es capaz de incrementar de forma significativa el porcentaje de espermatozoides capacitados de moruecos de la zona ecuatorial,** al igual que sucede en los de latitudes medias, aunque se observaron diferencias entre razas y épocas. La melatonina, tanto a concentración 100 pM como 1 µM evitó parcialmente el incremento de espermatozoides capacitados después de la incubación en las tres razas estudiadas, a diferencia de los descrito en moruecos de latitudes medias, donde sólo la concentración alta previno la capacitación. En las tres razas de la zona ecuatorial se observaron diferencias entre épocas para el efecto de la melatonina, siendo mayor en general en la época lluviosa, pero en cualquier caso este efecto de la melatonina fue menos aparente en la raza Criolla de lana. Esto podría estar relacionado con la menor densidad del receptor MT_2, que fue observada en el artículo 3 de esta tesis. La adición de melatonina en el medio de capacitación también tuvo un efecto positivo sobre el porcentaje de espermatozoides vivos sin inversión de fosfatidilserina, que también fue más evidente en la estación de lluvias. Se ha descrito previamente que el receptor MT_1 es el encargado de mediar el efecto antiapoptotico de la melatonina, al igual que el MT_2 se ha relacionado en la especie ovina con su efecto modulador de la capacitación. Es probable que la mayor concentración de melatonina en el plasma seminal en época de sequía, como se ha demostrado en el artículo 1, de lugar a una menor respuesta de los espermatozoides a la adición exógena de la misma, por la desensibilización de los receptores tras la exposición previa a la alta concentración melatonina presente en este fluido.

En conclusión, los resultados obtenidos en esta tesis doctoral evidencian variaciones en la concentración de melatonina en plasma seminal de moruecos situados en latitudes de fotoperiodo constante, además de la presencia de receptores de melatonina MT_1 y MT_2 en sus espermatozoides. Estos resultados, junto con los obtenidos tras capacitar *in vitro* los espermatozoides en presencia de melatonina, sugieren que, en estos animales, la melatonina ejercería otras funciones distintas a la regulación de la estacionalidad, como la modulación de la capacitación espermática o un efecto antiapoptótico, lo que abre nuevas líneas de investigación sobre el uso de melatonina para la mejora de la fertilidad en razas ovinas en latitudes ecuatoriales.

1.2 Abstract

Reproductive seasonality, regulated by photoperiod, is considered a limiting factor of sheep production in those countries whose latitude determines the existence of well-differentiated astronomical seasons. In these areas, changes in the photoperiod, through variations in nocturnal melatonin secretion, regulate ovine reproductive activity in both females and males. In rams, although semen production is continuous throughout the year, significant changes are observed in the seminal plasma components and sperm quality and functionality between the breeding and non-breeding seasons.

Over the past years, our research group has studied the effect of melatonin on ram reproduction under variable or seasonal photoperiodic conditions. These studies have shown variations in the concentration of melatonin in the ram seminal plasma between seasons. In addition, extra-pineal melatonin synthesis has been demonstrated in the testis and also the presence of melatonin membrane receptors (MT_1 and MT_2) in this organ and the sperm plasma membrane, suggesting that melatonin is related to sperm quality. This hypothesis is supported by the fact that the incubation of ram spermatozoa with melatonin decreases apoptotic markers and ROS levels. Moreover, this hormone can modulate sperm capacitation depending on its concentration, likely through the MT_2 receptor.

However, sheep are a worldwide distributed species, and some breeds are located in latitudes where the photoperiod is constant; that is, the daylength variation throughout the year is minimal or almost zero. **Thus, this thesis hypothesises that, even though melatonin is not implicated in seasonal reproduction in rams under the equatorial photoperiod, this hormone plays an essential role in the reproduction of these males.** Therefore, the aim of this thesis was **to evaluate the effect of constant photoperiod and melatonin on the seminal characteristics of rams located in the equatorial region.** Colombia is situated on the equator and has a constant photoperiod (12L: 12D), with only 32 minutes' difference between the longest and shortest days of the year. However, climatic factors such as rainfall can vary throughout the year, presenting a bimodal pattern of rains alternated with two periods of drought. This fact and other external factors could, in some way, modulate the reproductive activity of sheep and other livestock species in this area. In Colombia, wool or hair sheep are raised, depending on the height above sea level. The three main wool sheep breeds located in high-altitude areas are the Creole wool breed, Romney Marsh and Hampshire. The Creole wool breed is a native breed obtained from indiscriminate crosses of breeds introduced by the colonisers since the 16th century. Romney Marsh and Hampshire are two specialised sheep breeds of British origin introduced in the country in the last century.

In rams located in mid-latitudes with variable photoperiod, variations in the concentration of melatonin in seminal plasma throughout the year have been observed. To determine whether equatorial conditions and constant photoperiod affect this parameter, **the first objective of this thesis was to evaluate melatonin concentration in the ram seminal plasma of breeds located in the equatorial zone throughout the year.** The study was conducted in the three breeds mentioned above (Creole, Romney Marsh and

Hampshire). **Testosterone levels and the activity of antioxidant enzymes were also analysed** since a correlation with melatonin levels had been observed in seasonal breeds. The results (paper 1) showed that, despite being in constant photoperiod conditions, **the melatonin concentration in the seminal plasma of these rams varies throughout the year** regardless of their breed. **The seminal plasma obtained during the dry season presented higher melatonin levels than that of the rainy season.** Since the rainfall distribution affects the availability and quality of feed, it is possible that these changes in melatonin concentration in seminal plasma were due to changes in the availability and concentration of phytomelatonin in the forage and would explain the lack of differences between breeds (paper 1). Furthermore, unlike mid-latitude rams, **the concentration of melatonin did not correlate with the testosterone or the activity of antioxidant enzymes** under equatorial photoperiodic conditions**.** Regarding testosterone concentration, variations though the year was only observed in the British breeds (Romney Marsh and Hampshire). However, the testosterone rising happened before the increase in melatonin concentration, so this variation would not reflect the melatonin activation of the hypothalamus-pituitary-gonadal axis.

To elucidate whether the photoperiodic regime can affect the composition of the seminal plasma (objective 2), a comparative study of the components of this fluid was carried out in rams located in the equatorial (Colombia: Creole and Romney Marsh breeds) and mid-latitude zones (Spain: Rasa Aragonesa breed) during the same period (paper 2). The seminal plasma was obtained from September to November, the beginning of the breeding season in Rasa Aragonesa breed, and the second rainy season in the equatorial rams. **Melatonin and testosterone concentrations detected in the seminal plasma of rams located in the equator were lower than those of the mid-latitude.** Differences were also found in the concentration of antioxidant enzymes, whereas neither total protein nor protein profile differed between zones.

Due to the differences found in the seminal plasma melatonin concentration between rams located in areas of constant and variable photoperiod and, since melatonin can be of pineal or testicular origin, we set **as the third objective of this thesis to determine the origin of the seminal plasma melatonin in rams located in mid-latitudes and the equatorial zone**. For this, the seminal plasma of intact and vasectomised rams from Creole and Romney Marsh breeds (located in Colombia) and the Rasa Aragonesa breed (located in Spain) was analysed, and the concentration of melatonin, testosterone and proteins, and the activity of antioxidant enzymes were evaluated (paper 2). The results showed that the melatonin concentration was significantly lower in vasectomised rams but only in those located in mid-latitudes. This suggests that the seminal plasma melatonin in intact males at these latitudes has a dual origin, testicular and pineal. However, **in rams located in the equatorial zone, no differences were observed in the concentration of melatonin in seminal plasma between intact and vasectomised males**, suggesting that there is no synthesis of testicular melatonin or that it is not secreted into the seminal plasma.

On the other hand, the testosterone concentration decreased after vasectomy in animals located in both latitudes. The protein concentration also diminished, especially at the high molecular weight proteins, that would have originated in the epididymis and testis.

Finally, the activity of the antioxidant enzymes was not affected by vasectomy, which corroborates its origin in the accessory glands.

Once the presence of melatonin was identified in the seminal plasma of rams from the equatorial zone, and due to this hormone exerts part of its functions through its membrane receptors, **the fourth objective of this thesis was to identify the presence and distribution of the melatonin receptors MT_1 and MT_2, on spermatozoa from rams located in the equatorial zone, and to evaluate their possible variation between the rainy and dry seasons** (paper 3). The results **showed that both receptors were present in the sperm of the three breeds (Creole, Romney Marsh and Hampshire),** presenting the same immunotypes described for MT_1 in mid-latitude males and two new ones for the MT_2 receptor. Differences in the percentages of immunotypes for both the MT1 and MT2 receptors were observed in relation to mid-latitude rams. **Differences in distribution for both receptors were observed between breeds and seasons**, unlike rams located under variable photoperiod, in which only seasonal differences for the MT_2 receptor were observed. However, the density of receptors, evidenced by densitometric studies, showed that only the Creole breed presented differences between seasons in the MT_1 receptor, which suggests that, with this exception, the differences in the distribution of this receptor would not be accompanied by changes in its quantity. **In addition, the Creole breed also presented a significantly lower MT_2 receptor density than the introduced breeds,** regardless of the climatic season, which could be related to its better adaptation to the equatorial photoperiod. The results also showed that MT_2 could forms homodimers or heterodimers with other receptors of the GPCR-family, but not with MT_1, unlike that was observed in mid-latitude rams, where heterodimers of MT_1/ MT_2 were evidenced.

Once the presence of melatonin in seminal plasma and its receptors in the sperm membrane were detected, we set as the fifth and final objective **to determine whether melatonin could regulate the sperm capacitation state in rams located in constant photoperiod, as it was demonstrated in mid-latitude zones.** Before achieving this objective, it was necessary **to study the requirements and the response to *in vitro* capacitation of spermatozoa from the Creole, Romney Marsh and Hampshire breeds located in equatorial latitudes** since they had not been studied so far. This study was conducted in both the rainy and dry seasons (paper 4).

Our results showed that the addition of substances that raise **the intracellular cAMP (cocktail) to the capacitation medium could significantly increase the percentage of capacitated spermatozoa from equatorial rams,** as occurs in mid-latitudes. However, differences were observed between breeds and seasons. Melatonin, at 100 pM and 1 µM concentration, partially prevented the increase in capacitated spermatozoa after incubation in the three breeds studied, unlike mid-latitude rams, where only 1 µM melatonin avoided capacitation. In these equatorial breeds, differences in the melatonin were observed between seasons. The decapacitating effect was more significant during the rainy season. However, it was less apparent in the Creole breed, which could be related to the lower density of the MT_2 receptor, observed in paper 3 of this thesis. The addition of melatonin to the capacitation medium also positively affected the percentage of live sperm without phosphatidylserine translocation, which was also more evident in the rainy season. It has

been previously reported that the MT_1 receptor mediates the melatonin anti-apoptotic effect, just as MT_2 has been related to the modulation of sperm capacitation in sheep. It is possible that the high melatonin concentration in the seminal plasma during the dry season, as demonstrated in paper 1, decreased the response of spermatozoa to the exogenous melatonin, due to a desensitization of its membrane receptors after the exposition to the melatonin levels in this fluid.

In conclusion, the results obtained in this doctoral thesis show variations in the melatonin concentration in seminal plasma of rams located at latitudes of constant photoperiod, and the presence of MT_1 and MT_2 melatonin receptors on their spermatozoa. These results, together with those obtained after *in vitro* sperm capacitation in the presence of melatonin, suggest that, in these animals, melatonin would exert other functions than the regulation of seasonality, like the modulation of sperm capacitation or an anti-apoptotic effect. This opens up new lines of research on the use of melatonin to improve fertility in sheep breeds in equatorial latitudes.

2. REVISIÓN BIBLIOGRÁFICA

2.1. Origen, distribución e importancia del ganado ovino

La especie ovina fue una de las primeras especies de producción animal en ser domesticada por el ser humano, hace 10.000 años (Figura 2.1) en la región que hoy es conocida como Irán (Chessa et al., 2009). La estabulación, el control de la reproducción, y la selección de los animales basada en su morfología, producción y adaptación a los diferentes ambientes y climas, dieron lugar a las razas ovinas contemporáneas, distribuidas a nivel mundial, y con una considerable variabilidad genética (Taberlet et al., 2011). Además, todos los animales de una misma raza empezaron a exhibir las mismas características fenotípicas, incluyendo el mismo color de la capa (Kijas et al., 2012; Taberlet et al., 2011).

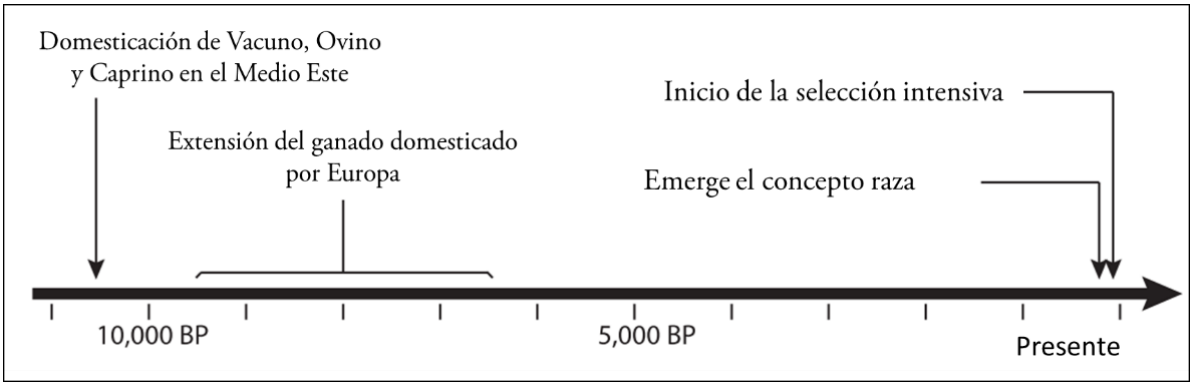


Figura 2. 1 Principales eventos en la domesticación del ganado vacuno, ovino y caprino (Adaptado de (Taberlet et al., 2011) BP: Before present (antes del presente)

Una vez doméstica en los valles de Oriente Próximo, y siguiendo las migraciones humanas, la especie ovina se expandió por el Danubio hacia el Centro y Norte de Europa, hacia la Europa mediterránea siguiendo la costa, y hacia los rincones más orientales de Asia o hacia el Sur de África (Delgado and Nogales, 2010). El norte de África se pobló de ovinos mediante una expansión propia por la costa mediterránea de África (Figura 2.2) (Delgado and Nogales, 2010).

Después de muchos siglos de asentamiento en el viejo continente, los ovinos viajaron a América en 1493 durante el periodo de conquista, en un principio razas españolas, y posteriormente ovinos africanos (Delgado and Nogales, 2010; Spangler et al., 2017). El comercio continuo de los colonos favoreció la diversidad de razas y su mezcla permitió a los ovinos del nuevo continente diferenciarse de las razas ibéricas, adaptándose a la climatología del lugar (Kijas et al., 2012).

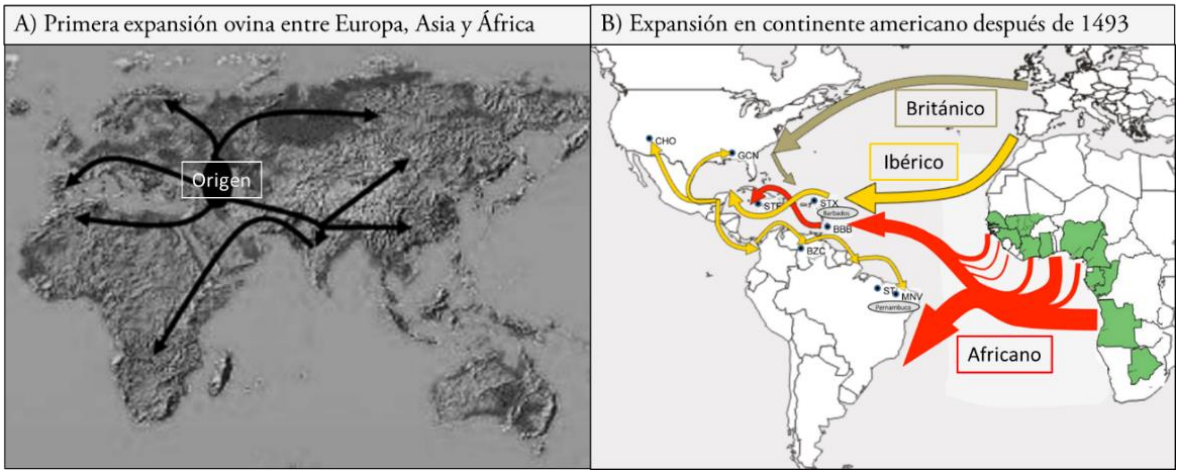


Figura 2. 2 Expansión de los ovinos domésticos desde su lugar de origen y posterior expansión al nuevo continente con el inicio de la colonización en 1493. *Tomado de* (Delgado and Nogales, 2010; Spangler et al., 2017).

La amplia dispersión de ovinos a través del tiempo, en las más variadas condiciones ambientales, ha hecho que esta especie sea considerada como uno de los animales domésticos más cosmopolitas, razón por la cual ha adquirido un papel cultural muy importante en la historia de las civilizaciones humanas. Los ovinos han logrado desarrollar una combinación única de respuestas para adaptarse a la presión de selección en diversas condiciones ambientales. Estas respuestas adaptativas incluyen: resistencia a enfermedades, tolerancia a la fluctuación de nutrientes en cuanto a disponibilidad y calidad, ajustando su metabolismo energético, capacidad de sobrevivir a condiciones climáticas extremas, y capacidad para reproducirse por largos periodos incluso con una baja calidad de alimento (Joy et al., 2020; Lv et al., 2014). Así, aproximadamente el 50% de la población ovina se encuentra en regiones de clima árido, además de estar bien adaptados al ambiente tropical (Gowane et al., 2017; Joy et al., 2020). Sin embargo, es muy difícil de establecer el número total de animales por continente, ya que los pequeños productores en países en desarrollo no suelen aportar datos oficiales. Por lo tanto, una forma de estimar la distribución ovina sería por la explotación de sus productos, como por ejemplo la producción de carne ovina a nivel mundial (Figura 2.3).

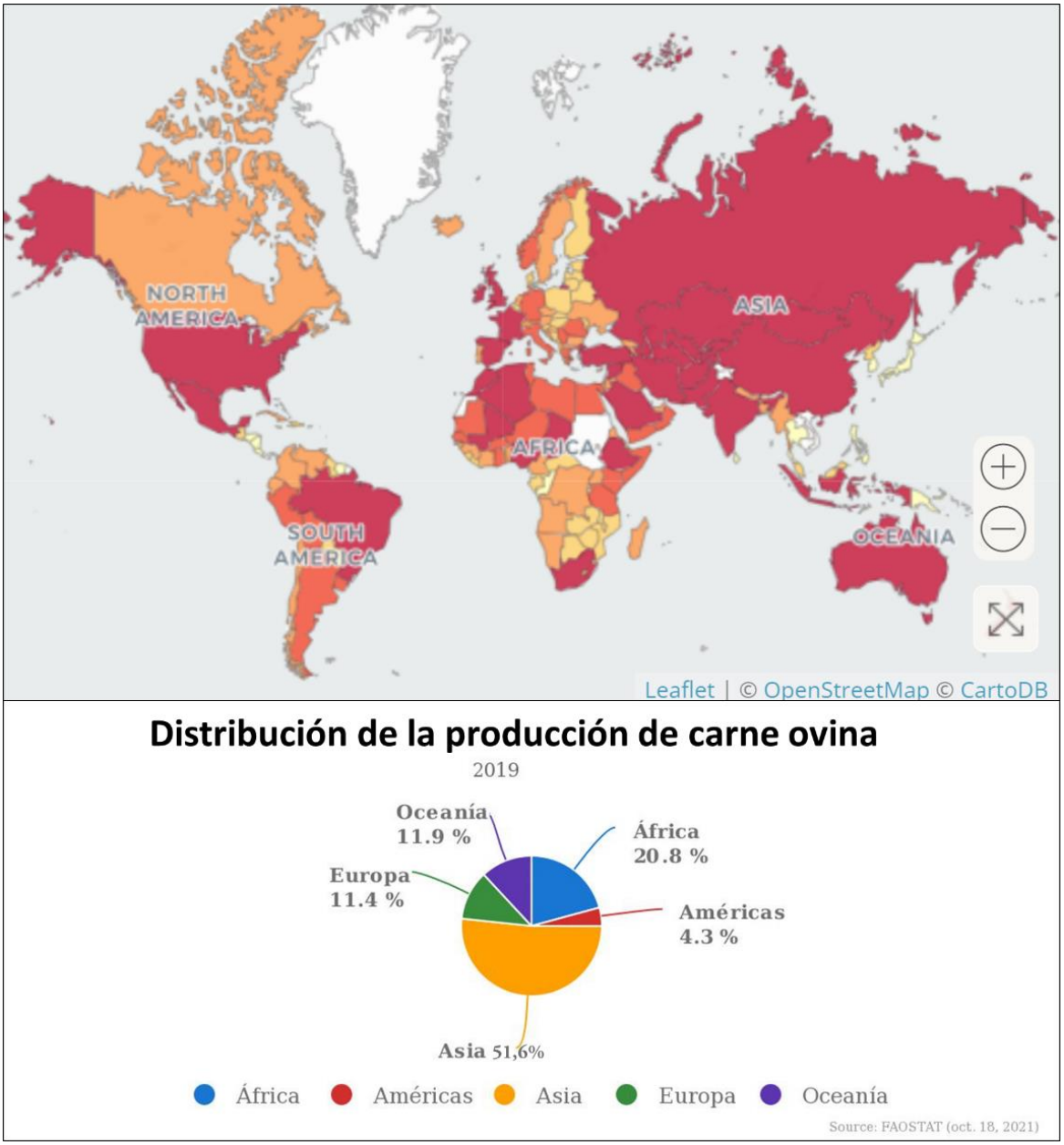


Figura 2. 3 Distribución mundial por continentes de la producción de carne ovina en el año 2019. Los datos fueron obtenidos en la página de la FAOSTAT https://www.fao.org/faostat/es/#data/QCL/visualize

A nivel mundial, tanto la producción de ganado ovino como el consumo de carne ovina se están incrementando. Los principales productores de carne y lana son China, India, y Australia (FAOSTAT, 2021). En cuanto a los países en vías de desarrollo, el mercado de la carne ovina y su consumo también se están incrementando. Particularmente en Sudamérica, la producción ovina empezó a disminuir drásticamente hasta el inicio del nuevo milenio, pero a partir de entonces se observó un cambio de tendencia con respecto al número de animales, incrementándose en los últimos 20 años alrededor de un 4% anual.

Por su parte, el calentamiento global ha generado cambios en las zonas climáticas de muchas regiones del mundo, con una expansión de las zonas con clima árido (desertificación) y una contracción de las zonas climáticas polares. Como consecuencia, muchas plantas y especies animales han experimentado cambios en su abundancia y en su estacionalidad (Shukla et al., 2019). En las especies animales, la disponibilidad de alimento y la temperatura determinan el balance energético, lo cual en última instancia determina el inicio de la estación reproductiva. En el caso de los herbívoros, una discordancia entre la época de nacimientos y el pico de disponibilidad de forraje podría afectar el ritmo de las estaciones reproductivas (Bronson, 2009). Esto es especialmente relevante en determinados países, como los sudamericanos, donde la producción de ganado está basada en sistemas de pastoreo extensivo, al igual que en otros países en vías de desarrollo ubicados en las zonas de trópico (Karthik et al., 2021). Un estudio prospectivo realizado en Sudamérica basándose en los diferentes escenarios de calentamiento global propuestos por el IPCC (*Intergovernmental Panel on Climate Change*) para el 2060 postula que, mientras la producción de ganado bovino, porcino, y aviar disminuirá, el ganado ovino se incrementará un 10% y se convertirá en el principal ganado de producción para el consumo humano en este continente (Seo et al., 2010).

2.2. Origen de las razas ovinas estudiadas en esta tesis: la Rasa Aragonesa y las principales razas existentes en Colombia

En este apartado se hablará del origen de las tres principales razas ovinas existentes en Colombia y se hará referencia también a la raza Rasa Aragonesa, principal raza ovina de Aragón, con la que se han hecho la mayoría de los estudios comparativos, considerando que esta raza está sujeta a un fotoperiodo variable característico de latitudes templadas.

En España se originaron los 4 principales troncos étnicos ovinos (Ibérico, Entrefino, Churro y Merino) de los que surgieron la mayoría de las razas españolas (Figura 2.4). Las razas ibéricas se fueron asentando por todo el territorio de la península de acuerdo con sus preferencias adaptativas. Actualmente existen un total de 10 razas autóctonas de fomento, 34 razas autóctonas en peligro de extinción y la integración de algunas otras pertenecientes a otros países de la Unión Europea. En la zona nordeste de España se cuenta con razas representantes de los cuatro grandes troncos distribuidos por las comunidades de Cataluña, Baleares, y Aragón. El principal representante del tronco Entrefino, distribuido ampliamente por la comunidad autónoma de Aragón, es por extensión, censo y por ser base primitiva en la formación de otras razas, la raza Rasa Aragonesa. Esta es una raza de aptitud cárnica, con una lana entrefina y de vellón blanco uniforme. Presenta un gran rusticidad y adaptación a las zonas áridas de la región, y los animales se mantienen en sistemas semi-extensivos, con pastoreo durante el día y estabulación en la noche. Aunque esta raza poco tuvo que ver con la formación de las razas iberoamericanas, se tomará como referencia comparativa al estar ubicada en una zona templada (mediterránea) con cambios en el fotoperiodo que dan lugar a una mayor actividad reproductiva durante el otoño y el invierno.

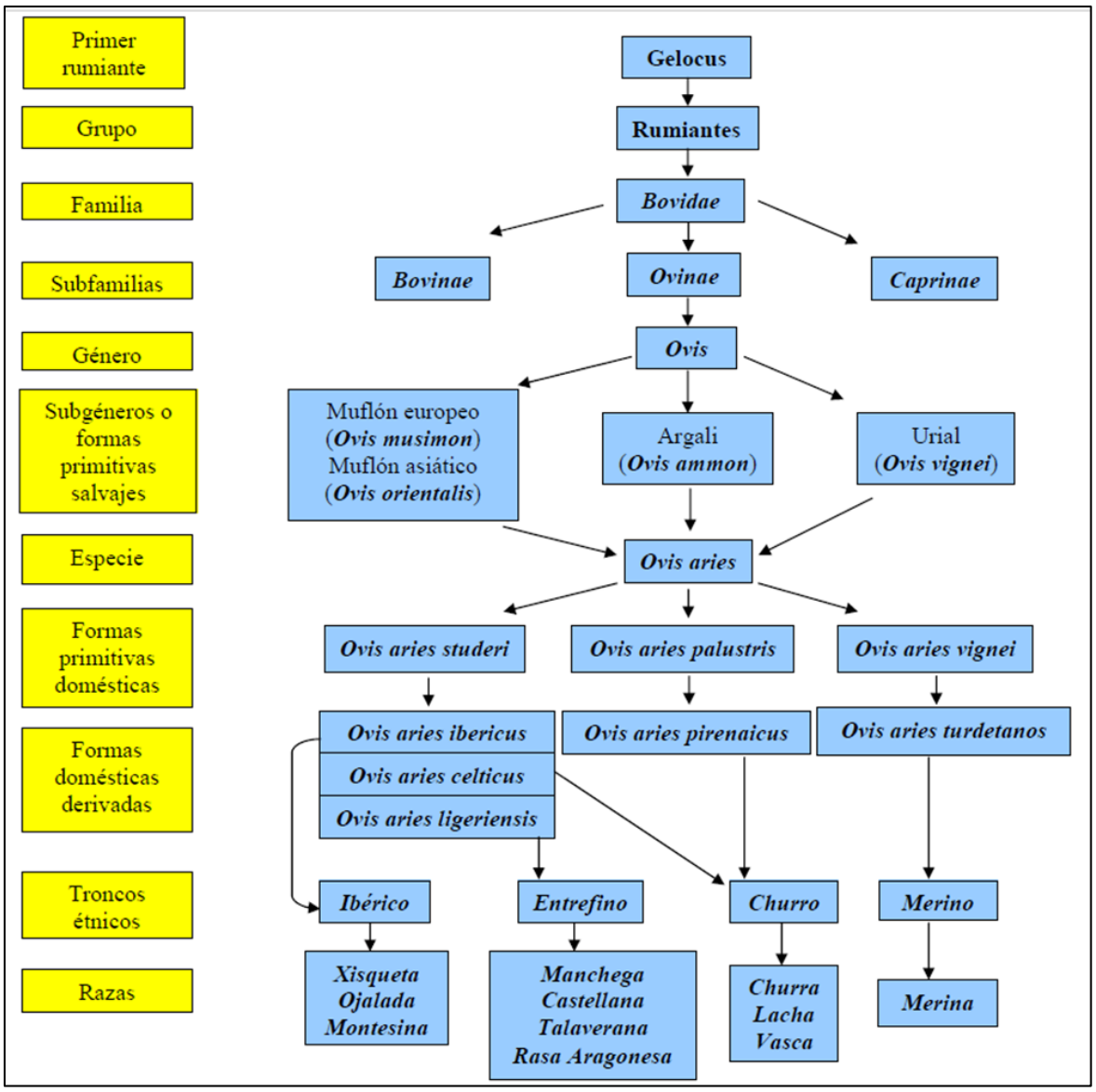


Figura 2. 4 Origen y evolución de los óvidos hasta las razas ibéricas. Tomado de: Conservación de recursos genéticos de la raza Xisqueta: caracterización estructural, racial y gestión de la diversidad en programas "in situ". (Torres, 2006)

En cuanto al origen de las razas colombianas, como se ha comentado anteriormente, los primeros ovinos llegaron al continente americano en el siglo XV. Dadas las limitaciones de espacio en los navíos durante el periodo de colonización (Delgado and Nogales, 2010), las poblaciones domésticas que atravesaron el atlántico eran pequeñas. Los primeros ovinos transportados hasta lo que hoy se conoce como República dominicana y Haití, traídos desde la Península Ibérica, contribuyeron a la formación de las razas iberoamericanas actuales. Estas poblaciones se diferenciaron genéticamente de las razas ibéricas por efecto de la deriva genética y los diferentes intereses productivos. El ingreso constante de animales, sin ningún criterio de raza, favoreció la diversidad genética y el incremento de tamaño de las poblaciones ovinas en los lugares de destino (Delgado and Nogales, 2010).

La mayor parte de las razas ovinas iberoamericanas están aún sin caracterizar, agrupándose bajo el término “criollas” a un gran número de razas perfectamente adaptadas a distintos ambientes (Revelo et al., 2020). De acuerdo a las comparaciones fenotípicas basadas en las características de la lana, se asumió que las razas fundadoras provenían de los troncos étnicos Ibérico, Churro (razas Lacha y Churra), y Merino. Sin embargo, los animales procedentes de este último tronco no se adaptaron a la zona tropical (de la Barra et al., 2011). También es probable que otras razas ibéricas del tronco Entrefino hayan participado en la creación de las razas criollas. Posteriormente, las razas africanas de pelo se extendieron por todas las regiones americanas, debido principalmente a que tuvieron una mejor adaptación al medioambiente, a las pobres condiciones alimenticias, y una mayor resistencia frente a parásitos (Revelo et al., 2020; Spangler et al., 2017), contribuyendo también a la formación de las razas criollas.

En Colombia, las razas criollas juegan un papel muy importante en la economía rural. Contrariamente a la tendencia observada en España, en Latinoamérica en general, y Colombia en particular, se ha observado un mínimo, pero constante crecimiento de las explotaciones ovinas (Ocampo et al., 2017). Las razas ovinas de lana se explotan principalmente en las zonas altas de montaña, a más de 2.000 m. sobre el nivel del mar, especialmente en el centro de las cordilleras Andinas. También existen núcleos de razas criollas de pelo, cuyo origen se debate entre la introducción de ovinos africanos o de ovinos de pelo de las Islas Canarias (Delgado and Nogales, 2010). Aunque las razas criollas de lana y de pelo son filogenéticamente distantes, recientemente se ha demostrado que comparten un origen común, al menos en la línea materna, con un ancestro de origen ibérico (Revelo et al., 2020).

Entre 1960 y 1980 se realizaron importaciones de razas ovinas de origen británico, con el fin de mejorar la producción y las características productivas de las razas criollas (Beaty and Williams, 1971). Respecto a las razas de lana introducidas, destacan la raza Corriedale (una raza sintética de Nueva Zelanda), Romney Marsh y Hampshire (Martínez et al., 2006). Estas razas son consideradas de nueva creación, ya que fueron desarrolladas hace alrededor de 150 años, en particular las dos últimas razas, cuyo nombre hace referencia a su zona inglesa de origen. Durante muchos años estas razas introducidas fueron manejadas como si conservaran la estacionalidad reproductiva, pese a que no estaban sujetas a cambios en el fotoperiodo. Sin embargo, con el paso del tiempo, y tras la experiencia de los pequeños productores de ovinos en las zonas de montaña, se concluyó que su reproducción era continua a lo largo del año, aunque la fertilidad variaba con las condiciones nutricionales (Vásquez-Romero, 1998).

En esta tesis nos centraremos en las razas Criolla de lana, Romney Marsh y Hampshire, cuyas características se detallan a continuación:

Criolla de lana: Es un animal rustico, y su finalidad productiva es en un 60% de carne y en un 40% de lana. Destacan por su alta fertilidad y aptitud maternal. Son animales livianos y delgados con poca densidad de lana, y vellón blanco y abierto. Pueden tener a su vez pelos de color marrón o castaño entremezclado en la lana, o incluso tener una

cobertura completamente negra (Pastrana-Bonilla and Calderón, 2001). La lana es usada de forma artesanal para la elaboración de productos típicos de la región andina.

Romney Marsh: Es una raza que se exporta desde Inglaterra a más de 43 países. Fue reconocida como raza en 1896 en Reino Unido, pero su origen se remonta a la edad media con la llegada de los romanos a las islas. Es una raza que procede de los ovinos de lana larga, y guarda gran parecido con los que actualmente se conocen como Merino (Ryder, 1962). Son animales rústicos, de tamaño intermedio, con extremidades cortas, y gruesas y con capacidad productiva de carne y lana. Se encuentran especialmente adaptados a regiones húmedas, frías y con vegetación abundante (Mujica, 2004).

Hampshire: Es resultado de una mezcla de razas ovinas que coexistían en la región de Hampshire, y que fue declarada como raza en 1889. Son animales rústicos y más pesados, por lo que se utilizan como línea paterna especializada en carne. Su piel es negra, pero la lana es blanca y de tamaño medio. Aunque su fertilidad no es destacable, son animales altamente prolíficos, siendo este el motivo por el cual, aunque en pequeña proporción, se conservan en las zonas de montaña (Vásquez-Romero, 1998).

2.3 La regulación de la reproducción en la especie ovina

Los pequeños rumiantes, entre los que se incluye la especie ovina, son considerados animales poliéstricos estacionales, con ciclos estrales que se suceden durante el periodo del año en el que los días son cortos. Esto quiere decir que en fotoperiodo decreciente (otoño), las ovejas entran en celo con el fin de concentrar los partos en primavera, y así asegurar la mayor disponibilidad de alimento a su progenie (Chemineau et al., 2008). Por el contrario, en las estaciones de primavera y verano hay una baja o ausente actividad reproductiva en las hembras conocida como anoestro estacional (Rosa and Bryant, 2003). Esta estacionalidad en la actividad reproductiva de los pequeños rumiantes se observa especialmente en zonas correspondientes a altas (>40º N o S) y medianas (>30º N o S) latitudes, donde los cambios en el fotoperiodo son muy marcados a lo largo del año (Chemineau et al., 1992). Así, las razas originarias de zonas de altas latitudes como los países del norte y centro de Europa, tienen mayor grado de foto-dependencia y mayor restricción de la actividad reproductiva, traduciéndose en un anoestro estacional más marcado que aquellas razas originarias de zonas más cercanas a los trópicos (Chemineau et al., 2004, 1992; Gómez-Brunet et al., 2012).

En las latitudes intermedias (<35º N y >30º N), las razas mediterráneas como la Merino, Manchega (Santiago-Moreno et al., 2000), Chios (Ibrahim, 1997), Serres (Avdi et al., 2004), Awassi (Kridli et al., 2007) o Moghani (Zamiri et al., 2010) se caracterizan por presentar una estación no reproductiva más corta, es decir que inician de nuevo su actividad reproductiva antes que finalice el verano. Estas razas expresan su máxima capacidad reproductiva entre el final del verano, el medio del otoño o durante el invierno, mientras que las razas ubicadas en latitudes más altas (>40º N) tales como Soay, île de France (Mandiki et al., 1998) o Texel (Hafez, 1952), presentan una estación no reproductiva más larga que se puede iniciar antes de la primavera y durar hasta más allá del final del verano. En el

hemisferio sur se presentan las mismas diferencias entre razas, con una mayor actividad reproductiva desde el final del verano hasta el final del otoño (Aller et al., 2012; Gastel et al., 1995).

Esas variaciones estacionales en la reproducción son menos pronunciadas en los machos que en las hembras (Chemineau et al., 2010; Rosa and Bryant, 2003). Aun así, se han observado cambios en el comportamiento sexual de los moruecos (Aller et al., 2012; Kafi et al., 2004), en el tamaño de los testículos (Avdi et al., 2004) y la ecotextura del tejido testicular (Hedia et al., 2020), en el perfil hormonal y en la composición del plasma seminal (Cardozo et al., 2006; Casao et al., 2010a; Marti et al., 2007), así como en las características morfométricas de los espermatozoides (Martí et al., 2012) y en la calidad seminal (Budai et al., 2013; Zamiri et al., 2010).

En la regulación estacional de la reproducción juega un papel fundamental el eje hipotálamo-hipofisario-gonadal del sistema neuroendocrino. La actividad de este eje estará influenciada tanto por el fotoperiodo, que actúa de forma directa (Malpaux et al., 1999), como por la nutrición, que actúa por medio de cambios en el balance energético (Bronson, 2009).

El fotoperiodo regula el ritmo endógeno circanual de la reproducción ajustando la actividad gonadal con las condiciones ambientales, y sincronizando el periodo reproductivo entre individuos. En el caso de los ovinos, el circuito neuronal que media la estacionalidad está comprendido por una vía multi-simpática en el área preóptica del hipotálamo para mediar la liberación de la GnRH (Lehman et al., 2010). De forma secuencial, los cambios en el fotoperiodo son percibidos por las células ganglio-retinales de la retina y la señal es transportada por el nervio óptico a los núcleos supraquiasmáticos (SCN) de la parte anterior del hipotálamo (Malpaux, 2006; Reiter et al., 2018) y de allí a la glándula pineal (Chemineau et al., 2008). La glándula pineal traduce la señal lumínica a una señal química mediante la síntesis y secreción de la melatonina (Reiter et al., 2009). La liberación de noradrenalina por parte de las neuronas simpáticas a los pinealocitos al inicio de cada noche regula el incremento en la producción y liberación de la melatonina al fluido cerebroespinal y a la circulación sanguínea (Figura 2.5). Por ello, la duración de la producción nocturna de melatonina viene condicionada por la variación anual del fotoperiodo y el patrón de secreción diaria de melatonina es considerada como el indicador endocrino de este fotoperiodo (Reiter et al., 2018).

Una vez liberada al fluido cerebroespinal, la melatonina actúa sobre el *pars tuberalis* del hipotálamo (Dardente, 2012; Vivid and Bentley, 2018) regulando la secreción de la hormona liberadora de gonadotropinas (GnRH) (Malpaux et al., 2001; Misztal et al., 2002).

La GnRH, a través del sistema porta-hipofisario alcanza la hipófisis, donde estimula la secreción de la hormona folículoestimulante (FSH) y la hormona luteinizante (LH) por parte de la adenohipófisis (Clarke and Cummins, 1985). Cuando estas hormonas se liberan al torrente sanguíneo, la FSH se une a sus receptores específicos en las células de Sertoli del testículo, regulando la espermatogénesis, mientras que la LH actúa sobre las células de

Leydig, favoreciendo producción de testosterona y estrógenos (Hess, 2003; Yu et al., 2018). A su vez, los esteroides ejercen un *feed-back* negativo sobre la secreción de gonadotropinas (Karsch et al., 1987).

Sin embargo, para la activación de la reproducción estacional en los ovinos, es necesaria la exposición previa a los días largos, que es considerada como la señal que

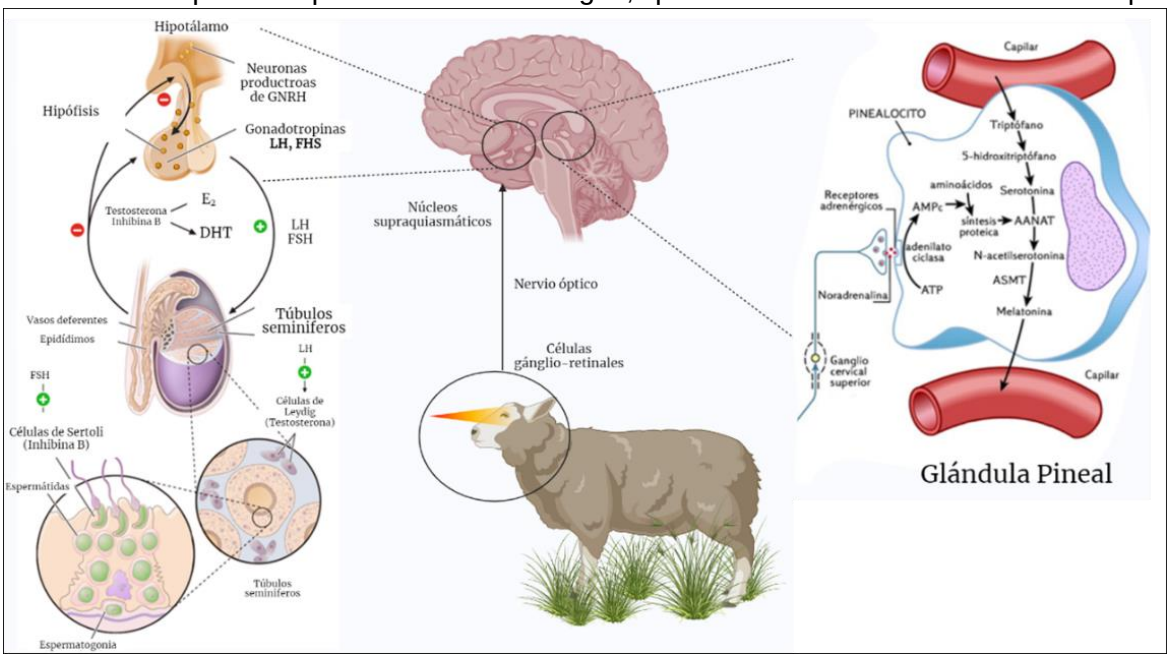


Figura 2. 5 Esquema de activación de la reproducción del morueco a través del eje hipotálamo-hipofisario-gonadal y acción de la glandula pineal para la producción de la melatonina. Modificado de (Dwyer & Quinton, 2019; Reiter et al., 2009)

conduce a activar una respuesta reproductiva en los días cortos. Así, se requiere un mínimo de 35 días de exposición a los días largos (con más de 12,5 horas de luz), para generar una respuesta al cambio del fotoperiodo (revisado en (Dardente, 2012).

En las zonas más cercanas al ecuador, con un régimen lumínico constante de 12 horas de luz y 12 de oscuridad a lo largo de todo el año (12L:12D, del inglés *light* y *darkness*, respectivamente), sin variación en el fotoperiodo, la reproducción está asociada a los niveles nutricionales del animal y se regula con otros factores medioambientales, como la variación de las lluvias en cada región, que a su vez afecta directamente la disponibilidad de alimento (Bronson, 2009; Rosa and Bryant, 2003). Una vez que las necesidades nutricionales están cubiertas, se ha demostrado que hay una ciclicidad continua en las hembras (Jackson et al., 1990), y una producción espermática constante en los machos tanto ovinos (Goshme et al., 2020) como caprinos (Chemineau, 1986). Si razas europeas, con marcada estacionalidad en origen, se introducen en zonas próximas al ecuador (<10º N y 10º S), donde no hay variación en el fotoperiodo, esa estacionalidad se va perdiendo (Jackson et al., 1990) aunque expresarían un patrón estacional normal si se viesen sujetas a cambios en el fotoperiodo (Chemineau et al., 2004; Gavojdian et al., 2015).

Aun así, en muchas zonas tropicales y sub-tropicales (entre 23º N y 23º S) las razas criollas, como la Pelibuey de México, la raza criolla de Martinica o la Santa Inés de Brasil,

presentan patrones estacionales menos marcados que los que presentan razas europeas introducidas como Suffolk (Arroyo et al., 2007), o Merino Australiano (Ismaya and Summers, 2005).

Por tanto, en zonas ecuatoriales existen otros factores que pueden contribuir a modular la actividad reproductiva, como son las variables medioambientales (temperatura, precipitación, etc.), la disponibilidad de alimento y el balance energético (Bronson, 2009; Dardente, 2012).

Esta tesis se centra en el estudio de la calidad seminal y determinados parámetros fisiológicos reproductivos de machos de las tres principales razas ovinas de lana existentes en Colombia: Criolla de lana, Romney Marsh y Hampshire. No sólo la procedencia de estas razas es distinta, sino también el tiempo que llevan introducidas en Colombia y, por tanto, los años de adaptación al fotoperiodo constante ecuatorial (12L:12D), lo que podría explicar las posibles diferencias reproductivas entre ellas.

2.4. El eyaculado del morueco: composición del plasma seminal y fisiología del espermatozoide

En esta tesis se analizaron las diferencias en la calidad y composición seminal, y en determinados parámetros reproductivos, entre moruecos de las razas Criolla de lana, Romney Marsh y Hampshire, en comparación con los de la raza Rasa Aragonesa. Para una mejor comprensión de los resultados, se explicará brevemente la composición del plasma seminal ovino, así como la morfología y fisiología del espermatozoide, centrándonos en los cambios que sufre en el tracto reproductor femenino.

2.4.1. Plasma Seminal

El plasma seminal es una mezcla de secreciones procedentes del testículo, el epidídimo y las glándulas accesorias, que se requiere como medio de supervivencia y para facilitar el transporte del espermatozoide durante la eyaculación y su paso por el primer tramo del tracto reproductor femenino (Juyena and Stelletta, 2012). En el morueco, las glándulas accesorias están compuestas por la ampolla, que es una elongación de los conductos eferentes que se abren directamente en la uretra pelviana; las glándulas vesiculares o vesículas seminales, que son pares y contribuyen a una gran proporción del eyaculado; las glándulas bulbouretrales y la próstata (Aisen, 2004; Galina and Valencia, 2008). La secreción de estas glándulas contiene una gran variedad de componentes que en su mayoría se encuentran también en sangre, incluyendo hormonas y enzimas. Otros productos de secreción en el plasma seminal son sustancias de bajo peso molecular como aminoácidos, glucosa, fructosa, fosfolípidos, al igual que iones como potasio y sodio, y en menor proporción zinc, cobre y hierro, además de vitaminas como la A, C y E (Juyena and Stelletta, 2012). Estos componentes orgánicos son esenciales para el mantenimiento del pH y la osmolaridad, y aportan nutrientes al espermatozoide (Juyena and Stelletta, 2012). Cabe resaltar la importancia de algunas proteínas del plasma seminal, que mantienen a los espermatozoides en un estado descapacitado, estabilizando la membrana plasmática y evitando una reacción acrosómica prematura (Desnoyers and Manjunath, 1992; Druart et

al., 2013). Aun así, los efectos del plasma seminal sobre la funcionalidad espermática todavía resultan controvertidos a día de hoy, como resultado de sus efectos inhibitorios (Moore et al., 2005) o estimuladores de la funcionalidad espermática (Kirkwood et al., 2008; Vadnais et al., 2005). Las funciones del plasma seminal se resumen en la Figura 2.6:

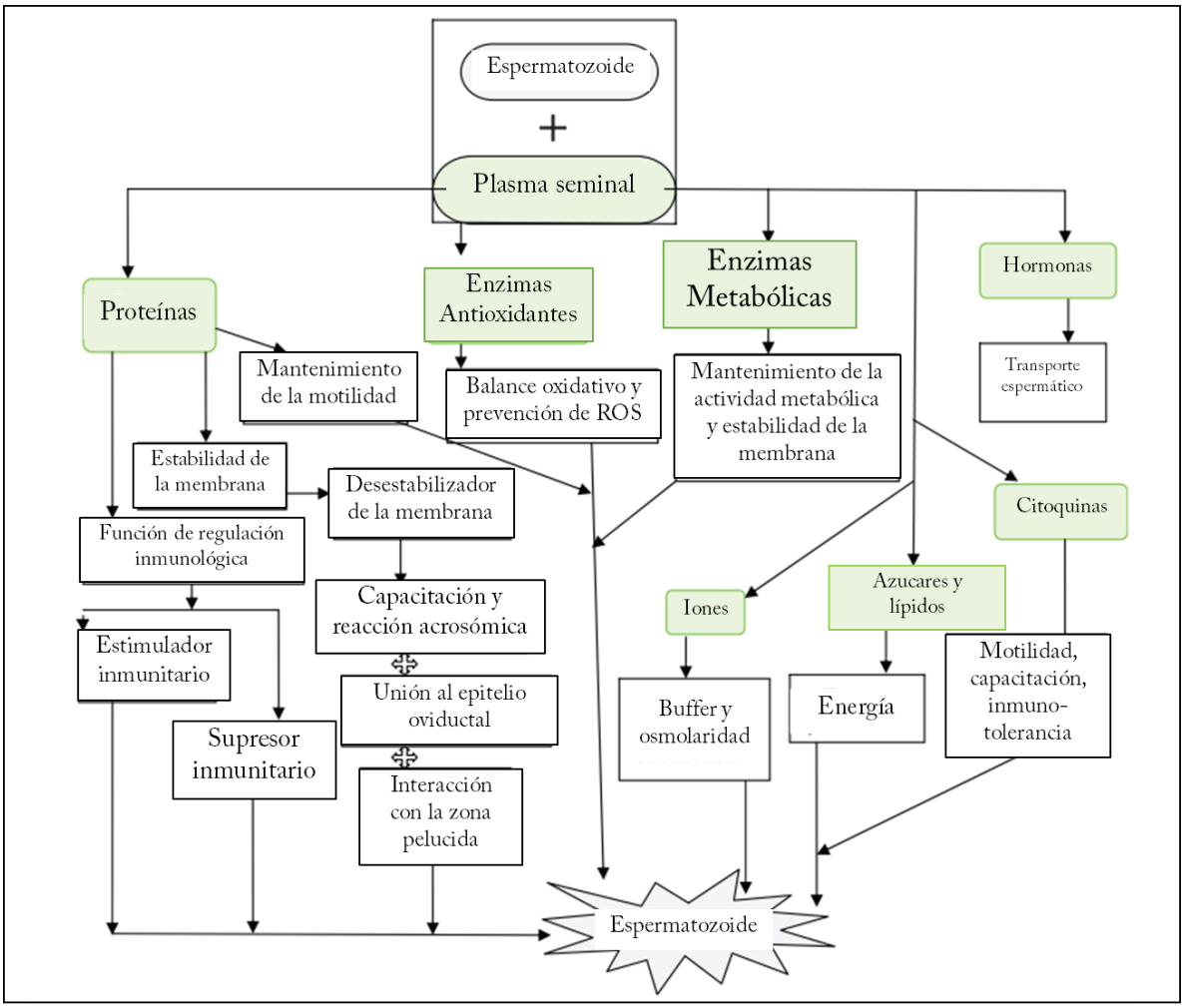


Figura 2. 6 Composición y funciones del plasma seminal de mamíferos. Tomado de (Juyena and Stelletta, 2012). El modelo está centrado en sus principales componentes. ROS: especies reactivas de oxígeno.

En el plasma seminal también existen agentes antioxidantes, principalmente enzimas (Pizarro et al., 2013; Tavilani et al., 2008), aunque también hormonas como la melatonina (Casao et al., 2010a), que protegen a los espermatozoides del daño oxidativo. Entre las enzimas antioxidantes se encuentran la superoxido dismutasa (SOD), la glutatión reductasa (GRD), la glutatión peroxidasa (GPx) y la catalasa.

Superóxido dismutasa (SOD): es la encargada de eliminar el radical superóxido ($O_2^{\cdot}$) de forma sinérgica con la catalasa. Existen tres isoformas, una citosólica (Cu/Zn-SOD) asociada a cobre y zinc; una intra-mitocondrial, asociada al magnesio (Mn-SOD) y una

extracelular igualmente asociada al cobre y zinc (EC-Cu/Zn-SOD). Las isoformas citosólica e intra-mitocondrial cumplen la función específica de eliminar radicales libres generados por el metabolismo de las mitocondrias (Stephenie et al., 2020). En el plasma seminal de rumiantes, la SOD se origina en las vesículas seminales y ejerce sus funciones después de la eyaculación. En los espermatozoides eyaculados se ha evidenciado a nivel acrosomal, post-acrosomal y flagelar (Juyena and Stelletta, 2012). De las tres isoformas, la actividad de la Cu/Zn-SOD es muy alta, y la de las Mn-SOD y EC-Cu/Zn-SOD es baja en el plasma seminal (Eghbali et al., 2008; Stephenie et al., 2020).

Catalasa (CAT): Es una enzima encargada de detoxificar el peróxido de hidrogeno (H_2O_2), que es una molécula altamente permeable, capaz de atravesar las membranas y ejercer daño en las estructuras distantes a su punto de origen (de Lamirande et al., 1997). Está enzima es secretada a nivel del epidídimo (Zini et al., 2002) y protege al espermatozoide del estrés oxidativo en el lumen epididimal (Juyena and Stelletta, 2012).

Glutatión peroxidasa (GPx): Es una enzima dependiente del selenio que actúa sobre los lípidos hidroperoxidados (Brigelius-Flohé and Maiorino, 2013) y el peróxido de hidrogeno. La GPx presente en el plasma seminal procede de las vesículas seminales, mientras que en el espermatozoide se ha localizado en el borde apical de la cabeza y en el post-acrosoma (Marti et al., 2008). El principal substrato de la GPx para eliminar el peróxido de hidrogeno es el glutatión reducido (GSH), el cual se encuentra en concentración relativamente alta en el plasma seminal. El GSH tiene gran capacidad antioxidante, y actúa como donador de electrones, y puede reaccionar directamente con el peróxido de hidrogeno, el anión superóxido, y los radicales del grupo hidroxilo. Para crear un equilibrio entre la reducción y oxidación del GSH, la enzima GPx actúa en sinergia con la enzima **Glutation reductasa (GRD),** que regenera el GSH desde el GSSG (la forma oxidada) con intervención del NADPH (Juyena and Stelletta, 2012)**.**

Otro componente de importancia en el plasma seminal son las hormonas, de las que destacaremos la melatonina y la testosterona, que son las que se estudiarán en esta tesis. La melatonina se secreta en la glándula pineal durante las horas nocturnas, por lo que el nivel de melatonina en el torrente sanguíneo es elevado durante la noche y bajo durante el día (Reiter et al., 2018). Sin embargo, la melatonina producida en la glándula pineal es solo una fracción de la melatonina total generada (Tan et al., 2010), ya que esta hormona también es sintetizada por los mamíferos en tejidos extra-pineales, como la retina, el cerebelo, el intestino, y las células del sistema inmune entre otros (Acuña-Castroviejo et al., 2014; Slominskia et al., 2013). En el caso del morueco, se ha descrito su síntesis en testículo y el epidídimo (González-Arto et al., 2016a) por lo que es probable que parte de la melatonina presente en el plasma seminal ovino (Casao et al., 2010a) provenga de estos órganos. Por otro lado, la testosterona en el plasma seminal de moruecos, presenta una secreción estacional, aunque ligeramente menor que la melatonina (Casao et al., 2010a). La testosterona, producida en los testículos, es importante para la espermatogénesis y funcionalidad espermática (Walker, 2009). Adicionalmente, la concentración de testosterona en el plasma seminal de moruecos se incrementa tras el tratamiento de los animales con melatonina exógena (Casao et al., 2010c), debido probablemente a la acción

de la melatonina en el eje hipotálamo-hipofisario-gonadal, y el subsecuente incremento de los niveles sanguíneos de testosterona (Kaya et al., 2000; Kokolis et al., 2000).

En moruecos de latitudes medias se han observado cambios estacionales en la composición de enzimas antioxidantes (Marti et al., 2007), proteínas en general (Cardozo et al., 2006), y de hormonas como la testosterona y la melatonina en el plasma seminal (Casao et al., 2010a). Estas variaciones podrían estar relacionadas con los cambios en la calidad seminal observados entre estación reproductiva y no reproductiva.

2.4.2. El espermatozoide ovino

La espermatogénesis tiene lugar en los testículos y consiste en la multiplicación de las células germinales y la diferenciación de las mismas para dar lugar finalmente a unas células haploides y altamente especializadas que son los espermatozoides (Staub and Johnson, 2018). Durante su proceso de formación, los espermatozoides van reduciendo su citoplasma y perdiendo gran parte de sus orgánulos, a la vez que se condensa y estabiliza su material genético (Ward, 2010), haciendo que la célula sea transcripcionalmente inactiva (Eddy, 2006).

La **cabeza** del espermatozoide está compuesta por el acrosoma y el núcleo. El **acrosoma** es una vesícula exocitótica que contiene un grupo de enzimas hidrolíticas necesarias para la penetración de la zona pelúcida (Eddy, 2006; Flesch and Gadella, 2000; Gerton, 2002). El acrosoma también contiene una gran variedad de moléculas funcionales requeridas para la interacción entre el espermatozoide y el ovocito, y que facilitan el proceso de fertilización (Toshimori, 2003). El **núcleo**, que ocupa la mayor parte de la cabeza, contiene el material genético, que, como se ha comentado anteriormente, está altamente condensado por la acción de un grupo de proteínas básicas llamadas protaminas (Ward, 2010), que reemplazan a las histonas (Eddy, 2006).

El **flagelo** se encuentra conectado a la cabeza por el **cuello** y contiene la estructura motora y la reserva energética para hacer del espermatozoide una célula motil (Lehti and Sironen, 2017). En la **pieza intermedia** se encuentran ensambladas las mitocondrias, formando una hélice alrededor de las fibras densas externas (Lehti and Sironen, 2017). Estas mitocondrias son las encargadas de proporcionar energía en forma de ATP para la activación del movimiento a través de la vía de la fosforilación oxidativa (Eddy, 2006; Turner, 2003). Sin embargo, en la **pieza principal**, la vía de producción de ATP es la glicólisis, menos eficiente que la fosforilación oxidativa, pero fundamental, ya que no está claro que la difusión de ATP desde la pieza intermedia pueda proporcionar toda la energía que necesitan las regiones más distales del flagelo (Miki et al., 2004).

Debido a la limitada actividad biosintética del espermatozoide, su funcionalidad se encuentra vinculada a los cambios a nivel de su membrana plasmática (Toshimori, 2011, 2003). Los lípidos y las proteínas, tanto las transmembranales como las proteínas periféricas, están organizados en microdominios en la cabeza del espermatozoide que separan zonas con funciones específicas. Por ejemplo: el sitio de unión a la zona pelúcida está restringido al borde apical del acrosoma y el punto de inicio de la fusión de membranas

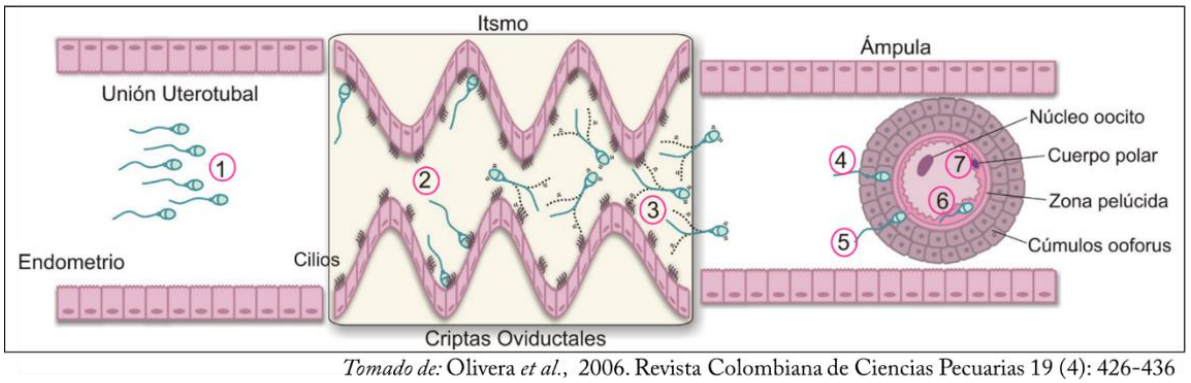
(acrosomal externa y plasmática) ocurre en el remanente de la membrana acrosomal que queda tras la reacción acrosómica, a la altura del borde pre-ecuatorial (Boerke et al., 2008).

Cuando los espermatozoides abandonan los testículos, continúan siendo funcionalmente inmaduros. Para poder adquirir la habilidad de fertilizar deben sufrir dos procesos de maduración post-testicular, el primero todavía en el tracto reproductor masculino, en su tránsito por el epidídimo, y el segundo ya en tracto reproductor de la hembra, la capacitación espermática. Durante la maduración epididimaria, los espermatozoides van incorporando proteínas secretadas por el epidídimo, y van perdiendo o reorganizando las que tenían. Las proteínas secretadas por la cabeza y cuerpo del epidídimo son las encargadas de favorecer la adquisición de la motilidad, la capacidad para unirse a la zona pelúcida, y para la fusión de membranas con el ovocito, mientras que las proteínas secretadas por la cola del epidídimo son las encargadas de mantener esa capacidad fecundante y la supervivencia de los espermatozoides durante su almacenamiento en este reservorio (Girouard et al., 2009). Además, van perdiendo el citoplasma residual, convertido en gota citoplásmica, y continúa la compactación de su cromatina. En el momento de la eyaculación, se mezclan con las secreciones que componen el plasma seminal, incorporando otros componentes, como factores descapacitantes.

2.4.3. Cambios sufridos por el espermatozoide en el tracto reproductor femenino

2.4.3.1. Capacitación espermática

Como se nombró antes, la capacitación es la segunda fase de maduración espermática y es un evento preparatorio para la fertilización en el tracto reproductor de la hembra (Chang, 1951). Es un proceso regulado intrínsecamente por reacciones pre-programadas dentro de la célula, pero que requieren ser activadas por eventos externos sin que exista un ligando específico (Bailey, 2010). La capacitación involucra una interacción dinámica entre los factores descapacitantes del plasma seminal y los factores estimulantes del ambiente uterino (Figura 2.7) (Nixon, 2006). La capacitación también puede inducirse *in vitro* en un medio bien definido y con unas condiciones de incubación adecuadas (Parrish et al., 1988).



Tomado de: Olivera *et al.*, 2006. Revista Colombiana de Ciencias Pecuarias 19 (4): 426-436

Figura 2. 7 Lugar donde suceden los eventos de capacitación espermática en condiciones *in vivo. Secuencia de los procesos que sufre el espermatozoide en el tracto reproductivo de la hembra: 1) Activación, 2) Capacitación, 3) Hiperactivación, 4) Reconocimiento entre gametos, 5) Reacción acrosomal, 6) Adhesión y 7) Fusión. Tomado de* (Olivera et al., 2006)

Uno de los eventos principales durante la capacitación es la perdida de colesterol desde la membrana. Para facilitar este proceso, el medio oviductal contiene albumina, lipoproteínas de alta densidad, y apolipoproteinas, que se encargan de secuestrar el colesterol, incrementando la fluidez de la membrana y su permeabilidad al calcio (Aitken and Nixon, 2013). Como resultado de estos cambios en su fluidez, la membrana espermática sufre una serie de pérdidas y redistribución de las proteínas y lípidos dentro de los diferentes microdominios (Boerke et al., 2008). El incremento de calcio (Ca^{2+}) y bicarbonato (HCO_3^-) intracelular activa la enzima adelinato ciclasa soluble (ACs) (Gadella and Luna, 2014; Xie et al., 2006). Esto da lugar a la producción de adenosín-monofosfato cíclico (AMPc), culminando en la activación de la proteína quinasa A (PKA), que promueve la fosforilación de residuos de tirosinas en ciertas proteínas (Aitken and Nixon, 2013) (Figura 2.8.). Además de la tirosina quinasa, también se han identificado otras quinasas activadas durante la capacitación, como serina/ treonina proteína quinasas, que regulan estos eventos (Visconti et al., 2011). Además, para que la capacitación tenga lugar, es necesario que exista un incremento en los niveles de especies reactivas de oxigeno (ROS) (Aitken and Curry, 2011).

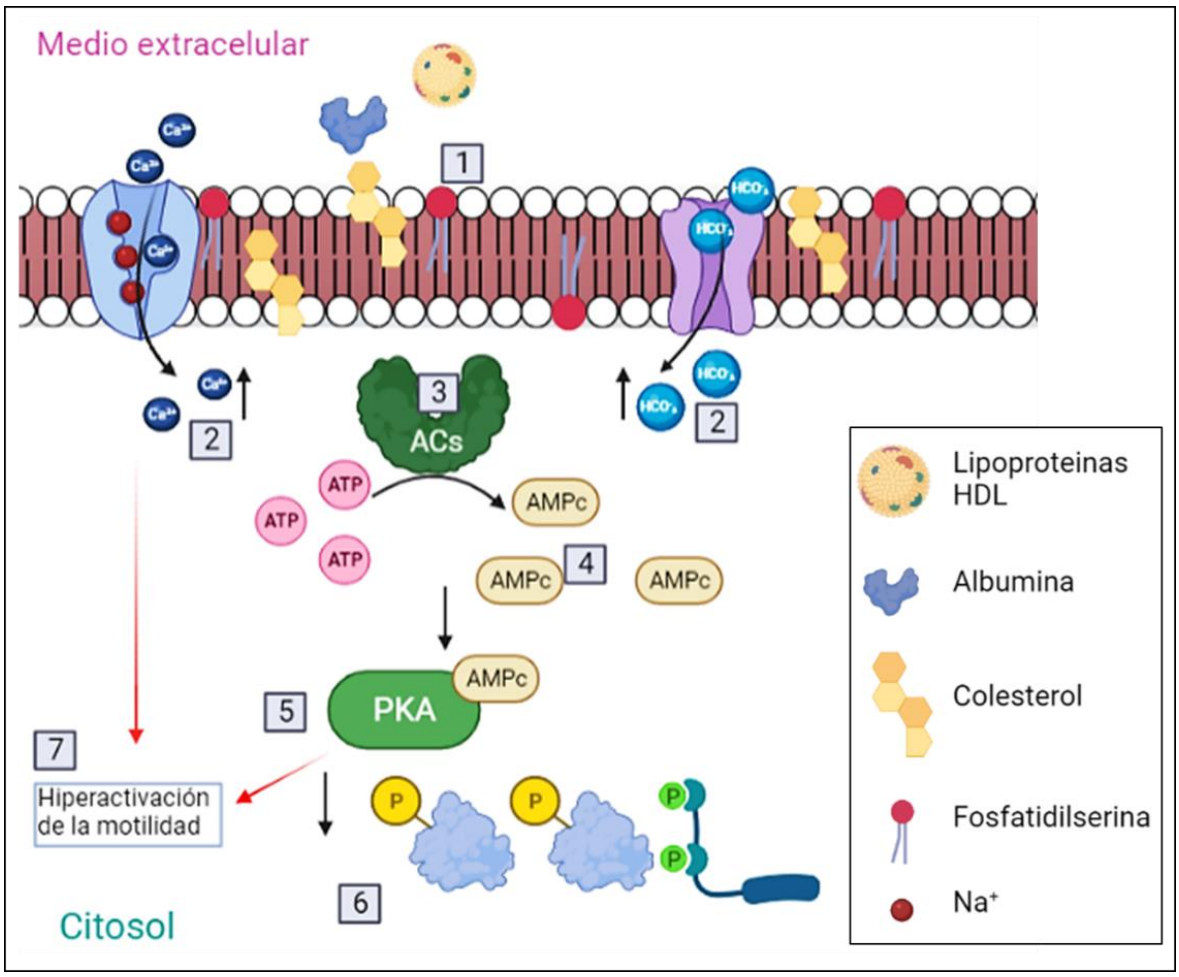


Figura 2. 8 Eventos de la capacitación espermática..1) Secuestro de colesterol por el medio ambiente uterino; 2) incremento en la concentración de calcio y bicarbonato intracelular; 3) activación de la adenilato ciclasa soluble (ACs); 4) incremento en la producción de AMPc; 5) activación de la proteína quinasa A (PKA); 6) Fosforilación de proteínas en residuos de tirosina; 7) El incremento de calcio y bicarbonato, junto con la activación de la PKA son rutas que inducen la hiperactivación de la motilidad. Imagen realizada en Biorende.com, basado en (Gadella and Luna, 2014)

Después de la activación de la PKA, la inducción especifica de fosforilación de residuos de tirosina en las proteínas del flagelo da lugar a un cambio en el patrón de movimiento flagelar (Turner, 2006). Esto da lugar a la hiperactivación de la motilidad, que es un cambio en la frecuencia de batido del flagelo dando lugar a movimientos vigorosos y perdida de la linealidad en la trayectoria (S T Mortimer and Maxwell, 1999). Este proceso ayuda a los espermatozoides a liberarse de las criptas oviductales y ayuda a penetrar la matriz extracelular del ovocito (Talevi and Gualtieri, 2010). La hiperactivación de la motilidad es un evento paralelo a la capacitación en la que intervienen canales específicos de calcio en el flagelo, que permiten el incremento del calcio intracelular, el cual se une a las dineínas del axonema para favorecer el movimiento asimétrico (Visconti et al., 2011).

2.4.3.2 Reacción acrosómica y unión a la zona pelúcida

La reacción acrosómica es un proceso de exocitosis enzimática que se requiere para atravesar la zona pelúcida (ZP) del ovocito. Como resultado de la capacitación, la membrana espermática adquiere la capacidad de acoplarse con la membrana acrosomal externa, liberando el contenido acrosomal (Gadella and Luna, 2014). Este proceso es inducido por la unión a glicoproteínas especie-específicas de la zona pelúcida, denominadas en general como ZP1, ZP2 y ZP3 (Balbach et al., 2020). A partir de esa unión, se provoca una despolimerización de la actina entre la membrana acrosomal externa y la membrana plasmática permitiendo su fusión (Balbach et al., 2020).

Una vez que el contenido acrosomal se libera, este se encarga de digerir la ZP (Gadella, 2008; Lasserre et al., 2003). Así, el espermatozoide puede entrar al espacio perivitelino y completar la fusión entre el remanente de la membrana espermática a nivel de la región ecuatorial de la cabeza, y el oolema (Gadella, 2008).

2.5 La melatonina

Como hemos visto en el apartado anterior, la melatonina es la hormona encargada de regular la estacionalidad reproductiva en ovinos de latitudes medias. La melatonina es una hormona lipofílica que se distribuye ampliamente en el sistema nervioso, la sangre y tejidos periféricos de los mamíferos.

La melatonina es una indolamina presente en todos los taxones (Zhao et al., 2019). La función inicial de la melatonina fue probablemente la de eliminar los radicales libres generados durante los procesos metabólicos y de fotosíntesis, tras la adaptación al uso del oxígeno atmosférico en los primeros estadios de la evolución de los microorganismos (Tan et al., 2015). Se especula que la melatonina evolucionó en bacterias que fueron fagocitadas por eucariotas primitivos. Según la teoría endosimbiótica, las bacterias ingeridas finalmente desarrollaron una asociación simbiótica con sus eucariotas hospedadores. Así, las α-proteobacterias ingeridas evolucionaron a mitocondrias, mientras que las cianobacterias se convirtieron en cloroplastos, y ambos orgánulos conservaron su capacidad para producir melatonina. Dado que estos orgánulos han persistido hasta el día de hoy, todas las especies que existen actualmente pueden continuar sintetizando melatonina en sus mitocondrias (animales y plantas) y cloroplastos (plantas) donde funciona como antioxidante (Zhao et al., 2019). La melatonina es muy eficiente en la eliminación de radicales libres, ya que tiene la habilidad de donar un electrón o un átomo de hidrogeno dependiendo del tipo de radical (Tan et al., 2002, 2000). Además, puede interactuar con los compuestos oxidativos produciendo metabolitos que también tienen actividad antioxidante (Zhao et al., 2019).

Adicionalmente, durante la evolución, esta hormona ha ido adquiriendo diversas funciones en los organismos entre las que destaca la modulación de los ritmos circadianos, la regulación de la estacionalidad reproductiva en mamíferos, una acción antiinflamatoria, antitumoral y estimuladora del sistema inmunológico en animales y de la tolerancia a estrés ambiental en plantas y hongos, entre otras (Zhao et al., 2019).

Como hemos visto anteriormente, la regulación de los ritmos biológicos es una de sus funciones claves. En los organismos unicelulares primitivos, la producción de radicales libres era mayor durante la fotofase (horas de luz) que en la escotofase (horas de oscuridad). por lo que la melatonina era consumida durante el día para la eliminación de esos radicales libres, dando como resultado un ritmo diurno primitivo de la melatonina. Esta utilización diferencial de la melatonina como antioxidante reflejaba de manera precisa los cambios en el ciclo de luz/oscuridad, por lo que muchos organismos adoptaron la melatonina como sistema de señalización fotoperiódico (Zhao et al., 2019). En los vertebrados, la glándula pineal evolucionó como lugar de producción y secreción cíclica de melatonina, lo que proporciona a todas las células del organismo información sobre los ciclos de luz/oscuridad (Zhao et al., 2019).

2.5.1 Biosíntesis y degradación de la melatonina en mamíferos.

En animales, la melatonina (N-acetil-5-metoxi-triptamina ($C_{13}H_{16}N_2O_2$) se produce principalmente en la glándula pineal y se forma a partir del aminoácido esencial triptófano (Figura 2.9). El principal sitio de producción de melatonina en las células es la mitocondria, donde a partir del triptófano que se hidroliza por la enzima triptófano hidroxilasa (TPH) da lugar a la 5-hidroxitriptófano. Posteriormente participan las enzimas L-aminoácido aromático descarboxilasa (AADC), arialkilamina N-acetiltransferasa (AANAT), y acetilserotonina O-meltiltransferasa (ASMT) para dar lugar a la melatonina (Tan et al., 2016).

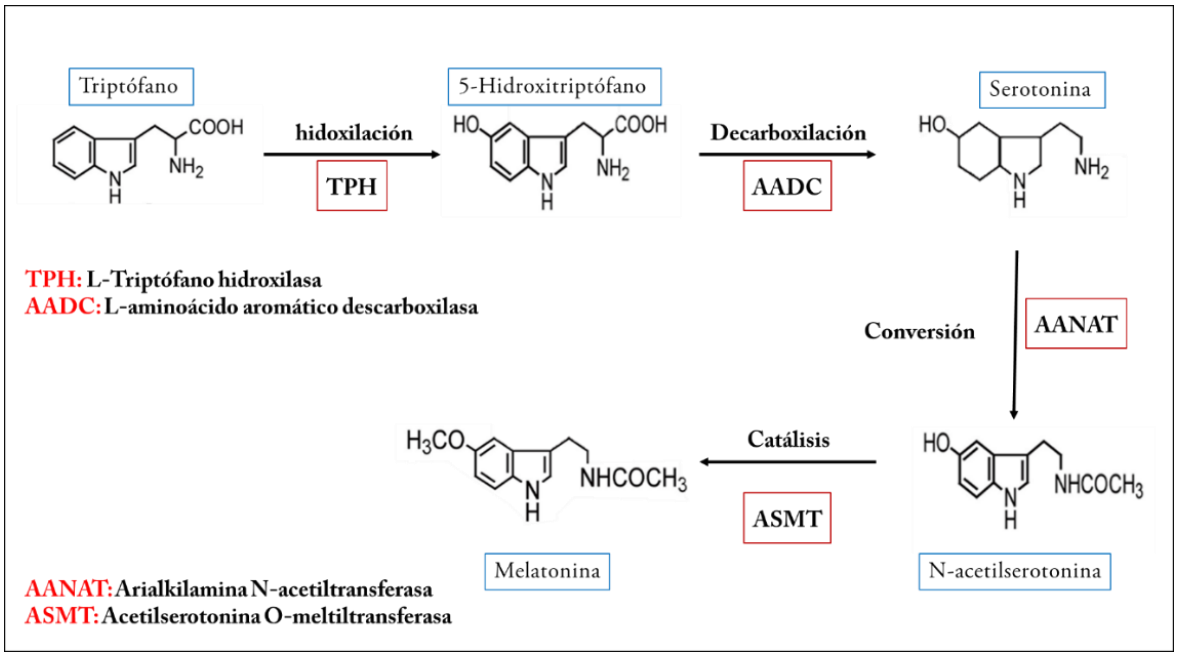


Figura 2. 9 Biosíntesis de la melatonina a partir del triptófano en mamíferos propuesta por (Tan et al., 2016)

Además, la melatonina también se produce en tejidos extrapineales, aunque esta melatonina extrapineal no está regulada por el fotoperiodo y no es liberada a la circulación sanguínea, sino que actúa a nivel local (Acuña-Castroviejo et al., 2014).

Una vez que la melatonina es liberada en el torrente sanguíneo o a nivel local, esta puede ser metabolizada por un proceso enzimático, o por su interacción con ROS y NOS (Especies reactivas de nitrógeno) (Galano et al., 2013; Tan et al., 2015). La mayor parte de la melatonina del plasma sanguíneo es metabolizada en el hígado mediante la vía enzimática de citocromo P_{450} mono-oxigenasa, dando origen a la 6-hidroximelatonina, que es eliminada a través de la orina. En los demás tejidos el proceso también es enzimático, y las principales enzimas descritas son: indoleamina 2,3-dioxigenasa (IDO), mieloperoxidasa (MPO) y eosinófilo peroxidasa (EPO), degradando la melatonina hasta 1-N_2-formil-5-metoxiquinuramina (AFMK) (Tan et al., 2007).

Además, la melatonina también se metaboliza mediante la interacción con productos oxidativos en una cascada de reacción, ya que sus propios metabolitos pueden interactuar con otros compuestos oxidativos neutralizándolos (Tan et al., 2015, 2007). Así, como producto de esta reacción se obtiene AFMK, y a partir de él se puede producir N_1-acetil-5-metoxiquinuramina (AMK) (Galano et al., 2013). Ambos metabolitos, tienen una potente acción protectora frente a los efectos nocivos de la peroxidación lipídica y el daño oxidativo del ADN en células neuronales (Manda et al., 2008).

2.5.2 Mecanismo de acción de la melatonina

En mamíferos, la melatonina puede actuar a través de la unión a sus receptores en la membrana de las células, o de forma independiente como antioxidante (Zhao et al., 2019). Así, la melatonina puede actuar directamente sobre los radicales libres cuando esta hormona se encuentra en cercanías al sitio de producción de ROS (Zhao et al., 2019). Tanto la melatonina pineal como la extra-pineal colaboran en el mantenimiento del balance oxidativo; sin embargo, mientras que en el tejido extra-pineal la melatonina puede alcanzar concentraciones micromolares, la melatonina circulante de origen pineal solo llega a concentraciones de 0,5 nM (Acuña-Castroviejo et al., 2014). De hecho, algunos autores proponen un papel diferente para melatonina pineal y la extra-pineal en mamíferos: agente sincronizador de las funciones del organismo para la primera, y agente protector de las células contra la oxidación y el daño oxidativo en el caso de la segunda, ya que ambas difieren en su mecanismo de acción y su regulación (Acuña-Castroviejo et al., 2014).

Tras la diversificación de las funciones de la melatonina en organismos pluricelulares, también fue necesaria la evolución de sus sitios de unión/receptores, así como de una vía de transducción de señales (Tan et al., 2010). Algunas de las acciones conocidas de la melatonina son mediadas por la unión a receptores acoplados a proteínas G (*G-protein-coupled receptors* o GPCRs). Estos receptores se denominaron receptor de melatonina 1 (MT_1, anteriormente MT1a) y receptor de melatonina 2 (MT_2, anteriormente MT1b), y según su afinidad por esta hormona se clasificaron como de alta y baja afinidad, respectivamente (Dubocovich and Markowska, 2005). En mamíferos existe un tercer receptor relacionado con la melatonina, que ha sido denominado GPR50. Este receptor pertenece a los denominados receptores huérfanos y, aunque la melatonina no puede unirse directamente

él, éste si puede regular su actividad tras formar heterodímeros con el receptor MT_1 (Gautier et al., 2018; Levoye et al., 2006). De hecho, se ha postulado que este receptor evolucionó a partir del receptor Mel1c (MT_3) reptiliano/aviar, y perdió su capacidad de unión a la melatonina en el proceso evolutivo, mientras que el Mel1c presente en anfibios, aves y peces, mantiene su afinidad por la melatonina (Gautier et al., 2018).

Así, la melatonina, a través de sus receptores, ejerce funciones como la modulación de los ritmos circadianos (MT_1/MT_2) (Dubocovich et al., 1998), de la vasoconstricción (MT_1) y vasodilatación (MT_2) (Doolen et al., 1998), y de la respuesta inflamatoria (MT_2), mejora de la inmunidad humoral y celular (MT_2) (Srinivasan et al., 2008), e inducción de la diferenciación celular (Bordt et al., 2001).

Como otros miembros de la familia de receptores GPCR, los receptores MT_1 y MT_2 son capaces de formar homodímeros y heterodímeros estables (Ayoub et al., 2004). La formación de heterodímeros es más habitual que la de homodímeros, pero los sitios de unión de la melatonina en ambos receptores se mantienen funcionales dentro del heterodímero (Dubocovich et al., 2010). Además, la actividad de estos receptores puede ser regulada mediante la desensibilización a la melatonina después de una larga exposición (Hazlerigg et al., 1993), internalización, desacoplamiento, eventos de fosforilación o una disminución en el número de receptores (Witt-Enderby et al., 2003).

Por otro lado, la melatonina también es capaz de atravesar directamente la membrana plasmática debido a su naturaleza anfipática, y llegar al citosol. Allí puede interaccionar con determinadas moléculas y compuestos o incluso unirse a receptores presentes en la membrana nuclear. En el citosol, la melatonina puede unirse con la calmodulina (CaM), implicada en el metabolismo del calcio, lo que permite regular la actividad de numerosas enzimas celulares (Zhao et al., 2019). A nivel nuclear, la melatonina también puede unirse a la familia de receptores del ácido retinoico ($ROR_{\alpha1}$, $ROR_{\alpha2}$ y RZR_{β}), aunque estos tienen menor afinidad por la melatonina que los receptores de membrana (Carlberg, 2000; Hardeland et al., 2006).

2.5.3 Melatonina en la reproducción ovina

Como se ha mencionado anteriormente, la melatonina pineal actúa en la regulación de la estacionalidad reproductiva. De esta forma, puede tener efectos inhibitorios en reproductores de días largos, como el hámster y el caballo, cuya época reproductiva es en primavera y verano, o efectos estimuladores en reproductores de días cortos, con actividad reproductiva en otoño, como es el caso de la mayoría de los pequeños rumiantes (oveja, cabra, ciervo) (Reiter et al., 2009). Los cambios en la secreción de melatonina nocturna, como respuesta a los cambios en el fotoperiodo, son el mensaje pasivo para que el eje hipotálamo-hipofisario-gonadal identifique en qué periodo del año se encuentra (Reiter et al., 2009). Sin embargo, el fotoperiodo es la señal común que determina el cambio de estaciones en los mamíferos de las latitudes más altas hasta el medio-trópico (Bronson, 2009), ya que las especies que habitan en zonas tropicales, más cercanas al ecuador y con

fotoperiodo constante, presentan menor o nula estacionalidad reproductiva, como hemos visto (Chemineau et al., 2008).

De este modo, la melatonina actúa sobre el control de la secreción de la GnRH, y consecuentemente con la liberación de LH y FSH, responsables de la actividad testicular en machos (Yu et al., 2018). Se han identificado receptores de melatonina en las neuronas del hipotálamo, en la hipófisis, y en los testículos y glándulas sexuales accesorias (Dubocovich and Markowska, 2005). En el hipotálamo, donde se modula la actividad reproductiva, la densidad de los receptores de melatonina podría determinar la sensibilidad a los cambios en el fotoperiodo. Así, especies (o razas) con altos niveles de receptores de melatonina en el SCN podrán utilizar la melatonina como clave en los ritmos circadianos y circanuales, mientras que especies (o razas) con bajos niveles, puede que utilicen otras claves de activación de la reproducción (Witt-Enderby et al., 2003).

Además, se han identificado los receptores de melatonina en distintos tejidos y órganos del tracto reproductor masculino. En nuestro grupo se identificaron ambos receptores, MT_1 y MT_2, en el tracto reproductor del morueco, especialmente en los testículos, la ampolla, las vesículas seminales y los conductos deferentes (Marta González-Arto et al., 2017). Previamente se había descrito la capacidad de síntesis de melatonina de las células de Leydig, espermatocitos y espermátidas del testículo ovino (González-Arto et al., 2016a). Así, aunque la melatonina de la glándula pineal modula la estacionalidad reproductiva ovina, la presencia de receptores a lo largo del tracto reproductor y la capacidad de síntesis de melatonina extrapineal del testículo, apuntan a una acción directa de la melatonina sobre la eficiencia reproductiva de esta especie. Como ya se ha comentado anteriormente, en la especie ovina la melatonina está presente en el plasma seminal. En razas situadas en latitudes medias, sujetas a cambios en el fotoperiodo, esta concentración es variable a lo largo del año y correlaciona con los niveles de testosterona y la actividad de las enzimas antioxidantes (Casao et al., 2010a). Esta melatonina del plasma seminal podría ser de origen pineal o testicular, aunque las evidencias hasta ahora apuntan hacia lo primero. Así, el uso de implantes subcutáneos de melatonina durante la época no reproductiva en moruecos de latitudes medias da lugar a un incremento en la concentración de melatonina en este fluido, posiblemente de origen sanguíneo (Casao et al., 2010c). Además, también se observó efecto sobre los niveles de otras hormonas en sangre (Kokolis et al., 2000; Lincoln and Ebling, 1985; Webster et al., 1991), la calidad espermática (Casao et al., 2010c; Kaya et al., 2000), parámetros testiculares (Rosa et al., 2012), la espermatogénesis y la fertilidad (Palacín et al., 2008), principalmente por su efecto estimulante del eje hipotálamo-hipofisario-testicular. También se ha descrito que el uso de estos implantes mejora la hemodinámica y la ecotextura testicular en la especie ovina (El-Shalofy et al., 2021), al igual que ocurre en caprino (Samir et al., 2020). Los implantes con melatonina también han resultado efectivos en la mejora de la calidad seminal incluso en animales ubicados en zonas tropicales, como demuestra un estudio llevado a cabo con búfalos (Ramadan et al., 2019).

La melatonina no solo actúa sobre la reproducción ovina modulando el eje hipotálamo-hipofisario-gonadal, sino que, gracias a su actividad antioxidante, la melatonina

también puede proteger a las células de los órganos sexuales, a los gametos, y al embrión durante su desarrollo (Abecia et al., 2019). En el caso de la hembra, la melatonina favorece el desarrollo de los folículos antrales y la maduración ovocitaria (Barros et al., 2020a, 2020b). Esta capacidad protectora contra el estrés oxidativo y apoptosis también ha sido observada *in vitro* en otras especies (Remião et al., 2016; Riaz et al., 2019).

Finalmente se ha observado que la adición de melatonina a cultivos *in vitro* de células madre espermatogónicas ovinas favorece su diferenciación a células haploides similares a espermatozoides y aumenta la concentración de testosterona en el medio de cultivo (Deng et al., 2016).

2.5.4 Acción de la melatonina en el espermatozoide ovino

Al igual que la melatonina exógena, administrada al morueco *in vivo* en forma de implantes, mejora la calidad seminal, este efecto se observa también *in vitro* sobre los espermatozoides, cuando se añade melatonina a los medios de incubación. En el caso de los espermatozoides de morueco, nuestro grupo fue el primero en evidenciar los efectos de la melatonina, sobre la apoptosis y la capacitación espermática, demostrando además que estos efectos eran dependientes de la concentración. Así, a concentraciones bajas de melatonina (100 pM) se observó un efecto promotor de la capacitación, mientras que a concentraciones altas (1 μM), se producía una acción descapacitante y anti-apoptotica (Casao et al., 2010b). De esta forma, altos niveles de melatonina, como los que existen en el plasma seminal, podrían prevenir la capacitación, posiblemente mediante la eliminación de ROS; sin embargo, a bajas concentraciones, como las que existen en el tractor reproductor de la hembra (Olcese, 2020), la melatonina podría controlar, sin llegar a eliminar, las especies reactivas de oxígeno y de nitrógeno, ya que son necesarias para que se produzca la capacitación (de Lamirande and O'Flaherty, 2008). Esta dualidad en el efecto de la melatonina se corroboró posteriormente tras confirmarse que, a bajas concentraciones de melatonina (100 pM), se estimulaba la producción de AMPc y finalmente la capacitación, mientras que altas concentraciones (1 μM) producían una disminución de los niveles de ROS (Gimeno-Martos et al., 2019). De igual forma, añadida a una concentración de 1 μM en el medio de congelación, la melatonina protege a los espermatozoides ovinos contra el daño causado por frio, de forma que presentan mejores valores de motilidad, integridad del ADN, concentración de ATP capacidad fecundante tras la descongelación (Succu et al., 2011).

La melatonina podría ejercer sus efectos, sobre todo su efecto antioxidante, tras atravesar libremente la membrana plasmática de los espermatozoides. Sin embargo, es importante señalar que los espermatozoides también presentan receptores para la melatonina y que los receptores MT_1 y MT_2 están presentes en la membrana espermática de especies tanto estacionales, como el ovino (Casao et al., 2012), como no estacionales (González-Arto et al., 2016c), lo que sugiere que podrían estar implicados en la regulación de las funciones espermáticas. Así, los efectos anti-apoptoticos de la melatonina en espermatozoides humanos están mediados por su unión al receptor MT_1 (Espino et al., 2011), mientras que los efectos sobre la capacitación en espermatozoides ovinos, estarían

modulados por el MT_2 (González-Arto et al., 2016b). Además, estos receptores pueden cambiar su densidad y distribución durante el proceso de capacitación *in vitro*, al menos en la especie ovina (Casao et al., 2012).

A la vista de los antecedentes expuestos, se observa que los mecanismos de acción de la melatonina son importantes en la reproducción del morueco, ya sea a nivel de la regulación de la estacionalidad, como a nivel directo sobre los espermatozoides. Sin embargo, prácticamente todos estos estudios se han realizado en animales ubicados en latitudes medias con fotoperiodo variable, y es posible que estos mecanismos no sean los mismos en moruecos situados en la zona ecuatorial y sometidos a fotoperiodo constante. Por lo tanto, se hace necesario ampliar los estudios de los efectos de la melatonina sobre la reproducción ovina en estas condiciones fotoperiódicas.

3 Hipótesis y Objetivos

Como se ha indicado anteriormente, los cambios en la longitud del día/noche, reflejados en la secreción de melatonina nocturna, sirven como mensajeros para dar inicio a la estación reproductiva en razas ovinas estacionales (Bittman et al., 1983), como la raza Rasa Aragonesa. Sin embargo, en zonas ecuatoriales (entre 10º Norte y 10º Sur), donde la longitud del día es igual a lo largo del año (12L:12D), la reproducción estaría regulada por factores externos como el ciclo anual de lluvias y disponibilidad de alimento (Rosa and Bryant, 2003), haciendo que el proceso de la activación en el eje hipofisario-gonadal difiera al descrito en ovinos que se encuentran en latitudes medias o altas.

Adicionalmente, la información sobre la reproducción ovina en zonas ecuatoriales no es muy amplia, especialmente en los países latinoamericanos donde la producción ovina es considerada una economía de subsistencia para campesinos y pequeños productores. Como regla general, la producción es extensiva, los animales no son suplementados con pienso, por lo tanto, dependen directamente del pastoreo. Colombia es un país ecuatorial con apenas variación del fotoperiodo a lo largo del año. Sin embargo, en algunas zonas, como la región andina, sí existen variaciones en el régimen de precipitaciones a lo largo del año, alternándose dos periodos de lluvias y dos de sequias, que afectan la disponibilidad de alimento.

Nuestro grupo de investigación ha estudiado el efecto de la melatonina sobre los diferentes aspectos de la reproducción de moruecos ubicados en latitudes medias que presentan estaciones astronómicas diferenciadas. Estos estudios demostraron que la concentración de melatonina en el plasma seminal era variable a lo largo del año, y su elevación correspondía con la época de mayor actividad reproductiva de estos animales (Casao et al., 2010a). También que el uso de melatonina exógena en forma de implantes tenía efectos positivos en la reproducción de los moruecos (Casao et al., 2013), y que su adición directa a los espermatozoides mejoraba la funcionalidad espermática y modulaba la capacitación (Casao et al., 2010b; Gimeno-Martos et al., 2019). Además, se demostró la existencia de receptores para melatonina en la membrana de los espermatozoides ovinos (Casao et al., 2012) y que muchas de sus acciones están mediadas por la unión de la hormona a los mismos (González-Arto et al., 2016b). Por otro lado, también se demostró la síntesis de melatonina extrapineal en diferentes tejidos del tracto reproductor masculino (González-Arto et al., 2016a).

En esta tesis se planteó como hipótesis que, aunque la melatonina no regule la estacionalidad reproductiva en los moruecos de la zona ecuatorial, esta hormona ejerce una función importante en la reproducción de estos animales.

Para elucidar esta hipótesis se planteó como **objetivo central evaluar el efecto del fotoperiodo constante y de la melatonina en las características seminales de moruecos ubicados en la zona ecuatorial**. Para llevarlo a cabo se plantearon los siguientes objetivos específicos:

1. Evaluar la concentración de melatonina en el plasma seminal de los moruecos ubicados en la zona ecuatorial a lo largo del año, al igual que la concentración de

testosterona y la activad de las enzimas antioxidantes, así como la posible correlación existente entre ellos.

2. Dilucidar si el régimen fotoperiódico puede afectar la composición del plasma seminal de los moruecos.

3. Determinar el origen de la melatonina del plasma seminal tanto en moruecos ubicados en latitudes medias como en la zona ecuatorial.

4. Identificar la presencia y la distribución de los receptores de melatonina MT_1 y MT_2, en la membrana de espermatozoides de moruecos de la zona ecuatorial y evaluar su posible variación entre las épocas de lluvia y de sequía.

5. Evaluar el efecto de la melatonina sobre la capacitación de espermatozoides de moruecos de la zona ecuatorial. Para ello hubo que:

   5.1. Estudiar previamente los requisitos y la respuesta a la capacitación *in vitro* de los espermatozoides de moruecos de la zona ecuatorial obtenidos en época de lluvia o de sequía

   5.2. Estudiar la respuesta de los espermatozoides a la adición de melatonina al medio de capacitación, evaluando su posible variación entre las épocas de lluvia y de sequía.

4 Materials and Methods

4.1 Reagents

Unless otherwise stated, all the reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA (a subsidiary of Merk KGaA, Darmstadt, Germany).

4.2 Semen collection and rams location

All animals used in this study were handled in strict accordance with the requirements of the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and the Colombian Animal Protection Regulations (Law 84/1989, modified by Law 1774/2016).

4.2.1 Rams in the Mediterranean climate

First ejaculates from intact and vasectomized *Rasa Aragonesa* rams (three and two, respectively) were collected once a week using an artificial vagina, for three months during the reproductive season (September to November). In order to eliminate individual differences and obtain enough sample volume for all the analyses, daily ejaculates from the rams of each experimental group (intact or vasectomized) were pooled and processed together (paper 2). All experimental procedures were performed under Project License PI19/17 approved by the Ethics Committee for Animal Experiments of the University of Zaragoza (approval date: 24 May 2017). The males were maintained under uniform nutritional conditions and natural photoperiod at the experimental farm of the University of Zaragoza, Spain (41°38'05.80" N 0°51'35.20" W). The local amplitude of the photophase during the period of study (September to November) varied from 12 h 35 min (i.e. 11 h 25 min of darkness) at the beginning of the experiment to 7 h 48 min (i.e. 16 h 12 min of darkness) at the end, i.e., 4 h 47 min of difference between the longest and the shortest day of this period.

4.2.2 Rams under Equatorial Photoperiod

Semen was obtained using an artificial vagina following the protocol approved by the Bioethics Committee of the National University of Colombia (CB-074-2014). Samples were obtained from 12 mature rams (2–5 years old) of three breeds (four Creole, four Romney Marsh and four Hampshire sires), in addition, two vasectomized males were included (one Creole and one Romney Marsh). Two successive ejaculates were collected once a week from each ram throughout the year (paper 1), or for three months (September to November) (paper 2) or during the rainy (April and May) and dry (June and July) seasons (paper 3 y 4). Ejaculates from rams of the same breed were pooled for all analysis in order to avoid individual differences. Rams were housed together with uniform nutritional conditions at the Centro de Investigación, Desarrollo Tecnológico y Extensión Ovina, CIDTEO), belonging to National University of Colombia, located in Mosquera (4°40’57’’ N 74°12’50’’ W) at 2510 m above sea level. The local amplitude of the photophase throughout the year varied from 12 h 21 min (i.e. 11 h 39 min of darkness) in the summer solstice to 11 h 49 min (i.e 12 h 11 min of darkness) in the winter solstice, i.e., 32 min of difference between the longest and the shortest day of the year.

4.3 Seminal plasma extraction and analysis

Seminal plasma was extracted by centrifugation at 7500 *x g* for 10 min in a microfuge at 4 °C (Eppendorf Centrifuge 5430R, Hamburg, Germany). The supernatant was centrifuged again, and the seminal plasma was then recovered and filtered through a 0.22 µm Millipore filter (Merck KGaA, Darmstadt, Germany). After adding 10% (v/v) protease and phosphatase inhibitor, seminal plasma was aliquoted and kept at -20 °C until analysis.

4.3.1 Protein analysis

The seminal plasma protein concentration was determined by Bradford colourimetric method (Bradford, 1976). This method is based on coloured complexes formed by Coomassie Brilliant Blue dye and sample proteins in an acid environment. For protein quantification, 2 µL of seminal plasma samples or known standards of bovine serum albumin (BSA) were loaded in a 96-wells microplate with 100 µL dye reagent (Quick Start Bradford protein assay, Bio-Rad Laboratories Ltd, Hercules, CA, USA). After 15 min of incubation, absorbance was measured at 595 nm (Tecan Spectrafluor plus, Tecan Group Ltd, Männedorf, Switzerland).

4.3.2 Hormonal analysis of seminal plasma

4.3.2.1 Melatonin concentration in seminal plasma

Melatonin concentrations in ram seminal plasma were quantified using a commercial competitive ELISA immunoassay (Direct saliva melatonin ELISA kit, Bühlmann Laboratories AG, Schönenbuch, Switzerland; sensitivity: 0.5 pg/mL, intra-assay variability: 5.2%, inter-assay variability: 11.2%), following the manufacturer's instructions. Briefly, 100 µL of each sample, blanks, controls, and calibrators were loaded in duplicate in a microtiter plate coated with an anti-melatonin antibody and incubated for 16–20 hours at 2–8 °C. After incubation, 50 µL of biotinylated melatonin were added to each well and incubated for another 3 hours at 2–8 °C. After incubation, the microtiter plate was washed three times with 300 µL of washing buffer per well. Then, 100 µL of streptavidin-conjugated horseradish peroxidase (HRP) were loaded into the wells and incubated for a further 60 min in a plate rotator set at 600 rpm at room temperature (RT). The wells were rewashed three more times using at least 300 µL of washing buffer, and 100 µL of tetramethylbenzidine substrate (TMB) were added to each well and incubated for 30 min in a plate rotator at 600 rpm at RT and protected from direct light. Finally, after incubation, 100 µL of 0.25 M SO_4H_2 solution (stop solution) were added, and a chromophore was formed in inverse proportion to the amount of melatonin present in the sample. After adding the stop solution, the colour turns from blue to yellow, and absorbance was measured on a microtiter plate reader (Tecan Spectrafluor plus, Tecan Group Ltd, Männedorf, Switzerland) at 450 nm within the next 10 min.

4.3.2.2 Testosterone concentration in seminal plasma

Testosterone concentrations in ram seminal plasma were quantified by use of a total testosterone commercial ELISA kit assay, based on the principle of competitivity binding (Testo-Easia, DiaSource Europe, Nivelles, Belgium; sensitivity: 0.05 ng/mL; intra-assay variability: 4.8%, inter-assay variability: 7.1%), following the manufacturer's instructions. First, 50 µL of each sample, control and calibrator, along with 100 µL of testosterone labelled with HRP, were loaded in duplicate in a microtiter plate coated with an anti-testosterone specific antibody and incubated for 1 hour at RT. After incubation, the wells were washed three times with 400 µL of wash solution per well. Then, 100 µL of the chromogenic substrate TMB were added to each well and incubated for 30 min at RT, protected from direct light. After incubation, 100 µL of 0.2 M HCl solution (stop solution) were added. The colourimetric reaction was stopped by the addition of the stop solution, and optical density of the resulting yellow product was measured on a microtiter plate reader (Tecan Spectrafluor plus, Tecan Group Ltd, Männedorf, Switzerland) at 450 nm within 10 min.

4.3.3 Antioxidant activity of seminal plasma

The seminal plasma antioxidant defence system was assessed by determining the activity of the following enzymes: glutathione reductase (GRD), glutathione peroxidase (GPx) and catalase (CAT). Each enzyme was evaluated by the protocol used previously described (Marti et al., 2007), but adapted for the microtiter plate (Casao et al., 2013). All samples were loaded in duplicate and analysed the same day.

4.3.3.1 Glutathione reductase (GRD. EC.1.6.4.2)

The GRD activity was evaluated by measuring the decrease in absorbance due to NADPH oxidation as a consequence of the GSSG (oxidised glutathione) reduction (Figure 4.1).

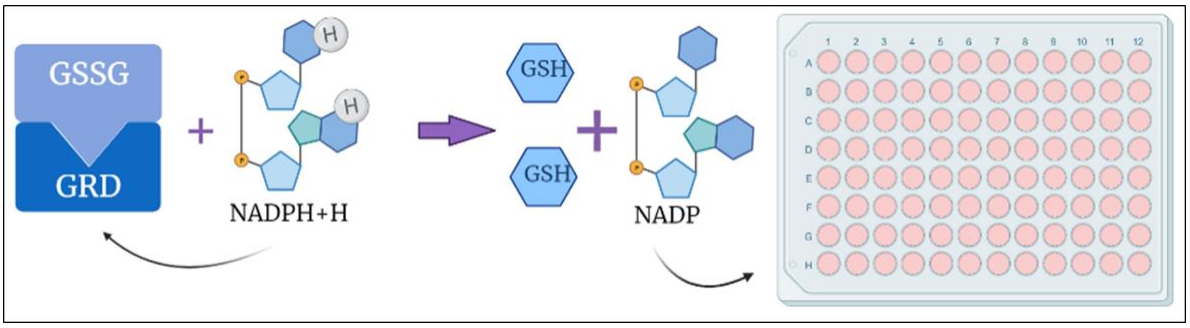


Figure 4. 1 Enzymatic reaction for the evaluation of glutathione reductase activity.GSSG: oxidised glutathione, GRD: glutathione reductase, GSH: reduced glutathione

The reaction mixture contained 300 mM sodium phosphate buffer at pH 7.2; 0.5 mM EDTA; 85 µM NADPH; 0.8 mM GSSG and 5 µL of seminal plasma to complete a final volume of 200 µL. The absorbance change at 340 nm was monitored for 3 min with a microtiter plate

reader (Tecan Spectrafluor plus, Tecan Group Ltd, Männedorf, Switzerland). One unit induces the oxidation of 1.0 µmole/min of NADPH at 25 °C, pH 7.2.

The enzymatic activity was determined as the difference in absorbance per minute between the first and the last measure, once the blank sample was subtracted, based on the following equation:

$$Activity = \frac{\Delta A_{340} \times DF}{6{,}22 \times V}$$

ΔA_{340}= change in absorbance
DF= Dilution factor
6,22=ε_{A340} of NADPH
V= Volumen

4.3.3.2 Glutathione peroxidase (GPx. EC.1.11.1.9)

The GPx activity was evaluated by measuring the oxidation of reduced glutathione (GSH) to oxidised glutathione (GSSG), catalysed by GPx and using ter-Butylhydroperoxide (t-BuO_2H) as an electron acceptor, coupled to the recycling of GSSG to GSH utilising glutathione reductase (GRD) and NADPH (Figure 4.2).

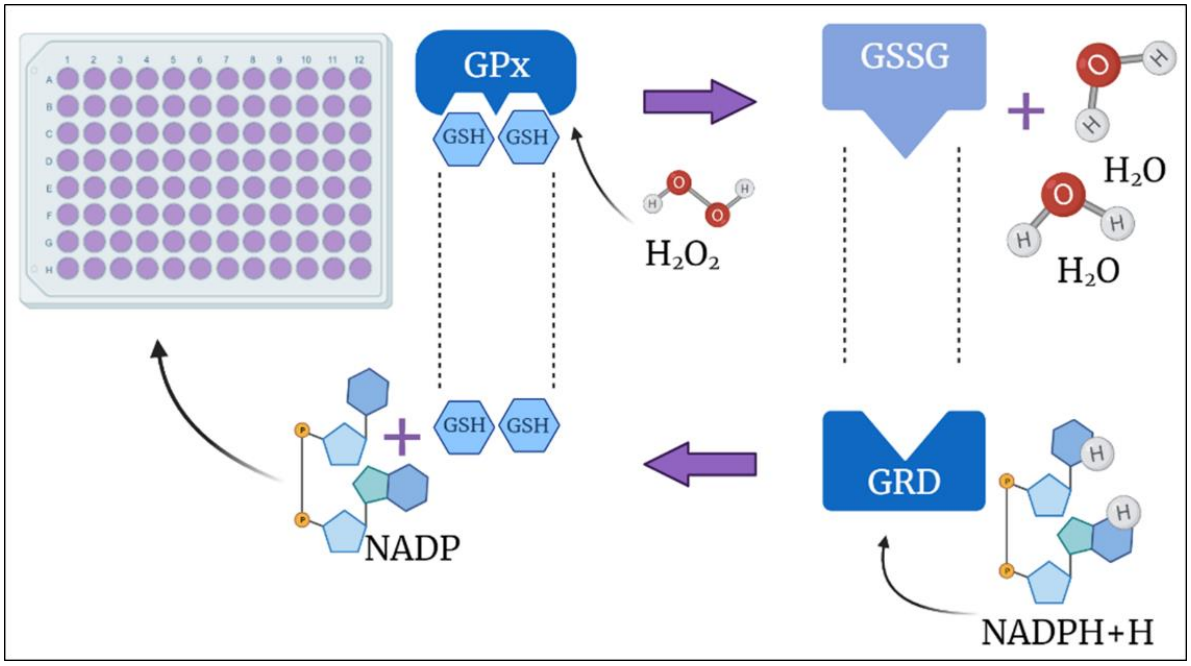


Figure 4. 2 Enzymatic reaction for the evaluation of glutathione peroxidase. GPx: glutathione peroxidase, GSH: reduced glutathione, GSSG: oxidised glutathione, and GRD: glutathione reductase

The reaction mixture contained 300 mM sodium phosphate buffer pH 7.2; EDTA 0.5 mM, 54 mUI of GRD; 85 µM NADPH; 2 mM GSH; 1.2 mM t-BuO_2H and 6 µL of seminal

plasma for a final volume of 200 µL. The absorbance change at 340 nm was monitored for 3 min with the microtiter plate reader Tecan Spectrafluor plus (Tecan Group Ltd, Männedorf, Switzerland). One enzyme unit (IU) is defined as the amount of GPx capable of transforming 1 µmole/min of NADPH at 25 °C, pH 7.2.

The enzymatic activity was determined as the difference in absorbance per minute between the first and the last measure, once the blank sample is subtracted, based on the following equation:

$$Activity = \frac{\Delta A_{340} \times DF}{6{,}22 \times V}$$

ΔA_{340}= change in absorbance
DF= Dilution factor
6,22=ε_{A340} of NADPH
V= Volumen

4.3.3.3 Catalase (CAT. EC.1.11.1.6)

Catalase enzymatic activity was evaluated by measuring the decrease in absorbance produced by the H_2O_2 reduction caused by this enzyme (Figure 4.3).

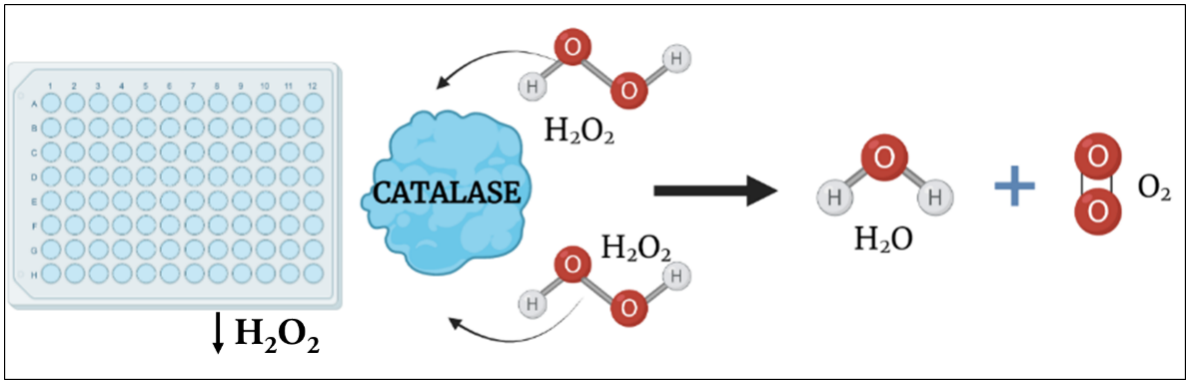


Figure 4. 3 Reaction for the evaluation of catalase enzymatic activity

The reaction mixture contained 50 mM sodium phosphate buffer (pH 7), 30 mM H_2O_2 and 4 µL seminal plasma (final volume of 200 µL). The change in absorbance was measured for 2 min at 240 nm in a plate reader (Tecan Spectrafluor plus, Tecan Group Ltd, Männedorf, Switzerland). One enzyme unit (IU) is defined as the amount of catalase capable of transforming 1.0 µmol/min of H_2O_2.

The enzymatic activity was determined as the difference in absorbance per minute between the first and the last measure, once the blank sample is subtracted, based on the following equation:

$$Activity = \frac{\Delta A_{240} \times DF}{43.6 \times V}$$

ΔA_{240}= change in absorbance
DF= Dilution factor
43.6=ε_{A240} of H_2O_2
V= Volumen

4.3.4 Seminal plasma protein analyses

4.3.4.1 SDS-PAGE

A total of 20 µg of protein was mixed with an electrophoresis sample buffer composed of 20% glycerol, 5% SDS, 0.125 M Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 10 mM EDTA and 0.1% bromophenol blue. Samples were loaded into the wells of a 10% (w/v) acrylamide gel and then were separated in one dimension following the Laemmli method (1970), using a Mini-PROTEAN II vertical electrophoresis system (Bio-Rad, Hercules, CA, USA). The conditions of electrophoresis were 130 V for 90 min at 4ºC. The electrophoresis buffer composition was 25 mM Tris, 192 mM glycine, and 0.1 % (v/v) SDS (pH 8.3). A mixture of pre-stained molecular weights ranging from 10 to 250 kDa (Bio-Rad, Hercules, CA, USA) was used as a standard.

After electrophoresis, the gels were stained with Coomassie Brilliant Blue (0.1% wt/v) in 45% (v/v) methanol and 10% (v/v) acetic acid for 10 minutes, and destained in 30% (v/v) methanol, 10% (v/v) acetic acid and distilled water until no background was detectable. Gel images were captured and analysed with the Odyssey Clx Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).

4.3.4.2 Proteomic analysis by difference gel electrophoresis (DIGE)

The comparative protein expression profile in seminal plasma from vasectomized and intact rams was analysed by difference gel electrophoresis (DIGE). This process consists of the initial separation of proteins by isoelectric focusing (IEF) as a first dimension followed by an orthogonal separation via SDS-PAGE. DIGE uses a direct labelling of proteins with fluorescent dyes (Cy2, Cy3 or Cy5) prior to IEF. Thus, two or more samples with different dyes are separated in the same gel, eliminating gel-to-gel variability (Unlu et al., 1997).

DIGE was performed in the Proteomics Unit of the Complutense University of Madrid, Spain, and analysed using the DeCyder 2-D Differential Analysis software (version 5.0, GE Healthcare, Chicago, IL, USA). Differentially expressed spots were excised, digested and identified by MALDI-TOF MS fingerprinting and the MASCOT algorithm v2.1 (Matrix Science, Boston, MA, USA).

4.4 Sperm selection and analysis

4.4.1 Sperm selection by dextran/swim-up method

The removal of seminal plasma is necessary due to its harmful effects on the viability of sperm cells. Seminal plasma contains several compounds that modify sperm plasma membrane, worsening motility and viability, as well as inhibiting both capacitation and fertilising ability.

A seminal plasma-free sperm population was obtained by the dextran/swim-up procedure, a method based on a technique for the separation of motile human spermatozoa, developed by Alvarez et al. (1993), and adapted for ram semen in our laboratory by García-López et al. (1996). This technique has the double advantage of obtaining a sperm population free from seminal plasma and enriched in highly viable and motile spermatozoa (García-López et al., 1996). The swim-up medium (SM) was composed by: 50 mM NaCl, 10 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM K_2HPO_4, 21 mM HEPES, 2.8 mM glucose, 0.33 mM sodium pyruvate, 18.6 mM sodium lactate, 200 mM sucrose, pH 6.5. 1.5 UI/mL of penicillin and 15 µg/mL of streptomycin were added to avoid contamination. To prevent premature capacitation of sperm cells, $CaCl_2$ and $NaHCO_3$ were omitted in the SM medium, as described by Grasa et al. (2004).

The procedure consisted of carefully pipetting 500 µL of semen in the bottom of a round 15 mm diameter tube. Over the semen, 500 µL of dextran/SM (30 mg dextran/mL SM), and then 1.5 mL BSA/SM (5 mg BSA/mL SM) were carefully deposited, avoiding mixing the different media. After 15 minutes of incubation at 37 °C, 750 µL of supernatant were removed and replaced by the careful addition of 750 µL of fresh BSA/SM medium. This incubation sequence was repeated 3 more times, so four consecutive supernatants were obtained (Figure 4.4). The first supernatant was rejected due to its low-quality spermatozoa content. The other three top layers obtained from the other three supernatants were combined to give 2.25 mL of sperm suspension (swim-up sample).

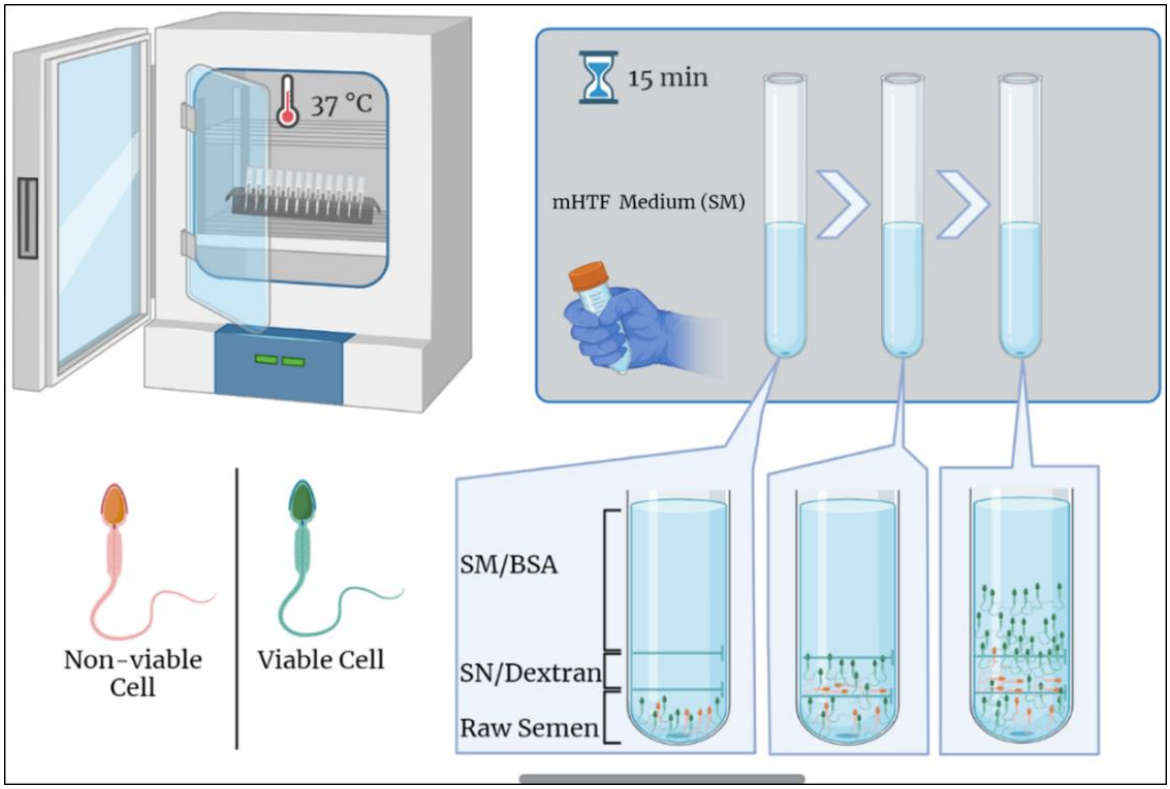


Figure 4. 4 Schematic representation of dextran/swim-up procedure.

4.4.2 Induction of in vitro sperm capacitation

In order to induce *in vitro* capacitation, swim-up-selected spermatozoa, in aliquots of 1.6 x 10^8 cells/mL, were incubated for 3 hours at 39 °C in a humidified incubator with 5% CO_2 in the air. Incubation was performed in a complete TALP (Tyrode's Albumin Lactate Pyruvate) medium based on the protocol from Parrish et al. (1988) and adapted by Colas et al.(2008). TALP medium was composed of 100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO3, 0.3 mM NaH_2PO_4, 21.6 mM sodium lactate, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM HEPES, 1 mM sodium pyruvate, 5 mM glucose and 5 mg/mL bovine serum albumin, pH adjusted to 7.2 with NaOH. As ram spermatozoa are difficult to capacitate *in vitro*, we also evaluated the addition of a cocktail of agents that increase intracellular cAMP to the TALP medium (Colás et al., 2010, 2008; Grasa et al., 2006). The cAMP-elevating compounds were 1 mM dibutyryl (dB)-cAMP, 1 mM caffeine and 1 mM theophylline, 0.2 mM okadaic acid and 2.5 mM methyl-b-cyclodextrin. We named this high cAMP medium as "cocktail medium".

To determine the role of melatonin on sperm capacitation in rams under equatorial photoperiod conditions, this hormone was added to samples in the cocktail medium at a final concentration of 100 pM or 1 µM. Melatonin was solubilized in dimethyl sulphoxide (DMSO) and phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.76 KH_2PO_4, pH 7.4). The final concentration of DMSO in all the melatonin samples was 0.1%. To account for the potential adverse effect of DMSO, the same concentration was included in cocktail-capacitated samples to which no melatonin had been added.

Thus, in paper 4, five experimental groups were analysed: swim-up (spermatozoa selected by the dextran/swim-up procedure before inducing the *in vitro* capacitation), cap-TALP (swim-up-selected spermatozoa incubated under capacitating conditions in TALP medium), cap-CK (swim-up-selected spermatozoa incubated under capacitating conditions in cocktail medium), and cap-CK-100 pM Mel and cap-CK-1 µM Mel (swim-up selected spermatozoa incubated under capacitating conditions in cocktail medium in the presence of 100 pM and 1 µM melatonin, respectively).

4.4.3 Evaluation of sperm quality parameters

4.4.3.1 Evaluation of cell concentration, motility and plasma membrane integrity

Swim-up and capacitated (in TALP and cocktail medium) samples were analysed using IVOS II CASA system (Hamilton Thorne, Beverly, MA, USA), which consists of an integrated optical system that provides automatic field selection and precise control of temperature (37 °C) during analysis. A previous dilution was required to guarantee a final concentration of 40 x 10^6 cells/mL, which fits into the operational range recommended by the IVOS II CASA system. The dilution medium was Phosphate HEPES Sucrose (PHS) (Mendoza et al., 2012), composed of 250 mM sucrose, 0.1 mM EGTA, 4 mM sodium phosphate, 5 mM glucose, 10 mM HEPES and 2 mM KOH; final pH 7.5. After this first dilution, aliquots were diluted (1:1 v/v) with the vital stain VIADENT™ (10 µg/mL freshly prepared before the experiment; supplied by Hamilton Thorne, Beverly, MA, USA) containing the fluorescent dye Hoechst 33258, and then incubated for 2 min at 37°C in the dark. Immediately after incubation, samples (3 µL) were placed onto pre-warmed 20 mm Leja slides (Leja Products BV, Nieuw-Vennep, Netherlands). Sperm concentration, motility and viability were sequentially analysed by the IVOS II CASA system. Standard motility analysis was performed on five fields under typical phase contrast illumination, and viability analysis was then performed on the same fields under fluorescent illumination.

4.4.3.2 Determination of translocation of phosphatidylserine (PS)

Annexin V (AnnV) is a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine (PS). The translocation of PS residues to the outer layer of the plasma membrane could be detected using annexin coupled with a fluorochrome. For microscopy evaluation of PS translocation, we use the Annexin V-Cy3.18 (Annexin V-Cy3™ Apoptosis Detection Kit). In order to differentiate between viable cells with or without PS translocation and nonviable cells, we used 6-CFDA along with Annexin V-Cy3.18. The non-fluorescent 6-CFDA enters the cell and is converted to the green fluorescent compound 6-carboxyfluorescein (6-CF). This conversion is produced by the esterases present only in living cells.

For PS evaluation, aliquots of 300 µL (6 x 10^6 cells diluted with 1 x commercial binding buffer) were stained with 5 µL 6-CFDA (1 mM in DMSO) and 2 µL Annexin V-Cy3.18 for 15 minutes at 37°C in darkness. Each stained sample was placed on a slide and analysed at 1000X magnification by epifluorescence microscopy. Viable sperm (6-CFDA+) were

visualised in green with a fluorescein (Nikon B-2A) filter and AnnV+ sperm in red with a rhodamine (Nikon G-2A) filter. Two hundred spermatozoa were counted per slide, and three populations were defined depending on their staining (Figure 4.5): 1) viable (green under fluorescein filter/non-stained under rhodamine filter); 2) apoptotic (green under fluorescein filter/red under rhodamine filter), and 3) dead cells (non-stained under fluorescein filter/red or non-stained under rhodamine filter).

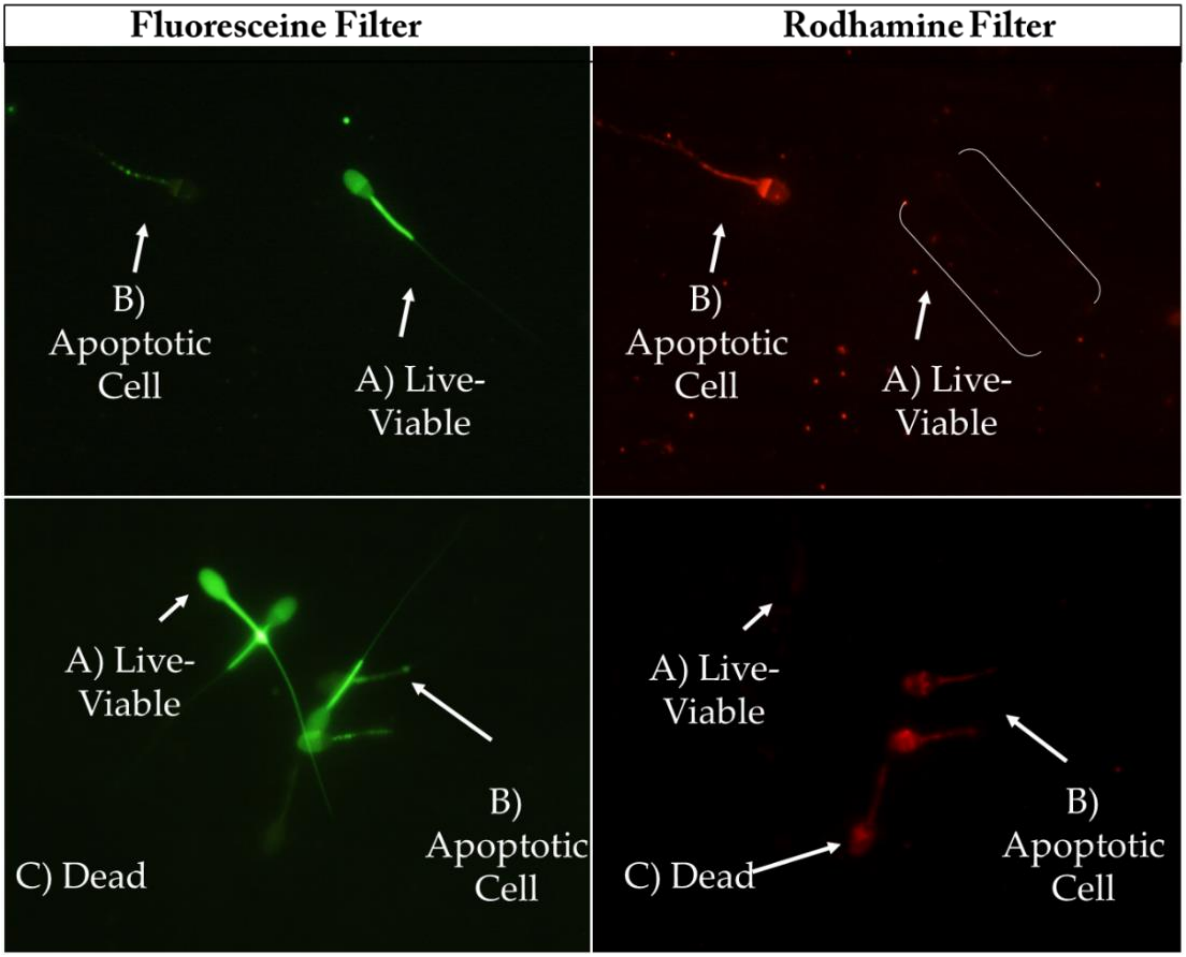


Figure 4. 5 Representative images of ram sperm stained with Annexin V-Cy3.18 and 6-CFDA. In the figure, live-viable (A), apoptotic (B), and dead cells (C) are identified. 1000x magnification.

4.4.3.3 Assessment of capacitation status by chlortetracycline (CTC) staining

Chlortetracycline (CTC) is an antibiotic which binds to membrane-associated cations, especially Ca^{2+} (Caswell and Hutchison, 1971). Upon entering the sperm, CTC binds free calcium, becoming more fluorescent (Ericsson, 1967). These CTC-Ca^{2+} complexes preferentially bind to the cell membrane, resulting in staining patterns characteristic of the spermatozoa's physiological state, which allows differentiating non-capacitated, capacitated, and acrosome reacted cells (Fraser et al., 1996). A modified version of the CTC assay (Grasa et al., 2006) al., 2006), described by Ward et al. (1984) and validated for ram

sperm by Gillan et al. (1997) was used in this work. A CTC solution (750 µM) was prepared daily in a buffer containing 20 mM Tris, 130 mM NaCl and 5 µM cysteine (pH 7.8), and sterilised with a 0.22 µm filter (Merck Millipore, Burlington, MA, USA). For each 20 µL sample (1.6 x 10^8 cells/mL), 20 µL of CTC solution and 5 µL (12.2% w/v) paraformaldehyde in 0.5 M Tris-HCl (pH 7.8), were added. Stained samples were incubated at 4 °C in the dark for 30 min. A 10 µL aliquot of each stained sample was placed on a glass slide and mixed with 2 µL of 0.22 M triethylenediamine (DABCO) diluted in 9:1 (v/v) glycerol: PBS. The samples were covered with 24 x 60 mm coverslips, sealed with colourless enamel, and stored in the dark at -20 ºC if evaluation could not be made the same day.

For the evaluation of the CTC patterns, samples were examined using a Nikon Eclipse E-400 microscope under epifluorescence illumination with a V-2A filter. All samples were processed in duplicate, and at least 200 spermatozoa per slide were evaluated. Three sperm types were estimated according to Gillan et al., (1997) (Figure 4.6): non-capacitated (NC, even distribution of fluorescence on the head, with or without a bright equatorial band), capacitated (C, with fluorescence in the anterior portion of the head) and acrosome-reacted cells (R, showing no fluorescence on the head).

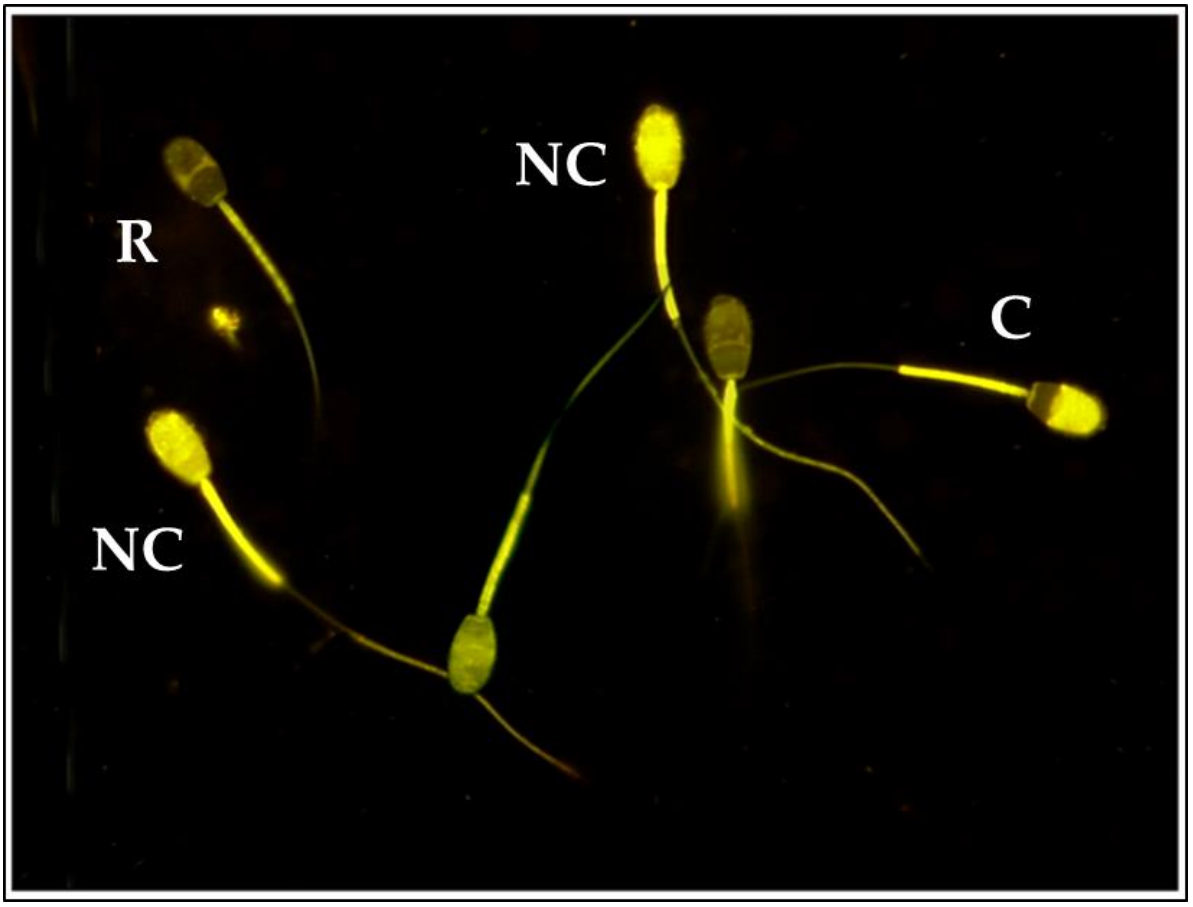


Figure 4. 6 Representative image of ram sperm capacitation state, evaluated by chlortetracycline (CTC) staining. Non-capacitated (NC), capacitated (C), and acrosome reacted (R) spermatozoa can be identified. Filter V-2A, 1000x magnification.

4.4.4 Identification and localisation of melatonin membrane receptors MT_1 and MT_2 by indirect immunofluorescence

Aliquots of 4×10^6 swim-up selected spermatozoa were fixed with 3.7% formaldehyde (v/v) in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH 7.2) for 20 min at room temperature (RT). The cells were then centrifuged for 6 min at 900×g at RT, and the pellet was resuspended in 500 µL of PBS. After that, 40 µL of cell suspension was smeared onto poly-L-lysine-coated slides and maintained at RT for 3 h to ensure good adhesion of the spermatozoa to the slide.

The slides were then washed three times with PBS, and non-specific binding sites were blocked with 5% BSA (w/v) in PBS overnight at 4 °C in a wet chamber. After three washes with PBS, the slides were incubated overnight at 4 °C in a wet chamber with the primary antibodies: MTNR1A mouse polyclonal antibody (Abnova Corporation, Taipei City, Taiwan; Cat# H00004543-A01, RRID: AB_462681) for the MT_1 receptor, and a rabbit polyclonal antibody for the MT_2 receptor (Origene Technologies, Rockville, MD, USA (formerly Acris Antibodies, GmbH, Herford, Germany); Cat# AP01322PU-N, RRID: AB_1619198), both diluted 1:50 in PBS containing 1% (w/v) BSA.

After three washes in PBS, the cells were incubated with secondary antibodies Alexa Fluor 594 chicken anti-mouse (Cat#A-21201, RRID: AB_2535787) for the MT_1 receptor and Alexa Fluor 488 chicken anti-rabbit (Cat# A-21441, RRID: AB_2535859) for the MT_2 receptor (Thermo Fisher Scientific, MA, USA), both diluted 1:800 in PBS containing 1% BSA (w/v) for 1.5 hours at RT in a humidity chamber and in the dark. The slides were then washed three times with PBS before adding 5 µL of 0.22 M DABCO to enhance and preserve cell fluorescence.

Finally, the preparations were covered with coverslips, sealed with colourless enamel and the immunotypes were evaluated using a Nikon Eclipse E-600 microscope (Tokyo, Japan) under epifluorescence illumination. All samples were processed in duplicate in a blind manner, and at least 200 spermatozoa were scored per slide. The different immunotypes and intensity of immunostaining are described in figure 4.7.

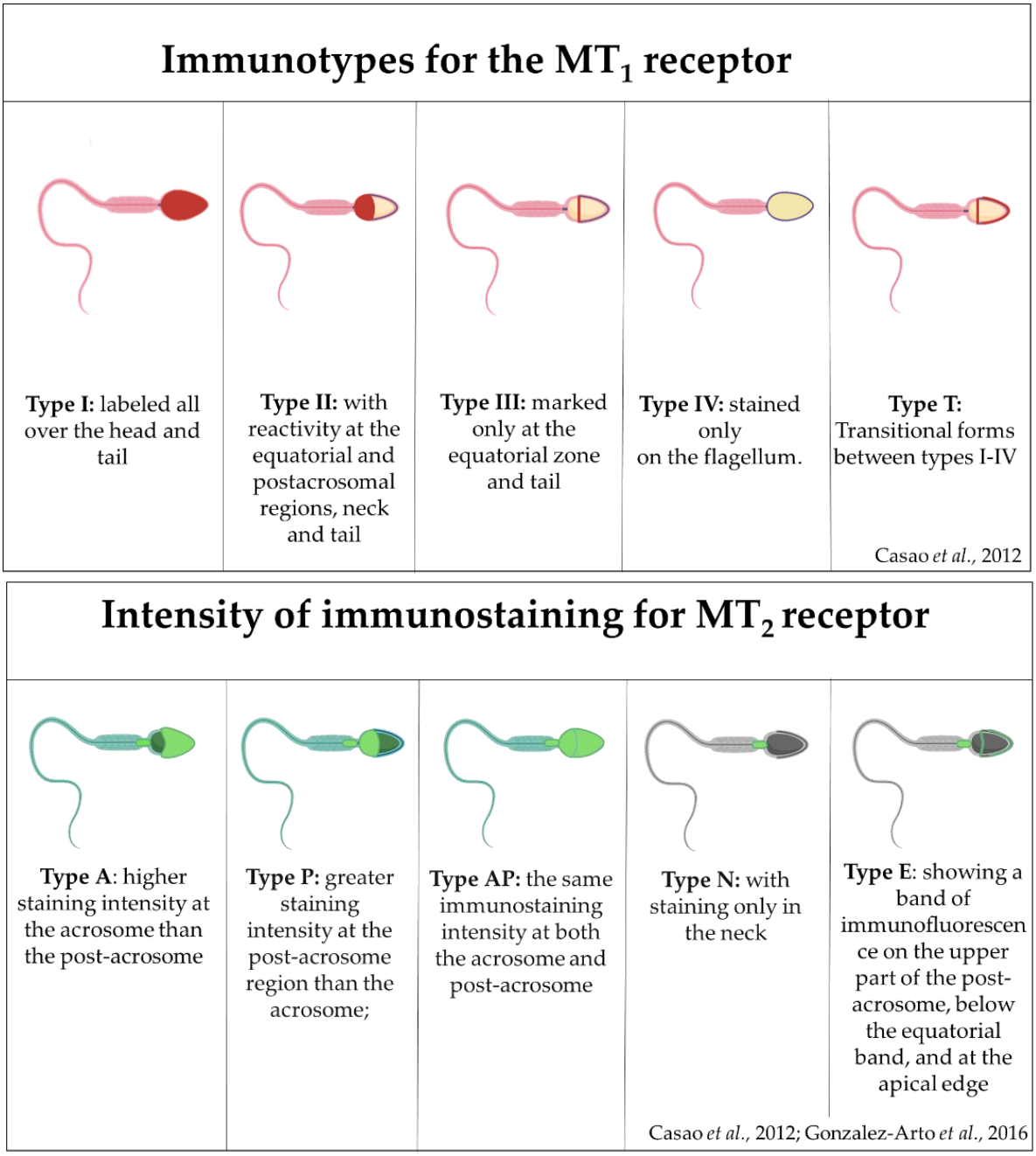


Figure 4. 7 Immunotypes for MT_1 and MT_2 melatonin receptors in ram spermatozoa, identified by indirect immunofluorescence. Base on Casao et al., 2012 and González-Arto et al., 2016)

4.4.5 Quantification of melatonin membrane receptors MT_1 and MT_2 and membrane tyrosine-phosphorylated proteins by western blot

Sperm membrane proteins were extracted by incubating swim-up selected (paper 3 and 4) and capacitated spermatozoa (paper 4) (320 × 10^6 cellsL in 200 µL) in 200 µL of extraction sample buffer (ESB; composed by 2% (w/v) SDS and 0.0626 M Tris-HCl, pH 6.8)

for 5 minutes at 100 °C in a sand bath. After incubation, the samples were centrifuged at 7500 x g for 5 minutes at 4 °C. The supernatant was recovered and 10% (v/v) protease and phosphatase inhibitors, 5% (v/v) β-mercaptoethanol, 1% (v/v) glycerol, and 0.002 % (v/v) bromophenol blue (in 10 % glycerol) were added. The supernatant was stored at -20 °C until its use.

Sperm membrane proteins (15 µg for melatonin receptors and 15 µL for tyrosine-phosphorylated proteins) were separated in one dimension in 10% (for MT_2 receptor and tyrosine-phosphorylated proteins) or 12% (MT_1 receptor) acrylamide gels by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) following the protocol described in the section 4.3.4.1.

After the electrophoresis, the separated proteins were blot onto 0.2 µm polyvinylidene difluoride (PVDF) membranes using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). Non-specific sites on the membrane were blocked with 5% (w/v) BSA in PBS for 4 hours at RT. The melatonin receptors were immunodetected by incubating overnight at 4 °C with the primary antibody Mel-1A-R rabbit polyclonal antibody against the MT_1 receptor (GeneTex Inc., Irvine, CA, USA; Cat# GTX100003, RRID: AB_1241048); or melatonin receptor 1B rabbit polyclonal antibody against the MT_2 receptor (Origene Technologies, Rockville, MD, USA (formerly Acris Antibodies, GmbH, Herford, Germany); Cat# AP01322PU-N, RRID: AB_1619198). Both were diluted 1:1000 in 0.1% Tween-20 PBS containing 1% (w/v) BSA. In addition, a mouse anti-tubulin antibody (Santa Cruz Biotechnology, Dallas, TX, USA; Cat# sc-398103/sc-5546, respectively), diluted 1:1000 (v/v) was used simultaneously as a loading control.

Proteins phosphorylated in tyrosine residues were identified with the primary mouse monoclonal anti-phosphotyrosine antibody (Clone 4G10; Millipore, Temecula, CA; Cat# 05–321, RRID: AB_309678), diluted 1:1000 (v/v) in 0.1% (w/v) Tween-20–PBS containing 1% BSA (w/v). Additionally, a rabbit anti-actin antibody (Sigma-Aldrich, St. Louis, MO, USA; Cat# A2066, RRID: AB_476693), diluted 1:1000 (v/v) was used as a loading control.

The membranes were washed three times in 0.1% (v/v) Tween-20 PBS and then incubated with a donkey anti-mouse IRDye 800CW (LI-COR Biosciences, Lincoln, NE, USA; Cat# 925-32212, RRID: AB_2716622) and donkey anti-rabbit IRDye 680CW (LI-COR Biosciences Cat# 925-68073, RRID: AB_2716687) secondary antibody, both diluted 1:20000 (v/v) for MT receptors or 1:15000 (v/v) for tyrosine-phosphorylated proteins, for 1 hour at RT. After extensive washing, the membranes were scanned, and the intensity of the detected bands was measured using the Odyssey CLx Imaging System and the Image Studio Acquisition Software (LI-COR Biosciences, Lincoln, NE, USA). With this software, the peak intensity of each band was evaluated and normalised to the loading control. For total MT_1 or MT_2 band intensity, the sum of the peak intensity of all bands in the lane was estimated. High (60-250 kDa) and low (10-45 kDa) molecular weight proteins were analysed separately for the tyrosine-phosphorylated proteins bands.

5. Resultados

5.1 Artículo 1: Changes in melatonin concentrations in seminal plasma are not correlated with testosterone or antioXidant enzyme activity when rams are located in areas with an equatorial photoperiod

Animal Reproduction Science 200 (2019) 22–30

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Changes in melatonin concentrations in seminal plasma are not correlated with testosterone or antioxidant enzyme activity when rams are located in areas with an equatorial photoperiod

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ARTICLE INFO

Keywords:
Ram
Equatorial
Melatonin
Testosterone
Antioxidant enzymes

ABSTRACT

In temperate climates, photoperiod and melatonin regulate ram reproduction, modulating hor- monal secretions, sperm quality, and seminal plasma composition. Information on the effect of an equatorial photoperiod (12L:12D) on ram reproduction, however, is scarce, and no data on hormonal concentrations and antioxidant enzyme activity in seminal plasma have been reported. Thus, the variation was investigated of melatonin and its relationship with testosterone and antioxidant enzyme activity in the seminal plasma of three sheep breeds in Colombia, when there was a consistent photoperiod during two dry and two rainy seasons per year. Semen was col- lected once a week from 12 mature rams (four of each breed: Colombian Creole, Hampshire, and Romney Marsh). Seminal plasma was obtained by centrifugation. The concentration of melatonin and testosterone were quantified along with the enzymatic activity of glutathione peroxidase (GPx), glutathione reductase (GRD), and catalase (CAT). Correlation analyses between melatonin and testosterone concentrations or enzymatic activity were also performed. Melatonin con- centration was affected by season ($P < 0.05$) but not breed, with lesser concentrations in the first rainy season. Testosterone concentration, however, was affected by breed and season, with greater concentrations ($P < 0.01$) in the Hampshire and Romney Marsh rams during the second dry season. Regarding antioxidant enzyme activity, there was only seasonal variation in GPx activity ($P < 0.05$). When correlation analyses were used for data assessments, there was a negative correlation between melatonin and testosterone concentrations in Hampshire rams. In conclusion, melatonin concentrations in seminal plasma of rams that were located in an area withan equatorial photoperiod was affected by the climatological season but there was no positive correlation with testosterone concentration or antioxidant enzyme activity.

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https://doi.org/10.1016/j.anireprosci.2018.11.007
Received 23 July 2018; Received in revised form 3 October 2018; Accepted 15 November 2018
Available online 16 November 2018
0378-4320/

1. Introduction

In temperate climates, photoperiod through modulation of melatonin secretion regulates reproductive activity in sheep. Photoperiodic signals reach the pineal gland and there is modulation of nocturnal melatonin secretion (Arendt, 1998; Bittman et al., 1983) and subsequent modulation of gonadotropin secretion (Malpaux et al., 1996), gonadal functions, and sexual behavior (Rosaand Bryant, 2003).

Seasonality effects in sheep decrease with the extent of domestication (Lincoln et al., 1990), and also depends on latitude at whichthe animals are located. Sheep breeds originating from England are highly seasonal (Tempest and Boaz, 1973), whereas those from intermediate latitudes such as Mediterranean breeds have a short anestrus (Forcada et al., 1992). Tropical and equatorial breeds are aseasonal or only have short periods of seasonally induced anestrus (Mahieu et al., 1989; Arroyo et al., 2016) and thus the re- productive pattern is regulated by food availability in relation to the annual rainfall cycle (Rosa and Bryant, 2003). Furthermore,ewes from temperate regions subjected to an equatorial photoperiod (12L:12D) soon afterwards begin to have non-seasonal re- productive patterns (Jackson et al., 1990).

The response of rams to seasonality is less marked than in ewes. In temperate regions ($> 30°$ and $< 45°$ north or south latitude), there are variations in hormonal concentrations (D'Occhio et al., 1984), sperm quality (Karagiannidis et al., 2000) and testicularvolume (Avdi et al., 2004) during different seasons of the year; however, there is no complete cessation of spermatogenesis and sexual activity. This variation in hormonal concentrations in rams during different seasons of the year is reflected in the seminal plasma composition, where concentrations of melatonin and testosterone also have a seasonal pattern. The melatonin concentration in this fluid correlates with that of testosterone and antioxidant enzyme activity (Casao et al., 2010). In tropical (below 23.5° north or south) and subtropical (between 23.5° and 35.0° north or south) regions, photoperiodic signals also regulate seasonality, although to a lesser extent. Nevertheless, variations in testicular size, testosterone concentrations (Milczewski et al., 2015) and several sperm parameters (Aguirre et al., 2007; Cardenas-Gallegos et al., 2012) have been reported. Information on ram reproduction in the equatorial zone (between 10 °N and 10 °S) with a 12L:12D light regimen, however, is scarce. Furthermore, there is no information on the effect of a tropical or equatorial photoperiod on variation in ram seminal plasma hormonal concentrations and antioxidant enzyme activity.

Colombia is an equatorial region where the differences in photoperiod throughout the year are minimal and as a consequence there are no short and long days. The Andean region, the most highly populated in the country, has a bimodal annual rain cycle, withtwo rainy and two dry seasons. This mountain area has cold and humid weather (Narváez-Bravo and León Aristizábal, 2001). Sheep currently represent 11% of the country's livestock, although in a climate change scenario, the choice of sheep as the primary livestock species is likely to increase (Seo et al., 2010). The Colombian Creole sheep is a native breed which is the result of indiscriminate breeding between European and African sheep since the Spanish conquest in the early 16th century, and thus animals of this breed are highly adapted to the Colombian climate (Ocampo et al., 2017). Additionally, imported wool sheep breeds, such as Hampshire, Romney Marsh and others, have been introduced in the country since 1963, when the first importation occurred. These breeds are characterized by a superior productive performance, but there is relatively little adaptation to the local climatic conditions in terms of fertility (Beaty, 1971).

The aim of this research was to elucidate the effect of an equatorial photoperiod on ram reproduction by investigating the yearly variation of melatonin concentration and its relationship with testosterone concentrations and antioxidant enzyme activity in theseminal plasma of a native (Colombian Creole) and two imported (Hampshire and Romney Marsh) sheep breeds, reared in Colombia when there was a 12L:12D light regimen during two dry and rainy seasons of the year.

2. Materials and methods

2.1. Animals and seminal plasma extraction

All animals used in this study were handled in strict accordance with Colombian Animal Protection Regulations (Law 84/1989,modified by Law 1774/2016). Rams were housed together with uniform nutritional conditions at the Center for Ovine Research, Technological Development and Extension (Centro de investigación, desarrollo tecnológico y extensión ovino –CIDTEO) National University of Colombia, located in Mosquera (4°40′57″ N 74°12′50″ W) at 2510 m above sea level. The ram's diet was based on pasture (*Pennisetum clandestinum*, *Lolium perenne*), supplemented with concentrate (400 g), corn silage (300 g) and mineralized salt (100 g). Local amplitude of the photo-phase throughout the year varies from 12 h 21 min (11 h 39′ of dark) in the summer solstice to 11 h 49 min (12 h 11 min of dark) in the winter solstice, i.e., 32 min of difference between the longest and the shortest day of the year (Fig. 1). The climate of the region is classified as Cfb following the Köppen Climate Classification System. The average temperature is 13.6 °C whereas the annual variation of temperature between coldest and hottest months is 0.7 °C. The mean relative air humidity ranges from 92% in the morning to 70% in the evening, and the mean annual rainfall is 960 mm, with a mean of 205 days with precipitation per year (Fig. 1).

Semen was obtained using an artificial vagina (AV) following the protocol approved by the Bioethics Committee of the National University of Colombia (CB-074-2014). Raw semen was obtained from 12 mature rams (2–5 years old) of three breeds (four Creole; four Romney Marsh and four Hampshire sires). Two successive ejaculates were collected once a week from each ram throughout theyear and mixed together for seminal plasma extraction.

Seminal plasma from each mixed semen sample was obtained by centrifugation at $7500 \times g$ for 5 min in a microfuge (HERMLE Labortechnik GmbH, Siemensstr 25, D-78564 Wehingen, Germany) at 4 °C. The supernatant was collected and centrifuged again, andthe recovered seminal plasma was filtered through a 0.22 mm Millipore membrane (Merck KGaA, Darmstadt, Germany). The seminal

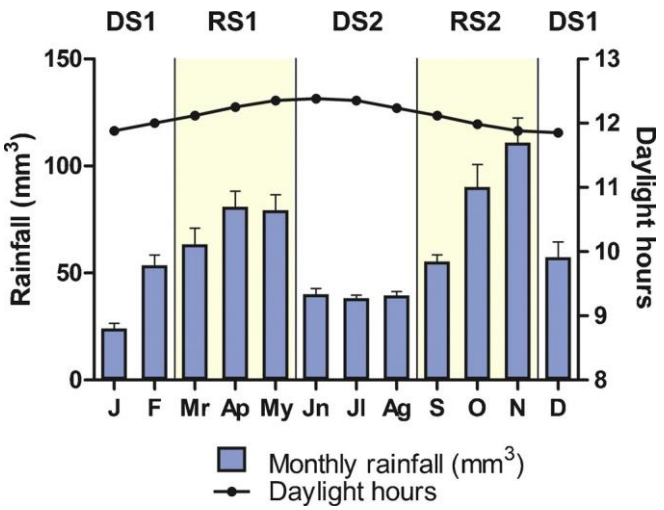

Fig. 1. Monthly rainfall (mm^3, bars) and daylight hours (line) in Mosquera, Colombia (4°40′57″ N 74°12′50″ W); Monthly rainfall is shown asmean ± S.E.M. of five consecutive years, whereas the daylight hours were recorded the 21st of each month.

plasma was stored at −20 °C after adding 10% protease and phosphatase inhibitor (Sigma Chemical Co, St. Louis, MO, USA).

2.2. Melatonin evaluation

Melatonin concentrations in ram seminal plasma were quantified using a commercial competitive immunoassay (Direct salivamelatonin ELISA kit, Bühlmann Laboratories AG, Switzerland, Sensitivity: 0.5 pg/mL, Intra-assay variability: 5.2%, Inter-assay variability: 11.2%), following the manufacturer's instructions. Briefly, 100 μL of each sample, control, and calibrator were loaded in duplicate in a microtiter plate coated with an anti-melatonin antibody and incubated for 16–20 h at 2–8 °C. After incubation, 50 μL of biotinylated melatonin were added to each well and incubated for 3 h at 2–8 °C. After three washes, 100 μL of streptavidin conjugatedto horseradish peroxidase (HRP) were loaded into the wells and incubated for a further 60 min in a plate rotator set at 600 rpm at 18–28 °C. The wells were rewashed three times, and 100 μL of tetramethylbenzidine substrate (TMB) were added to each well, and incubated for 30 min in a plate rotator at 600 rpm and 18–28 °C, protected from direct light. After incubation, 100 μL of 0.25 M SO_4H_2 solution were added, and absorbance was measured on a microtiter plate reader (TECAN Spectrafluor plus, Switzerland) at 450 nm.

2.3. Testosterone assays

Testosterone concentrations in ram seminal plasma were quantified by use of a total testosterone commercial ELISA kit assay (Testo-Easia, BioSource Europe, S.A., Belgium; Sensitivity: 0.05 ng/mL; Intra-assay variability: 4.8%, Inter-assay variability: 7.1%),following the manufacturer's instructions. Briefly, 50 μL of each sample, control, and calibrator, along with 100 μL of testosterone labeled with horseradish peroxidase (HRP) were loaded in duplicate in a microtiter plate coated with an anti-testosterone specific antibody and incubated for 1 h at room temperature. After incubation, the wells were washed three times, and 100 μL of chromogenic substrate (TMB) were added to each well and incubated for 30 min at room temperature, protected from direct light. After incubation, 100 μL of 0.2 M HCl solution were added, and absorbance was measured on a microtiter plate reader (TECAN Spectrafluor plus,Switzerland) at 450 nm.

2.4. Antioxidant enzyme activity assays

The seminal plasma antioxidant defense system was assessed by determining the activity of the following enzymes: Glutathione reductase (GRD), glutathione peroxidase (GPx) and catalase (CAT). Measurements were performed as previously described (Casaoet al., 2013) with adaption occurring for the microtiter plate using a spectrophotometric method with a microtiter plate reader (TECAN Spectrafluor plus, Tecan Grup Ltd., Männedorf, Switzerland). All samples were loaded in duplicate and analyzed in the same assay.

2.4.1. Glutathione reductase (GRD. EC.1.6.4.2)

The GRD activity was measured by following the decrease in absorbance due to NADPH oxidation as a consequence of the GSSGreduction. The reaction mixture contained 300 mM sodium phosphate buffer at pH 7.2; 0.5 mM EDTA; 85 μM NADPH; 0.8 mM oxidized glutathione (GSSG) and 5 μL of seminal plasma to complete a final volume of 200 μL. The absorbance change at 340 nm was monitored for 3 min with the microtiter plate reader. One unit induces the oxidation of 1.0 μmole/min of NADPH at 25 °C, pH 7.2.

2.4.2. Glutathione peroxidase (GPx. EC.1.11.1.9)

The GPx activity was measured following the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) as a result of catalysis with GPx and using ter-Butylhydroperoxide (t-BuO_2H) as an electron acceptor, coupled to the recycling of GSSG to GSHutilizing GRD and NADPH. The reaction mixture contained 300 mM sodium phosphate buffer at pH 7.2; EDTA 0.5 mM, 54 mUI ofGRD; 85 μM NADPH; 2 mM GSH; 1.2 mM t-BuO_2H and 6 μL of seminal plasma for a final volume of 200 μL. The absorbance change at340 nm was monitored for 3 min with the microtiter plate reader. One enzyme unit (IU) is defined as the amount of GPx capable of transforming 1 μmol/min of NADPH at 25 °C, pH 7.2.

2.4.3. Catalase (CAT. EC.1.11.1.6)

Catalase activity was measured by determining the decrease in absorbance due to H_2O_2 reduction to H_2O and O_2 in the presenceof catalase. The reaction mixture contained 50 mM sodium phosphate buffer at a pH of 7; 11 mM t-BuO_2H and 4 μL of seminal plasmato complete a final volume of 200 μL. The absorbance change at 240 nm was monitored for 120 s with the microtiter plate reader. One enzyme unit (IU) is defined as the amount of catalase capable of transforming 1.0 μmol/min of H_2O_2.

2.5. Statistical analyses

Monthly data (mean ± S.E.M. of the weekly seminal plasma samples) from each breed were pooled together for illustrative purposes. Also, data from each breed were grouped for the four climatic seasons (Dry season 1: From December to February; Rainy season 1: March to May; Dry season 2: June to August and Rainy season 2: September to November) for statistical analyses. First, normality was evaluated by the Kolmogorov-Smirnov test, and homogeneity of variance by the Levene's test. The effect of the breed and the season on hormonal concentration and antioxidant enzyme activity was subsequently analyzed using a two-way ANOVA
followed by use of a Bonferroni or a Games-Howell *post-hoc* test, as appropriate. The possible correlation between the melatonin concentration in seminal plasma and testosterone concentration or antioxidant enzyme activity was evaluated using a Pearson correlation test. GraphPad Software (La Jolla, CA, USA) and SPSS Statistics (IBM Analytic, Armonk, NY, USA) were used, and$P < 0.05$ was considered statistically significant.

3. Results

There were monthly variations in melatonin concentrations in seminal plasma in all three breeds (Fig. 2a), with the least values during the two rainy seasons between March and May and again between October and December. There were no differences among breeds during the yearly evaluations. Thus, when the data were grouped into the four climatological seasons, the use of the two-way ANOVA indicated there was an effect of the season but not of the breed on the melatonin concentration in seminal plasma. During the first rainy season (from March to May), the melatonin concentration was less in the Creole ($P < 0.05$ when compared with the second dry season) and the Hampshire rams ($P < 0.01$ when compared with both dry seasons). In the Romney Marsh rams, the melatonin concentration also was less during the first rainy season and there were no differences during the other seasons of the year (Table 1).

Surprisingly, there was a different pattern in testosterone concentration in ram seminal plasma as compared with that for mel-atonin with two distinct peaks occurring. The first increase in testosterone concentration was in May–June, and the second in November (Fig. 2b). There was a distinct and sustained increase in testosterone concentration; however, this was less in the Creole rams. There was a difference in testosterone concentration in ram seminal plasma among seasons and breeds. In particular, thetestosterone concentration was greater in the second dry season when compared with the first in the Romney Marsh and Hampshire
($P < 0.01$), but not in the Creole (Table 2) rams. Furthermore, the testosterone concentration in Creole rams during the second dry season was less than the values in the rams of the other two breeds ($P < 0.01$).

For the antioxidant enzyme activity, there was only a monthly variation in GPx activity during the year (Fig. 3a), with lesser activity from January to April which increased from May to December. Consequently, GPx activity was less during the first dry and rainy seasons when compared with the second seasons ($P < 0.05$) in all the breeds (Table 3). The results from the statistical analysis indicated there were differences between breeds for GPx activity, or between breed or season for GRD or CAT activity (Tables 4 and5). There were no differences in the yearly pattern of GRD and CAT activity (Fig. 3b and c).

Correlation analyses indicated that there was no relationship between melatonin concentrations in ram seminal plasma and antioxidant enzyme activity in any of the breeds. There was only a negative correlation ($P < 0.05$) between melatonin and testos- terone concentrations in seminal plasma of Hampshire rams (Table 6).

4. Discussion

Even though there is considerable knowledge of the role of melatonin in sheep reproduction in temperate climates, little is known about the involvement of this hormone in tropical sheep breeds. To begin to clarify this subject, the melatonin concentration was quantified, and its relationship with testosterone concentration and antioxidant enzyme activity was determined, in the seminal plasma of Creole, Romney Marsh, and Hampshire rams reared in Colombia when there was an equatorial light regimen (12L:12D)during two dry and rainy seasons.

In the present study, there were no differences in the melatonin concentrations in the seminal plasma of these breeds. These results are consistent with previous reports where there was a lack of differences between sheep breeds in terms of pituitary response

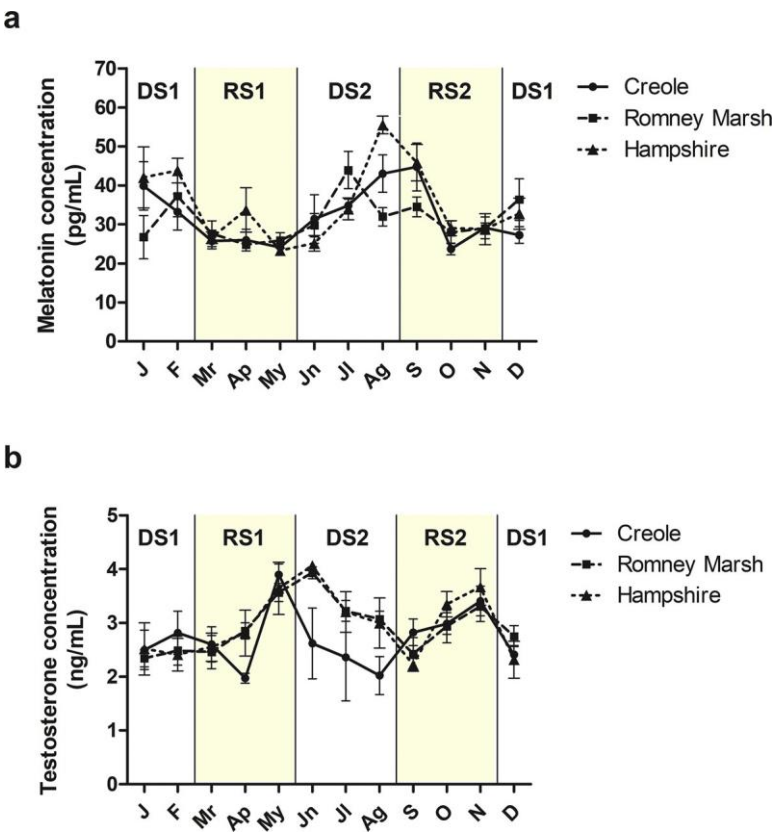

Fig. 2. Monthly values of melatonin (a) and testosterone (b) concentrations in seminal plasma of Creole, Romney Marsh, and Hampshire rams (four rams of each breed) when there was an equatorial photoperiod (12L:12D); Values are depicted as mean ± S.E.M. of four seminal plasma samples/ ram and month; Dry (DS) and rainy (RS) seasons are also indicated.

Table 1
Melatonin concentration (pg/mL) in ram seminal plasma, obtained from three different breeds (four rams of each breed) when there was an equatorial photoperiod during four climatic seasons (dry season 1: from December to February; rainy season 1: from March to May; dry season 2: from June to August and rainy season 2: September to November); Values are shown as mean ± S.E.M; *n* indicates the number of seminal plasma samples per breed and season; Different letters in the same row indicate differences between seasons.

	Dry season 1 ($n = 52$)	Rainy season 1 ($n = 52$)	Dry season 2 ($n = 52$)	Rainy season 2 ($n = 60$)
Creole	$32.98 \pm 2.73^{a,b}$	25.35 ± 1.08^{a}	36.04 ± 2.97^{b}	$32.77 \pm 3.38^{a,b}$
Romney Marsh	33.68 ± 3.15	26.22 ± 1.34	34.81 ± 2.54	30.14 ± 1.5
Hampshire	38.99 ± 3.09^{b}	26.37 ± 1.14^{a}	38.23 ± 4.04^{b}	$34.50 \pm 2.80^{a,b}$

Table 2
Testosterone concentration (ng/mL) in ram seminal plasma, obtained from three different breeds (four rams of each breed) when there was an equatorial photoperiod during four climatic seasons (dry season 1: from December to February; rainy season 1: from March to May; dry season 2: from June to August and rainy season 2: September to November); Values are depicted as mean ± S.E.M; *n* indicates the number of seminal plasma samples per breed and season; Different lowercase letters in the same row indicate differences between seasons, whereas different capital letters in the same column indicate differences among breeds.

	Dry season 1 ($n = 52$)	Rainy season 1 ($n = 52$)	Dry season 2 ($n = 52$)	Rainy season 2 ($n = 60$)
Creole	2.56 ± 0.16	2.80 ± 0.25	2.35 ± 0.34^{A}	3.06 ± 0.14
Romney Marsh	2.54 ± 0.16^{a}	$2.91 \pm 0.18^{a,b}$	3.45 ± 0.13^{Bb}	$2.89 \pm 0.18^{a,b}$
Hampshire	2.40 ± 0.12^{a}	$2.96 \pm 0.24^{a,b}$	3.47 ± 0.19^{Bb}	$3.10 \pm 0.21^{a,b}$

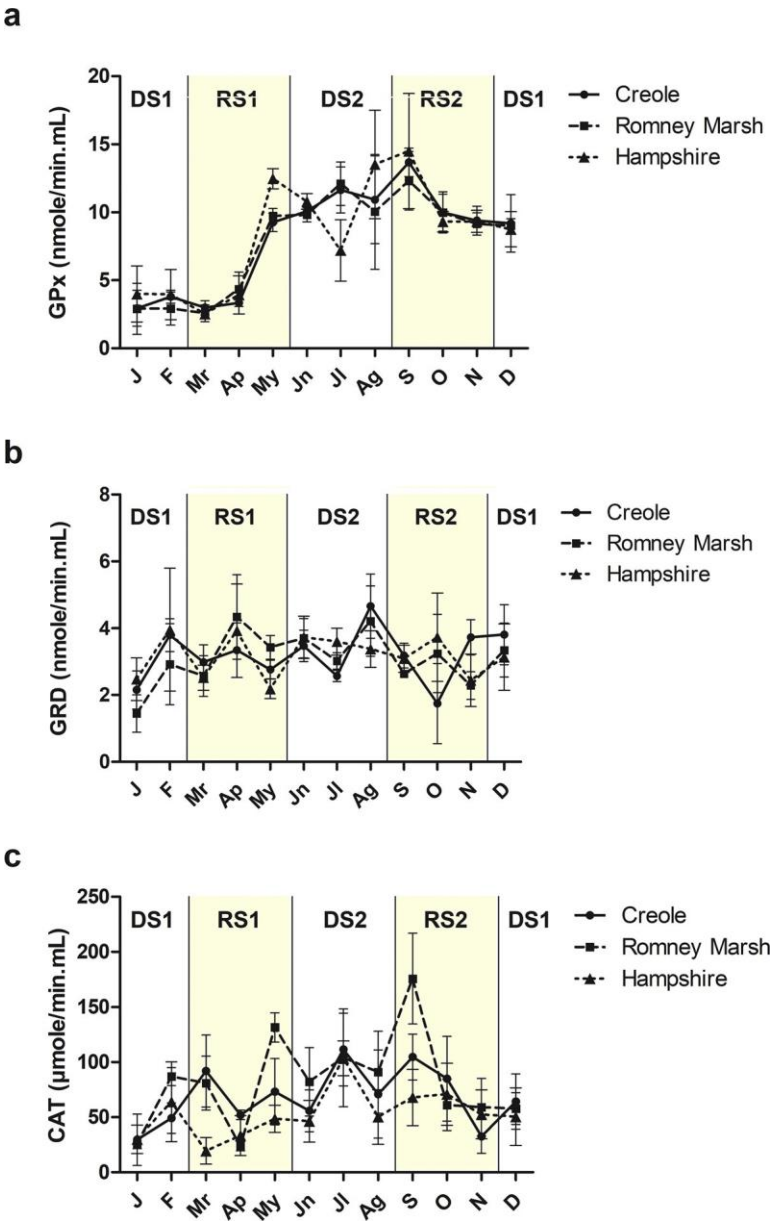

Fig. 3. Monthly values of glutathione peroxidase (GPx, panel a) glutathione reductase (GRD, panel b), and catalase (CAT, panel c) activities in seminal plasma of Creole, Romney Marsh, and Hampshire rams (four rams of each breed) when there was an equatorial photoperiod (12L:12D); Values are depicted as mean ± S.E.M. of four seminal plasma samples/ram and month; Dry (DS) and rainy (RS) seasons are also indicated.

Table 3
Glutathione peroxidase (GPx) activity (nmole/min mL) in ram seminal plasma, obtained from three different breeds (four rams of each breed) when there was an equatorial photoperiod during four climatic seasons (dry season 1: from December to February; rainy season 1: from March to May; dryseason 2: from June to August and rainy season 2: September to November); Values are shown as mean ± S.E.M; *n* indicates the number of seminal plasma samples per breed and season; Different letters in the same row indicate differences between seasons.

	Dry season 1 ($n = 52$)	Rainy season 1 ($n = 52$)	Dry season 2 ($n = 52$)	Rainy season 2 ($n = 60$)
Creole	4.66 ± 0.79[a]	5.02 ± 0.89[a]	10.82 ± 1.03[b]	10.61 ± 0.91[b]
Romney Marsh	5.26 ± 1.07[a]	5.32 ± 0.98[a]	10.59 ± 1.3[b]	10.15 ± 0.79[b]
Hampshire	5.81 ± 1.12[a]	6.01 ± 1.33[a]	10.51 ± 1.96[b]	10.56 ± 1.24[b]

Table 4
Glutathione reductase (GRD) activity (nmole/min mL) in ram seminal plasma, obtained from three different breeds (four rams of each breed) when there was an equatorial photoperiod during four climatic seasons (dry season 1: from December to February; rainy season 1: from March to May; dryseason 2: from June to August and rainy season 2: September to November); Values are shown as mean ± S.E.M/ *n* indicates the number of seminal plasma samples per breed and season.

	Dry season 1 ($n = 52$)	Rainy season 1 ($n = 52$)	Dry season 2 ($n = 52$)	Rainy season 2 ($n = 60$)
Creole	3.29 ± 0.44	3.03 ± 0.31	3.56 ± 0.40	2.91 ± 0.47
Romney Marsh	2.63 ± 0.52	3.64 ± 0.54	3.35 ± 0.27	2.73 ± 0.39
Hampshire	3.17 ± 0.67	2.41 ± 0.18	3.58 ± 0.30	3.05 ± 0.51

Table 5
Catalase (CAT) activity (μmole/min mL) in ram seminal plasma, obtained from three different breeds (four rams of each breed) when there was an equatorial photoperiod during four climatic seasons (dry season 1: from December to February; rainy season 1: from March to May; dry season 2: from June to August and rainy season 2: September to November); Values are shown as mean ± S.E.M; *n* indicates the number of seminal plasma samples per breed and season.

	Dry season 1 ($n = 52$)	Rainy season 1 ($n = 52$)	Dry season 2 ($n = 52$)	Rainy season 2 ($n = 60$)
Creole	49.41 ± 11.68	50.38 ± 10.62	50.38 ± 10.67	79.41 ± 18.09
Romney Marsh	59.07 ± 15.36	65.70 ± 15.36	82.41 ± 18.94	91.21 ± 19.50
Hampshire	37.15 ± 10.62	29.66 ± 12.80	59.82 ± 12.80	61.86 ± 12.46

Table 6
Pearson's correlation (*r*) between melatonin and testosterone concentrations and antioxidant enzyme activity in seminal plasma of Creole, Romney Marsh, and Hampshire rams; $^{*}P < 0.05$.

Melatonin	Testosterone	GPX	GRD	Catalase
Creole	−0.403	0.309	0.328	0.502
Romney Marsh	0.089	0.470	−0.319	0.415
Hampshire	−0.606*	0.443	−0.077	0.398

to the photoperiod (Poulton and Robinson, 1987). Seasonal sheep breeds subjected to an equatorial light regimen revert to a non- seasonal breeding pattern or a breeding pattern where there is a minimal effect of seasonality similar to what occurs in the localbreeds (Wodzicka-Tomaszewska et al., 1967; Jackson et al., 1990).

Furthermore, the melatonin concentration in the seminal plasma of these Colombian rams is less than when these rams are locatedin temperate climates, even during the non-reproductive season (Casao et al., 2010). Even with the relatively small concentration of melatonin, the results of the present study indicate it fluctuates throughout the year, with lesser values during rainy seasons and increases during the dry seasons. There was the least melatonin concentration during the first rainy season, from March to May,which is coincident with the minimum concentrations observed in rams located in temperate climates (Casao et al., 2010). This initial decrease in the seminal plasma melatonin concentration also occurs at a time coincident with the anovulatory season in Black-Belly sheep (Chemineau et al., 2004) when there are tropical photoperiodic conditions. The melatonin concentration in seminal plasma decreases again a second time in October and November, the months during whichthe concentrations of this hormone are maximumin rams located in temperate climates.

Several factors can affect the melatonin concentration in ram seminal plasma. The concentration of this hormone in this fluid reflects the seasonal variations of blood melatonin (Casao et al., 2010) and there are also increases after exogenous melatonin treatment (Casao et al., 2013). Thus, the changes in seminal plasma concentrations of melatonin between dry and rainy seasons couldbe due to differences in nocturnal melatonin secretion. The rams used in the present experiment were located at 4°40′N and were subjected to the natural light regimen, with no photoperiodic changes that could modify nocturnal melatonin secretion from the pineal gland. The fluctuation in these factors, therefore, do not explain these seasonal differences. Although in some species, such asthe European Hamster, nocturnal melatonin secretion, when there is a long photoperiod, can be affected by temperature (Vivien- Roels et al., 1997) however, there have been no reports that ambient temperatures modulate melotonin secretion in sheep (Wodzicka- Tomaszewska et al., 1967). Furthermore, in the mountain region where the rams used in the present study were located, mean temperatures do not oscillate throughout the year. As previously suggested, this variation in seminal plasma melatonin could also beof testicular origin (Gonzalez-Arto et al., 2016). Previous results with European rams indicated that there was not variation in melatonin synthesis by the testis that was related to season of the year (Cebrian-Perez et al., 2017) which is similar to what occurs in other organs with extra-pineal melatonin (Acuña-Castroviejo et al., 2014).

The most likely source of variation in seminal plasma melatonin in rams located in areas with an equatorial photoperiod could bethe variation in content of this hormone in the feed. Vegetal melatonin, known as phytomelatonin, has been detected in a wide variety of plant families (Koca Caliskan et al., 2017). The endogenous phytomelatonin increases when there are stressful conditions,

such as high salinity, cold temperatures or drought (Arnao and Hernandez-Ruiz, 2013a,b). Thus, during the dry seasons, the mel- atonin concentration in pasture could be greater than during the rainy seasons, therefore, a greater amount of melatonin could be ingested with the feed of the rams and could contribute to increases in the concentrations of circulating melatonin (Hattori et al., 1995) and consequently its concentration in seminal plasma. The presence of phytomelatonin in the feed of the rams would also explain the lack of differences among the breeds in the present study.

Regardless of its origin, the melatonin concentration in the seminal plasma of rams when they were located in an area with an equatorial photoperiod does not correlate positively with the testosterone concentration or antioXidant enzyme activity, unlike what occurs in rams located in temperate climates (Casao et al., 2010). The testosterone concentration in the rams of the present study was affected by breed and season. Only the rams of the British breeds had distinct differences in profiles of testosterone concentrations in both dry seasons with greater values during the second dry season. In temperate regions, the increase in nocturnal melatonin se-

cretion at the beginning of the reproductive season stimulates the hypothalamus-pituitary-testicular axis (D'Occhio et al., 1984; Lincoln et al., 1981), and blood testosterone concentrations increase 2–4 weeks later (Rosa and Bryant, 2003). This hormonal changeis reflected in the pattern of testosterone in ram seminal plasma throughout the year (Casao et al., 2010) or after melatonin treatment
(Casao et al., 2013). In the Romney Marsh or Hampshire rams in the present study that were located in an area where there was an equatorial photoperiod, the testosterone increase in seminal plasma occurred before the melatonin increase, so a melatonin stimu- latory effect is unlikely. It is possible that in these rams there was stimulation by non-photoperiodic cues to synchronize the re- productive functions. Rams subjected to constant short or long photoperiods for several years had an endogenous reproductive rhythm in terms of testicular volume and prolactin secretion (Howles et al., 1982), although the pattern did not occur at the time ofyear when the natural breeding cycle prevailed. Social factors may also have affected the outcomes in the present study where the rams with British breeding also had greater concentrations of testosterone than the Creole rams. Creole rams are smaller and less aggressive than Hampshire and Romney Marsh rams, and thus probably have a lesser rank in their social group, as indicated by their behavior during semen collection. Results of previous studies indicate that subordinate males have lesser testosterone concentrations than dominant animals (Ungerfeld and Lacuesta, 2015) and fewer increases in testosterone during the breeding season (Aguirre et al., 2007).

For antioXidant enzyme activity, there was only variation in GPx activity during the year, although this variation was not cor-
related with that of melatonin. This result is consistent with previous findings with Simmental bulls raised in tropical climates, inwhich there was only a seasonal profile in GPx (Nichi et al., 2006). In the present study, GPx activity increased in May, at the end ofthe first rainy season, and remained greater during the following dry and rainy seasons. Under tropical conditions, where there is a relatively greater temperature or humidity, antioXidant enzyme activity increases and this minimizes oXidative damage to the spermatozoa (Soren et al., 2016). Although the temperature remains constant throughout the year in the equatorial region where the rams of the present study were located, this increase in GPx activity could be due to the greater ambient humidity during the rainy season. In a previous study with Duroc boars in a tropical region results for sperm morphology patterns indicated that with relatively lesser temperatures and greater humidity there was the same effect that occurred with relatively greater temperatures and lesser humidity (Suriyasomboon et al., 2005).

5. Conclusions

In conclusion, melatonin is present in the seminal plasma of Creole, Romney Marsh, and Hampshire rams when they are located inan area where there is an equatorial photoperiod (12L:12D), with there being differences in concentration between rainy and dry seasons. The melatonin concentration in this fluid, however, does not correlate with the testosterone concentration or antioXidant enzyme activity, unlike what occurs in rams located in areas where that are temperate climates. These findings provide new per- spectives on the use of melatonin in ram reproduction when rams are located in areas where there are tropical or equatorial climates.

Conflicts of interest

None.

Acknowledgments

This research was supported by CICYT-FEDER AGL 2014-57863-R, DGA A-26 (Spain) and the 110 157 635 854 Project, funded by Colciencias in the call Col576 2012 (Universidad Nacional de Colombia, Colombia). The authors thank the Corporación Colombianade investigacion agropecuaria "Agrosavia" for their assistance.

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5.2 Artículo 2: Vasectomy and Photoperiodic Regimen Modify the Protein Profile, Hormonal Content and Antioxidant Enzymes Activity of Ram Seminal Plasma

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Vasectomy and Photoperiodic Regimen Modify the Protein Profile, Hormonal Content and Antioxidant Enzymes Activity of Ram Seminal Plasma

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Received: 14 September 2020; Accepted: 28 October 2020; Published: 29 October 2020

Abstract: This work aimed to determine the contribution of the testis and epididymis and the effect of the photoperiodic regimen on ram seminal plasma (SP). Semen was collected from 15 mature rams located in an equatorial (Colombian Creole and Romney Marsh, eight intact and two vasectomized) or a temperate climate (Rasa Aragonesa, three intact and two vasectomized). SP proteins were analyzed by Bradford, SDS-PAGE and difference gel electrophoresis (DIGE). Melatonin and testosterone concentrations were quantified by ELISA,and activity of glutathione peroxidase (GPx), glutathione reductase (GRD), and catalase by enzymatic assays. Vasectomy increased protein concentration and the intensity of high molecular weight bands ($p < 0.001$), with no differences between breeds. DIGE revealed the absence of six proteins in vasectomized rams: angiotensin-converting enzyme, lactotransferrin, phosphoglycerate kinase, sorbitol dehydrogenase, epididymal secretory glutathione peroxidase and epididymal secretory protein E1. Vasectomy also decreased melatonin concentrations in seasonal rams, and testosterone in all of them ($p < 0.001$), but did not affect antioxidant enzyme activity. Equatorial rams showed lower melatonin and testosterone concentration ($p < 0.01$) and catalase, but higher GPx activity ($p < 0.05$). In conclusion, vasectomy modifies the protein profile and hormonal content of ram seminal plasma,whereas the exposure to a constant photoperiod affects hormonal concentration and antioxidant enzymes activity.

Keywords: melatonin; testosterone; antioxidant enzymes; vasectomy; DIGE; seminal plasma; ram

1. Introduction

Seminal plasma (SP) is a complex fluid secreted mainly by the testis, epididymis and accessory glands, although some contribution from the spermatozoa [1] has also been identified. SP composition varies among species, mainly due to differences in size and secretory capacity of the accessory glands [2]. SP is composed of proteins, amino acids, enzymes, ions, lipids, sugars, hormones and cytokines, each of these components having essential functions for the spermatozoa (reviewed in [3]). Moreover, several elements of seminal plasma, specially proteins [4], but also others, such as fructose [5] and noncoding microRNAs [6], are related with fertility. Seminal plasma proteome analyses has been used to identify fertility markers in several species, such as human [7], boar [8], bull [9,10]or dromedary camel [11].

Additionally, SP analysis after vasectomy is a useful tool for identifying which constituents of this fluid originate from the testis and epididymis. A previous study of human vasectomized patients identified several proteins unique either to control or post-vasectomized patients [12]. Vasectomy also decreased alkaline phosphatase in alpacas [13], and several biochemical components, including protein composition, in dogs [14]. In the ram, vasectomy increases SP protein concentration [15], while it decreases the high molecular weight proteins [16], and eliminates the membrane vesicles, which suggests a testicular or epididymal origin. However, there areno reports on the effect of vasectomy on the proteomic composition of ram seminal plasma.

Besides proteins, other SP components necessary for the sperm function are antioxidant enzymes and hormones. SP antioxidant enzymes, namely glutathione peroxidase (GPx), glutathione reductase (GRD), superoxide dismutase (SOD) and catalase, protect the spermatozoa from oxidative damage, and have been related with male fertility [17,18]. The origin of these SP antioxidant enzymes is unclear. Several isoforms of GPx are secreted by the epididymis [19], although some contribution of the accessory gland has also been identified [20,21]. GRD, SOD and catalase are likely secreted by the accessory sex glands [21,22]. To the best of our knowledge, no report on the source of ram SP antioxidant enzymes has been published.

As regards hormones, testosterone and melatonin can be found in ram seminal plasma [23]. Testosterone is secreted by the Leydig cells of the testes; thus, a testicular origin of the seminal plasma testosterone is expected. Nonetheless, testosterone also enters the general circulation after its secretion [24], and a systemic source cannot be ruled out either. In contrast, melatonin is secreted mainly by the pineal gland [25]; thus, a pineal origin of seminal plasma melatonin is likely. However, we recently demonstrated the presence of melatonin-synthesizing enzymes in the testis [26], thus this SP melatonin could also be of testicular origin. The levels of testosterone [27] and melatonin [28] in seminal plasma have been connected to male fertility. The beneficial effects of SP melatonin could been related to its antioxidant properties [29,30], whereas the levels of SP testosterone has been proposed as a spermatogenesis marker [31] in human.

Ovine is a seasonal species, and its reproductive activity is regulated by the photoperiod through melatonin secretion [32,33]. In rams located in temperate regions (>30 °C and <45 °C northor south latitudes, 18L:6D light regime), differences in seminal plasma protein and hormonal content, and in antioxidant enzyme activity, vary between reproductive and non-reproductive seasons [23,34]. However, in rams located in equatorial climates (between 10° N and 10° S, 12L:12D), variations in the composition of SP are most likely related to food intake or climatic factors [35]. Nonetheless, the differences in SP between rams located in temperate and equatorial climates has never been studied before.

Thus, the objectives of this work were: 1) to determine the contribution of the testis, epididymis and accessory sex glands secretions to protein and hormonal concentrations, and to antioxidant enzymeactivity in ram seminal plasma and 2) to elucidate whether the photoperiodic regimen can affect the seminal plasma composition of intact and vasectomized rams.

2. Results

No differences were found in protein, hormonal concentration or antioxidant enzyme activity between the two analyzed non-seasonal breeds (Creole and Romney Marsh), either intact or vasectomized (Appendix A, Table A1). Thus, data from intact or vasectomized rams of both non-seasonal breeds were pooled for further analysis.

1.1. Vasectomy Modifies the Protein Profile of Ram Seminal Plasma

Protein concentration was higher ($p < 0.001$) in SP from vasectomized than from the intact rams, (Table 1). SDS-PAGE, followed by band quantification, showed that vasectomy decreased highmolecular weight bands ($p < 0.001$), with no differences in medium or low molecular bands in either the seasonal or equatorial rams (Table 1 and Figure 1). The photoperiodic regime did not affect protein concentration or band quantification.

Table 1. Protein concentration (mg/mL, $n = 12$ seminal plasma samples for seasonal rams, and $n = 24$ seminal plasma samples for non-seasonal) and densitometric quantification ($\times 10^3$ arbitrary units, $n = 4$ seminal plasma samples) of high, medium and low molecular weight (MW) bands of seminal plasma from intact and vasectomized rams subjected to a temperate (seasonal rams) or equatorial (non-seasonal rams) photoperiod. Results are shown as mean ± SEM. [a], [b] indicate $p < 0.001$.

		Band Intensity × 10^3 (Arbitrary Units)		
	Total Protein (mg/mL)	**High MW Bands (250–75 kDa)**	**Medium MW Bands (75–37 kDa)**	**Low MW Bands (37–10 kDa)**
Intact seasonal rams	34.20 ± 5.42 [a]	3.90 ± 1.43 [a]	11.25 ± 3.54	9.95 ± 2.80
Vasectomized seasonal rams	39.60 ± 1.66 [a]	3.54 ± 0.53 [a]	9.45 ± 1.21	11.80 ± 1.46
Intact non-seasonal rams	64.63 ± 6.36 [b]	0.44 ± 0.08 [b]	7.57 ± 1.0	12.20 ± 1.47
Vasectomized non-seasonal rams	53.40 ± 2.10 [b]	0.60 ± 0.13 [b]	10.56 ± 5.29	10.85 ± 3.44

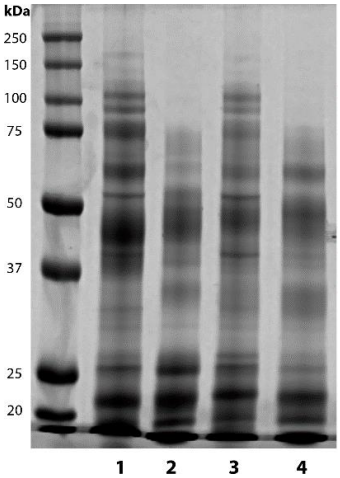

Figure 1. Representative image of SDS-PAGE of seminal plasma proteins from intact and vasectomized rams subjected to temperate (seasonal rams) or equatorial (non-seasonal rams) photoperiod. 1: intact seasonal, 2: vasectomized seasonal, 3: intact non-seasonal, 4: vasectomized non-seasonal.

Due to the lack of significant differences between seasonal and equatorial rams, we only analyzed differences in the seminal plasma protein composition by difference gel electrophoresis (DIGE)in the seasonal males. DIGE revealed that the abundance of 40 spots increased (0.6%) and of144 spots decreased (2.0%) in vasectomized animals ($p < 0.01$). DIGE also detected six proteins that were only present in intact rams (−6.0 Log volume ratio, Supplementary Figure S1). These proteins were identified (Supplementary File S2) as angiotensin-converting enzyme (ACE, Figure 2a),

lactotransferrin (Figure 2b), sorbitol dehydrogenase (Figure 2c), phosphoglycerate kinase (Figure 2d), epididymal secretory glutathione peroxidase (Figure 2e) and epididymal secretory protein E1 (Figure 2f). Among the spots that increased in the vasectomized rams, we identified the inactive ribonuclease-like protein 9 (RNase9, Figure 2g) as the most abundant.

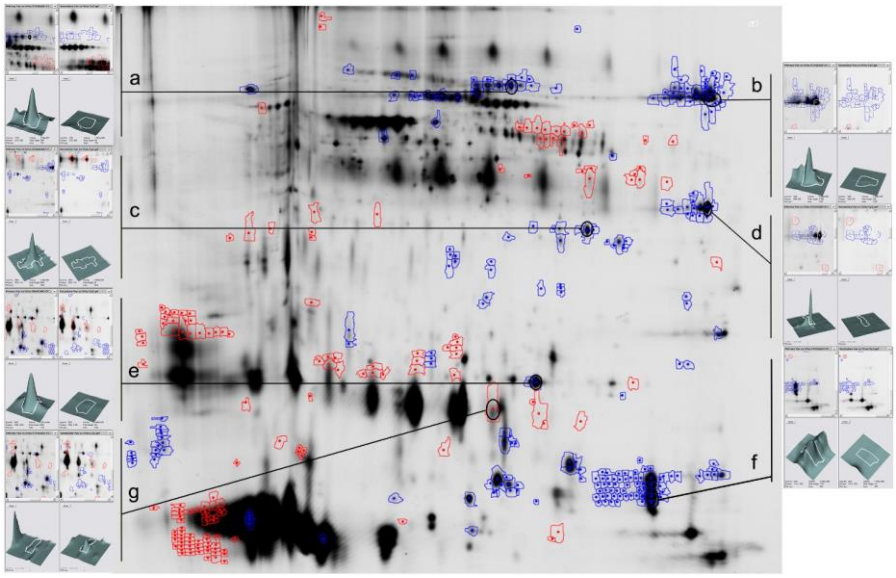

Figure 2. Representative image of difference gel electrophoresis (DIGE) analysis of seminal plasma proteins from intact and vasectomized rams. Angiotensin-converting enzyme (**a**), lactotransferrin (**b**), sorbitol dehydrogenase (**c**), phosphoglycerate kinase (**d**), epididymal secretory glutathione peroxidase (**e**), epididymal secretory protein E1 (**f**) and inactive ribonuclease-like protein 9 (**g**) spots are identified.

1.2. Vasectomy Decreases Melatonin and Testosterone Concentrations, but Not Antioxidant Enzyme Activity inRam Seminal Plasma

Melatonin analyses revealed that the vasectomized rams in temperate climate had lower SP melatonin concentrations than the intact rams (264.3 ± 19.9 vs. 124.3 ± 12.3 pg/mL for intact and vasectomized rams, respectively, $p < 0.001$). This vasectomy effect was not found in males located in the equatorial photoperiod. Moreover, the melatonin concentration was significantly higher in seasonal than in non-seasonal rams, regardless of the state of their reproductive tract (Figure 3a).

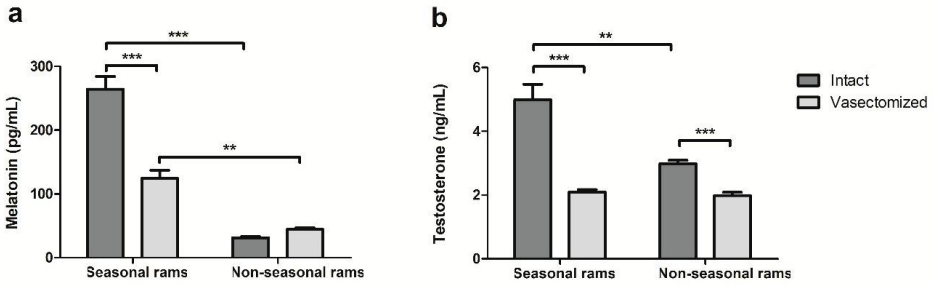

Figure 3. (**a**) Melatonin and (**b**) testosterone concentration in seminal plasma of intact and vasectomizedrams subjected to temperate (seasonal rams) or equatorial (non-seasonal rams) photoperiod. Results areshown as mean ± S.E.M of $n = 12$ seminal plasma samples for seasonal rams, and $n = 24$ seminal plasmasamples for non-seasonal. ** $p < 0.01$, *** $p < 0.001$.

On the other hand, vasectomy reduced the testosterone concentration in the seminal plasma ofboth seasonal and non-seasonal vasectomized rams when compared with their intact counterparts(Figure 3b, $p < 0.001$) This hormone was higher in the intact animals located in a temperate climate than in those in an equatorial photoperiod; however, this difference was not found in the vasectomized rams (Figure 3b)

The photoperiod but not vasectomy affected the antioxidant enzyme activity, but vasectomy did not.Both the intact and vasectomized rams located in an equatorial photoperiod showed a higher GPx activity than the seasonal ones (Figure 4a, $p < 0.001$). In contrast, the intact rams located in atemperate climate had more catalase enzymatic activity than their equatorial counterparts (Figure 4c, $p < 0.05$).No differences were found in GRD activity.

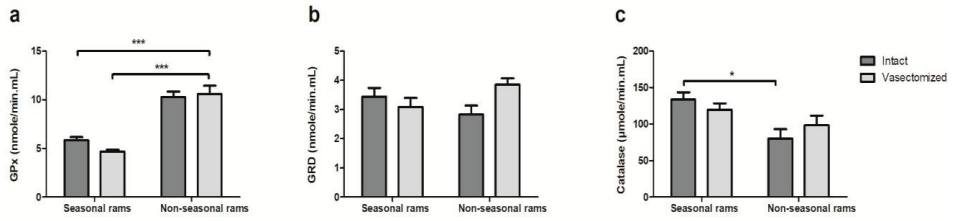

Figure 4. (**a**) Glutathione peroxidase (GPx), (**b**) glutathione reductase (GRD) and (**c**) catalase enzymatic activity in seminal plasma of intact and vasectomized rams subjected to temperate (seasonal) or equatorial (non-seasonal rams) photoperiod. Results are shown as mean ± S.E.M of $n = 12$ seminal plasma samples for seasonal rams, and $n = 24$ seminal plasma samples for non-seasonal. * $p < 0.05$, *** $p < 0.001$.

3. Discussion

Previous studies revealed no differences in seminal plasma protein concentration between Colombian Creole and Romney Marsh rams [36]. In this work, we have also found that the photoperiodic regime (temperate vs. equatorial) does not affect protein concentration or the SDS-PAGE band profile.

However, vasectomy increased the protein concentration in seminal plasma, irrespective of the location of the rams. Nonetheless, this variation was not uniform, and a decrease in high molecular weight proteins was detected. These results were in concordance with those of Ghaoui et al. [15,16]and further support the hypothesis that most of the protein constituents of seminal plasma are derived from the accessory sex glands, whereas the high molecular weight proteins would have a testicular and epididymal origin in the form of membrane vesicles [15].

DIGE analyses allowed us to delve into these differences, and revealed an increase in the abundance of 40 spots and a decrease of 144 in seminal plasma from vasectomized rams, along with the absenceof six proteins, subsequently identified as angiotensin-converting enzyme (ACE), lactotransferrin, phosphoglycerate kinase, sorbitol dehydrogenase, epididymal secretory glutathione peroxidase and epididymal secretory protein E1. Lactotransferrin [37], epididymal secretory glutathione peroxidase and epididymal secretory protein E1 [38] are secreted in the epididymis, and phosphoglycerate kinase in the testis [39]. In contrast, ACE and sorbitol dehydrogenase had been postulated to be of sperm origin [40] and released in the caput epididymis [1], although in human seminal plasma ACE could also be secreted by the prostate [41].

These proteins have multiple effects on spermatozoa: epididymal secretory protein E1 binds tothe sperm surface, especially in the acrosome and midpiece [42], and it is possibly related to spermmotility; phosphoglycerate kinase is also associated with sperm motility in several species [43,44]; sorbitol dehydrogenase, which has been linked with cryopreservation resistance [45] and also with sperm motility during the epididymal transit [46]; ACE, which binds to the spermatozoa and is crucial for sperm-egg binding [47]. Finally, epididymal secretory glutathione peroxidase has antioxidant activity, and lactotransferrin is an iron carrier that prevents sperm lipid peroxidation [48]. These proteins

could be useful as markers of fertility: in human semen, an increase in lactotransferrin levels has been related to a decrease in leukocyte levels [49], whereas epididymal secretory protein E1 was increased in asthenozoospermic samples [50].

Surprisingly, the protein with the biggest increase in the seminal plasma of vasectomized ramswas inactive ribonuclease-like protein 9 (RNase9). RNase9, which has antibacterial activity [51] andwas previously identified in the epithelium of the epididymis in human [52], mouse [53] and rat [54],and also in the postacrosomal region of human spermatozoa [52]. However, its abundance in the seminal plasma of the vasectomized rams suggests that in this species it would not be secreted by the epididymis, but in one of the accessory glands, possibly the seminal vesicles [55].

On the other hand, vasectomy reduced, but did not suppress, the melatonin and testosterone concentrations in the seminal plasma, which suggests that a portion of the seminal plasma hormonal content has a testicular origin.

The decrease in the melatonin concentration caused by vasectomy was only detected in seasonal rams. In rams located in temperate climates, the melatonin concentration in seminal plasma undergoes seasonal variation [23]. Additionally, the melatonin-synthesizing enzymes aralkylamine N-acetyltransferase (AANAT) and N-Acetylserotonin O-methyltransferase (ASMT) are present in ram testes [26]. In this work, we have demonstrated that around half of the melatonin found in the seminal plasma of seasonal rams during the reproductive season has a testicular origin, and thepineal gland might be the source of the other half. However, the lack of differences in the melatoninconcentration between intact and vasectomized rams located in a constant photoperiod suggests that,in these males, either the testicular melatonin synthesis is eliminated, or the testicular melatonin isnot secreted to the seminal plasma. This could be due to the different photoperiodic environment the rams are exposed to. In seasonal rams, melatonin receptors MT1 and MT2 are also present in the testes [56], thus variations in pineal melatonin could regulate testicular melatonin secretion, as in other extrapineal melatonin-synthesizing organs or cells [57]. In contrast, equatorial rams are subjected to a constant 12L:12D light regimen and the lack of seasonal variations in pineal melatonin could be reflected in testicular melatonin secretion. However, more studies on the presence and functionality of melatonin-synthesizing enzymes and melatonin receptors in the testes of equatorial rams are needed to test this hypothesis. Apart from the lack of differences between intact and vasectomized rams, non-seasonal males had a significantly lower concentration of seminal plasma melatonin than theirseasonal counterparts. We had previously detected these low melatonin levels in a previous study [35].

This difference in melatonin concentration could be due to either the shorter night length (12 h 10^{J} vs. 16 h 12^{J} of darkness for equatorial and Mediterranean rams at the end of the experiment) or the static photoperiodic signal caused by a 12L:12D light regimen [58].

Vasectomy also decreased the testosterone concentration in the seminal plasma of seasonal and non-seasonal rams, although this change was more marked in the Mediterranean males. This seminal plasma testosterone decrease has been previously identified in human [59] and boar [60]. Our results also suggest a dual origin of the testosterone present in the ram seminal plasma, with testicular (from rete testes fluid [61]) and non-testicular (likely from circulating blood or, to a lesser extent, from adrenal glands [62]) contribution. We also detected differences in testosterone concentrationbetween intact rams, but not between the vasectomized ones from different light regimens, which suggests a possible photoperiodic regulation of the local testosterone secretion by the testis. In the seasonal ram, testosterone secretion is regulated by the photoperiod [63,64], thus, it is possible that,as previously discussed for melatonin concentration, the differences in the dark/light regimen between the rams would explain the differences in the testis contribution to seminal plasma testosterone found in this work.

Finally, vasectomy did not affect antioxidant enzymatic activity. Previous studies in humans revealed that vasectomy did not affect catalase and superoxide dismutase activities in human seminal plasma [65], and that most antioxidant enzymes present in this fluid had a prostatic origin [22].

Thus, an accessory gland source would explain the presence of GPx in vasectomized rams although DIGE analyses revealed the absence of epididymal secretory glutathione peroxidase in these animals.

GPx was significantly higher in equatorial than in Mediterranean rams, whereas catalase activity was higher in seasonal males. We had previously detected this increase in GPx activity during therainy season [35] in tropical climates, which would likely protect the spermatozoa from oxidative damage in high humidity or temperature conditions. We had also previously detected higher levels of catalase activity, although not significant, during the autumn months in seasonal rams, and a positive correlation with seminal plasma melatonin [23], which could explain the results found in this work.

4. Materials and Methods

4.1 Animals and Seminal Plasma Extraction

4.1.1 Rams in the Mediterranean Climate

Seminal plasma was obtained weekly for three months during the reproductive season (Septemberto November) from first ejaculates of five Rasa Aragonesa rams (three intact and two vasectomized)in compliance with the requirements of the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. All experimental procedures were performed under Project License PI19/17 approved by the Ethics Committee for Animal Experiments of the University of Zaragoza (approval date: 24 May 2017). The males were maintained under uniform nutritional conditions and natural photoperiod at the Experimental Farm of the University of Zaragoza, Spain ($41^{\circ}38^{J}05.8^{JJ}$ N $0^{\circ}51^{J}35.2^{JJ}$ W). Local amplitude of the photophase during the period of study (September to November) varies from 12 h 35^{J} (11 h 25^{J} of darkness) at the beginning of the experimentto 7 h 48^{J} (16 h 12^{J} of darkness) at the end, i.e., 4 h 47^{J} of difference between the longest and the shortest day of this period.

Ejaculates from intact and vasectomized animals were collected once a week using an artificial vagina. In order to eliminate individual differences and obtain enough volume of sample forall the analyses, daily ejaculates from the rams of each experimental group were pooled and processed together [66]. Seminal plasma was extracted by centrifugation at 7500× *g* for 10 minin a microfuge at 4 °C (Eppendorf Centrifuge 5430R, Hamburg, Germany). The supernatant was centrifuged again; the seminal plasma was then recovered and filtered through a 0.22 µm Milliporemembrane (Merck KGaA, Darmstadt, Germany). After adding 10% protease and phosphatase inhibitor (Sigma Chemical Co., St. Louis, MO, USA), SP was aliquoted and kept at −20 °C until analysis.

4.1.2 Rams under Equatorial Photoperiod

All procedures used in this study were in strict accordance with Colombian Animal Protection Regulations (Law 84/1989, modified by Law 1774/2016) and were approved by the Bioethics Committeeof the Faculty of Veterinary Medicine and Zootechnics, Bogotá Headquarters, National University of Colombia (Project license: CB-074-2014, approval date: 5 November 2014). The rams were locatedat the Center for Ovine Research, Technological Development and Extension (CIDTEO), of theNational University of Colombia, Mosquera ($4^{\circ JJ}$ $40^{J}57^{JJ}$ N $74^{\circ JJ}$ $12^{J}50^{JJ}$ W). Local amplitude of the photophase during the period of study (September to November, rainy season) varies from 12 h 09^{J}(11 h 51^{J} of darkness) to 11 h 50^{J} (12 h 10^{J} of darkness), i.e., 19^{J} of difference between the longest and the shortest day.

Semen was obtained using an artificial vagina from 10 mature rams of two breeds (Creole and Romney Marsh, eight intact and two vasectomized). Ejaculates were collected once a week, and daily samples from each breed and experimental group were pooled together for seminal plasma extraction.

Seminal plasma was obtained following the same protocol as in the rams in the Mediterranean Climate, and sent to Spain, where the SP was analyzed, in dry ice.

4.2 Seminal Plasma Protein Analyses

The seminal plasma protein concentration was calculated by Bradford's method [67] using a commercial kit (Quick Start Bradford protein assay, Bio-Rad, Hercules, CA, USA), whereas the protein composition was analyzed by SDS-PAGE and difference gel electrophoresis (DIGE).

For SDS-PAGE, 20 µg of SP proteins were mixed with a loading buffer (Tris/HCl 0.045 M, EDTA 0.8 mM, SDS 3% (*wt*/*v*), glycerol 10% (*v*/*v*), β-mercaptoethanol 5% (*v*/*v*) and bromophenol blue 0.004% (*wt*/*v*)) and loaded in a 10% polyacrylamide gel. The electrophoresis was performed at 130 V for 90 min at 4 °C.
A mixture of prestained molecular weights ranging from 10 to 250 kDa (Bio-Rad, Hercules, CA, USA)was used as a standard. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (0.1% *wt*/*v*) in 45% (*v*/*v*) methanol and 10% (*v*/*v*) acetic acid, and de-stained in 30% (*v*/*v*) methanol,10% (*v*/*v*) acetic acid and distilled water until no background was detectable. Gel images were capturedand analyzed with the Odyssey Clx Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA).Finally, the proteomic analysis by difference gel electrophoresis (DIGE) was performed in the Proteomics Unit of the Complutense University of Madrid, Spain, and analyzed using the DeCyder 2-D Differential Analysis software (version 5.0, GE Healthcare, Chicago, IL, USA). Differentially expressed spots were excised, digested and identified by MALDI-TOF MS fingerprinting and the MASCOT algorithm v2.1 (Matrix Science, Boston, MA, USA).

4.3 Seminal Plasma Hormonal Analyses

4.3.1 Melatonin Evaluation

Seminal plasma melatonin concentration was measured using a competitive commercial immunoassay (Direct saliva melatonin ELISA kit, Bühlmann Laboratories AG, Schönenbuch, Switzerland). Assay specifications were: Sensitivity: 0.5 pg/mL, Intra-assay variability: 5.2%, Inter-assay variability: 11.2%. Following the manufacturer's instructions, 100 µL of seminal plasmasample, control, or calibrator were loaded in duplicate in a microtiter plate coated with an anti-melatonin antibody. After 16 to 20 h incubation at 2 to 8 °C, 50 µL of biotinylated melatonin were added to each well.Following another 3 h of incubation at 2 to 8 °C and three washes, 100 µL of streptavidin-conjugated horseradish peroxidase (HRP) was added into the wells. The plate was then incubated for 60 min at 600 rpm at room temperature in a plate rotator. After the other three were washed, 100 µL of tetramethylbenzidine substrate (TMB) was added and the plate was incubated for 30 min at the same conditions but protected from direct light. Finally, 100 µL of 0.25 M SO_4H_2 solution was added, and absorbance was measured at 450 nm on a plate reader (TECAN Spectrafluor plus, Tecan Group Ltd.,Männedorf, Switzerland).

4.3.2 Testosterone Evaluation

Seminal plasma total testosterone concentration in the ram seminal plasma was evaluated by meansof a commercial ELISA kit assay (Testo-Easia, BioSource Europe, SA, Belgium; Sensitivity: 0.05 ng/mL; Intra-assay variability: 4.8%, Inter-assay variability: 7.1%). Following the manufacturer's instructions, 50 µL of seminal plasma, control or calibrators was loaded in duplicate in an anti-testosterone coated microtiter plate. After the addition of 100 µL of HRP-labelled testosterone, the plate was incubated for one hour at room temperature. At the end of the incubation, the wells were washed three times, 100 µL of TMB were added to each well and the plate was incubated for 30 min at room temperature, protectedfrom direct light. Finally, 100 µL of 0.2 M HCl solution was added, and absorbance was measured on a plate reader (TECAN Spectrafluor plus, Tecan Group Ltd., Männedorf, Switzerland) at 450 nm.

4.4 Seminal Plasma Antioxidant Enzymes Activity

In these assays, all samples were loaded in duplicate and analyzed the same day.

4.4.1 Glutathione Peroxidase (GPx)

Glutathione Peroxidase enzymatic activity was evaluated in 6 µL of seminal plasma, measuring the oxidation of glutathione (GSH, 2 mM) to oxidized glutathione (GSSG) catalyzed by GPx. Ter-Butylhydroperoxide (t-BuO2H, 1.2 mM) was used as an electron acceptor, and GSSG was recycled back to GSH using GRD (54 mUI) and NADPH (85 µM), in a 300 mM sodium phosphate buffer (pH 7.2) that also contained EDTA 0.5 mM. Final volume was 200 µL. The enzymatic activity was monitored for 3 min at 340 nm in a microtiter plate reader (TECAN Spectrafluor plus, Tecan Group Ltd., Männedorf, Switzerland).

4.4.2 Glutathione Reductase (GRD)

Glutathione Reductase enzymatic activity was evaluated measuring the decrease in absorbance produced by NADPH oxidation because of the oxidized glutathione (GSSG) reduction. The reaction mixture contained 300 mM sodium phosphate buffer at pH 7.2; 0.5 mM EDTA; 85 µM NADPH; 0.8 mM GSSG. Five µL of seminal plasma were added to complete a final volume of 200 µL. The enzymatic activity was evaluated for 3 min at 340 nm with a microtiter plate reader (TECAN Spectrafluor plus,Tecan Group Ltd., Männedorf, Switzerland).

4.4.3 Catalase

Catalase enzymatic activity was evaluated by the decrease in absorbance due to the H_2O_2 reduction produced by this enzyme. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7), 30 mM H_2O_2 and 4 µL seminal plasma (final volume of 200 µL). The change in absorbance was measured for 2 min at 240 nm in a plate reader (TECAN Spectrafluor plus, Tecan Group Ltd., Männedorf, Switzerland).

4.5 Statistical Analyses

The number of evaluated samples was 12 from each breed (Rasa aragonesa, Creole or Romney Marsh) and experimental group (intact or vasectomized). The distribution of the data and homoscedasticity were evaluated by the Kolmogorov-Smirnov and Bartlett's tests, respectively. When data showed a normal distribution and equal variances (i.e., total protein, band intensityand GRD), differences between experimental groups were compared by means of ANOVA, followed by Tukey's Multiple Comparison Test. When the studied data failed the normality or homoscedasticity test (i.e., hormonal data, GPx and catalase), differences between groups were analyzed by means of the Kruskal-Wallis test, followed by Dunn's post-test. All statistical analysis was performed with GraphPad Prism version 5.01 for Windows (GraphPad Software, La Jolla, CA, USA).

5. Conclusions

In conclusion, vasectomy modifies the protein profile and hormonal content of ram seminal plasma, but not antioxidant enzyme activity. The exposure to a constant photoperiod resulted in decreased melatonin and testosterone concentrations in this fluid, and increased GPx activity, with no effect on the protein profile.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/21/8063/s1.

Author Contributions: Conceptualization, A.C., J.A.A. and J.Á.C.-P.; methodology, A.C., J.A.A. and J.Á.C.-P.; formal analysis, M.C.-S., M.F. and A.C.; investigation, M.C.-S., M.F. and F.T.-R.; resources, J.Á.C.-P., R.P.-P., J.A.A., M.H. and H.G.-L.; data curation, M.C.-S., M.F. and A.C.; writing—original draft preparation, A.C.; writing—review and editing, all the authors; visualization, M.C.-S., M.F. and A.C.; supervision, A.C., R.P.-P., T.M.-B., M.H. and J.Á.C.-P.; project administration, R.P.-P., T.M.-B., J.Á.C.-P., J.A.C. and H.G.-L.; funding acquisition, R.P.-P., J.Á.C.-P., J.A.C. and H.G.-L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by CICYT-FEDER AGL 2017-83799-R, CICYT-FEDER AGL 2013-43328-P and DGA A-07_17R (Spain), the 110 157 635 854 Project, funded by Colciencias Code Research Program 110157635854, contract 0607-2013 (Colombia) and the Tunisian Ministry of Higher Education and Scientific Research, University of Gabès.

Acknowledgments: The proteomic analysis was performed in the Proteomics Unit of Complutense University of Madrid, a member of ProteoRed, and is supported by grant PT17/0019, of the PE I+D+i 2017-2020, funded by ISCIII and ERDF. The authors thank the Corporación Colombiana de investigacion agropecuaria "Agrosavia" andthe staff of the Livestock and Wildlife Laboratory, IRA Médenine, Tunisia for their assistance.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Abbreviations

SP	Seminal plasma
GPx	Glutathione peroxidase
GRD	Glutathione reductase SOD
	Superoxide dismutase
DIGE	Difference gel electrophoresis

Appendix A

Table A1. Protein, melatonin and testosterone concentration, and antioxidant enzymes activity in seminal plasma of intact and vasectomized rams of two breeds (Creole and Rommey Marsh) located in equatorial photoperiod. Results are shown as mean ± SEM of n = 12 seminal plasma samples.

	Intact		Vasectomized	
	Creole	**Romney Marsh**	**Creole**	**Romney Marsh**
Total protein (mg/mL)	36.51 ± 2.30	42.69 ± 2.18	52.05 ± 2.98	55.01 ± 10.12
Melatonin (pg/mL)	32.78 ± 3.38	30.15 ± 1.50	46.63 ± 3.05	42.51 ± 4.52
Testosterone (ng/mL)	3.06 ± 0.14	2.89 ± 0.18	1.92 ± 0.13	2.05 ± 0.14
Glutathione peroxidase (nmole/min mL)	10.44 ± 0.82	10.15 ± 0.79	12.68 ± 1.43	9.58 ± 0.71
Glutathione reductase (nmole/min mL)	2.41 ± 0.46	2.73 ± 0.42	4.50 ± 0.43	3.60 ± 0.29
Catalase (µmole/min mL)	68.84 ± 16.00	91.21 ± 19.50	104.04 ± 20.06	93.75 ± 17.71

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5.3 Artículo 3: Melatonin membrane receptors MT1 and MT2 are expressed in ram spermatozoa from non-seasonal breeds

Tropical Animal Health and Production (2020) 52:2549–2557
https://doi.org/10.1007/s11250-020-02289-4

REGULAR ARTICLES

Melatonin membrane receptors MT_1 and MT_2 are expressed in ram spermatozoa from non-seasonal breeds

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Received: 21 November 2019 / Accepted: 13 May 2020 / Published online: 22 May 2020

Abstract

In mammals, many melatonin biological functions are mediated through its interaction with the membrane receptors MT_1 andMT_2. We have previously reported their presence in ram spermatozoa from males located in temperate climates, but there is no information on their presence in spermatozoa from rams in areas with an equatorial photoperiod (12L:12D). Thus, we haveinvestigated the existence and cellular distribution of melatonin receptors in spermatozoa from three sheep breeds in Colombia(Colombian Creole, Hampshire, and Romney Marsh) during dry and rainy seasons, using indirect immunofluorescence andwestern blot. Our results indicated the presence of melatonin receptors in spermatozoa from these rams, and that their distributiondiffers from that previously found in spermatozoa from rams in temperate climates. Moreover, two new immunotypes of MT_2were identified: type N, with staining only in the neck, and type E with a band of immunofluorescence in the upper part of thepost-acrosome and the apical edge. Likewise, differences between breeds and climate seasons were detected for both receptors.However, densitometry analysis of western blot bands only revealed differences between seasons in the Creole rams for MT_1 andthe Romney Marsh rams for MT_2, whereas differences between breeds were only detected for MT_2. It could be inferred thatmelatonin receptors in rams subjected to an equatorial photoperiod might be more closely related to sperm quality than seasonal control. Therefore, the presence of these receptors suggests that melatonin could be a useful tool to increase the fertility of ramslocated in tropical or equatorial climates.

Keywords Ram · Equatorial · Melatonin receptors

Introduction

Melatonin is an indolamine hormone produced at nighttime not only by the pineal gland (Reiter 1991) but also by extra-pineal tissues (Acuña-Castroviejo et al. 2014) during the day. It is broadly distributed among all taxa, and it performs a wide range of biological roles: as an antioxidant and antiapoptotic molecule, an immunomodulator, a regulator of circadian cycles and sleep, a signal for seasonal reproduction, among others (see reviews by Pandi-Perumal et al. (2006) and Tan et al. (2010)). In mammals, many of the actions of melatonin are mediated through its interaction with the melatonin receptors MT_1 and MT_2, which belong to the G protein-coupled receptor superfamily (Dubocovich et al. 2010).

In most species, including sheep, seasonality, regulated by melatonin secretion, is the most critical factor that controls the reproductive function (Malpaux et al. 2001). In seasonal sheep breeds located in temperate climates, photoperiod differences between short and long days affect the duration of nocturnal

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11250-020-02289-4) contains supplementary material, which is available to authorized users.

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melatonin secretion, which modulates the reproductive activ-ity through the hypothalamic-pituitary-gonadal axis (Chemineau et al. 2010). However, in tropical and subtropical latitudes with minimal photoperiod variations during the year, some sheep breeds have been reported to be almost or entirely unseasonal, although other environmental cues, such as rainfall, could affect their reproduction due to the amount and quality of forage (Rosa and Bryant 2003). Moreover, most ewes from temperate climates subjected to an equatorial photoperiod (12L:12D) exhibit a non-seasonal reproductive pattern after a short period of adaptation (Jackson et al. 1990).

In rams located in temperate regions, testicular volume, sexual behavior, and semen quality vary between the reproductive and non-reproductive seasons (Lincoln et al. 1990; Rosa et al. 2012). These differences between seasons have been related to the modulatory effect of melatonin on the hypothalamic-pituitary-testicular axis (Fitzgerald and Stellflug 1991). Nevertheless, the presence of melatonin re-ceptors MT_1 and MT_2 in the testis, accessory glands (González-Arto et al. 2017) and spermatozoa (Casao et al. 2012) also suggest a direct action of this hormone in the ramreproductive tract. However, despite the considerable knowl-edge of the role of melatonin and melatonin receptors in sheepreproduction in temperate climates, little is known about thefunctions of this hormone in tropical or equatorial regions. Likewise, there is no information on the presence of melatonin receptors in spermatozoa from rams subjected to an equatorialphotoperiod.

The functions of melatonin receptors in the spermatozoaare likely to be other than seasonal regulation, as they can alsobe found in the sperm of other non-seasonal species (Gonzalez-Arto et al. 2016b; van Vuuren et al. 1992). Likewise, the MT_1 melatonin receptor has been related withoxidative damage and apoptosis protection in human sperm(Deng et al. 2017; Espino et al. 2011) whereas MT_2 has beenassociated with the modulation of ram sperm capacitation un- der in vitro conditions (Gonzalez-Arto et al. 2016a).

In a previous study, we demonstrated the presence of melatonin in the ram seminal plasma from three sheepbreeds (Colombian Creole, Romney Marsh, and Hampshire) subjected to an equatorial photoperiod, with dif- ferences between rainy and dry seasons (Carvajal-Sernaet al. 2019), although the source of this variation remains unknown. The Colombian Creole sheep (Creole) is a native sheep breed, present in the country for five centuries, where-as Romney Marsh and Hampshire were introduced in the early 1960s. Thus, in order to further investigate how mela-tonin affects ram reproduction in an equatorial photoperiod(12L:12D), we examined the presence and distribution of melatonin receptors in spermatozoa obtained from one native (Creole) and two imported sheep breeds (Hampshire and Romney Marsh) reared in Colombia, during dry and rainy seasons.

Materials and methods

Animals and location

All rams used in this study were handled in strict accor- dance with the Colombian Animal Protection legislation (Law 84/1989, modified by Law 1774/2016). Males were housed under uniform nutritional conditions at the Center for Ovine Research, Technological Development and Extension of the National University of Colombia, located in Mosquera (4° 40′ 57″ N. 74° 12′ 50″ W) at 2510 m above sea level. Indirect immunofluorescence assays were performed at the "Tibaitatá" research center, part of Colombian Corporation for Agricultural Research (AGROSAVIA).

The rams were kept under natural photoperiod condi- tions with 32 min of difference between the longest andthe shortest day of the year. Their diet was based on pasture (*Pennisetum clandestinum*, *Lolium perenne*), sup- plemented with commercial pellets (400 g, 87% dry mat- ter, 25% fiber, 12% protein, and 3% fat), corn silo (300 g), and mineralized salt (100 g). The climate of the region is classified as Cfb following the Köppen Climate Classification System (Peel et al. 2007), and the experi- ments were performed during the rainy (April-May) and dry seasons (June-July).

Semen collection and sperm selection

Semen was collected by artificial vagina once a week following the protocol approved by the Bioethics Committee of the Faculty of Veterinary Medicine and Zootechnics, Bogotá Headquarters, National University of Colombia (CB-074-2014). All experiments were car-ried out with fresh semen obtained from twelve mature rams (2-5 years old) of three different breeds (Creole, Romney Marsh, and Hampshire, four rams of each breed). The ejaculates were kept at 37 °C until laboratory analy- sis, and semen samples from rams of the same breed were mixed and processed together to eliminate individual dif- ferences (Ollero et al. 1996). After collection, sperm mo- tility, viability, and morphology were evaluated with the IVOS II system (Hamilton Thorne, Beverly, MA, USA) (Carvajal-Serna et al. 2018).

A seminal plasma-free sperm population was obtained using a dextran/swim-up procedure (Garcia-Lopez et al. 1996), performed in a medium with the following composition: 200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate,21 mM HEPES, 10 mM KCL, 4 mM $NaHCO_3$, 3 mM $CaCl_2$, 2.8 mM glucose, 0.4 $MgSO_4$, 0.3 mM sodium pyruvate, 0.3 mM K_2HPO_4, 30 mg/mL dextran, and 5 mg/mL BSA (pH adjusted to 7.2).

IIF assays

Aliquots of 4 × 10^6 swim-up selected spermatozoa were fixed with 3.7% formaldehyde (v/v) in phosphate- buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH 7.2) for20 min at room temperature. The cells were then centri- fuged for 6 min at 900×*g* at room temperature and thepellet resuspended in 500 μL of PBS. After that, 40 μLof cell suspension was smeared onto poly-L-lysine-coated slides and maintained at room temperature for 3 h to ensure good adhesion of the spermatozoa to the slide. The slides were then washed three times with PBS, and non-specific binding sites were blocked with 5% BSA (w/v) in PBS overnight at 4 °C in a humidity chamber. After another three washes with PBS, the slides were incubated over- night at 4 °C in a humidity chamber with the primary an- tibodies: MTNR1A mouse polyclonal antibody (Abnova Corporation, Taipei City, Taiwan; Cat# H00004543-A01, RRID: AB_462681) for the MT_1 receptor and a rabbit polyclonal antibody for the MT_2 receptor (Acris Antibodies G mbH, Herford, Germany; RRID: AB_1619198), both diluted 1:50 in PBS containing 1% (w/v) BSA. After three washes in PBS, the cells were in- cubated with secondary antibodies Alexa Fluor 594 chick- en anti-mouse (Cat#A-21201, RRID: AB_2535787) for the MT_1 receptor and Alexa Fluor 488 chicken anti-rabbit (Cat# A-21441, RRID:AB_2535859) for the MT_2 receptor (Thermo Fisher Scientific, MA, USA), both diluted 1:800 in PBS containing 1% BSA (w/v) for 1.5 h at room tem- perature in a humidity chamber and in the dark. The slides were then washed three times with PBS before the addition of 5 μL of 0.22 M triethylenediamine (DABCO; Sigma- Aldrich Corporation, St. Louis, MO, United States) to en- hance and preserve cell fluorescence. Finally, the prepara- tions were covered with coverslips, sealed with colorless enamel and the immunotypes were evaluated using a Nikon Eclipse E-600 microscope (Tokyo, Japan) under epifluorescence illumination. All samples were processedin duplicate in a blind manner, and at least 200 spermato- zoa were scored per slide.

The melatonin receptor distribution in Creole, Romney Marsh and Hampshire spermatozoa was classified follow- ing a previous study performed on ejaculated spermatozoa from Rasa Aragonesa rams (Casao et al. 2012). Thus, the spermatozoa were categorized in four immunotypes forthe MT_1 receptor: type I, labeled all over the head andtail; type II, with reactivity at the equatorial and post-acrosomal regions, neck and tail; type III, marked only at the equatorial zone and tail, and type IV, stained onlyon the flagellum. Transitional forms between types I-IV were classified as type T (Fig. 1a–c). For the MT_2 recep- tor, spermatozoa were classified according to the intensity of the immunostaining (Casao et al. 2012; Gonzalez-Artoet al. 2016a): Type A, higher staining intensity at the acrosome than the post-acrosome; type P, greater staining intensity at the post-acrosome region than the acrosome; and type AP, the same immunostaining intensity at boththe acrosome and post-acrosome (Fig. 1d–f).

Western blotting

Sperm membrane proteins were extracted by incubation of 3 × 10^7 cells in 100 μL extraction buffer (125 mM TRIS- HCl, 4% (w/v) sodium dodecyl sulfate, 10% (v/v) β- mercaptoethanol) at 100 °C in a sand bath for 5 min. After incubation, samples were centrifuged at 7500×*g* for 5 min at4 °C. The supernatant was recovered, 10% (v/v) protease in-hibitor cocktail (Sigma-Aldrich Corporation, St. Louis, MO,USA), 20% (v/v) glycerol, and 0.02% (v/v) bromophenol bluewere added, and the protein samples were stored at − 20 °C until their use.

For sodium dodecyl sulfate-polyacrylamide gel electropho- resis (SDS-PAGE), 15 μg of protein were loaded on 12% and 10% (*w*/*v*) acrylamide gels for MT_1 and MT_2 receptors, re- spectively. The proteins were separated by standard electro- phoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). After the blocking of non-specific sites on the membrane with 5% (w/v) BSA in PBS for 4 h, the melatonin receptors were immunodetected by incubating overnight at 4 °C with the primary antibody Mel-1A-R rabbit polyclonal antibody against the MT_1 receptor (GeneTex Inc., Irvine, CA, USA; Cat# GTX100003, RRID: AB_1241048) or rabbit polyclonal antibody against the MT_2 receptor (Acris Antibodies, GmbH, Herford, Germany; Cat# AP01322PU-N, RRID: AB_1619198), both diluted 1:1000 in 0.1% Tween-20 PBS containing 1% (w/v) BSA. In addition, a mouse anti-tubulin antibody (Sta Cruz Biotechnology, Dallas, TX, USA; Cat# sc-398103), diluted 1:1000 (v/v) was used simultaneously as a loading control. The membranes were washed three times in 0.1% (v/v) Tween-20 PBS and then incubated with a donkey anti-mouse IRDye 800CW (LI-COR Biosciences, Lincoln, NE, USA; Cat# 925-32212, RRID:AB_2716622) and donkey anti-rabbit IRDye 680CW (LI-COR Biosciences Cat# 925-68073, RRID:AB_2716687) secondary antibody, both diluted 1:20000 (v/v), for 1 h at room temperature. After extensive washing, the membranes were scanned, and the intensity of the detected bands was measured using the Odyssey CLx Imaging System and the Image Studio Acquisition Software (LI-COR Biosciences, Lincoln, NE, USA). With this soft- ware, the peak intensity of each band was evaluated and nor- malized to the loading control. For total MT1 or MT2 band intensity, the sum of the peak intensity of all bands in the lane was estimated.

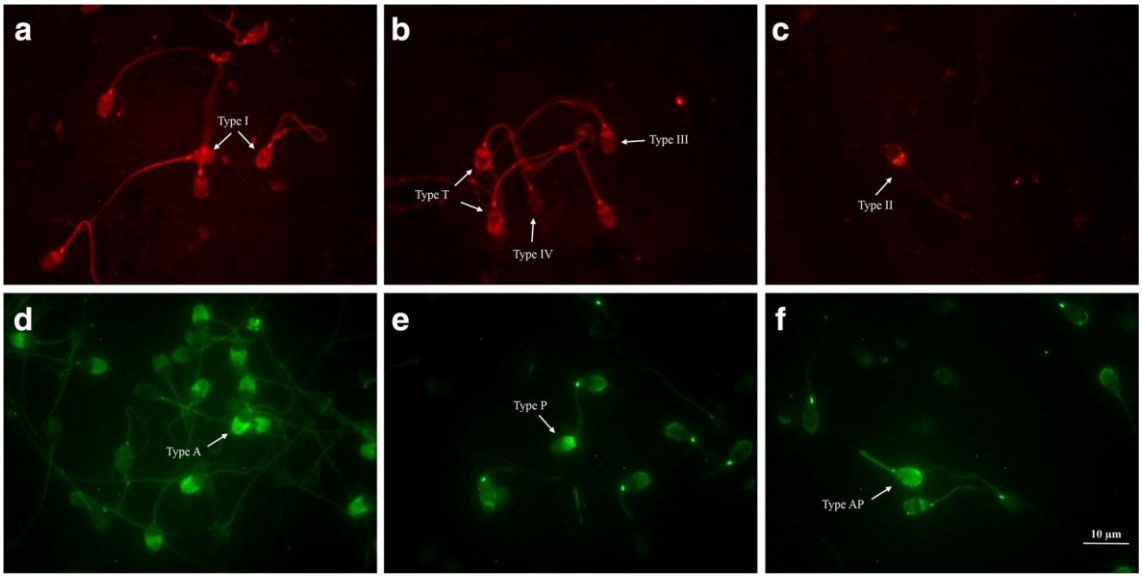

Fig. 1 Immunotypes for melatonin receptor MT_1 (a-c) and MT_2 (d-f), first described in seasonal rams (Casao et al. 2012), and identified in the spermatozoa of three sheep breeds (Colombian Creole, Hampshire and Romney Marsh) raised under equatorial photoperiod (12L:12D)

Statistical analyses

The results are shown as mean ± SEM of 4 experiments for each season. The differences in the distribution of the melatonin receptors between breeds and seasons, analyzed by indirect immunofluorescence (IIF) assays, were evaluated by means of the Chi-square test, whereas the differences in band intensity, assessed by western blotting, were analyzed by two-way ANOVA, followed by the Bonferroni post hoc test. All statistical analyses were performed using SPSS 14.0 (SPSS Inc., Chicago, IL, USA).

Results

Sperm quality parameters of fresh semen from Creole, Romney Marsh and Hampshire rams before melatonin receptor evaluation are shown in Online resource 1.

Changes in melatonin receptor distribution by IIF

Indirect immunofluorescence assays against the melatonin receptors revealed the presence of both MT_1 and MT_2, in the three studied breeds. Moreover, statistical analyses showed significant differences between breeds and seasons for both of them.

For the MT_1 receptor, type I was the predominant immunotype in the three breeds and for both seasons (Table 1). Furthermore, Romney Marsh showed a higher percentage of type I spermatozoa during the rainy season ($P<0.05$) when compared with Creole and Hampshire, although this percentage decreased significantly during the dry season when no significant differences between the breeds were found. Immunotypes II, III, and IV were significantly lower in Romney Marsh than in Creole and Hampshire during the rainy season, although these differences only remained for type IV Romney Marsh spermatozoa during the dry season. In this season, Hampshire rams also showed a lower percentage of type III spermatozoa when compared with the other two breeds ($P<0.05$), and Creole rams showed a higher rate of type IV spermatozoa when compared with Romney Marsh ($P<0.05$). Regarding transitional spermatozoa, Creole rams showed significantly higher levels during the rainy season, and Hampshire rams during the dry season. Finally, statistical analysis revealed significant differences between seasons within all immunotypes and breeds, except for immunotypes II and IV in Hampshire, and immunotype T in Creole rams.

For the MT_2 melatonin receptor, IIF revealed not only the presence of the immunotypes A, P, and AP but also two new immunotypes not previously found in ram ejaculated spermatozoa. We named them type N, with staining only in the neck (Fig. 2a) and type E, showing a band of immunofluorescence on the upper part of the post-acrosome, below the equatorial band, and at the apical edge (Fig. 2c).

IIF analyses also revealed that type A was the most frequent MT_2 immunotype in the three breeds and for both seasons, although it was significantly higher in Creole rams than in Romney Marsh and Hampshire (Table 2). Conversely, Creole rams showed a lower percentage of type N immunotype than the other two during the rainy season, but not during the dry season when no differences between breeds were detected. For the AP immunotype, Creole rams also showed a significantly lower percentage when compared with Hampshire during the rainy season and with Romney Marsh

Table 1 Different immunotypes, assessed by indirect immunofluorescence (IIF) for melatonin MT_1 receptor, in spermatozoa from Creole, Romney Marsh, and Hampshire rams located in an equatorial photoperiod (12L:12D), during the rainy and dry season. Results are shown as mean ± SEM (n = 4). Different lowercase letters between columns represent differences ($P < 0.05$) between breeds. *Differences between seasons within the same breed. Type I, labeling all over the head and tail; type II, reactivity at the equatorial, post-acrosomal, neck and flagellum; type III, equatorial region and tail labeling; type IV, staining only on the flagellum; transitional forms between types I–IV were classified as type T (transitional)

MT_1	Type I (%)	Type II (%)	Type III (%)	Type IV (%)	Transitional
Rainy season					
Creole	45.77 ± 13.54 a*	18.22 ± 4.99 a*	18.92 ± 7.44 a*	5.65 ± 3.11 a*	11.42 ± 2,54 a
Romney Marsh	77.26 ± 2.55 b*	12.23 ± 0.17 b*	5.26 ± 1.59 b*	0.70 ± 0.69 b*	4.56 ± 2,34 b*
Hampshire	49.35 ± 17.52 a*	21.55 ± 10.24 a	18.60 ± 4.49 a*	4.50 ± 2.59 a	6.02 ± 2.29 b*
Dry season					
Creole	37.25 ± 2.92*	24.50 ± 2.95*	13.00 ± 3.53 a*	10.25 ± 2.86 a*	14.00 ± 3.80 a
Romney Marsh	36.14 ± 6.25*	24.95 ± 4.04*	16.66 ± 2.24 a*	5.33 ± 2.02 b*	13.23 ± 5.47 a*
Hampshire	39.00 ± 5.55*	22.25 ± 9.47	10.00 ± 1.52 b*	7.00 ± 1.68	24.25 ± 10.03 b*

and Hampshire rams during the dry season. Additionally, Hampshire rams showed a significantly higher rate of type P spermatozoa during the rainy season than the other two breeds, and Romney Marsh during the dry season but only when compared with Creole rams ($P < 0.05$). No differences in type E spermatozoa were detected between breeds for either season.

Statistical analyses also revealed differences between the seasons ($P < 0.05$) in type A and N spermatozoa in all three studied breeds, in type P in Creole and Hampshire rams, and type E only in Creole rams. No differences between seasons were detected for type AP spermatozoa.

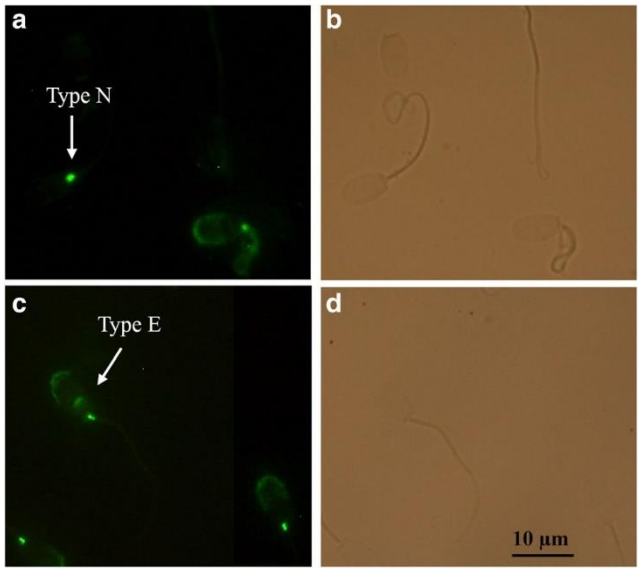

Fig. 2 New immunotypes N (a) and E (c) for melatonin receptor MT_2, identified using indirect immunofluorescence in the spermatozoa of three sheep breeds (Colombian Creole, Hampshire and Romney Marsh) raised under equatorial photoperiod (12L:12D). The bright-field is also shown (b, d)

Melatonin receptor identification by Western blot andquantification

Western blot analysis was carried out to identify and evaluate differences in receptors density between breeds and seasons. Western blot against the MT_1 melatonin receptor revealed adistinct 39 kDa band, compatible with this receptor, and a weaker 32 kDa band (Fig. 3a). The Western Blot against themelatonin receptor MT_2 identified a strong band at 50 kDa,two weaker ones at 45 and 39 kDa, the latter compatible with the molecular weight of MT_2 receptor, a double band between 32 and 30 kDa, and a very faint band at 78 kDa (Fig. 4a).

Densitometry analysis of the 39 kDa band for the MT_1 receptor revealed no differences between breeds or seasons,whereas the total band intensity increased ($P < 0.05$) duringthe dry season, but only in Creole rams (Fig. 3b). For the MT_2 receptor, the 39 kDa band intensity was higher in Hampshirethan in Creole rams ($P < 0.05$) in both dry and rainy seasons,with no difference between seasons (Fig. 4b). When the MT_2receptor total band intensity was evaluated, Creole rams showed lower band intensity than Hampshire and Romney Marsh rams, but only during the dry season, whereas the MT_2 receptor total band intensity was higher ($P < 0.05$) in the Romney Marsh rams during the rainy season when com-pared with the dry season.

Discussion

We have previously identified the presence of melatonin recep-tors MT_1 and MT_2 in spermatozoa obtained from Rasa Aragonesa rams (Casao et al. 2012), a seasonal Mediterranean sheep breed, during reproductive and non-reproductive seasons (Gonzalez-Arto et al. 2016a). In this study, we have demonstrat-ed that both melatonin receptors, MT_1 and MT_2, are also present

Table 2 Different immunotypes, assessed by indirect immunofluorescence (IIF) for melatonin MT_2 receptor, in spermatozoa from Creole, Romney Marsh, and Hampshire rams located in an equatorial photoperiod (12L:12D), during the rainy and dry season. Results are shown as mean ± SEM (n = 4). Different lowercase letters between columns represent differences (P < 0.05) between breeds.*Differences between seasons within the breed. Type A, more intense staining on acrosome than post-acrosome; typeP, more intense staining on post-acrosome than acrosome; type AP, equalstaining on both acrosome and post-acrosome; type N, with staining only inthe neck; type E, immunofluorescence in the upper part of the post-acrosome and apical edge.

MT_2	Type A (%)	Type P (%)	Type AP (%)	Type N (%)	Type E (%)
Rainy season					
Creole	59.15 ± 5.67 a*	5.03 ± 3.38 a*	9.10 ± 5.12 a	22.87 ± 7.81 a*	6.83 ± 2.60*
Romney Marsh	41.57 ± 6.42 b*	2.95 ± 1.12 a	13.25 ± 6.87	39.35 ± 13.67 b*	3.80 ± 2.07
Hampshire	38.92 ± 5.12 b*	7.60 ± 5.10 b*	14.55 ± 7.66 b	33.57 ± 9.70 b*	7.10 ± 2.59
Dry season					
Creole	74.75 ± 8.26 a*	1.50 ± 0.50 a*	5.50 ± 1.19 a	17.00 ± 6.87*	4.50 ± 0.50*
Romney Marsh	65.00 ± 3.74 b*	5.00 ± 3.15 b	13.50 ± 4.17 b	13.5 ± 1.84*	6.33 ± 2.18
Hampshire	68.00 ± 4.74 b*	2.50 ± 1.19*	10.00 ± 1.95 b	15.25 ± 3.77*	7.50 ± 0.50

in the spermatozoa of three sheep breeds raised under equatorial photoperiodic conditions. This finding suggests that the melato-nin receptor function in the spermatozoa could be other than the regulation of seasonal breeding. Also, the presence of these receptors in the spermatozoa of non-seasonal species, such asboar (Gonzalez-Arto et al. 2016b) or even human (van Vuurenet al. 1992), supports this hypothesis.

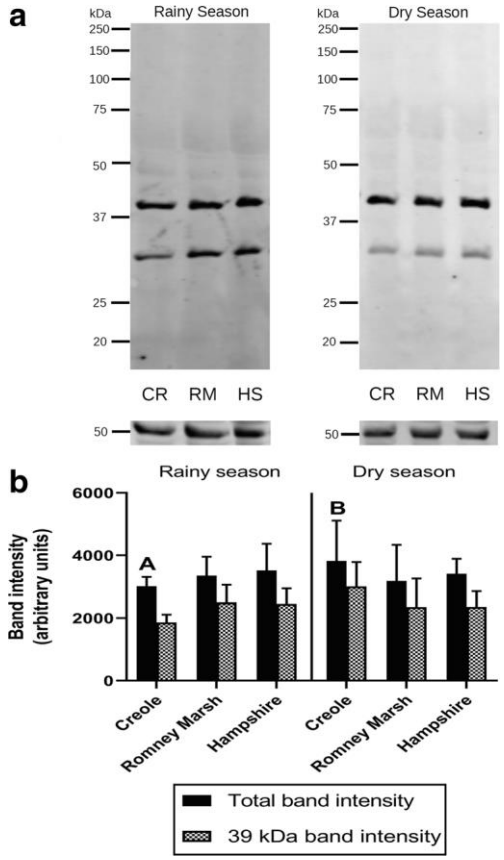

Fig. 3 Presence of MT_1 melatonin receptor, identified by Western blotting, in sperm protein extracts from Creole (CR), Romney Marsh (RM), and Hampshire (HS) rams located in an equatorial photoperiod (12L:12D), during the rainy and dry season (a, α-tubulin as a loading control is shown in the lower panel) and quantified by densitometry (b). Results are shown as mean ± SEM (n = 4). Different uppercase letters represent differences (P < 0.05) between seasons

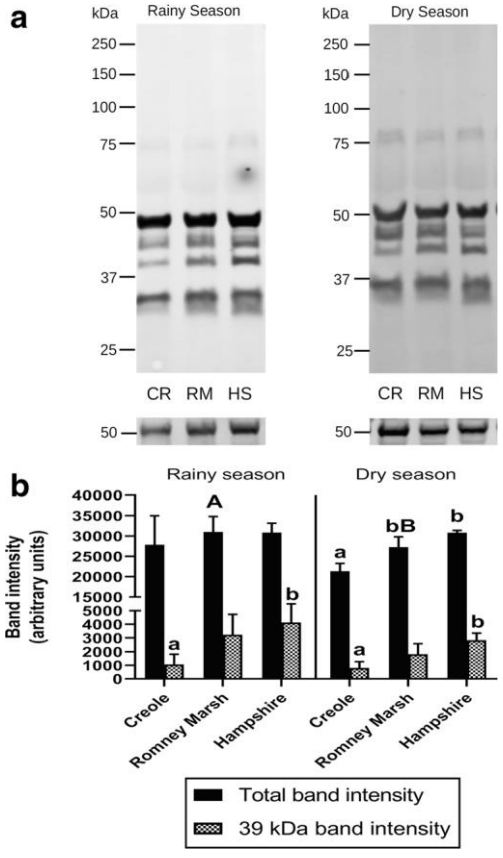

Fig. 4 Presence of MT_2 melatonin receptor, identified by Western blotting, in sperm protein extracts from Creole (CR), Romney Marsh (RM), and Hampshire (HS) rams located in an equatorial photoperiod(12L:12D), during the rainy and dry season (a, α-tubulin as a loading control is shown in the lower panel) and quantified by densitometry (b).Results are shown as mean ± SEM (n = 4). Different lowercase letters represent differences (P< 0.05) between breeds, whereas uppercase let-ters represent differences (P< 0.05) between seasons

However, the melatonin receptor distribution in Creole, Romney Marsh and Hampshire rams differs from that de-

scribed by Gonzalez-Arto et al. (2016a) for Rasa Aragonesa rams. In these three breeds, experiencing an equatorial photoperiod, the predominant immunotypes were type I for MT_1, and type A for MT_2, whereas in seasonal rams in a temperate climate they were type II and type AP for MT_1 and MT_2, respectively. Differences in the rate of MT_1 and MT_2 immunotypes between Creole, Romney Marsh and Hampshire were also detected. Moreover, two new immunotypes for MT_2, not previously identified in ram ejaculated spermatozoa, were identified in this study: type N, located only in the sperm neck and previously reported in epididymal ram spermatozoa (González-Arto et al. 2017), and type E, placed in the equatorial band and apical edge and not detected previously in this species.

The physiological significance of the variation in the melatonin receptor distribution between breeds remains unclear. For MT_1, these differences could be related with the allelic frequencies of the MlnI and RsaI polymorphisms in the MT_1 receptor gene (Notter and Cockett 2005), which has been associated with variations in the seasonal reproductive patterns in several sheep breeds (Carcangiu et al. 2009; Martínez-Royo et al. 2012; Trecherel et al. 2010). However, because all these studies were focused on the effect of MT_1 receptor gene polymorphism on seasonal reproduction in the ewe, there is no information on the impact of MlnI or RsaI polymorphisms on the ram. To date, no MT_2 gene receptor polymorphism has been associated with reproductive traits in any species. Nevertheless, there are also profound differences in the distribution of melatonin receptors in spermatozoa between species, even between those closely related, such as horse and donkey (Gonzalez-Arto et al. 2016b), with no apparent implications for the reproductive physiology or seasonality.

The results obtained also revealed differences in the percentages of MT_1 and MT_2 immunotypes between dry and rainy seasons in the three studied breeds, unlike in rams from temperate climates for which seasonal differences were only found in the MT_2 receptor (Gonzalez-Arto et al. 2016a). These seasonal differences observed in equatorial rams, subjected to a 12L:12D light regime, cannot be caused by changes in the photoperiod, but to climatic factors. Although there is no clear breeding seasonality in sheep under tropical or equatorial photoperiod, it has been observed that rainfall could control reproduction, probably by nutritional influence (Galina et al. 1996). The rainfall effect on small ruminants has also been described in the Mediterranean climatic region, in which it affects the artificial insemination outcome in ewes and goats (Abecia et al. 2016; Arrebola et al. 2009). Moreover, in tropical and equatorial climates, differences in the biochemical components of seminal plasma from bucks have been detected between seasons (Aguiar et al. 2013), as well as a decrease in sperm functionality after cryopreservation during the dry season (Aguiar et al. 2013; Silva et al. 2011). It is well known that spermatozoa undergo a process of maturation during their transit along the epididymis, most likely controlled by their surrounding environment (Dacheux and Dacheux 2014). In previous work, we have demonstrated that the melatonin receptor locations in ram spermatozoa vary during sperm maturation and epididymal transit (González-Arto et al. 2017). Thus, rainfall, through its nutritional influence, might affect the final melatonin receptor distribution during sperm maturation in the epididymis.

Another cause of the variation of melatonin receptor distribution between seasons could be the melatonin itself. We havepreviously determined that the melatonin concentration in the seminal plasma of rams reared in an equatorial photoperiod was higher during the dry season (Carvajal-Serna et al. 2019). The exposure to a high level of melatonin can desensitize melatonin receptors (Witt-Enderby et al. 2003) by internaliza- tion events (Gerdin et al. 2003; Trecherel et al. 2010). Therefore, differences in the distribution of MT_1 and MT_2 receptors between climatic seasons could be related to theirinternalization that results in their desensitization.

Western blot analysis of the extracted ram sperm proteinsrevealed a 39 kDa protein band, compatible with both MT_1 and MT_2 receptors (Dubocovich et al. 2010), and additionally, another band at 32 kDa in both melatonin membrane recep-tors. This 32 kDa protein band might be related to the activa-tion of the melatonin receptors, given that G proteins dissoci-ate their α subunit after receptor activation for cell signalingtransduction (Dubocovich and Markowska 2005; Gilman 1995). The western blot also revealed a weak band at 75 kDa for MT_2, but not MT_1. In our previous work with rams of the seasonal Rasa aragonesa breed, we identified this75 kDa band in both MT_1 and MT_2 western blots (Casaoet al. 2012), which led us to suggest that both receptors couldform heterodimers in the ram spermatozoa. However, the 75 kDa band found in the present study could be the result of melatonin receptor homodimerization instead of an interac- tion between both receptors, despite the high tendency to- wards MT_1/MT_2 heterodimer formation in somatic cells under in vitro conditions (Ayoub et al. 2004). Nevertheless, in therams from areas with an equatorial photoperiod, only the MT_2 homodimer seems to be present. Moreover, the blots for the MT_2 receptor also revealed a strong signal, which appeared to be formed by a double band of 45–50 kDa. Thus, it is possible that, in the ram spermatozoa, the MT_2 melatonin receptor forms heterodimers with orphaned melatonin receptors GPR50, MT3 and QR2, or other members of the GPCR re- ceptor family (Oishi et al. 2018). Heterodimerization of the GPCR and melatonin receptors may have significant conse- quences in the receptor function, signaling, and regulation (Oishi et al. 2018). In HEK293T transfected cells, melatonindiminished elevated cAMP levels in cells expressing GPR61/MT_2 or GPR62/MT_2 heterodimers (Oishi et al. 2017). Given

that high cAMP levels induce ram sperm capacitation, the formation of MT_2 receptor heterodimers could explain the decapacitating melatonin effect in ram spermatozoa (Gimeno-Martos et al. 2019; Gonzalez-Arto et al. 2016a).

Despite the differences detected between breeds and seasons in the melatonin receptor distribution evaluated by IIF, western blot densitometry analysis only revealed differences between seasons in the Creole rams for MT_1. This suggests that, except for the Creole, differences in MT_1 distribution are not accompanied by changes in receptor quantity. However, the analysis for the MT_2 receptor revealed that this receptor density was lower in the Creole rams than in the other two breeds, which could be due to the origin of the breed. The Colombian Creole sheep is a native breed (Ocampo et al. 2017) and thus is more adapted to the Colombian equatorial climate and photoperiod than the Hampshire and Romney Marsh, which were introduced into the country 50 years ago. This adaptation to a non-seasonal photoperiod could be reflected in the reduction of the melatonin receptor density observed in this study. However, the fact that after five centuries of breeding in a 12L:12D photoperiod the melatonin receptor density has been reduced but not disappeared in the Creole rams further supports the hypothesis that melatonin has other physiological functions in the spermatozoa than seasonal control. There is a growing body of evidence in multiple species which indicates that melatonin and its receptors play an essential role in sperm fertility (Cebrian-Perez et al. 2014). Melatonin can exert antiapoptotic actions in human spermatozoa (Espino et al. 2011), increases its viability after cryopreservation (Deng et al. 2017) and enhances hamster sperm hyperactivation (Fujinoki 2008) through the MT_1 receptor, whereas it seems to modulate ram sperm capacitation through the MT_2 receptor (Gonzalez-Arto et al. 2016a). Thus, melatonin receptors might also be involved in sperm fertility, which would explain their presence in the spermatozoa from both seasonal and non-seasonal species (Gonzalez-Arto et al. 2016b; van Vuuren et al. 1992), as well as in the rams reared in an equatorial photoperiod.

In conclusion, the melatonin receptors MT_1 and MT_2 are present in spermatozoa from Creole, Romney Marsh, and Hampshire rams subjected to an equatorial photoperiod (12 L:12D), with differences in their distribution and density between breeds and seasons. The presence of these receptors suggests that melatonin could be a useful tool to increase the fertility of rams located in tropical or equatorial climates.

Acknowledgments This research was supported by CICYT-FEDER AGL 2014-57863-R, DGA A07_17R (Spain) and the 110 157 635 854 Project, funded by Colciencias in the call Col576 2012 (Universidad Nacional de Colombia, Colombia). The authors thank the Corporación Colombiana de Investigacion Agropecuaria "Agrosavia" for their assistance.

Author contributions R.P-P, J.A.C., H.G-L., J.A.C-P., and A.C. con-ceived and designed the experiments; M.C-S. and E.N-R. performed theexperiments; A.C., R.P-P., and M.C-S. analyzed the data and A.C., M.C- S., and T.M-B. wrote and corrected the paper. All authors have approved the final article.

Compliance with ethical standards

Statement on the welfare of animals All applicable international, national, and/or institutional guidelines for the care and use of animals werefollowed. Methodologies for semen collection were approved by the Bioethics Committee of the Faculty of Veterinary Medicine and Zootechnics, Bogotá Headquarters, National University of Colombia (CB-074-2014).

Conflict of interest The authors declare that they have no conflict of interest.

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5.4 Artículo 4: Sperm Behavior and Response to Melatonin under Capacitating Conditions in Three Sheep Breeds Subject to the Equatorial Photoperiod

animals

MDPI

Article

Sperm Behavior and Response to Melatonin under Capacitating Conditions in Three Sheep Breeds Subject to the Equatorial Photoperiod

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Citation: Carvajal-Serna, M.; Cardozo-Cerquera, J.A.; Grajales-Lombana, H.A.; Casao, A.; Pérez-Pe, R. Sperm Behavior and Response to Melatonin under Capacitating Conditions in Three Sheep Breeds Subject to the Equatorial Photoperiod. *Animals* **2021**, *11*, 1828. https://doi.org/10.3390/ani11061828

Academic Editor: Gianluca Neglia

Received: 17 May 2021
Accepted: 17 June 2021
Published: 18 June 2021

Simple Summary: In temperate regions, sheep demonstrate seasonal reproduction regulated by changes in photoperiod. This regulation is mediated by nocturnal melatonin secretion. However, in equatorial regions, with no photoperiodic changes, sheep tend to breed in all seasons of the year. Despite this, changes in seminal composition or sperm quality have been reported throughout the year. We demonstrated that melatonin concentration in seminal plasma varies between rainy and dry seasons in three Colombian breeds (Colombian Creole, Romney Marsh, and Hampshire). As melatonin can exert direct effects on ram spermatozoa, in this study we hypothesized that melatonin could modulate sperm capacitation in equatorial-located breeds as we had previously reported in seasonal breeds from temperate regions. First, we assayed two media for in vitro capacitation and found that the increment in capacitated and acrosome-reacted sperm was higher in the so-called "cocktail medium" for the three breeds and in rainy and dry seasons. The addition of melatonin to the cocktail medium partially prevented the increase in capacitated spermatozoa from all breeds and during all seasons. This study could help in understanding how melatonin affects ram reproduction in the equatorial photoperiod.

Abstract: In this study, we demonstrated that, in seasonal Mediterranean ovine breeds, supplement- ing the TALP medium with cAMP-elevating agents (the cocktail medium) is effective for achievingram sperm capacitation, and that melatonin is able to regulate this phenomenon. We investigated the behavior under capacitating conditions using the TALP and cocktail mediums, and the response to melatonin, of spermatozoa from three sheep breeds (Colombian Creole, Romney Marsh, and Hampshire) subject to the equatorial photoperiod, during the dry and the rainy seasons. The cocktail medium was able to induce sperm capacitation, assayed by chlortetracycline staining and phospho-tyrosine levels, to a greater extent than TALP, without a higher loss of viability (membrane integrity and viable spermatozoa without phosphatidylserine (PS) translocation). The addition of melatoninat 100 pM or 1 μM in the cocktail medium partially prevented the decrease in viability without PS translocation and the increase in capacitated spermatozoa from all breeds, with no significant effect on phosphotyrosine levels. Differences between breeds and seasons were evidenced. This study shows that melatonin is able to exert direct effects on spermatozoa in ovine breeds under equatorial photoperiod conditions, as it does in seasonal breeds located in temperate regions.

Keywords: ram sperm; equatorial; photoperiod; capacitation and melatonin

1. Introduction

Sperm capacitation in mammalians is a pre-requirement for fertilization and it occurs physiologically in the female reproductive tract, in the vicinity of the oocyte [1,2]. Sperm capacitation can be carried out in vitro, by adding substances that trigger the cAMP/PKA pathway [3,4]. Most spermatozoa from domestic species are easily capacitated in vitro in the presence of substances such as calcium, bicarbonate, and cholesterol acceptors [5–8]. However, ram spermatozoa are very difficult to capacitate in vitro and also require other substances that increase intracellular cAMP or avoid its degradation [4]. Our group previously demonstrated that a cocktail of substances added to the TALP medium success- fully promotes capacitation of spermatozoa from Rasa Aragonesa [4,9], a Mediterranean breed raised in temperate latitudes with seasonal reproductive behavior. In temperate regions (>30° and <45° north or south latitude), sheep demonstrate seasonal reproduction, regulated mainly by melatonin according to changes in photoperiod (reviewed in [10]). Melatonin is secreted during the night by the pineal gland and displays a circadian rhythm with a nocturnal maximum and diurnal basal levels [11]. Sheep are short-day breeders, which means that higher levels of nocturnal melatonin secretion during autumn and win- ter nights in temperate regions have a stimulatory effect on their reproduction [12–14]. Although this seasonality is less marked in the ram than in the ewe [10,15], variations in testicular volume, sexual behavior, and semen quality have been detected between reproductive and non-reproductive seasons [16–18]. In the equatorial region (between 10° north and 10° south), with no changes in day length during the year, there are no changes in nocturnal melatonin levels acting as a cue for the timing of breeding. Thus, ovine breeds subject to equatorial photoperiod conditions tend to breed all-year-round [19]. Neverthe- less, changes in seminal composition or sperm quality have also been reported in small ruminants throughout the year at this latitude, which could be attributed to environmental factors other than the photoperiod, such as rainfall [20–22].

However, melatonin can influence sheep reproduction through additional mechanisms other than photoperiod translation. This hormone can also be synthesized in the ramtestes [23], it is present in seminal plasma and the female reproductive tract, and it can exert direct effects on ram spermatozoa by binding to specific membrane receptors (MT_1 and MT_2) [24]. Our group has demonstrated that melatonin is able to protect spermatozoa from Mediterranean seasonal rams against oxidative damage and apoptosis [25,26] and modulatesperm capacitation under in vitro conditions [25,27]. In a medium with a cocktail of cAMP-elevating agents, melatonin at micromolar concentrations was able to prevent sperm capacitation, whereas at lower concentrations it modified motile sperm subpopulations [28]. Despite the understanding of melatonin's role in ram reproduction in temperate regions, little is known about this hormone's functions in males under the equatorial

photoperiod. Colombia, located at 4.5° N, has a 12L:12D photoperiod and an isothermal climate with a bimodal precipitation pattern, with two rainy and two dry seasons. Among ovine breeds in the country, Wool Creole, Romney Marsh, and Hampshire are predominant in high altitude areas [29]. Wool Creole is a native breed that has emerged from extensive crossbreeding since the 16th century [30]. This breed, adapted to the local environmental conditions, represents a vital genetic resource for small-scale agriculture in Andean re-gions [31]. As an alternative to this native breed, imported breeds such as Romney Marsh and Hampshire have been introduced since 1963. These breeds have a higher productive performance but are less adapted than the native breeds in terms of fertility [32].

In a previous study with these three Colombian breeds under equatorial photoperiod conditions, we observed differences in the melatonin concentration in seminal plasma obtained in the rainy and dry seasons [33,34]. As there was no change in night length that would modify the nocturnal secretion of melatonin, we hypothesized that these differences between seasons could be attributed to the content of phytomelatonin in the pasture [33]. Moreover, our group also demonstrated that spermatozoa from these Colombian rams also contain MT_1 and MT_2 receptors, and there are differences between breeds and seasons in the locations and densities of these receptors [34]. However, no previous studies have

investigated how spermatozoa from these breeds, located in equatorial latitudes, respond under in vitro capacitating conditions, and whether exogenous melatonin could modulate this process.

Thus, the first objective of this study was to evaluate the response to in vitro capacita- tion in spermatozoa obtained from one native (Wool Creole) and two imported (Hampshire and Romney Marsh) sheep breeds reared in Colombia under a photoperiodic regimen of 12L:12D. The second objective was to elucidate whether melatonin can regulate ram sperm capacitation in these breeds in a medium with cAMP-elevating agents. Both evaluations were conducted during the rainy and dry seasons. This study could help in understanding how melatonin affects ram reproduction in the equatorial photoperiod.

2. Materials and Methods

2.1. Animals

Semen was obtained from twelve mature rams (2–5 years old) of three different sheep breeds (Wool Creole, Romney Marsh, and Hampshire; four rams of each breed). The animals were kept under uniform nutritional conditions at the Center for Ovine Research, Technological Development and Extension of the National University of Colombia, located in Mosquera (4°40′57′′ N. 74°12′50′′ W) at 2510 m above sea level. The rams' diet was based on pasture (*Pennisetum clandestinum, Lolium perenne*), supplemented with 200 g of pellets (Leche Standard 70) and 15 g of mineralized salt (Universal F), both from FINCA
S.A. The rams were kept under natural photoperiod conditions. The local amplitude of the photophase throughout the year fluctuates from 12 h 21′ (11 h 39′ of darkness) in the summer solstice to 11 h 49′ (12 h 11′ of darkness) in the winter solstice; i.e., with a totalof 32′ of difference between the longest and the shortest days of the year. The climate of the region is classified as Cfb according to the Köppen Climate Classification System. The medium temperature is 13.6 °C, the annual variation between the coldest and hottestmonths being 0.7 °C. The daily temperature and relative air humidity varies from 18 °C to 7 °C and from 92% to 70%, respectively. The mean annual rainfall is 960 mm, with a mean of 205 rainy days per year. The experiments were performed in the rainy season (April–May) and dry season (June–July) based on precipitation data from the Institute ofHydrology, Meteorology, and Environmental Studies (IDEAM).

2.2. Semen Collection and Processing

Semen was collected once a week with the aid of an artificial vagina during four weeksin the rainy season and four weeks in the dry season. All procedures were performed inaccordance with the Colombian Animal Protection Regulations (Law 84/1989, modified by Law 1774/2016) and under approval of the Bioethics Committee of the Faculty ofVeterinary Medicine and Zootechnics of Bogotá, National University of Colombia (Projectlicense: CB-074-2014). Before including rams in the study, individual ejaculates of eachram were analyzed separately during several months. All ejaculates showed ≥70% spermmotility (evaluated by an IVOS II CASA system; Hamilton Thorne, Beverly, MA, USA) and ≥75% normal sperm morphology. For the experiments described in this study, ejaculates from rams of the same breed were pooled and processed together in order to eliminate individual differences, [35].

After semen collection, the ejaculates were kept at 37 °C upon arrival at the laboratory located at the Tibaitatá research center which belongs to the Colombian Corporation for Agricultural Research (AGROSAVIA).

A seminal plasma-free sperm population was obtained using a dextran/swim-up procedure based on the modification proposed by Garcia-Lopez et al. 1996 [36]. It was performed in a swim-up medium (SM) devoid of $NaHCO_3$ and $CaCl_2$ [37] with the follow-ing composition: 200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate, 21 mM HEPES,10 mM KCL, 2.8 mM glucose, 0.4 mM $MgSO_4$, 0.3 mM sodium pyruvate, 0.3 mM K_2HPO_4, and 5 mg/mL de BSA (pH adjusted to 6.5).

2.3. In Vitro Sperm Capacitation

Swim-up-selected spermatozoa (1.6 x 10^8 cells/mL) were incubated in a humidified incubator for 3 h at 39 °C and with 5% CO_2 in the air. Incubations were performed in a complete TALP medium [4,38] composed of 100 mM NaCl, 25 mM $NaHCO_3$, 21.6 mMNa lactate, 10 mM HEPES, 3.1 mM KCl, 2 mM $CaCl_2$, 1 mM Na pyruvate, 0.4 mM $MgCl_2$,and 0.3 mM NaH_2PO_4 with 5 mM glucose, 5 mg/mL bovine serum albumin (BSA) and a pH of 7.2. As ram spermatozoa are difficult to capacitate in vitro, we also evaluated the addition of a cocktail of agents that increase intracellular cAMP to the TALP medium [4,7,9]. The cAMP-elevating compounds were 1 mM dibutyryl (dB)-cAMP, 1 mM caffeine, 1 mM theophylline, 0.2 mM okadaic acid, and 2.5 mM methyl-b-cyclodextrin (Sigma-Aldrich, Merck KGaA, St. Louis, MO, USA). We named this high cAMP medium the "cocktail medium". This medium has already been proven for capacitating ram spermatozoa in certain seasonal breeds located in temperate regions but not in non-seasonal ones located in equatorial regions.

Melatonin was solubilized in DMSO (dimethyl sulphoxide) and PBS (phosphate-buffered saline: 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 1.76 KH_2PO_4; pH 7.4) and then added to samples in the cocktail medium at a final concentration of 100 pM or 1 µM. The final concentration of DMSO in all the melatonin samples was 0.1%. To take into consideration the potential adverse effect of DMSO, the same concentration was included in cocktail- capacitated samples to which no melatonin had been added.

Thus, five experimental groups were analyzed in the present study: swim-up (sperma- tozoa selected by the dextran/swim-up procedure before inducing in vitro capacitation),cap-TALP (swim-up-selected spermatozoa incubated under capacitating conditions in TALP medium), cap-CK (swim-up-selected spermatozoa incubated under capacitating conditions in cocktail medium), and cap-CK-100 pM Mel and cap-CK-1 µM Mel (swim-up-selected spermatozoa incubated under capacitating conditions in cocktail medium in thepresence of 100 pM and 1 µM melatonin, respectively).

2.4. Evaluation of Motility and Plasma Membrane Integrity

Motility and viability, assayed as plasma membrane integrity, were sequentially analyzed using an IVOS II CASA system (Hamilton Thorne, Beverly, MA, USA) on the same five fields under phase contrast and fluorescent illumination (using the Viadent filter), respectively [39]. For viability analysis, samples were previously stained using theVIADENT™ stain biz-benzamide trihydrochloride (Hoeschst 33258, 5 µg/mL; Hamilton Thorne, Beverly, MA, USA) which penetrates only in cells with a damaged membrane andattaches to the DNA, emitting fluorescence.

2.5. Detection of Membrane Phosphatidylserine Translocation

Annexin V (AnnV) is a protein with a high affinity for phosphatidylserine (PS). PS translocation is a marker of apoptotic-like changes in spermatozoa [40]. Simultaneous staining with 6-CFDA and Annexin V-Cy3.18 (Apoptosis Detection Kit; Sigma-Aldrich, St. Louis, MO, USA) was used in order to differentiate between viable spermatozoa with or without PS translocation and non-viable cells. The non-fluorescent 6-CFDA enters the cell and is converted to the green fluorescent compound 6-carboxyfluorescein (6-CF) onlyin viable cells, whereas red fluorescence can be observed only in cells with translocation of PS (AnnV+).

Aliquots of 300 µL (6 x 10^6 cells diluted with 1 x binding buffer) were stained with5 µL 6-CFDA (1 mM in DMSO) and 2 µL Annexin V-Cy3.18. Viable cells (6-CFDA+)were visualized in green with a standard fluorescein (Nikon B-2A) filter and AnnV+ cells (labeling PS exposure, Annexin V-Cy3.18+) in red with a rhodamine (Nikon G-2A) filter under an epifluorescence microscope (1000x magnification). At least 200 spermatozoa were counted per slide.

2.6. Assessment of Capacitation Status by Chlortetracycline (CTC) Staining

The capacitation status was determined by a modified chlortetracycline (CTC) fluorescence assay [41]. A CTC solution was prepared daily, adding 750 µM of CTC to a buffer composed of 130 mM NaCl, 20 mM Tris, and 5 µM cysteine (pH 7.8), which was then filtered through a 0.22 mm filter (Merck Millipore, Darmstadt, Germany). For a 20 µL sample (1.6 x10^8 cells/mL), 20 µL of CTC solution and 5 µL (12.2% *w*/*v*) paraformalde-hyde (prepared in 0.5 M Tris-HCl, pH 7.8) were added. For evaluating CTC patterns,the samples were examined using a Nikon Eclipse E-200 microscope (Nikon Instruments, Kanagawa, Japan) under epifluorescence illumination in the V-2A filter (excitation filter 380–425 nm) at 1000x magnification. At least 200 spermatozoa per slide were classifiedinto three categories [42]: non-capacitated (NC, with uniform fluorescence on the head, with or without a bright equatorial band), capacitated (C, showing fluorescence in the anterior region of the head), and acrosome-reacted (AR, showing no fluorescence on thehead) spermatozoa.

2.7. Tyrosine Phosphorylation as Capacitation Assay

Sperm samples were subjected to protein membrane extraction. Proteins were obtained by suspending sperm (1.6 x 10^8 cells/mL in 200 µL) in 200 µL of extraction sample buffer (ESB; composed of 2% SDS (sodium dodecyl sulfate-polyacrylamide (*w*/*v*) and 0.0626 M Tris-HCl, pH 6.8) [4]. The samples were incubated for 5 min at 100 °C and then centrifuged at 7500x *g* for 5 min at 4 °C. After recovering the supernatant, a mix of phosphatase and protease (10% *v*/*v*) inhibitors (Sigma-Aldrich, St. Louis, MO, USA), β-mercaptoethanol (5% *v*/*v*), glycerol (1% *v*/*v*), and bromophenol blue (0.002% (*v*/*v*) in 10% glycerol) were added and it was then stored at -20 °C until its use.

For sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 15 µLof samples were loaded on 10% (*w*/*v*) SDS-PAGE gels for detection of phosphorylated protein in tyrosine residues. Proteins were separated by standard SDS-PAGE [43] and trans-ferred onto polyvinylidene difluoride (PVDF) membrane using a transfer unit (Trans-Blot pack and Trans-Blot Turbo Transfer System, respectively, both from Bio-Rad Laboratories, Hercules, CA, USA).

Non-specific sites on the membrane were blocked with 5% BSA in PBS (*w*/*v*) for 4 h and membranes were incubated overnight at 4 °C with the mouse monoclonal anti-phosphotyrosine primary antibody (Clone 4G10; Millipore, Temecula, CA; Cat# 05–321, RRID: AB_309678), 1:1000 (*v*/*v*) in 0.1% (*w*/*v*) Tween-20–PBS containing 1% BSA (*w*/*v*). Additionally, a rabbit anti-actin antibody (Sigma-Aldrich, St. Louis, MO, USA; Cat# A2066, RRID: AB_476693), diluted 1:1000 (*v*/*v)* was used at the same time as a loading control.After three 15 min washes with 0.1% (*w*/*v*) Tween-20–PBS, membranes were incubatedwith a secondary donkey anti-rabbit IRDye 680-CW (LI-COR Biosciences; Cat# 926–32,223, RRID: AB_621845) and donkey anti-mouse IRDye 800-CW (LI-COR Biosciences, Lincoln,NE, USA; Cat# 926-32213, RRID: AB_621848) conjugated antibodies, both diluted 1:15,000 (*v*/*v*) in 0.1% (*w*/*v*) Tween-20–PBS containing 1% BSA (*w*/*v*) for 1 h at room temperature. After extensive washing with 0.1% (*w*/*v*) Tween-20–PBS, membranes were scanned, and the intensity of the bands was measured with the Odyssey CLx Imaging System (LI-COR Biosciences, Lincoln, NE, USA). For densitometric evaluation, the signals cor- responding to the high (60–250 kDa) and low (10–45 kDa) molecular weight phosphoty-rosine proteins were considered, and the middle bands corresponding to the BSA signal were omitted.

2.8. Statistical Analysis

The obtained results were presented as means ± S.E.M. The number of replicates was four for all analyses (n = 4), except for PS translocation (n = 3). To determine whether there were significant differences in protein tyrosine phosphorylation between the treatments(swim-up, cap-TALP, cap-CK, cap-CK + 100 pM Mel, and cap-CK + 1 µM Mel), breeds,or seasons, two-way ANOVA tests followed by Bonferroni post hoc tests were used after

the normality of the data was evaluated by the Kolmogorov–Smirnov test (Graph-Pad InStat software 3.01; San Diego, VA, USA). The percentage of total and progressive motility, viability, viable sperm without PS translocation, and CTC staining patterns were compared by means of Pearson's chi-square test using SPSS software version 24.0, (IBM Corp, Armonk,NY, USA). $p < 0.05$ was used to indicate significant differences.

3. Results

3.1. Evaluation of the Changes after In Vitro Capacitation in Spermatozoa from Different Breedsand Seasons

3.1.1. Changes in Motility after In Vitro Capacitation

Creole rams generally presented better total and progressive motility than the other two breeds in both seasons ($p < 0.05$, Table 1). After 3 h of incubation in capacitating conditions, the percentages of total and progressive motility decreased ($p < 0.05$) in relationto swim-up, and mainly when incubation was in the cocktail medium, for all breeds and seasons, except for the Creole breed during the rainy season. During this season, the percentages of total and progressive motility after in vitro capacitation remained high inthe Creole breed and decreased significantly in the Hampshire breed ($p < 0.05$). The decline in motility was more pronounced during the dry season except for Hampshire spermatozoa (Table 1).

Table 1. Percentage of total and progressive sperm motility in the rainy and dry seasons in three ram breeds before (swim-up) and after 3 h of incubation at 39 °C and 5% CO_2 (capacitating conditions) in TALP (cap-TALP) or TALP with cAMP-elevating agents (cap-CK).

Variables	Total Motility			Progressive Motility		
Breed	**Creole**	**Romney Marsh**	**Hampshire**	**Creole**	**Romney Marsh**	**Hampshire**
			Rainy season			
Swim-up	77.9 ± 3.7 ^{aA}	72.8 ± 3.0 ^{bA}	65.7 ± 3.9 ^{*cA}	64.7 ± 11.7 ^{aA}	41.3 ± 9.0 ^{*bA}	43.9 ± 5.1 ^{*bA}
Cap-TALP	68.6 ± 3.0 ^{*aB}	55.4 ± 6.2 ^{*bB}	29.0 ± 5.2 ^{*cB}	44.4 ± 4.9 ^{*aB}	27.5 ± 5.9 ^{*bB}	5.8 ± 3.0 ^{*cB}
Cap-CK	76.7 ± 2.8 ^{*aA}	47.2 ± 6.8 ^{*bC}	22.0 ± 3.8 ^{*cC}	32.8 ± 7.0 ^{*aC}	17.0 ± 4.1 ^{*bC}	1.2 ± 0.3 ^{*cC}
			Dry season			
Swim-up	78.8 ± 3.4 ^{aA}	69.2 ± 4.0 ^{bA}	72.9 ± 3.3 ^{bA}	59.8 ± 3.9 ^{aA}	53.3 ± 5.0 ^{bA}	54.2 ± 0.6 ^{abA}
Cap-TALP	46.9 ± 13.6 ^{aB}	39.8 ± 3.6 ^{bB}	36.7 ± 9.6 ^{bB}	23.0 ± 11.5 ^{aB}	13.0 ± 4.6 ^{bB}	13.2 ± 5.3 ^{bB}
Cap-CK	33.8 ± 5.8 ^{C}	33.8 ± 5.2 ^{B}	28.1 ± 8.5 ^{C}	5.7 ± 1.9 ^{C}	3.9 ± 1.6 ^{C}	5.9 ± 3.0 ^{C}

Values are expressed as means ± S.E.M. ($n = 4$). * represents significant differences ($p < 0.05$) between seasons within the same treatment and breed; different lowercase letters in the same row represent significant differences between breeds within a treatment and season; different capital letters in the same column represent significant differences between treatments (swim-up, Cap-TALP, and Cap-CK) within a breed and season.

3.1.2. Changes in Plasma Membrane after In Vitro Capacitation

The percentages of sperm viability (membrane integrity) in swim-up, cap-TALP, and cap-CK samples were higher in the rainy than in the dry seasons for all breeds ($p < 0.05$)except for Romney Marsh swim-up samples (Table 2). A significant decrease ($p < 0.05$) was observed after 3 h incubation in capacitating conditions both in TALP and cocktail media,with a difference between media for Creole and Hampshire breeds in the rainy season. Moreover, during both seasons the sperm viability after in vitro capacitation, with either media, was higher in the Creole breed than in the other breeds.

When PS translocation was evaluated simultaneously with plasma membrane in- tegrity, significant differences between breeds were also revealed (Table 2). Swim-up sam- ples from Creole rams showed a higher percentage of spermatozoa without PS translocation than those from the other two breeds. However, incubation in capacitating conditions sig- nificantly affected the Creole sperm in both seasons ($p < 0.05$), unlike the other two breeds that were affected only in the dry season (Romney Marsh) or not affected (Hampshire). Nonetheless, the rate of viable sperm without PS translocation remained higher than in the other breeds after the incubation in capacitating conditions in the Creole breed, especially when compared with the Hampshire breed ($p < 0.05$). On the other hand, there were no

significant differences between seasons in any breed, except for cocktail samples from Hampshire rams, in which this parameter decreased even more during the dry season.

Table 2. Percentages of viability (plasma membrane integrity) and viable sperm without PS translocation in the rainy and dry seasons in three ram breeds before (swim-up) and after 3 h of incubation at 39 °C and 5% CO_2 (capacitating conditions) in TALP (cap-TALP) or TALP with cAMP-elevating agents (cap-CK).

Variables	Viability (Plasma Membrane Integrity %)			Viable Sperm without PS Translocation (%) Breed		
	Creole	Romney Marsh	Hampshire	Creole	Romney Marsh	Hampshire
			Rainy season			
Swim-up	88.6 ± 3.6 ^{*aA}	80.4 ± 5.3 ^{bA}	86.5 ± 4.2 ^{*aA}	62.4 ± 5.2 ^{aA}	47.0 ± 3.5 ^{bA}	40.4 ± 9.2 ^{bA}
Cap-TALP	80.1 ± 1.3 ^{*aB}	68.2 ± 5.9 ^{*bB}	49.1 ± 4.5 ^{*cB}	53.6 ± 5.9 ^{aB}	53.0 ± 5.0 ^{aA}	35.4 ± 0.7 ^{bA}
Cap-CK	86.0 ± 1.0 ^{*aA}	63.0 ± 6.5 ^{*bB}	40.6 ± 3.9 ^{*cC}	56.3 ± 6.4 ^{aAB}	55.5 ± 7.5 ^{aA}	40.7 ± 1.3 ^{*bA}
			Dry season			
Swim-up	85.7 ± 2.9 ^{aA}	79.2 ± 3.3 ^{bA}	80.8 ± 3.9 ^{abA}	70.4 ± 11.9 ^{aA}	50.0 ± 6.5 ^{bA}	39.8 ± 0.2 ^{cA}
Cap-TALP	68.9 ± 8.5 ^{aB}	54.6 ± 3.9 ^{bB}	55.6 ± 2.2 ^{bB}	70.0 ± 2.6 ^{aA}	42.0 ± 10.6 ^{bB}	37.0 ± 3.0 ^{bA}
Cap-CK	71.7 ± 4.8 ^{aB}	56.7 ± 3.9 ^{bB}	53.5 ± 8.3 ^{bB}	51.3 ± 12.0 ^{aB}	43.0 ± 7.2 ^{aAB}	32.5 ± 4.5 ^{bA}

Values are expressed as means ± S.E.M. (n = 4 and n = 3 for viability and PS translocation, respectively). * represents significant differences (p < 0.05) between seasons within the same treatment and breed; different lowercase letters in the same row represent significant differencesbetween breeds within a treatment and season; different capital letters in the same column represent significant differences between treatments (swim-up, Cap-TALP and Cap-CK) within a breed and season.

3.1.3. Changes in Capacitation Status after In Vitro Capacitation

Swim-up samples from Hampshire rams presented much higher (p < 0.05) percentagesof capacitated (35.5±4.74 vs. 18.56±2.72 and 19.75±3.75 for Creole and Romney Marsh, respectively) and acrosome-reacted (6.00±1.47 vs. 1.98±0.57 and 1.00±0.70 for Creole and Romney Marsh, respectively) spermatozoa in the rainy season than the other twobreeds (Figure 1).

The incubation in TALP medium under capacitating conditions led to a significant decrease in the percentage of non-capacitated spermatozoa in both seasons for all three breeds (Figure 1). This decrease was concomitant with an increase in the rate of capacitated spermatozoa, except for Romney Marsh and Hampshire in the rainy season, and with an increment in acrosome reacted spermatozoa in most of the experimental groups. These changes were more evident when incubations were performed in the cocktail medium inboth seasons for the three breeds. Moreover, during the dry season, a greater differencebetween the effect of the TALP and cocktail medium on the rate of capacitated spermatozoa was observed. Also, no differences between breeds were observed during the dry seasonwhen spermatozoa were incubated with the cocktail medium.

The more noteworthy dissimilarities were found when the effect of the season was analyzed. We found a substantial difference between dry and rainy seasons (p < 0.05) in the Romney Marsh and Hampshire spermatozoa response to the high cAMP medium, but notfor the Creole spermatozoa. However, the Creole spermatozoa showed a higher responsein the TALP medium during the rainy season (p < 0.05).

3.1.4. Changes in Phosphorylation in Tyrosine Residues after In Vitro Capacitation

Incubation in the TALP medium did not significantly increase the phosphotyrosine signal compared to the swim-up samples, except for Creole in the rainy season (Figure 2). However, when the incubation was performed in the cocktail medium, a significant increment (p < 0.05) in the signal was observed in all breeds and seasons except for Romney Marsh in the rainy season. This increase was much higher in the dry than in the rainy seasonfor the three breeds (Creole:2335.86±331.94 vs. 7211.04±617.25; RM: 1254.89±203.88 vs. 4247.92±677.23; HS:2091.26±332.26 vs. 5468.36±325.20; rainy and dry season, respectively) (Figure 2), with significant differences between seasons (p < 0.05). The incre- ment was lower in spermatozoa from Romney Marsh than in spermatozoa from the other two breeds.

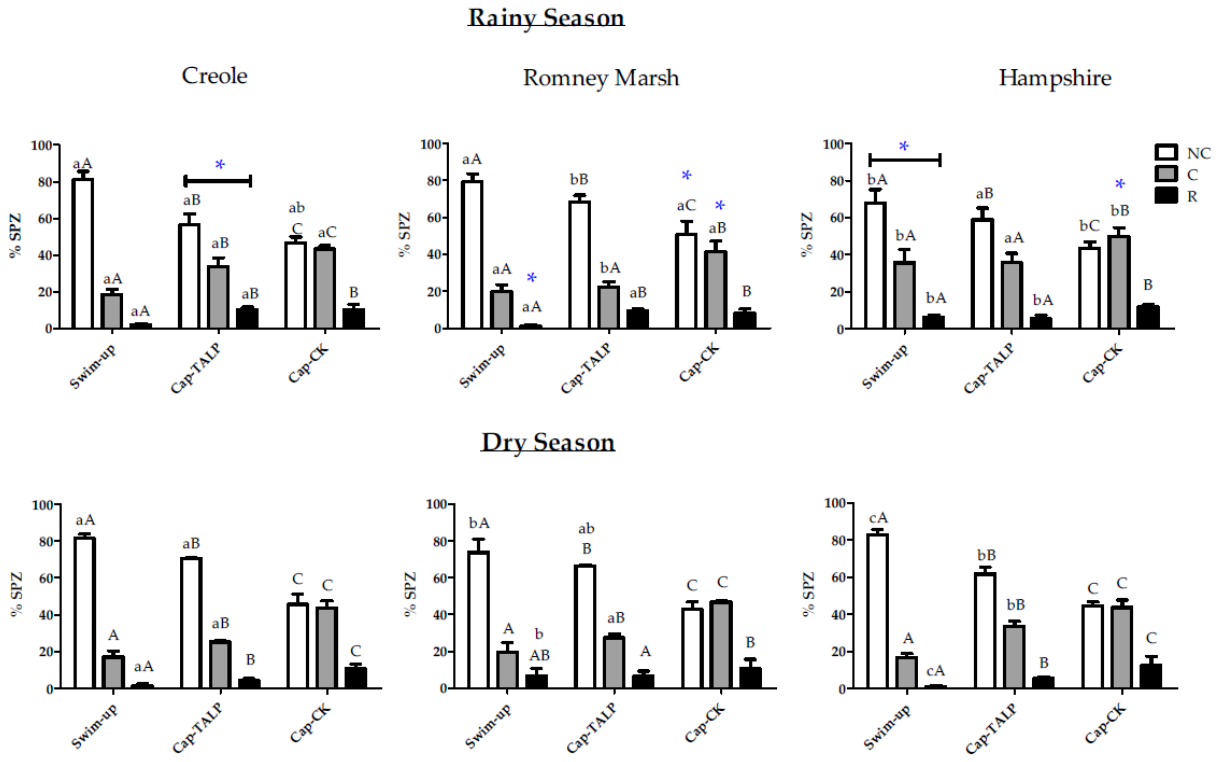

Figure 1. Assessment of capacitation status of ram spermatozoa, evaluated by chlortetracycline (CTC) staining, before (swim-up) and after 3 h incubation at 39 °C and 5% CO_2 (capacitating conditions) in TALP (cap-TALP) or TALP with cAMP-elevating agents (cap-CK). The distributions were done by breed and season (rainy or dry). Data for percentages of non-capacitated (NC), capacitated (C), and acrosome-reacted (R) spermatozoa are expressed as means ± S.E.M. (n = 4).
* represents significant differences (p < 0.05) between seasons within the same treatment and breed; different capital letters represent significant differences between treatments (swim-up, Cap-TALP, and Cap-CK) within a breed and season; different lowercase letters represent significant differences between breeds within a treatment and season.

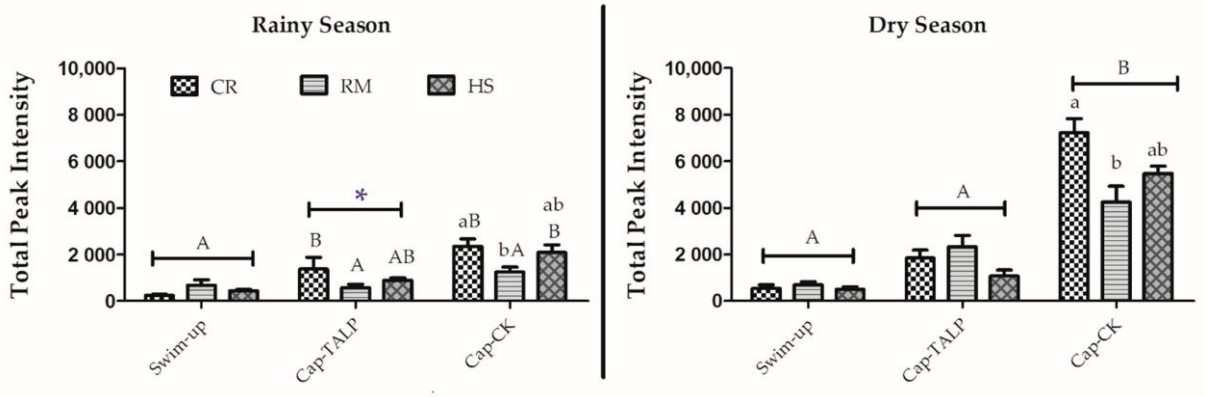

Figure 2. Protein tyrosine phosphorylation evaluated by densitometry in samples before (swim-up) and after 3 h of incubation at 39 °C and 5% CO_2 (capacitating conditions) in TALP (cap-TALP) or TALP with cAMP-elevating agents (cap-CK). Data for the Creole (CR), Romney Marsh (RM), and Hampshire (HS) breeds are distributed by season (rainy ordry) and expressed as means ± S.E.M (n = 4). * represents significant differences (p < 0.05) between seasons within the same treatment and breed; different capital letters represent significant differences between treatments (swim-up, Cap-TALP, and Cap-CK) within a breed and season; different lowercase letters represent significant differences between breeds within atreatment and season.

3.2. Evaluation of the Effect of Melatonin on In Vitro Capacitation in Spermatozoa from Different Breeds and Seasons

3.2.1. Effects on Motility during In Vitro Capacitation

When samples were incubated in capacitating conditions in the cocktail medium, the presence of melatonin had no effect on total motility in most experimental samples compared to the cap-CK without the hormone, except for a decrease in Creole and Hamp- shire spermatozoa at 100 pM in the rainy season and the dry season, respectively, and an increase in Romney Marsh spermatozoa at 1 µM in the rainy season (Table 3). Regarding progressive motility, there were also no significant effects, except for a slight decrease ($p < 0.05$) in Hampshire spermatozoa when melatonin was added at both concentrations in the dry season (Table 3).

Table 3. Percentages of total and progressive sperm motility in the rainy and dry seasons in three ram breeds after 3 h of incubation at 39 °C and 5% CO_2 (capacitating conditions) in TALP with cAMP-elevating agents without (cap-CK) or with100 pM and 1 µM melatonin (Cap-CK-100 pM MEL and Cap-CK-1 µM MEL).

Variables	**Total Motility**			**Progressive Motility**		
Breed	**Creole**	**Romney Marsh**	**Hampshire**	**Creole**	**Romney Marsh**	**Hampshire**
			Rainy season			
Cap-CK	76.7 ± 2.8 ^{*aA}	47.2 ± 6.8 ^{*bA}	22.0 ± 3.8 ^{*cA}	32.8 ± 7.0 ^{*aA}	17.0 ± 4.1 ^{*bA}	1.2 ± 0.3 ^{*cA}
Cap-CK-100 pM MEL	67.8 ± 5.1 ^{*aB}	51.0 ± 5.4 ^{*bA}	22.9 ± 3.9 ^{cA}	30.0 ± 4.9 ^{*aA}	17.7 ± 4.1 ^{*bA}	2.3 ± 0.9 ^{cA}
Cap-CK-1 µM MEL	77.5 ± 2.1 ^{*aA}	57.1 ± 2.4 ^{*bB}	18.6 ± 1.5 ^{cA}	32.3 ± 2.5 ^{*aA}	18.1 ± 1.8 ^{*bA}	1.7 ± 0.6 ^{cA}
			Dry season			
Cap-CK	33.8 ± 5.8 ^{A}	33.8 ± 5.2 ^{A}	28.1 ± 8.5 ^{A}	5.7 ± 1.9 ^{A}	3.9 ± 1.6 ^{A}	5.9 ± 3.0 ^{A}
Cap-CK-100 pM MEL	30.5 ± 5.6 ^{aA}	33.9 ± 6.5 ^{aA}	21.9 ± 5.0 ^{bB}	5.1 ± 2.3 ^{A}	3.8 ± 1.3 ^{A}	2.4 ± 0.7 ^{B}
Cap-CK-1 µM MEL	35.1 ± 6.6 ^{aA}	30.0 ± 4.0 ^{aA}	23.3 ± 6.1 ^{bA}	5.9 ± 2.8 ^{A}	3.6 ± 1.3 ^{A}	3.0 ± 1.3 ^{B}

Values are expressed as means ± S.E.M. ($n = 4$). * represents significant differences ($p < 0.05$) between seasons within the same treatment and breed; different lowercase letters in the same row represent significant differences between breeds within a treatment and season;different capital letters in the same column represent significant differences between treatments (Cap-CK, Cap-CK-100 pM MEL, and Cap-CK-1 µM MEL) within a breed and season.

3.2.2. Effects on Plasma Membrane during In Vitro Capacitation

In the dry season, the presence of melatonin in cap-CK samples did not affect the membrane integrity (viability). In the rainy season, 1 µM of melatonin ($p < 0.05$) had a positive effect in Romney Marsh spermatozoa and a negative one in Hampshire ones, whereas 100 pM had a slightly negative effect in the Creole breed compared to cap-CK without the hormone (Table 4), in concordance with its impact on motility in these breeds (Table 3).

Table 4. Percentages of viability (plasma membrane integrity) and viable sperm without PS translocation in the rainy and dry seasons in three ram breeds after 3 h of incubation at 39 °C and 5% CO_2 (capacitating conditions) in TALP with cAMP-elevating agents without (cap-CK) or with 100 pM and 1 µM melatonin (Cap-CK-100 pM MEL and Cap-CK-1µM MEL).

Variables	Viability (Plasma Membrane Integrity %)			Viable Sperm without PS Translocation (%) Breed		
	Creole	Romney Marsh	Hampshire	Creole	Romney Marsh	Hampshire
			Rainy season			
Cap-CK	86.0 ± 1.0 ^{*aA}	63.0 ± 6.5 ^{*bA}	40.6 ± 3.9 ^{*cA}	56.3 ± 6.4 ^{aA}	55.5 ± 7.5 ^{aA}	40.7 ± 1.3 ^{*bA}
Cap-CK-100 pM MEL	80.3 ± 3.7 ^{*aB}	65.7 ± 7.1 ^{*bA}	44.0 ± 6.3 ^{cA}	72.9 ± 6.4 ^{*aB}	57.0 ± 6.2 ^{bA}	49.7 ± 7.5 ^{*bB}
Cap-CK-1 µM MEL	84.8 ± 1.6 ^{*aAB}	73.7 ± 3.5 ^{*bB}	36.1 ± 5.8 ^{*cB}	64.7 ± 3.3 ^{*aA}	62.7 ± 7.7 ^{*aB}	44.2 ± 8.4 ^{bA}
			Dry season			
Cap-CK	71.7 ± 4.8 ^{aA}	56.7 ± 3.9 ^{bA}	53.5 ± 8.3 ^{bA}	51.3 ± 12.0 ^{aA}	43.0 ± 7.2 ^{aA}	32.5 ± 4.5 ^{bA}
Cap-CK-100 pM MEL	72.2 ± 4.4 ^{aA}	54.7 ± 4.5 ^{bA}	50.0 ± 5.7 ^{bA}	51.7 ± 5.4 ^{aA}	50.0 ± 1.5 ^{aAB}	41.0 ± 4.0 ^{bAB}
Cap-CK-1 µM MEL	71.5 ± 4.2 ^{aA}	54.0 ± 2.60 ^{bA}	50.1 ± 3.8 ^{bA}	46.0 ± 4.6 ^{aA}	55.7 ± 0.7 ^{bB}	47.5 ± 10.5 ^{aB}

Values are expressed as means ± S.E.M. (n = 4 and n = 3 for viability and PS translocation, respectively). * represents significant differences (p < 0.05) between seasons within the same treatment and breed; different lowercase letters in the same row represent significant differencesbetween breeds within a treatment and season; different capital letters in the same column represent significant differences between treatments (Cap-CK, Cap-CK-100 pM MEL, and Cap-CK-1 µM MEL) within a breed and season.

However, when the percentage of intact spermatozoa without PS translocation was evaluated, the effect of melatonin was positive in all breeds in both seasons, except in the Creole breed during the dry season. The effective melatonin concentration depended on the breed and season. During the rainy season, an increase in this parameter in Creole and Hampshire spermatozoa was observed with 100 pM melatonin. In the Romney Marsh breed, 1 µM melatonin was the effective concentration (p < 0.05) in both seasons (Table 4).

3.2.3. Effects on Capacitation Status during In Vitro Capacitation

The addition of melatonin at both concentrations (100 pM and 1 µM) in the cocktail medium partially prevented the increase in capacitated sperm provoked by the incubation in capacitating conditions in all breeds (Figure 3). Thus, in the cap-CK samples with melatonin, a higher percentage of non-capacitated sperm (p < 0.05) was observed comparedto the cap-CK samples without the hormone. This effect was concomitant with a lower rate of capacitated sperm (p < 0.05), except for Romney Marsh in the dry season. Although the preventive effect of melatonin on sperm capacitation was observed in the three breeds, itwas more noticeable in the Romney Marsh and Hampshire rams during the rainy season.An impact on reacted spermatozoa was observed only in the rainy season; the Cap-CK samples with melatonin showed a lower percentage than the Cap-CK without the hormone. This effect was evident at both concentrations in the Hampshire breed and at 100 pM or1 µM for the Creole and Romney Marsh breeds, respectively.

In general, the effect of melatonin was more evident in the rainy season than in thedry season for all breeds.

3.2.4. Effects on Phosphorylation in Tyrosine Residues during In Vitro Capacitation

In general, the addition of melatonin to samples incubated under capacitating condi- tions had no significant effects on phosphotyrosine levels, except for an increase in Creole spermatozoa in the rainy season (Figure 4). Despite the decrease in the signal observed in the dry season in the Creole and Hampshire breeds, it was not significant when compared with Cap-CK without the hormone (Figure 4). Significant differences between seasons were observed (p < 0.05).

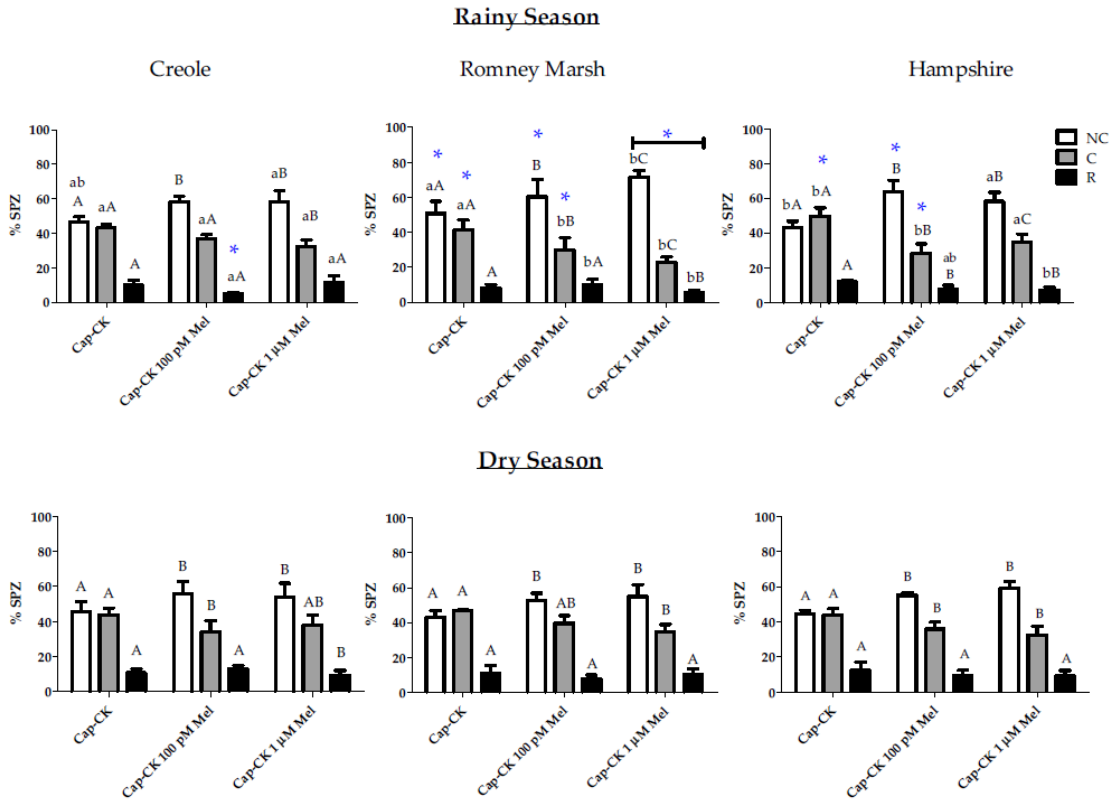

Figure 3. Assessment of capacitation status of ram spermatozoa, evaluated by chlortetracycline (CTC) staining, after 3 h incubation at 39 °C and 5% CO_2 (capacitating conditions) in TALP with cAMP-elevating agents without (cap-CK) or with100 pM and 1 µM melatonin (cap-CK-100 pM MEL and Cap-CK-1 µM MEL). The distributions were done by breed and season (rainy or dry). Data for percentages of non-capacitated (NC), capacitated (C), and acrosome-reacted (R) spermatozoa are expressed as means ± S.E.M. (n = 4). * represents significant differences ($p < 0.05$) between seasons within the same treatment and breed; different capital letters represent significant differences between treatments (Cap-CK, Cap-CK-100 pM MEL and Cap-CK-1 µM MEL) within a breed and season; different lowercase letters represent significant differences between breeds within a treatment and season.

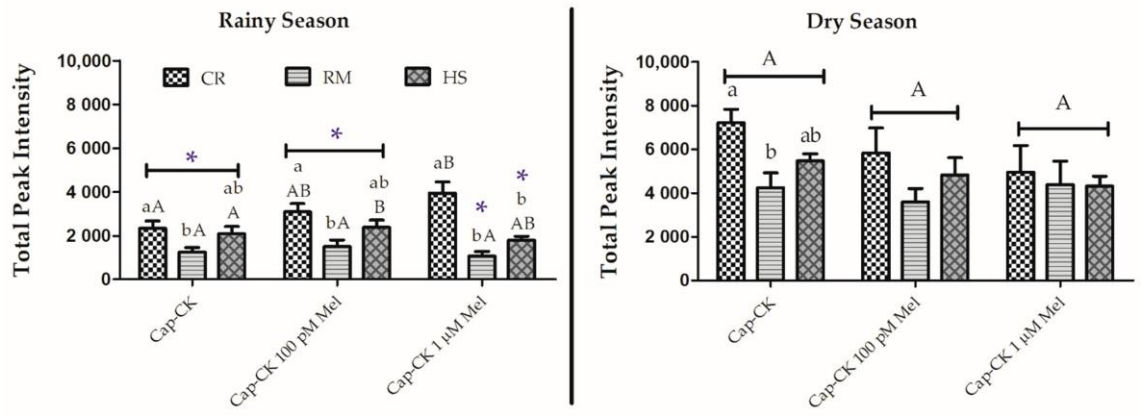

Figure 4. Protein tyrosine phosphorylation evaluated by densitometry in samples after 3 h of incubation at 39 °C and 5%CO_2 (capacitating conditions) in TALP with cAMP-elevating agents without (cap-CK) or with 100 pM and 1 µM melatonin(Cap-CK-100 pM MEL and Cap-CK-1 µM MEL). Data for the Creole (CR), Romney Marsh (RM), and Hampshire (HS) breedsare distributed by season (rainy or dry) and expressed as means ± S.E.M (n = 4). * represents significant differences ($p < 0.05$) between seasons within the same treatment and breed; different capital letters represent significant differences betweentreatments (Cap-CK, Cap-CK-100 pM MEL, and Cap-CK-1 µM MEL) within a breed and season; different lowercase lettersrepresent significant differences between breeds within a treatment and season.

4. Discussion

In this study, we evaluated the response to in vitro capacitation in ram spermatozoafrom three Colombian breeds located in the equatorial region under two different seasonal climatic conditions and in natural grazing. The evaluation of some sperm quality variables before capacitation revealed significant differences between the native breed (Creole) and the introduced ones (Romney Marsh and Hampshire). Spermatozoa from Creole rams showed higher total and progressive motility, better plasma membrane integrity, and less PS translocation than spermatozoa from the introduced breeds, in both rainy and dry seasons. When comparing between seasons, sperm quality variables were generally better in the rainy season for Creole and Romney Marsh and in the dry season for Hampshire.

After incubation in capacitating conditions, both in TALP and in TALP with a cocktail of agents that increased the cAMP (cocktail medium), most of these variables decreased, especially with the cocktail medium. These changes were more acute in the dry season, except for in the Hampshire breed. In the rainy season, total and progressive motility and viability in Creole spermatozoa remained higher after incubation in the cocktail mediumthan those of the imported breeds. Our previous results in Rasa Aragonesa rams in the Mediterranean region also showed a decrease in these variables but with no statistical differences between both media [28].

Regarding the capacitation status, incubation in both media led to a significant de- crease in the percentages of non-capacitated spermatozoa in both seasons and for the three breeds. This decrease was significantly more acute when incubation was performed in the cocktail medium than in TALP, this being concomitant with increases in the rates of capacitated and acrosome-reacted spermatozoa. Changes in capacitation status would explain the decrease in motility, especially progressive motility, due to the sperm hyperac- tivation associated with capacitation [9,28,44]. The decrease in spermatozoa without PS translocation could also be attributed to capacitation. Translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane has been defined as one of the earliest signs of apoptosis [45,46], but it is also associated with plasma membrane scrambling related to capacitation and the acrosome reaction [47]. Moreover, it is worth noting that capacitation and apoptosis share signal transduction pathways [48]. When comparing between breeds, it can be seen that spermatozoa from Hampshire rams obtained in the rainy season were significantly more capacitated before incubation than spermatozoa from the other breeds. This would explain the levels of capacitated sperm after incubation in the cocktail medium being significantly higher in this breed. However, in the dry sea-son, no differences in the percentages of capacitated spermatozoa between breeds were observed either before or after incubation in the cocktail medium.

A significant increase in the phosphorylation in tyrosine residues of proteins from samples incubated in the cocktail medium was also observed in both seasons for all breeds, unlike what occurred when incubation was carried out in TALP. Previously, we demonstrated that protein tyrosine phosphorylation of membrane proteins is related tothe capacitation state in ram spermatozoa [7,49]. Also, we showed that the presence ofcAMP-elevating agents in the capacitation medium is necessary to achieve a high increasein the phosphorylation levels in Mediterranean seasonal rams [4,50]. In the present study,the effect of the cocktail medium was more evident in the dry season. However, thisincrease was much less marked in the Romney Marsh breed than in the other two breeds.Therefore, the cocktail medium was able to induce sperm capacitation to a greater extent than TALP, without a higher loss of sperm quality, in these three breeds in bothseasons. Consequently, we used the cocktail medium to test the ability of melatonin to exertdirect effects on spermatozoa from breeds subjected to the equatorial period, as occurs in breeds of reproductive seasonality located in other latitudes [4,9,28,51].

In the present study, we assayed two concentrations of melatonin, 100 pM and 1 μM, because we have previously observed different effects on Rasa Aragonesa spermatozoa depending on its concentration. In this Mediterranean seasonal breed, melatonin at 100 pM had a positive impact on viability, whereas 1 μM provoked a slight decrease in progressive

motility when added to the cocktail medium in capacitating conditions [28]. In Colom- bian breeds, the positive effect of 100 pM of melatonin was only observed in the RomneyMarsh breed in the rainy season and the effect on progressive motility was evidenced in the Hampshire breed in the dry season at both concentrations of the hormone. The effect of melatonin was more evident when the percentage of capacitated spermatozoa was evaluated. The addition of melatonin at both concentrations in the cocktail mediumpartially prevented the increase in capacitated sperm provoked by the incubation in ca- pacitating conditions in all breeds. Our group has previously shown that melatonin can regulate in vitro capacitation mainly via the MT_2 receptor in sperm from temperate-located rams [27]. In a previous study [34], we demonstrated that both melatonin receptors, $MT_1$and MT_2, are also present in the spermatozoa of these three Colombian breeds raised under equatorial photoperiodic conditions. In the present study, the effect of melatonin was, in general, more evident in the rainy season than in the dry season for all breeds. This could be attributed to previously reported differences in the melatonin concentration inseminal plasma between seasons, with higher levels in the dry season for the three studied breeds [33]. Thus, the previous exposure of spermatozoa to high endogenous melatonin levels during the dry season could mitigate the effect of this hormone when it is added in vitro. This effect has been previously reported in somatic cells, where the exposure toa high level of melatonin can desensitize melatonin receptors [52]. Differences between breeds could also be attributed to previously described differences in the density of mela-tonin receptors [34]. The lesser effect of melatonin on sperm capacitation in Creole rams could be due to a lower MT_2 density in spermatozoa from this breed than the other two, as we described in a previous study [34]. Colombian Creole sheep are a native breed that,after five centuries of breeding in a 12L:12D photoperiod, are much more adapted to the equatorial climate and photoperiod than the Hampshire and Romney Marsh breeds, which were introduced into the country only 50 years ago. This adaptation to a non-seasonal photoperiod could be reflected in the reduction of the MT_2 receptor density, but the fact that it has not disappeared further supports the idea that melatonin has other physiological functions than seasonal control, as we pointed out in our previous work [23,53]. In the studied Colombian breeds, both assayed melatonin concentrations partially prevented the increase in capacitated sperm, whereas in Rasa Aragonesa spermatozoa only the higher concentration was effective. However, when phosphotyrosine levels were analyzed, no significant effect of the hormone was observed, in contrast with the decrease evidenced in Rasa Aragonesa spermatozoa in the presence of 1 μM melatonin [28].

Melatonin also had a significant effect on the percentage of viable sperm without PS translocation, which remained high in capacitating conditions in all breeds and in both seasons, except in the Creole breed during the dry season. As mentioned above, PS translocation could be related to capacitation or apoptosis [48]. The decapacitating effect of melatonin could explain the lower levels of sperm with PS translocation, but an antiapoptic effect of melatonin has also been reported in somatic cells [49] as well as in spermatozoa [25,54]. This antiapoptotic action seems to be mediated by melatonin binding to its receptors, specifically MT_1 [55,56]. The observed variations between seasons, the effect being more evident in the rainy than in the dry season, could equally be attributed to the higher endogenous levels of melatonin in seminal plasma in the dry season [33], and consequently to a desensitization of the melatonin receptors.

5. Conclusions

To sum up, the present study shows that incubation in a medium with cAMP-elevating agents effectively achieves in vitro capacitation in spermatozoa from different ram breeds under the equatorial photoperiod. However, the response to the in vitro capacitation was different between breeds and seasons. The addition of melatonin to the medium with cAMP-elevating agents partially prevented the increase in capacitated spermatozoa and the decrease in viable spermatozoa without PS translocation in the three studied breeds, with differences between breeds and seasons. This study shows that melatonin is able to exert

direct effects on spermatozoa in ovine breeds located under the equatorial photoperiod, asit does in seasonal breeds located in temperate regions.

Author Contributions: Conceptualization, R.P.-P., A.C., and J.A.C.-C.; methodology, R.P.-P., A.C.,and J.A.C.-C.; formal analysis, M.C.-S., A.C., and R.P.-P.; investigation, M.C.-S.; resources, R.P.-P., A.C., H.A.G.-L., and J.A.C.-C.; data curation, M.C.-S. and A.C.; writing—original draft preparation,M.C.-S. and R.P.-P.; writing—review and editing, M.C.-S., A.C., and R.P.-P.; supervision, R.P.-P., A.C., H.A.G.-L., and J.A.C.-C.; project administration, R.P.-P., A.C., H.A.G.-L., and J.A.C.-C.; funding acquisition, R.P.-P., H.A.G.-L., and J.A.C.-C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by CICYT-FEDER AGL2017-83799-R, Gobierno de Aragón, grant number DGA A07_20R (Spain), and the 110 157 635 854 Project of the Colciencias Code Research Program (Colombia).

Institutional Review Board Statement: All procedures used in this study were in strict accordance with the Colombian Animal Protection Regulations (Law 84/1989, modified by Law 1774/2016) and were approved by the Bioethics Committee of the Faculty of Veterinary Medicine and Zootechnics,Bogotá Headquarters, National University of Colombia (Project license: CB-074-2014, approval date:5 November 2014).

Data Availability Statement: The datasets generated for this study can be found in the figshare repository 10.6084/m9.figshare.14528916.

Acknowledgments: Special thanks to Emma Conde and Esneider Rivera for the technical sup- port with the rams, and the sample collection and transportation. In addition, special thanks to Yolanda Goméz Vargas from the Molecular Genetics Laboratory of AGROSAVIA (Colombian Cor- poration of Agriculture Research) for providing the facilities and equipment to perform part of the experimental activities.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the designof the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, orin the decision to publish the results.

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6. Discusión general

6.1. Cambios en la concentración de melatonina en el plasma seminal de moruecos de la zona ecuatorial a lo largo del año (artículo 1)

Aunque el efecto de la melatonina en la reproducción ovina se ha estudiado ampliamente, la mayor parte de la información publicada se basa en estudios realizados en ovinos bajo condiciones climáticas templadas y con fotoperiodo variable dependiente de las estaciones del año (Rosa and Bryant, 2003). En estas latitudes medias, el fotoperiodo, a través de la secreción de melatonina nocturna por la glándula pineal, regula la actividad reproductiva ovina tanto en hembras como en machos (Bittman et al., 1983; Malpaux et al., 1996). En trabajos anteriores de nuestro grupo se observó que la concentración de melatonina en el plasma seminal de moruecos de la raza Rasa Aragonesa, que es una raza estacional de zonas templadas, variaba a lo largo del año, presentando un máximo durante la estación reproductiva (Casao et al., 2010a). Sin embargo, poco se conoce sobre el papel de la melatonina en ovinos ubicados en las zonas ecuatoriales, donde el fotoperiodo no presenta variación y es aproximadamente de 12 horas de luz y 12 horas de oscuridad (12L:12D). Por ello, **el primer objetivo de la presente tesis doctoral fue cuantificar la concentración de melatonina en el plasma seminal de moruecos de tres razas ovinas ubicadas en Colombia, sometidas a fotoperiodo constante a lo largo del año.** Adicionalmente se evaluó la concentración de testosterona y la actividad de las enzimas antioxidantes del plasma seminal, y se analizó su correlación con la concentración melatonina.

En este primer objetivo se demostró por primera vez que en **moruecos ubicados en la zona ecuatorial también se producen cambios en la concentración de melatonina del plasma seminal a lo largo del año**. Además, el estudio comparativo entre razas reveló que no hubo diferencias para la concentración de melatonina en el plasma seminal entre las tres razas estudiadas (Criolla de lana, Romney Marsh, y Hampshire). Es decir, que las razas introducidas recientemente (Romney Marsh y Hamprshire), presentaron valores similares de concentración de melatonina a los obtenidos para la raza Criolla de lana, establecida en Colombia desde hace cinco siglos. Por tanto, y **en lo que respecta a la concentración de melatonina en el plasma seminal, el periodo de adaptación a las condiciones dadas en la zona ecuatorial no influyó en los valores obtenidos.**

Estudios previos ya habían demostrado que, en términos de respuesta pituitaria, no existen diferencias entre razas cuando son sometidas a fotoperiodo artificial (Poulton and Robinson, 1987). Además, cuando ovinos de latitudes medias eran sometidos a un régimen de luz de la zona ecuatorial (12L:12D), se revertía el patrón estacional de actividad ovárica (Jackson et al., 1990; Wodzicka-Tomaszewska et al., 1967). En nuestro estudio, la concentración de melatonina detectada en plasma seminal de moruecos de razas criadas en la zona ecuatorial fue menor que la descrita en machos de Rasa Aragonesa, incluso más bajos que los encontrados durante la estación no reproductiva de esta raza (Casao et al., 2010a). Sin embargo, cabe destacar que, aunque inferiores en concentración, **nuestros resultados también reflejan una fluctuación en la concentración de melatonina en**

plasma seminal a lo largo del año, con menores valores en las estaciones de lluvias y un incremento en las estaciones de sequía. El descenso observado en las razas colombianas entre los meses de marzo y mayo coincidiría con el descrito en la raza Rasa Aragonesa en latitudes medias (Casao et al., 2010a). Sin embargo, la concentración de melatonina en plasma seminal de razas ubicadas en Colombia desciende de nuevo en octubre y noviembre, los meses en que esta concentración es máxima en machos de clima templado.

La cantidad de melatonina existente en el plasma seminal podría ser debida, en parte, a la secreción de esta hormona a nivel pineal, como demostraría su concordancia con la concentración presente en sangre, tal y como se ha descrito en animales situados en un fotoperiodo estacional (Casao et al., 2010a; Pool et al., 2020). Dado que, en nuestro experimento, los moruecos no estuvieron sometidos a cambios en el fotoperiodo por su ubicación cercana al ecuador, la variación en secreción pineal de melatonina regulada por la duración del periodo de oscuridad no sería la responsable de los cambios en la concentración de esta hormona en el plasma seminal.

Pero la melatonina también puede ser sintetizada en el tracto reproductor del morueco (González-Arto et al., 2016a), y por tanto pasar a formar parte del plasma seminal. Sin embargo, a este respecto, Acuña-Castroviejo *et al* (2014), observaron que la melatonina sintetizada en tejidos extrapineales no sigue necesariamente un patrón estacional como la melatonina producida por la glándula pineal. En concreto, la síntesis de melatonina por el testículo en moruecos de Rasa Aragonesa no muestra variaciones estacionales (Cebrián-Pérez et al., 2017). Por esta razón, parece poco probable que los cambios en la concentración de esta hormona observados a lo largo del año en el plasma seminal de los moruecos de razas colombianas sean atribuibles a cambios en la síntesis de melatonina a nivel testicular. Sin embargo, para confirmar esto, habría que analizar la síntesis de melatonina a nivel testicular en los moruecos de zonas ecuatoriales.

Si los cambios observados no son imputables a cambios en el fotoperiodo, puede que haya otros factores externos que influyan sobre los niveles de melatonina en plasma seminal, como por ejemplo la temperatura. En algunas especies como el hámster europeo, se ha descrito que la temperatura puede afectar la secreción de melatonina (Vivien-Roels et al., 1997), pero este efecto no se ha demostrado en ovinos (Wodzicka-Tomaszewska et al., 1967). Además, una característica de los países tropicales cercanos al ecuador es la escasa variación en la temperatura ambiental a lo largo del año (Narváez and León, 2001), por lo que la temperatura no sería la causa de variación en la concentración de melatonina en el plasma seminal observado en este trabajo.

Una explicación más probable sería atribuible a un diferente aporte de melatonina exógena debido a la variación en el contenido de esta hormona en el alimento. Al igual que en los animales, las plantas pueden sintetizar melatonina, la cual es denominada fitomelatonina (Koca Çalişkan et al., 2017; Tan and Reiter, 2020). Además, diferentes factores estresantes para las plantas pueden incrementar la producción endógena de fitomelatonina. Entre ellos se destaca, una alta salinidad, bajas temperaturas, y periodos de

sequias (Arnao, 2014). En animales, el consumo de dietas vegetales ricas en fitomelatonina puede contribuir al incremento en la concentración de melatonina circulante (Hattori et al., 1995) y en consecuencia en el plasma seminal. En nuestro caso, la concentración de melatonina en el pasto sería mayor en épocas de sequía que de lluvia, y por tanto los moruecos ingerirían más fitomelatonina en estas épocas, lo que se explicaría el aumento de esta hormona en el plasma seminal durante los meses de sequía. Además, el origen nutricional de esta melatonina también explicaría la falta de diferencias entre razas observada en este trabajo.

A diferencia de los estudios realizados en moruecos de latitudes medias (Casao et al., 2010a), en este trabajo realizado en la zona ecuatorial, la concentración de melatonina en el plasma seminal no correlacionó con otros componentes del plasma seminal como la testosterona, ni con la actividad de las enzimas antioxidantes. Los cambios en el patrón de secreción de testosterona a lo largo del año son un reflejo de la activación del eje hipotálamo-hipofisario-gonadal mediado por la melatonina en condiciones de estacionalidad (D'Occhio et al., 1984), produciéndose un aumento de testosterona 2-4 semanas tras el aumento de melatonina (Rosa and Bryant, 2003). En nuestro trabajo, sólo se observaron diferencias a lo largo del año en la concentración de testosterona para las razas británicas introducidas más recientemente (Romney Marsh y Hampshire), pero el aumento ocurrió antes del incremento en los niveles de melatonina, así que no es probable que la melatonina sea la responsable de este efecto. La raza Criolla de lana no presentó cambios estadísticos en la concentración de testosterona a lo largo del año, y además presentó concentraciones menores de testosterona que las razas introducidas. Los machos de esta raza son más pequeños y menos agresivos que los de las razas británicas, y ocupan los rangos inferiores en el grupo social. Dado que los machos subordinados presentan niveles de testosterona menores que los machos dominantes y menores variaciones estacionales de esta hormona (Ungerfeld and Lacuesta, 2015), las diferencias en la concentración de testosterona entre razas de este estudio podrían deberse a factores sociales (Aguirre et al., 2007). En cuanto a las enzimas antioxidantes, únicamente la enzima glutatión peroxidasa (GPx) mostró variaciones a lo largo del año, con un especial incremento en la primera época de lluvias del año. También se observó que esta variación no correlacionaba con la variación de concentración de melatonina. La actividad de GPx aumentó en el mes de mayo, durante la primera época de lluvias, y se mantuvo así en las siguientes épocas de sequía y de lluvia. En otros mamíferos en condiciones climáticas tropicales, se ha observado que una humedad ambiental o una temperatura alta incrementan la actividad de esta enzima para minimizar el daño en los espermatozoides (Nichi et al., 2006; Soren et al., 2016; Suriyasomboon et al., 2005).

6.2. Comparación en la composición del plasma seminal de moruecos de latitudes templadas y zona ecuatorial (artículo 2).

Con objeto de dilucidar si el régimen fotoperiódico puede afectar a la composición del plasma seminal, se llevó a cabo un estudio comparativo de los componentes de este fluido en moruecos ubicados en Colombia (4°40'57'' N 74°12'50'' O) (razas Criolla de lana, Romney Marsh,) y en España (41°38'05.80" N 0°51'35.20" O) (raza Rasa Aragonesa)**.** Este estudio se realizó entre los meses de septiembre y noviembre, lo que representa para los moruecos de latitudes medias el inicio de la época reproductiva, y para los moruecos de latitudes ecuatoriales, el inicio de la segunda época de lluvias del año.

En relación con la melatonina, la concentración detectada en plasma seminal de los moruecos ubicados en la zona ecuatorial fue menor que la de moruecos de latitudes medias. Esto estaría en concordancia con lo apuntado en el apartado anterior, tras la comparación de la concentración de melatonina de los machos ubicados en la zona ecuatorial (artículo 1) con lo reportado en la bibliografía para otras razas de latitudes medias. En el presente estudio (artículo 2), hay que destacar que la toma de muestras se realizó en las mismas condiciones y en los mismos meses del año, pero, lógicamente la escotofase fue más corta en esa época en la zona ecuatorial que en la zona templada (12 h 10' vs. 16 h 12', respectivamente), lo que podría explicar, en parte, estas diferencias en la concentración de melatonina (Chemineau et al., 2008).

Además, **se observaron diferencias en la concentración de testosterona en plasma seminal entre machos de latitudes medias y ecuatoriales, siendo también mayor en los primeros**, lo que reforzaría el hecho de que la melatonina regula la secreción de testosterona por el testículo en condiciones de estacionalidad (D'Occhio et al., 1984; Lincoln et al., 1981).

En cuanto a las enzimas antioxidantes, la actividad de la enzima GPx en plasma seminal fue mayor en los moruecos de latitudes ecuatoriales, posiblemente para contrarrestar el daño oxidativo producido por la alta humedad durante la época de lluvias (Soren et al., 2016), como se observó en el anterior trabajo (artículo 1). Sin embargo, la actividad de la catalasa fue mayor en machos estacionales. En trabajos previos de nuestro grupo, ya se habían detectado niveles aumentados de la actividad de la catalasa durante la época reproductiva en moruecos estacionales, y una correlación positiva con los niveles de melatonina en plasma seminal (Casao et al., 2010a), lo que estaría en concordancia con los resultados del presente trabajo.

Sin embargo, no se encontraron diferencias entre machos ubicados en diferentes latitudes al estudiar las proteínas del plasma seminal, ni en la concentración total de proteína ni en la composición proteica. Estos resultados se basan en el análisis densitométrico de las bandas obtenidas tras electroforesis SDS-PAGE. Quizás un análisis

comparativo más exhaustivo, utilizando electroforesis en dos dimensiones u otras técnicas, revelase diferencias entre machos de distintas latitudes, pero, en cualquier caso, no es el objetivo de la presente tesis.

6.3. Determinación del origen de la melatonina de plasma seminal en moruecos ubicados en la zona ecuatorial (artículo 2).

Con objeto de indagar el origen de la melatonina del plasma seminal, se realizó un estudio comparativo de la composición de este fluido en moruecos enteros y vasectomizados de las razas Criolla de lana y Romney Marsh ubicados en la zona ecuatorial**.** Además, para obtener más información en relación con el efecto del fotoperiodo, se llevó a cabo un estudio simultaneo con machos de la rasa Rasa Aragonesa ubicados en una latitud media. Este estudio se realizó en las mismas condiciones del apartado 6.2.

Los moruecos vasectomizados de latitudes medias presentaron una disminución en la concentración de melatonina en el plasma seminal en comparación con los moruecos enteros, lo cual sugiere que, al menos parte de esta hormona tiene un origen testicular, ya que, tras la vasectomía, las secreciones testiculares no forman parte del plasma seminal. Esta posibilidad de síntesis extrapineal de melatonina a nivel testicular fue planteada tras identificar las enzimas de síntesis de esta hormona en el testículo de moruecos de razas estacionales (González-Arto et al., 2016a). A la vista de los presentes resultados, se puede inferir que al menos la mitad de la concentración de melatonina en el plasma seminal de moruecos con marcada estacionalidad reproductiva tiene origen testicular y la otra mitad probablemente pineal, ya que, si fuese totalmente testicular, no se detectaría tras la vasectomía.

En contraste, **en los moruecos de la zona ecuatorial no se observaron diferencias en la concentración de melatonina entre machos enteros y vasectomizados**, lo que sugiere que, en estos machos, o bien no hay síntesis de melatonina testicular, o bien esta no es secretada al plasma seminal. Esto podría deberse también a las diferencias de fotoperiodo a las que los animales están sometidos. En el tracto reproductor de moruecos estacionales se han identificado los receptores de melatonina MT_1 y MT_2 (M. González-Arto et al., 2017), que podrían estar implicados en la regulación de la secreción testicular de melatonina a través de las variaciones de secreción de la melatonina pineal, como se ha sugerido para otros tejidos extra-pineales (Acuña-Castroviejo et al., 2014). En moruecos situados en zonas ecuatoriales, sometidos a un régimen fotoperiódico 12L:12D, la ausencia de variaciones en la secreción pineal de melatonina podría reflejarse en una ausencia de secreción de melatonina testicular (Barrell et al., 2000). Sin embargo, se requerirían más estudios sobre la posible síntesis de melatonina y la presencia de receptores para la misma en el tracto reproductor de los moruecos en condiciones de latitudes ecuatoriales para corroborar esta hipótesis.

Además de la concentración de melatonina, se analizaron diferencias entre machos vasectomizados y enteros en otros componentes del plasma seminal. Respecto a **la**

concentración de testosterona, los todos machos vasectomizados presentaron una menor concentración de la misma en comparación con los machos enteros, independientemente de la latitud. Por tanto, nuestros resultados sugieren un origen dual de la testosterona del plasma seminal de moruecos, con una contribución testicular (desde la *rete testis* (Cooper and Waites, 1966), y no testicular, principalmente desde circulación sanguínea o, en menor medida, la glándula adrenal (Georgiadis et al., 1992). No se encontraron diferencias en cuanto a la actividad de las enzimas antioxidantes en el plasma seminal de moruecos enteros y vasectomizados de ambas latitudes, lo que corrobora el origen de estas enzimas en las glándulas accesorias. Sin embargo, si se observó una menor concentración proteica en el plasma seminal de machos vasectomizados de ambas latitudes, debida principalmente a una disminución de las proteínas de alto peso molecular. Este resultado corrobora trabajos previos de Ghaoui et al (2007) donde identifica la mayoría de los componentes del plasma seminal como derivados de las glándulas accesorias, mientras que las proteínas de alto peso molecular tendrían un origen testicular y epididimal.

6.4. Identificación y localización de los receptores de melatonina MT_1 y MT_2 en la membrana espermática de moruecos de la zona ecuatorial (artículo 3)

Una vez demostrado que los moruecos de latitudes ecuatoriales presentaban cambios en el patrón de secreción de melatonina del plasma seminal a lo largo del año (artículo 1), para seguir profundizando en los mecanismos de acción de esta hormona **nos planteamos como cuarto objetivo investigar la presencia y distribución de los receptores de melatonina MT_1 y MT_2 en la membrana plasmática de espermatozoides de moruecos de raza Criolla de lana, Hampshire y Romney Marsh y estudiar posibles variaciones entre las épocas de lluvia y sequía.** Esta diferenciación entre épocas obedece al hecho de que, ya que el fotoperiodo es constante en estas latitudes, la principal clave medioambiental que pudiese determinar cambios en la reproducción son los periodos de lluvias, debido a su consecuente efecto en la disponibilidad y calidad del forraje (Bronson, 2009; Rosa and Bryant, 2003). Además, tal y como se demostró en el artículo 1, la concentración de melatonina en plasma seminal varía entre épocas de lluvia y sequía.

En la primera parte de este objetivo se constató que ambos receptores MT_1 y MT_2 están presentes en los espermatozoides de las tres razas ovinas estudiadas (artículo 3). Con estos resultados, se refuerza la idea de que la melatonina cumpliría otras funciones en la reproducción de los mamíferos, más allá de las relacionadas a la estacionalidad, como sugerían la presencia de ambos receptores en los espermatozoides de especies no estacionales, como el cerdo (González-Arto et al., 2016c), y humano (Espino et al., 2011).

En este estudio también se determinaron los inmunotipos predominantes en las razas de latitudes ecuatoriales, basados en los descritos previamente por nuestro grupo en la raza Rasa Aragonesa (Casao et al., 2012). En las razas colombianas estudiadas el inmunotipo predominante para el receptor MT_1 fue el tipo I (marcaje en toda la cabeza y

flagelo) y para el receptor MT_2 fue el tipo A (mayor intensidad en el acrosoma), mientras que en Rasa aragonesa predomina el tipo II (marcaje en la región ecuatorial, post-acrosoma y flagelo) y el tipo AP (intenso en acrosoma y post-acrosoma), respectivamente (González-Arto et al., 2016b). En este estudio también se observaron diferencias entre las razas Criolla de lana, Romney Marsh y Hampshire en el porcentaje de inmunotipos para ambos receptores. Otra diferencia destacable de los espermatozoides de estas razas con respecto a los espermatozoides de Rasa aragonesa, fue la presencia de dos inmunotipos para MT_2 que no habían sido descritos previamente en espermatozoides eyaculados: el inmunotipo N (localización del receptor únicamente en el cuello del espermatozoide) identificado hasta el momento sólo en espermatozoides ovinos epididimarios (Marta González-Arto et al., 2017), y el inmunotipo E, con localización en el borde apical y banda ecuatorial, no descrito previamente en esta especie.

En estudios previos, realizados en el grupo, se observaron diferentes subtipos de espermatozoides en función de la distribución de los receptores tanto en especies estacionales como no estacionales (González-Arto et al., 2016c). Esto apunta a que, aun cuando la importancia fisiológica de la variación en la distribución de los receptores de melatonina en la membrana plasmática todavía se desconoce, no estaría, en principio, vinculada con la existencia de estacionalidad reproductiva.

Estas diferencias de distribución podrían ser debidas a cambios en los genes que codifican estos receptores. En el gen que codifica el receptor MT_1 (*MTRN1A)* se han evidenciado, al menos, dos polimorfismos mediante las enzimas de restricción *Mln*I y *Rsa*I, (asociados a la posición 606 y 612 del gen, respectivamente) en diferentes razas ovinas estacionales como la Sarda (Carcangiu et al., 2009) y la Rasa Aragonesa (Martínez-Royo et al., 2012). Recientes estudios de nuestro grupo apuntan a que los polimorfismos del gen *MTNR1A* podrían estar relacionados con la distribución de los receptores MT_1 y MT_2 en el espermatozoide ovino. Por tanto, sería necesario llevar a cabo estudios similares en moruecos de la zona ecuatorial para indagar en la relación de la presencia de estos polimorfismos con la distribución de los receptores de melatonina en estas razas no estacionales.

Por su parte, los estudios sobre posibles polimorfismos del receptor MT_2 son escasos. Esto se debe, en parte, a que el gen *MTNR1B* que codifica el receptor MT_2 en ovinos, no fue completamente clonado hasta 2009 (Cogé et al., 2009). Estudios previos, amplificando únicamente el exón 2 del gen, habían caracterizado diferentes polimorfismos de este gen tanto en razas consideradas estacionales (Texel y Corriedale) como no estacionales (Hu, Small Tail Han y Dorset). Estos polimorfismos no se asociaron con la estacionalidad reproductiva o la prolificidad, pero se sugirió que podrían afectar la interacción ligando-receptor (Xiao et al., 2007). Por tanto, al igual que en el caso del receptor MT_1, se requieren más estudios para entender el efecto del genotipo en la distribución del receptor MT_2 en la membrana espermática, y una posible regulación entre ambos receptores.

Además de diferencias entre razas, **nuestros resultados también revelaron diferencias en los porcentajes de inmunotipos de ambos receptores entre las épocas**

de lluvias y sequía, para las tres razas estudiadas. Esto difiere de lo observado previamente en los espermatozoides de moruecos de latitudes medias, en los cuales únicamente se observaron diferencias entre la estación reproductiva y no reproductiva para el receptor MT_2 (González-Arto et al., 2016b). En nuestro caso, las diferencias entre las dos épocas observadas en los moruecos de la zona ecuatorial no estarían ocasionadas por cambios en el fotoperiodo, sino, posiblemente, por factores ambientales, especialmente el efecto nutricional que pueden causar los ciclos de lluvias (Galina et al., 1996), como ya apuntaban los resultados del artículo 1 de esta tesis doctoral. Los espermatozoides sufren un proceso de maduración durante su tránsito por el epidídimo, y la distribución de los receptores espermáticos de melatonina va cambiando durante el mismo (Marta González-Arto et al., 2017). Dado que esta maduración epididimaria está controlada, muy posiblemente, por factores ambientales (Dacheux, 2014), es posible que la pluviometría, a través de su influencia sobre los niveles de alimento, pueda afectar a la distribución final de los receptores de melatonina durante la maduración espermática. Otra posible causa de variación en la distribución de receptores entre épocas podría ser la concentración de melatonina en el plasma seminal, ya que altas concentraciones de melatonina pueden causar desensibilización de los receptores por internalización (Gerdin et al., 2003; Trecherel et al., 2010; Witt-Enderby et al., 2003). Como se ha descrito en el artículo 1 de esta tesis, la concentración de melatonina en el plasma seminal de las razas situadas en la zona ecuatorial varia a lo largo del año, con una mayor concentración en la época de sequía. **Por lo tanto, las diferencias en la distribución de los receptores MT_1 y MT_2 entre distintas estaciones climáticas podría estar regulada por la exposición a la melatonina del plasma seminal.**

Para corroborar los resultados evidenciados por inmunofluorescencia, también se analizaron las proteínas de la membrana espermática mediante western blot usando anticuerpos frente a los dos receptores. Los resultados demostraron la presencia de una banda de 39 kDa compatible con ambos receptores (Dubocovich et al., 2010) junto con una banda adicional de 32 kDa. Esta última banda se podría relacionar con la forma activa del receptor, ya que en las proteínas G la subunidad α se disocia después de la activación del receptor (Dubocovich and Markowska, 2005; Gilman, 1995). En un estudio similar realizado en la raza Rasa Aragonesa (Casao et al., 2012), se identificó también una banda de 75 kDa, tanto para los western blots de MT_1 como los de MT_2, lo que apuntaba a la existencia de heterodímeros de estos receptores, tal y como sucedía en células somáticas cultivadas *in vitro* (Ayoub et al., 2004). Sin embargo, en el presente trabajo, esta banda de 75 kDa, se observó solo en los western blots de MT_2, no en los de MT_1, por lo que parece resultado de la formación de homodímeros del receptor MT_2. Así, **nuestros resultados sugieren, que en espermatozoides de moruecos de latitudes ecuatoriales únicamente se forman homodímeros MT_2/ MT_2.** Además, la presencia de una doble banda de 45 – 50 kDa en los westerns de MT_2 sugiere su heterodimerización con otros miembros de la familia de receptores GPRC, como MT3, QR2, GPR61, GPR62, GPR135 y otros, lo que posiblemente afecta la función, activación y regulación de estos receptores (Oishi et al., 2018). En células HEK293T transfectadas, la melatonina inhibe la elevación de los niveles de AMPc en las células que expresan los heterodímeros GPR61/MT_2 o GPR62/MT_2 (Oishi and Jockers,

2017). En el caso de espermatozoides de morueco, la formación de estos heterodímeros podría explicar la acción descapacitante de la melatonina a determinada concentración (Casao et al., 2010b; González-Arto et al., 2016b) al inhibir el aumento de los niveles de AMPc durante la capacitación *in vitro*, como se ha demostrado previamente (Gimeno-Martos et al., 2019).

El análisis densitométrico de los western blot, evidenció únicamente diferencias entre épocas para la raza Criolla de lana en el receptor MT_1. Este resultado sugiere que, con la excepción de la raza Criolla de lana, las diferencias en la distribución del receptor MT_1 no están acompañadas de cambios en la cantidad del mismo. Por otro lado, el análisis densitométrico del receptor MT_2 reveló que la densidad de este receptor fue más baja en los espermatozoides de la raza Criolla de lana que en las otras dos razas, independiente de la época. Esta disminución en la densidad del receptor MT_2 podría deberse al hecho de la Criolla de lana es una raza nativa de Colombia, y está más adaptada al clima y al fotoperiodo constante que Hampshire y Romney Marsh, que fueron introducidas hace 50 años. No obstante, el hecho de que después de cinco siglos de cría en fotoperiodo ecuatorial los receptores de melatonina sigan presentes en moruecos criollos, refuerza la evidencia de que la melatonina a través de sus receptores cumple otras funciones esenciales en la fertilidad más allá de la regulación de la estacionalidad (Cebrián-Pérez et al., 2014).

6.5. Respuesta a la capacitación *in vitro* y efecto de la adición de melatonina en espermatozoides de moruecos de la zona ecuatorial (artículo 4).

Una vez cuantificados los cambios en la concentración de melatonina en el plasma seminal de moruecos de la zona ecuatorial, y demostrada la presencia y los cambios en la distribución de los receptores de melatonina MT_1 y MT_2 en su membrana espermática, nos planteamos como último objetivo de esta tesis doctoral **evaluar si la melatonina podía regular la capacitación en estas razas, al igual que lo hace en razas estacionales.** Previamente a la consecución de este objetivo, se hizo necesario **estudiar los requisitos y la respuesta a la capacitación *in vitro* de las razas Criolla de lana, Romney Marsh y Hampshire ubicadas en latitudes ecuatoriales,** ya que no se había estudiado hasta el momento. Este estudio se llevó a cabo tanto en época de lluvias como de sequía (artículo 4).

A pesar de que los espermatozoides de la mayoría de las especies domesticas se capacitan fácilmente *in vitro*, los espermatozoides ovinos requieren de un conjunto de sustancias que incrementen el AMPc y eviten su degradación (Colás et al., 2008). Esta mezcla de sustancias, que denominamos comúnmente *cocktail*, añadidas a un medio usado para la capacitación de otras especies, como es el medio TALP, son necesarias para promover la capacitación espermática de moruecos de latitudes medias (Colás et al., 2010, 2008), pero hasta la fecha no se había estudiado su necesidad en moruecos de latitudes ecuatoriales.

Por lo tanto, en primer lugar, nos planteamos evaluar la respuesta a la capacitación *in vitro* de las razas Criolla de lana, Hampshire y Romney Marsh en condiciones de fotoperiodo ecuatorial y época de lluvia y sequía, En el presente estudio se observaron diferencias entre razas y entre épocas para los parámetros de calidad seminal evaluados antes de la capacitación, siendo la raza Criolla de lana la que presentó los mejores valores de motilidad total, motilidad progresiva, integridad de la membrana, y de espermatozoides vivos sin inversión de la fosfatidilserina (PS), posiblemente por su mejor adaptación a las condiciones climáticas y de fotoperiodo. Tras la capacitación, tanto en TALP como en medio *cocktail* (TALP con sustancias que incrementan el AMPc), la gran mayoría de los parámetros de calidad espermática disminuyeron, especialmente en la época seca y con el medio *cocktail*, igual que ocurre en espermatozoides de razas ovinas en latitudes medias (Gimeno-Martos et al., 2019). Sin embargo, durante la estación de lluvias, la motilidad total y progresiva y la viabilidad se mantuvieron altas en la raza Criolla de lana en comparación con las otras dos.

La incubación bajo condiciones capacitantes produjo una disminución en el porcentaje de espermatozoides no capacitados, y un incremento concomitante del porcentaje de espermatozoides capacitados y que habían sufrido la reacción acrosómica, más marcado en los espermatozoides incubados en medio *cocktail* que en el medio TALP. Estos cambios en el estado de capacitación explicarían la disminución de la motilidad, especialmente la motilidad progresiva, como resultado de la hiperactivación espermática asociada a la capacitación (Colás et al., 2010; Gimeno-Martos et al., 2019; Sharon T. Mortimer and Maxwell, 1999). La disminución en los espermatozoides vivos sin inversión de la PS también puede ser atribuida a la reorganización de la membrana espermática durante la capacitación y la reacción acrosómica (Martin et al., 2005). Al comparar entre razas, los eyaculados de la raza Hampshire obtenidos en la estación de lluvias tenían mayor porcentaje de espermatozoides capacitados que los de las otras dos, lo que explicaría que, tras su incubación en medio *cocktail,* este porcentaje también fuese superior. En cambio, durante la época seca no se observaron diferencias entre razas en el porcentaje de espermatozoides capacitados, ni antes ni después de la incubación con medio *cocktail*. Nuestros resultados también evidenciaron un incremento en la fosforilación de proteínas en residuos de tirosina, superior en las muestras incubadas en medio *cocktail* que en TALP, en ambas épocas y en las tres razas, aunque menos marcado en los espermatozoides de Romney Marsh. Por tanto, al igual que lo que ocurre en moruecos de raza Rasa Aragonesa (Grasa et al., 2006; Pérez-Pé et al., 2002) el medio *cocktail* fue capaz de inducir la capacitación *in vitro* de los espermatozoides de moruecos de la zona ecuatorial, en ambas épocas y en las tres razas estudiadas.

Una vez que demostramos que el medio *cocktail* es capaz de inducir la capacitación en espermatozoides de razas ovinas situadas en una zona de fotoperiodo ecuatorial, **nos planteamos evaluar el efecto de la adición de dos concentraciones de melatonina sobre la capacitación *in vitro* en espermatozoides de moruecos en condiciones ecuatoriales.** Estudios previos de nuestro grupo en razas de latitudes medias habían demostrado un efecto dual de la melatonina durante la capacitación espermática en función

de su concentración, ya fuese 100 pM (baja) o 1 µM (alta) (Casao et al., 2010b; Gimeno-Martos et al., 2019), por lo que en este estudio ensayamos ambas concentraciones de melatonina.

Los resultados obtenidos al incubar espermatozoides de razas situadas en la zona ecuatorial con ambas concentraciones de melatonina difieren de los obtenidos en la raza Rasa Aragonesa. En esta raza, 100 pM de melatonina tiene un efecto positivo sobre la viabilidad, mientras que 1 µM provoca una disminución de la motilidad progresiva en medio *cocktail* (Gimeno-Martos et al., 2019). En nuestras razas de la zona ecuatorial, el efecto positivo de 100 pM de melatonina solo se observó en Romney Marsh durante la época de lluvias, mientras que la melatonina a ambas concentraciones sólo afecto a la motilidad progresiva de los espermatozoides de Hampshire en ambas épocas.

El efecto de la adición de melatonina fue más evidente cuando se evaluó el estado de capacitación mediante tinción con CTC. La adición de melatonina a ambas concentraciones previno parcialmente el incremento de espermatozoides capacitados después de la incubación en todas las razas estudiadas, a diferencia de lo que ocurre con los espermatozoides de moruecos de latitudes medias en los que solo la concentración alta tiene un efecto descapacitante (Gimeno-Martos et al., 2019).

Estudios previos de nuestro grupo en razas estacionales habían demostrado que la melatonina regula la capacitación espermática a través del receptor MT_2 (González-Arto et al., 2016b). En el artículo 3 de esta tesis demostramos la presencia tanto de MT_1 como de MT_2, en la membrana de los espermatozoides de estas tres razas situadas en condiciones de fotoperiodo ecuatorial, por lo que este mecanismo de acción sería posible.

El hecho de que el efecto de la melatonina fuese, en general, más evidente durante la época de lluvias que durante la de sequía para todas las razas, podría deberse a las diferencias en la concentración de melatonina en el plasma seminal que se dan entre la época de lluvias y sequía en estas razas de la zona ecuatorial, como hemos demostrado en el artículo 1 de esta tesis doctoral. Es probable que la exposición previa de los espermatozoides a la melatonina endógena del plasma seminal, más alta durante la época de sequía, disminuya el efecto de la melatonina cuando es añadida en el medio de capacitación, como se ha observado en células somáticas, en las que altos niveles de melatonina pueden causar una desensibilización de los receptores para la misma (Witt-Enderby et al., 2003).

Las diferencias de respuesta a la melatonina entre razas observadas en este trabajo se podrían también atribuir a las diferencias en la densidad de los receptores, descritas en el artículo 3. Así, el bajo efecto de la melatonina sobre la capacitación espermática en la raza Criolla de lana podría ser debido a la menor densidad del receptor MT_2 que presentan los espermatozoides de esta raza, en comparación con las otras dos, y que podría estar relacionado con la mayor adaptación de esta raza a condiciones de fotoperiodo ecuatorial, como se ha comentado en el apartado anterior. Sin embargo, el hecho de tras 500 años de adaptación a un fotoperiodo constante, mantenga activos sus receptores MT_2 y sus

espermatozoides sean capaces de responder a la melatonina, refuerzan la idea, tal y como ya se ha comentado, de que la melatonina tiene otras funciones en la reproducción ovina aparte de la regulación de la estacionalidad (González-Arto et al., 2016b, 2016a). Finalmente, la adición de melatonina también tuvo un efecto positivo en el porcentaje de espermatozoides vivos sin inversión de PS, que se mantuvieron altos en todas las razas y ambas épocas, excepto en la Criolla de lana durante la estación seca. Aunque, como se mencionó antes, la inversión de la PS está relacionada a la capacitación, también es un proceso relacionado con la apoptosis en células somáticas (Koopman et al., 1994) y espermáticas (Martí et al., 2006). El efecto anti-apoptótico de la melatonina parece estar mediado por su unión al receptor MT_1 en espermatozoides humanos (Espino et al., 2011). Por tanto, las diferencias en el efecto de la melatonina sobre la inversión de PS entre épocas observadas en este trabajo, más evidente en la estación de lluvias que en la seca, también podrían ser atribuidas a la concentración de melatonina en plasma seminal que produciría una desensibilización del receptor, en este caso el MT_1.

7. Conclusiones

7.1 Conclusiones

1. En condiciones de fotoperiodo constante, la concentración de melatonina en el plasma seminal de moruecos ubicados en la zona ecuatorial varía entre épocas climáticas, con mayor concentración durante la época de sequía, sin diferencias entre las tres razas estudiadas (Criolla de lana, Romney Marsh y Hampshire)

2. En moruecos ubicados en la zona ecuatorial, en condiciones de fotoperiodo constante, la concentración de melatonina en plasma seminal no correlaciona con la de testosterona ni con la actividad de las enzimas antioxidantes, a diferencia de lo observado en moruecos de latitudes medias con fotoperiodo variable**.**

3. Tanto la concentración de melatonina como de testosterona en plasma seminal es menor en moruecos de las razas estudiadas ubicadas en la zona ecuatorial que la existente en moruecos de las razas estudiadas de latitudes medias.

4. La melatonina presente en el plasma seminal de moruecos ubicados en la zona ecuatorial procede principalmente de la glándula pineal, a diferencia de la de moruecos ubicados en zonas de latitudes medias, que tiene un origen tanto pineal como testicular.

5. Los receptores de melatonina MT_1 y MT_2 están presentes en los espermatozoides de las tres razas estudiadas de la zona ecuatorial, la Criolla de lana, Romney Marsh y Hampshire, con diferencias en su distribución entre razas y entre épocas de lluvia y sequía.

6. Los espermatozoides de la raza Criolla de lana, la ubicada en la zona ecuatorial desde hace más de cinco siglos, presentan menores densidades del receptor MT_2, en comparación con las razas británicas introducidas en el siglo pasado.

7. Al igual que sucede en moruecos ubicados en latitudes medias, en moruecos de la zona ecuatorial, la adición de agentes elevadores del AMPc induce la capacitación espermática en mayor medida que en ausencia de estos compuestos, siendo este efecto más pronunciado en la época de sequía.

8. La adición de melatonina al medio de capacitación previene parcialmente el incremento de espermatozoides capacitados y disminuye el porcentaje de espermatozoides con características apoptóticas, siendo estos efectos más evidentes en la época de lluvia, cuando la concentración de melatonina en plasma seminal es menor.

9. El efecto de la melatonina sobre la capacitación espermática es menor en espermatozoides de la raza Criolla de lana, que son los que presentan una menor densidad del receptor MT_2 en su membrana plasmática.

7.2 Conclusions

1. Under constant photoperiod conditions, the melatonin concentration in the seminal plasma of rams located in the equatorial zone varies between climatic seasons, with a higher concentration during the dry season and without differences between the three studied breeds (Creole, Romney Marsh, and Hampshire)

2. In rams located in the equatorial zone, under constant photoperiod conditions, the melatonin concentration in seminal plasma does not correlate with testosterone concentration or the activity of antioxidant enzymes, unlike that observed in mid-latitude rams under a variable photoperiod.

3. The melatonin and testosterone concentration in seminal plasma are both lower in rams of the three studied breeds located in the equatorial zone than the observed in rams located in mid-latitude zones.

4. The melatonin present in the seminal plasma of rams located in the equatorial zone originates mainly in the pineal gland, unlike rams located in mid-latitude zones, in which has a pineal and testicular origin.

5. The melatonin receptors MT_1 and MT_2 are present in the spermatozoa of the three studied breeds from the equatorial zone, the Creole, Romney Marsh, and Hampshire, with differences in their distribution between breeds and seasons.

6. The spermatozoa from the Creole breed, located in the equatorial region for more than five centuries, present lower MT_2 receptor density than the spermatozoa from British breeds, introduced the last century.

7. In rams located in the equatorial zone, the addition of cAMP-elevating agents induces sperm capacitation at a greater level than in the absence of these compounds, as observed in mid-latitudes rams; this effect being more pronounced in the dry season.

8. The addition of melatonin to the capacitation medium partially prevents the increase in capacitated spermatozoa and decreases the percentage of spermatozoa with apoptotic characteristics, these effects being more evident in the rainy season when melatonin concentration in the seminal plasma is lower.

9. The effect of melatonin on sperm capacitation was lower in spermatozoa from the Creole breed, which are the ones that show the lowest density of the MT_2 receptor in their plasma membrane.

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