TESIS DOCTORAL

Mejora de la eficiencia reproductiva en ovejas Rasa Aragonesa mediante el uso del alelo *FecX*^{R} del gen *BMP15* y de la hormona anti-Mülleriana (AMH) como marcadores de su población folicular

Belén Lahoz Crespo

2013

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Improving reproductive efficiency in Rasa Aragonesa ewes using the FecX^R allele on the BMP15 gene and anti-Müllerian hormone (AMH) as markers of follicular population

Memoria presentada por **Belén Lahoz Crespo** para optar al grado de Doctora en Veterinaria con mención Internacional por la Universidad de Zaragoza

2013

A mi madre

GOBIERNO DE ARAGON
Departamento de Industria e Innovación

CERTIFICACIÓN DE LOS DIRECTORES DE TESIS

cita

D. **José Luis Alabart Álvarez**, Doctor en Ciencias Químicas, y D. **José Folch Pera**, Doctor en Veterinaria, ambos investigadores de la Unidad de Tecnología en Producción Animal del Centro de Investigación y Tecnología Agroalimentaria (CITA) del Gobierno de Aragón,

HACEN CONSTAR

Que Dña. **Belén Lahoz Crespo**, licenciada en Veterinaria, ha realizado bajo nuestra dirección los trabajos correspondientes a su Tesis Doctoral titulada "**Mejora de la eficiencia reproductiva en ovejas Rasa Aragonesa mediante el uso del alelo *FecX*^{R} del gen *BMP15* y de la hormona anti-Mülleriana (AMH) como marcadores de su población folicular**", que se corresponde con el proyecto de Tesis aprobado por la comisión de Doctorado, y que cumple los requisitos exigidos para optar al grado de Doctora por la Universidad de Zaragoza, por lo que autorizan su presentación como compendio de publicaciones y con mención de "Doctor Internacional", para que pueda ser juzgada por el Tribunal correspondiente.

Lo que suscribimos como directores del trabajo en Zaragoza, a uno de julio de dos mil trece.

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Fdo. José Luis Alabart Álvarez

Fdo. José Folch Pera

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Castanet, July 8, 2013

Object: Evaluation of the PhD thesis presented by *Belén Lahoz Crespo*

To whom it may concern,

The present evaluation concerns the thesis entitled "Mejora de la eficiencia reproductiva en ovejas Rasa Aragonesa mediante el uso del alelo FecXR del gen BMP15 y de la hormona anti-Mülleriana (AMH) como marcadores de su población follicular".

This work aims at studying the influence of a natural genetic variant in the BMP15 gene evidenced in the Rasa Aragonesa population of hyperprolific sheep. Prolificacy has an important economic purpose in meat sheep populations, but special management consideration must be made when mutations affecting this trait segregate in a flock.

The present thesis provides some crucial information (mutation effect on prolificacy, lamb parameters, assisted reproduction biotechnologies) and tools (AMH quantification) for a quick increase of prolificacy and optimal fecundity of Rasa Aragonesa flocks. In parallel with these applied research data, this work brings also important data in the basic research on BMP and AMH in the control of ovarian function.

The manuscript is a well-organized document starting with a general introduction of 20 pages replacing the work in the general context of ovine production in Spain and Aragon and describing the importance of reproduction parameters and strategies used for reproductive efficiency. Thereafter, stand the description and the genetic diffusion of the mutation in the BMP15 gene affecting prolificacy of Rasa Argonesa sheep, which is at the centre of this thesis. It is well replaced in the context of other major mutations affecting prolificacy in sheep, with information on folliculogenesis and hypothesis about the ovarian mode of action of these kinds of mutations. Finally, the description of the AMH, closely related to BMP15 ended this

general introduction that brings all the important information needed to understand the thesis work and context.

The thesis objectives are clearly established and their implementation is attested by the publication of 4 peer-reviewed articles and a last one under submission. In each of these papers, the working hypothesis, materials and methods used to answer the question and data analyses were well planned and realized. Three articles (and the one submitted) have been published in journals with an excellent reputation in the "Agricultural, Dairy and Animal Science" category. Even if, one article stands lower in "Agricultural Economics and Policy" category, it proves the excellent valorisation of the work done by *Belén Lahoz Crespo* during this thesis.

A very much appreciated clear and concise summary part with final conclusion and considerations ended this excellent document.

Regarding the overall comments, I conclude on the fact that this thesis brought very interesting and important contribution to production of Rasa Aragonesa sheep and more largely to reproductive physiology of the ovarian function.

Thus, I warmly recommended this thesis manuscript from *Belén Lahoz Crespo* to be favourably evaluated by the commission to obtain "al grado de Doctora en Veterinaria con mención Internacional por la Universidad de Zaragoza"

Dr. Stéphane FABRE
PhD, Accreditation to supervise Research (HDR)

Instituto Nacional de Investigación Agropecuaria
URUGUAY

9 de Julio de 2013

Propósito: Evaluación de la tesis doctoral presentada por Belén Lahoz Crespo.

A quien corresponda,

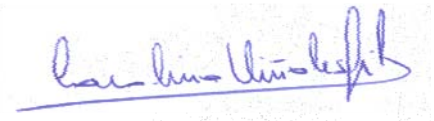
La presente evaluación involucra la tesis titulada " Mejora de la eficiencia reproductiva en ovejas Rasa Aragonesa mediante el uso del alelo *FecX*^{R} del gen BMP15 y de la hormona anti-müleriana (AMH) como marcadores de su población folicular".

La tesis tiene por objetivo estudiar el efecto de una mutación genética sobre parámetros de crecimiento folicular y hormonales, que tienen impacto en la edad a la pubertad, calidad ovocitaria y fertilidad en ovejas Rasa Aragonesa. La temática abordada en la tesis de Beléz Lahoz Crespo es de gran relevancia para la producción ovina a nivel Nacional e Internacional, ya que la búsqueda de marcadores de eficiencia reproductiva es la base del éxito económico de los sistemas productivos.

La seriedad del trabajo queda en evidencia si consideramos que en las 21 páginas dedicadas a los antecedentes, se han citado 330 artículos publicados en revistas arbitradas. La estrategia experimental ha sido adecuada y reconocida por la calidad de los resultados obtenidos, dando origen a 5 trabajos publicados y o enviados a publicar en revistas arbitradas por pares, de buen impacto en el área de Producción animal.

Por este motivo, recomiendo que el trabajo presentado por Belén Lahoz Crespo sea considerado por la comisión evaluadora para obtener el grado de Doctora en Veterinaria con mención Internacional por la Universidad de Zaragoza.

Sin otro particular, saluda a Uds. atentamente.



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La presente Tesis Doctoral se ha realizado en el Centro de Investigación y Tecnología Agroalimentaria del Gobierno de Aragón (CITA-Aragón) en el marco de los siguientes proyectos de investigación:

1) "*Mejora de la prolificidad de las ganaderías de Oviaragón SCL asociando la difusión de la variante génica BMP15 (Alelo ROA) y la mejora poligénica*" (Ministerio de Educación y Ciencia; MEC TRACE PET 2008-0076).

2) "*Marcadores de productividad de la oveja medidos en edad prepúber*" (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria; INIA RTA 2011-00128-C03-01).

Carnes Oviaragón SCL ha co-financiado empresarialmente el proyecto "TRACE PET 2008-0076) a través del convenio de colaboración técnica con el CITA-Aragón titulado "*Homogenización de la oferta comercial de corderos de Rasa Aragonesa con garantías sanitarias a lo largo del año"* (CDTI Ref IDI 20070880). El Institut National de la Recherche Agronomique (Nouzilly-Tours, Francia) ha financiado parte de los análisis hormonales.

La doctoranda ha disfrutado de una Beca PreDoctoral de formación de personal investigador del Subprograma FPI-INIA, en el tema "*Producción in vitro de embriones a partir de oocitos de OPU (Ovum pick-up) en la oveja*", según convocatoria de 22 de febrero de 2008 (BOE nº50, de 27 de febrero de 2008), resolución del 20 de agosto de 2008 (BOE nº266, de 4 de noviembre de 2008) y de un contrato de duración determinada concedido por el CITA-Aragón el 1 de octubre de 2012.

La Tesis es un compendio de las siguientes publicaciones:

1. Lahoz B., Alabart J.L., Jurado J.J., Calvo J.H., Martínez-Royo A., Fantova E., Folch J. 2011. Effect of the *FecX*^{R} polymorphism in the bone morphogenetic protein 15 gene on natural or equine chorionic gonadotropin-induced ovulation rate and litter size in Rasa Aragonesa ewes and implications for on-farm application. Journal of Animal Science 89, 3522–3530.

2. Lahoz B., Folch J., Sánchez P., Alabart J.L. 2013. Caracterización del pico preovulatorio de LH en ovejas Rasa Aragonesa portadoras o no del alelo *FecX*^{R} (ROA®) / Preovulatory LH surge of Rasa Aragonesa ewes carrying or not the *FecX*^{R} allele (ROA®). ITEA-Información Técnica Económica Agraria DOI: http://dx.doi.org/10.12706/itea.2013.025.

3. Lahoz B., Alabart J.L., Folch J., Sánchez P., Echegoyen E., Cocero M.J. 2013. Influence of the *FecX*^{R} allele in heterozygous ewes on follicular population and outcomes of IVP and ET using LOPU-derived oocytes. Reproduction in Domestic Animals 48, 717–723.

4. Lahoz B., Alabart J.L., Cocero M.J., Monniaux D., Echegoyen E., Sánchez P., Folch J. 2013. Anti-Müllerian hormone (AMH) concentration in sheep and its dependence of age and independence of BMP15 genotype: an endocrine predictor to select the best donors for embryo biotechnologies. Theriogenology DOI: 10.1016/j.theriogenology.2013.09.033.

5. Lahoz B., Alabart J.L., Monniaux D., Mermillod P., Folch J. 2012. Anti-Mullerian hormone plasma concentration in prepubertal ewe lambs as a predictor of their fertility at a young age. BMC Veterinary Research 8, 118.

Agradecimientos

Al echar la vista atrás, tomo consciencia del largo camino recorrido para llegar hasta aquí. Un camino con desniveles y llanos, con piedras y hermosas flores, pero sobre todo un camino que siempre he recorrido acompañada. Quiero expresar mi más profunda gratitud a todas estas personas que me han guiado, orientado, avituallado, ayudado y acompañado, sin las cuales esta Tesis no habría sido posible. El camino de quién soy, y la vista puesta hacia dónde voy...

A mis directores de Tesis, José Folch y José Luis Alabart, por haber confiado en mí y haberme brindado esta oportunidad de crecer en lo profesional y en lo personal. Por su generosidad, sus inestimables consejos, por escucharme, y por su vital comprensión en los momentos difíciles.

A Mª Jesús Cocero, por ser mi "tercera directora", por haber estado tan cerca aún estando lejos, siempre dispuesta a echarme una mano, y por haber compartido sin reservas sus extensos conocimientos sobre biología. A las almas de este equipo de Reproducción sin los cuales esta Tesis no sería lo mismo: A Piluca, por su intachable eficiencia en los trabajos de laboratorio y por su incansable predisposición; y a Elías, por su pragmatismo y por amenizar los trabajos con esos cánticos que hacen que las experiencias con las ovejas queden "niqueladas".

A Jorge Calvo, por sus consejos en la parte genética, y por los amenos ratos de microondas compartido. A Pepenacho, por su inestimable ayuda en la parte de FIV, y por las distendidas charlas.

A mis compañeros de fatigas y de tertulias, los becarios-precarios: A Juan Pablo Vico y a Rebeca Calavia, por tantas "mochilas" juntos. A Albert Martínez, René Mozo, Raimón Ripoll, Lucía Dieste, a "las Lauras" (mis compañeras de isla), a Samir y a Elda Dervishi. A los compañeros de desayunos y de Tupper, ¡por compartir comidas y risas! A Chus De Miguel, Pili Muñoz, "las Pilares", "las Saras", a María, a Carlos y a tantos otros. A Nancy d'Cruz, Olga Mitjana, y al resto de compañeros que pasaron por aquí, por las gratas experiencias compartidas. A Fernando Muñoz, a Albi y al resto de compañeros de la casa amarilla.

Al personal laboral del CITA, en especial a Miguel Ángel Navarro, por su gran profesionalidad y predisposición. A Ismael, Fernando, Céspedes, Adrián, Pablo, Oscar, Enrique, Javier, y a todos aquellos que en algún momento han tenido que venir "fuera de horario". Al propio CITA de Aragón, por permitirme disfrutar de sus instalaciones y del preciado tesoro que tiene, que son los animales.

A los chicos de la recepción, por haberme hecho empezar tantos días con una sonrisa: a Albertico, el chico de las natillas, y a Francisco, que se ha ido pero siempre estará ahí.

A toda la gente del INRA, por sus calurosas acogidas.
Je veux remercier à Alice Fatet, Sandrine Freret, et à Juliette Cognié, pour m'avoir facilité tellement les choses, pour les manips des échographies, et pour m'accueillir si chaleureusement. Pour avoir été plus que des collègues !
Je souhaite exprimer ma gratitude à Pascal Mermillod, Danielle Monniaux, Stèphane Fabre et Loys Bodin, pour leurs conseils précieux.
À Natti Poulin, Jean-Luc Touzé, Maite Pellicer-Rubio et Aschraf.
Et à mes amies de place Plum et Guingette, María Catalá, Prya, Hannnah et Stephanie !

A los técnicos y ganaderos de UPRA-Carnes Oviaragón, por su colaboración y por haberme enseñado a ver la problemática del sector desde otro punto de vista.

A Carolina Viñoles, por aceptar dedicar parte de su preciado tiempo a revisar esta Tesis.

Indudablemente a mis padres, porque sin su incansable esfuerzo no hubiera llegado hasta aquí.
Muy especialmente a mi madre, la mujer más bella que conocí. Por su generosidad sin límites y por toda una vida... que no es poco. Porque seguro estaría muy orgullosa de este trabajo.
Con gran cariño a mi padre, por haberme enseñado el valor del esfuerzo y de la constancia, y por sus sabios consejos.

A mi hermana Blanca, por ser mucho más que una hermana, por estar sin llamar y por dar sin esperar recibir. A mi hermano Carlos, porque nos parecemos demasiado, y a mi hermano Miguel Ángel, por su entrega en el diseño gráfico de esta Tesis y en todo lo que hace. A mis otros hermanos, los "políticos": a Gaby por ser un sol, a Nerea, ¡porque algún día haremos el experimento de la judía!, y a Tere. A mis "pequeñajos" del alma: Miguel, Claudia, Pedro, Dani, Paula, Juan y Carla, por su inocente y contagiosa sonrisa, y por enseñarme lecciones que no salen en los libros.

A Carmen, que siempre la llevaré conmigo, por tratarme como a una hija y por su bondad. A Miguel. A mi Tía Pili por su ternura, a Ivi, a Jarilla, a los veterinarios y a todos los que me dejo, pero no olvido.

Y por supuesto a Michel, mi compañero infatigable de viaje y de viajes. Porque esta Tesis es también suya. Por estar siempre ahí, por apoyarme, por animarme, por aguantarme, por entenderme, por hacerme reír y por enseñarme a defender la alegría como una trinchera...

Si puedes mirar, ve

Si puedes ver, repara

(José Saramago)

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INTRODUCCIÓN GENERAL

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AI	Artificial insemination
ALK6	Activin-like kinase 6
AMH	Anti-Müllerian hormone Hormona anti-Mülleriana
BMP15	Bone Morphogenetic Protein 15
BMPR-1B	Bone morphogenetic protein receptor 1B
BMPs	Bone Morphogenetic Proteins
BSA	Bovine Serum Albumin Albúmina sérica bovina
CC	Condición corporal
CL	Corpus luteum Cuerpo lúteo
COCs	Cumulus-oocyte complexes Complejos cumulus-oocito
eCG	Equine chorionic gonadotropin Gonadotropina coriónica equina
EDTA	Etilendiaminotetraacétic acid Ácido etilendiaminotetraacético
ELISA	Enzyme-Linked ImmunoSorbent Assay
ET	Embryo transfer
FF	Follicular fluid Fluido folicular
FGA	Fluorogestone acetate Acetato de fluorogestona
FIV	Fertilización In Vitro
FSH	Follicle stimulating hormone Hormona foliculoestimulante
GDF9	Growth differentiation factor 9
GnRH	Gonadotropin-releasing hormone Hormona liberadora de gonadotropinas
h	Hours / horas
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IA	Inseminación artificial
IGP	Indicación Geográfica Protegida
im	Intramuscularly / intramuscular
IU	International Units
IVF	In Vitro Fertilization
IVM	In Vitro Maturation
IVP	In Vitro Production
kg	Kilograms / kilogramos
LSMEANS	Least squares means / Medias corregidas por mínimos cuadrados
LH	Luteinizing hormone Hormona luteinizante
LOPU	Laparoscopic Ovum Pick-Up Punción laparoscópica de oocitos
LS	Litter size
MFS	Mean Follicle Size Tamaño folicular medio
MIS	Müllerian inhibiting substance
MIV	Maduración In Vitro
MOET	Multiple ovulation and embryo transfer
OPU	Ovum Pick-Up Punción de oocitos
OR	Ovulation rate
PAC	Política Agraria Común
PAG	Plasmatic pregnancy-associated glycoprotein Proteína plasmática asociada a la gestación
PCR	Polymerase Chain Reaction Reacción en cadena de la polimerasa
PFMO	Partial Failure of Multiple Ovulations
PIV	Producción In Vitro
PMSG	Pregnant mare serum gonadotropin Gonadotropina sérica de yegua preñada
R	*FecX*^{R} hemicigous ram Macho hemicigoto para *FecX*^{R}
R+	*FecX*^{R} heterocigous ewe Hembra heterocigota para *FecX*^{R}
R+0	R+ ewe eCG untreated Hembra R+ sin tratar con eCG
R+240	R+ ewe treated with 240 IU eCG Hembra R+ tratada con 240 UI eCG
R+480	R+ ewe treated with 480 IU eCG Hembra R+ tratada con 480 UI eCG
ROA	"Rasa OviAragón"
ROC	Receiving-Operating Characteristic
RR	*FecX*^{R} homocigous ewe Hembra homocigota para *FecX*^{R}
RR	Recovery rate Tasa de recuperación
SD	Standard Deviation Desviación Estándar
SE	Standard Error / Error estándar
SOC	Heat-inactivated estrous sheep serum Suero de oveja en celo
TA	Ternasco de Aragón
TE	Transferencia de embriones
TGF	Transforming Growth Factor
TO	Tasa de ovulación
UE	Unión Europea
UI	Unidades internacionales
UTH	Unidad de Trabajo Hombre
VG	Valor genético
vs.	Versus
X g	Gravity force
++	Wild-type ewe Hembra portadora del alelo salvaje
++0	++ ewe eCG untreated Hembra ++ sin tratar con eCG
++480	++ ewe treated with 480 IU eCG Hembra ++ tratada con 480 UI eCG

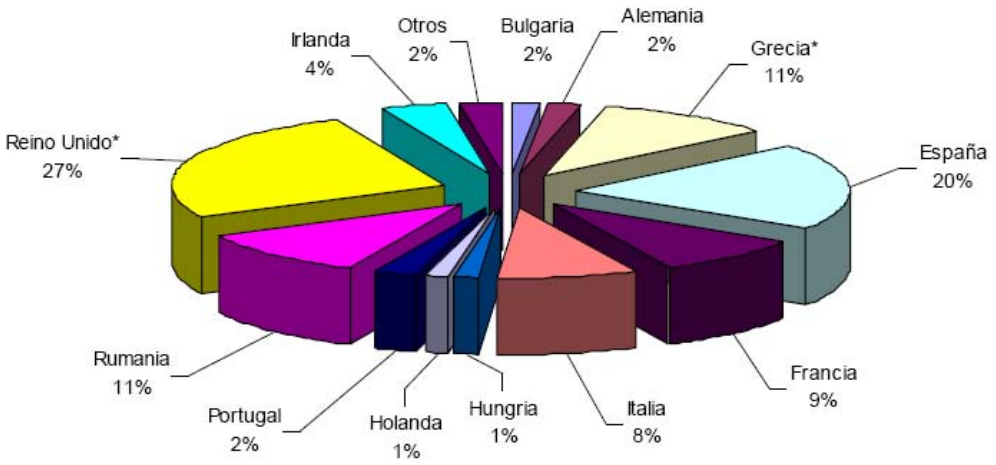
INTRODUCCIÓN GENERAL

1.

Introducción General

1.1. El sector ovino en España y en Aragón

España cuenta con el 20% del total de la cabaña ovina comunitaria, superado únicamente por Reino Unido (*Figura 1*), lo que le sitúa como el segundo país productor de ovino de la UE-27. A 1 de enero de 2013, el censo ovino español se situaba en 16.609.069 cabezas (MAGRAMA, 2013). El 11,4% de este censo se localiza en la Comunidad Autónoma de Aragón (1.906.655 cabezas), donde constituye una actividad de gran importancia para su economía agraria. El sector ovino en Aragón cuenta con una gran tradición histórica, que se remonta al menos hasta el año 4.000 a.C., tal y como pone de manifiesto la existencia de pinturas rupestres en la Comunidad (Sierra, 1992). Su relevancia va más allá de la actividad económica, siendo destacable su repercusión sobre aspectos sociales y medioambientales. Sus características de rusticidad han sido clave para su adaptación tanto a zonas áridas como a zonas de montaña, contribuyendo históricamente al asentamiento de la población rural en zonas desfavorecidas que ofrecen escasas alternativas laborales. En la actualidad es una actividad de vital importancia para evitar la despoblación del medio rural y mantener vivo el patrimonio.



*Fuente: EUROSTAT. Elaboración SGPG. *Dato de 2011*

Figura 1. **Distribución del censo ovino por países en la UE-27 en 2012 (MAGRAMA, 2013)**

La Rasa Aragonesa es la principal raza ovina de la Comunidad Autónoma de Aragón, siendo la segunda en importancia a nivel nacional después de la raza Merina (*Figura 2*). En la actualidad cuenta con 456.515 ovejas (MAGRAMA, 2011) inscritas en el Libro Genealógico, que es gestionado por dos Asociaciones de Productores de Rasa Aragonesa reconocidas oficialmente por el Ministerio de Agricultura, Alimentación y Medio Ambiente: UPRA–Grupo Pastores (Unión de productores de raza Rasa Aragonesa) y ANGRA (Asociación Nacional

de criadores de Rasa Aragonesa). Es una raza autóctona mediterránea de aptitud cárnica orientada a la producción de cordero ligero comercializado bajo la indicación geográfica protegida (IGP) “Ternasco de Aragón” (BOA, 2009).

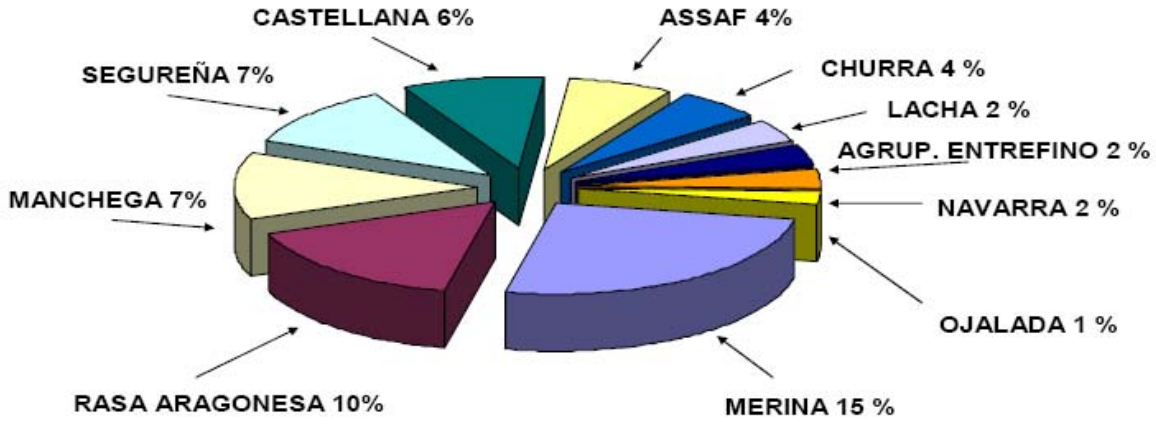


Figura 2. **Distribución del censo ovino por razas en España en 2011 (MARM, 2009; MAGRAMA, 2011)**

En los últimos años se ha registrado una disminución constante del censo de ganaderías y de ovejas tanto en Europa como en España. Esta disminución es una continuación de la inercia a la baja de los últimos diez años, habiéndose registrado a nivel nacional un descenso de unos 3 millones de cabezas (alrededor de un 15%) y de unas 13.500 explotaciones entre 2008 y 2011 (*Figura 3*). En Aragón, donde se concentra la mayor parte de la raza Rasa Aragonesa, se observa la misma tendencia. Si comparamos el número de cabezas actual con los 2,8 millones de ovejas del año 1999, se observa que el censo ovino aragonés se ha reducido prácticamente a la mitad y se han perdido más de 2.000 explotaciones ganaderas, permaneciendo en la actualidad 3.900. Estos descensos son preocupantes, particularmente si tenemos en cuenta que en Aragón la actividad ganadera predomina sobre la agricola en la Producción Final Agraria (Gobierno de Aragón, 2013).

Este descenso continuado demuestra las dificultades económicas y sociales por las que está atravesando el sector. En España, el valor de la producción se ha estancado en los últimos años, mientras que los costes han crecido casi un 50% en una década, principalmente por el incremento del precio de las materias primas utilizadas para la alimentación y el aumento de los costes generales, como gasóleo y electricidad. Las importaciones de cordero extranjero con menores costes de producción, el bajo consumo de carne por habitante agravado por la crisis económica global, la falta de relevo generacional, y los continuos cambios de la Política Agraria Común (PAC) no han hecho más que agravar el problema.

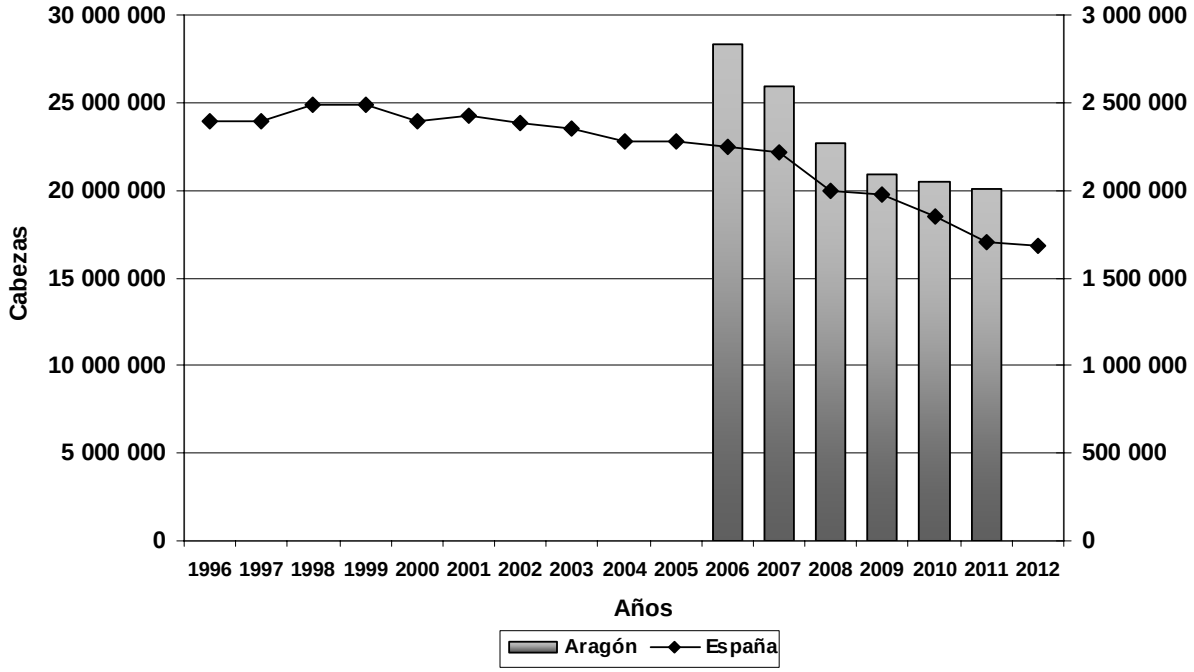


Figura 3. **Evolución de los censos de ovino en España entre los años 1996-2012 (línea de puntos) y en Aragón entre los años 2006-2011 (barras). Adaptado de MAGRAMA (2013)**

Todos estos factores han llevado irremediablemente a una pérdida de rentabilidad continuada. En las explotaciones de ovino de carne de Aragón, en sólo seis años (periodo 2002-2007) los resultados económicos disminuyeron un 32,9% por oveja (Pardos y Fantova, 2009). Si tenemos en cuenta que este estudio se llevó a cabo con las explotaciones más tecnificadas y productivas, y que el panorama de los años posteriores no ha sido mejor, la pérdida de rentabilidad de las explotaciones ovinas en Aragón es un problema alarmante. Si bien no existe una solución única para el sector ovino en España y Aragón, debido a la gran diversidad de sistemas de producción existentes que van desde los más extensivos hasta los netamente intensivos, la mejora de la eficiencia reproductiva permitiría en la mayoría de los casos mejorar la rentabilidad de las explotaciones, permitiendo bien producir lo mismo con menos animales, o bien aumentar la producción sin necesidad de incrementar su censo.

1.2. Eficiencia reproductiva en ovino

1.2.1. Importancia de la eficiencia reproductiva

La mayor parte de las explotaciones que están manteniendo su actividad y que tienen más posibilidades de permanecer son las más eficientes desde el punto de vista de la productividad, lo que les permite asegurar un alto margen bruto por oveja. Esta misma tendencia es la que se observa en los rebaños de ovino de carne en Francia (Benoit y

Laignel, 2011). La eficiencia en la producción es fundamental para mejorar los resultados económicos y optimizar la mano de obra, pero además limita el impacto ambiental en términos de consumo de energía y de emisión de gases de efecto invernadero por kilogramo de carne producida (Benoit y Laignel, 2011). En el caso de la Rasa Aragonesa, los resultados económicos, y por tanto la viabilidad de las explotaciones, dependen principalmente del número de corderos vendidos por oveja (Pardos et al., 2008, 2012). Una función de beneficio elaborada para determinar la influencia de las diferentes variables sobre el Margen Bruto por UTH, demostró que la prolificidad (corderos/parto) y la fertilidad (partos/oveja y año) eran las variables con mayor influencia sobre la rentabilidad económica de las explotaciones, de manera que por cada 1% que aumentaban cualquiera de ellas, aumentaba un 4,3% el Margen Bruto por UTH (Pardos et al., 2007a, 2007b; *Figura 4*). Por tanto, dado su gran impacto económico, muchos ganaderos están interesados en aumentar la fertilidad y la prolificidad de sus ovejas.

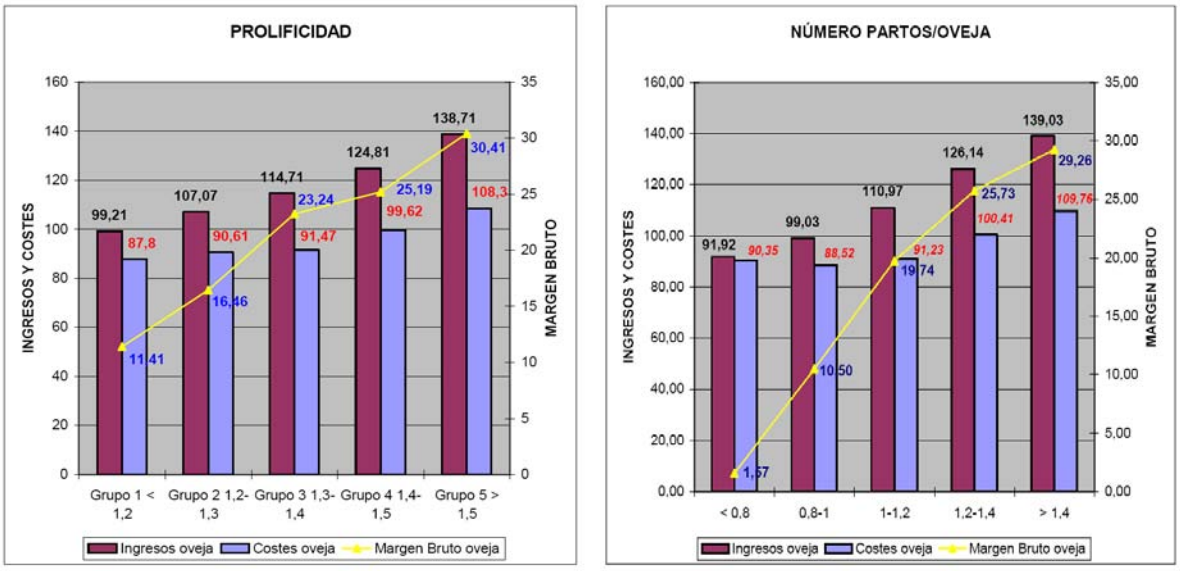


Figura 4. **Margen bruto, ingresos y costes por oveja en función de la prolificidad y del número de partos por oveja y año (Pardos et al., 2007b)**

El número de partos de una oveja durante toda su vida (vida productiva) es junto con la prolificidad y la fertilidad el parámetro reproductivo con mayor influencia sobre la rentabilidad económica de las explotaciones. Además de numerosos factores como la ordenación de cubriciones, el fotoperiodo, la alimentación o la edad de la oveja (Gordon, 1999), uno de los factores que más influye en la vida productiva de la oveja es la edad al primer parto. Conseguir que las corderas se queden gestantes dentro de su primer año de edad reduce el coste de la reposición, aumenta la vida productiva de las ovejas en el rebaño, reduce el

intervalo entre generaciones (Fogarty et al., 2007), y reduce los riesgos de sacrificio de los animales menos productivos (Kern et al., 2010). En Rasa Aragonesa se describió ya en 1989 que adelantar la edad al primer parto daba lugar a una mayor productividad de la oveja a lo largo de su vida (Gabiña, 1989) y se ha vuelto a demostrar en un trabajo reciente de Jurado y Jiménez (2013).

Sin embargo, en la Rasa Aragonesa, a pesar de que las hembras alcanzan un peso vivo adecuado para parir a los 12-13 meses, la edad media al primer parto es de 19 meses (Gabiña, 1989), lo que da lugar a largos periodos improductivos y consecuentes pérdidas económicas. Se calcula que el coste de mantenimiento de una oveja Rasa Aragonesa es de 80,15 €/año (dato de 2007; Pardos y Fantova, 2009). Estudios muy recientes sobre un gran número de partos en Rasa Aragonesa (Jurado y Jiménez, 2013) han demostrado que adelantar el primer parto antes de los 15 meses no afecta negativamente a la vida productiva de la ovejas, sino que supone un incremento significativo del número de partos durante la vida del animal, y por lo tanto una mayor duración de su vida productiva.

1.2.2. Factores que afectan a la eficiencia reproductiva

La prolificidad y fertilidad son, a nivel práctico, las principales medidas del rendimiento reproductivo en ovino, y se ven afectadas tanto por factores genéticos como por una gran variedad de factores ambientales.

La **tasa de ovulación** (TO) es el número de oocitos maduros liberados en un determinado ciclo, y se estima contabilizando el número de cuerpos lúteos en el ovario. Variaciones en la tasa de ovulación se traducen, una vez descontadas las pérdidas embrionarias, en variaciones en el mismo sentido en la prolificidad. La **prolificidad** es el número de corderos nacidos en relación al número de partos. Entre los factores ambientales que afectan a la tasa de ovulación y a la prolificidad cabe destacar el estado nutricional, el balance energético de la dieta, el fotoperiodo y la estacionalidad, factores socio-sexuales entre los que se incluye el “efecto macho”, la temperatura y la humedad relativa, y el estrés (Gordon, 1999; Scaramuzzi y Martin, 2008). Los efectos nutricionales tienen una función bidireccional: la suplementación nutricional puede estimular la foliculogénesis (Webb et al., 2004; Scaramuzzi et al., 2006) y por tanto incrementar la prolificidad, mientras que una nutrición insuficiente inhibe la liberación de GnRH y puede conducir a la falta de ovulación. Además influyen sensiblemente la edad del animal y el manejo de cada explotación con sus diferentes sistemas de ordenación de cubriciones. En cuanto al control genético de la tasa de ovulación, puede estar regulada por un efecto poligénico (efecto de varios genes en conjunto) y/o por la acción de genes mayores que tienen un gran efecto sobre el carácter prolificidad, como veremos más adelante. La herencia mixta de la prolificidad como la suma la herencia poligénica más la de un gen mayor, ha sido demostrada por diversos autores (Bradford et al., 1986; Bodin et al., 2002).

La aparición de la **pubertad** va a influir sobre la edad al primer parto, lo que va a tener un impacto sobre la vida productiva del animal. La pubertad es el proceso de adquisición de la competencia reproductiva, y en la hembra, su inicio depende principalmente de la capacidad de respuesta de las neuronas del hipotálamo al feedback positivo del estradiol para producir GnRH que cause la ovulación. La pubertad en la hembra se puede definir de muchas maneras: momento de aparición del primer estro, edad de la primera ovulación, o edad a la que es capaz de quedar gestante y llevar a término la gestación. En la presente Tesis, cuando hablemos de pubertad generalmente nos referiremos a esta última definición, ya que nos parece la más adecuada teniendo en cuenta los objetivos a nivel de explotación. Al hablar de **fertilidad** del rebaño, nos referiremos a número de partos en relación al número de ovejas puestas a cubrir. Al igual que ocurre con la tasa de ovulación y la prolificidad, existen múltiples factores que determinan la aparición de la pubertad. Existen los factores medioambientales (fotoperiodo y señales socio-sexuales) o los metabólicos endógenos (balance energético), que pueden ser manipulados por el ganadero, mientras que los factores genéticos son intrínsecos al animal (Valasi et al., 2012).

En este sentido, en la presente Tesis se consideró de interés tener en cuenta el potencial reproductivo de la cordera al nacimiento. Las ovejas, como otros mamíferos, nacen con un número limitado y muy variable de oocitos en el ovario que va disminuyendo drásticamente con la edad. Al nacer, estos ooocitos se encuentran en su mayoría contenidos en folículos primordiales en reposo o al inicio de su crecimiento, y constituyen la **reserva folicular** ovárica. Tanto los aspectos genéticos como las variaciones en la alimentación de la madre durante la gestación afectan al desarrollo folicular en el estadio fetal y subsecuentemente a su reserva folicular al nacimiento (McNatty et al., 1995; Borwick et al., 1997; Da Silva et al., 2002; Grazul-Bilska et al., 2009; Kotsampasi et al., 2009). Estudios recientes en vacuno sugieren que la población folicular presente en los ovarios de las terneras parece tener un gran impacto en su fertilidad en la edad adulta (Ireland et al., 2011).

1.2.3. Estrategias de mejora de la eficiencia reproductiva

Teniendo en cuenta la gran variedad de factores que afectan a la prolificidad y a la fertilidad, es evidente que existen múltiples alternativas de mejora, tanto nutricionales y de manejo, como genéticas.

El uso de **tratamientos hormonales** de sincronización e inducción del celo, principalmente de esponjas intravaginales de FGA (Acetato de Fluorogestona) y eCG, es una práctica muy extendida que ofrece buenos resultados productivos a corto plazo. En Rasa Aragonesa, es frecuente utilizarlos en ovejas adultas para mejorar la fertilidad y la prolificidad durante la época de anestro estacional (finales de invierno y principios de verano), así como para realizar la IA a tiempo fijo durante todo el año, tal y como lleva a cabo UPRA-Grupo Pastores dentro de su programa de mejora genética. Se pueden usar también para

adelantar la pubertad en las corderas. Otros tratamientos utilizados son los implantes de melatonina o las prostaglandinas (revisado por Abecia et al., 2012). Sin embargo, la utilización de estos productos tiene varios inconvenientes. Los tratamientos hormonales dejan residuos en carne y leche, y aunque no supone un riesgo para la salud, no beneficia la imagen del producto por parte de un consumidor cada día más exigente y más sensibilizado, que demanda productos que no impliquen ningún riesgo para la salud humana, y que estén producidos respetando el medio ambiente y el bienestar animal (Scaramuzzi y Martin, 2008). Los tratamientos hormonales se encuentran actualmente en el punto de mira de la UE con las nuevas políticas de limitación creciente de productos veterinarios en producción animal, y presumiblemente a medio plazo se tenderá a una regulación estricta o incluso a su prohibición, como ya ocurre en las ganaderías ecológicas. Los corderos que han recibido este tipo de tratamientos no pueden comercializarse bajo el etiquetado de "producto ecológico", lo que también les puede restar valor añadido. Además, la eCG tiene un origen animal, existiendo riesgo de transmisión de enfermedades interespecíficas, y su uso reiterado induce la aparición de anticuerpos en las ovejas, lo que afecta negativamente a los resultados reproductivos (Bodin et al., 1997). Por último, la aplicación de los tratamientos implica manejos adicionales del rebaño que pueden suponer una complicación y tienen un cierto coste económico.

Por ello, ante un posible marco de prohibición, es importante ir desarrollando nuevos métodos libres de hormonas que puedan suplir en un futuro la utilización de los actuales tratamientos. En los últimos años, se ha empezado a potenciar el uso de estrategias libres de hormonas que permitan al ganadero mejorar la eficiencia reproductiva de una manera respetuosa con el medio ambiente y con el consumidor. Es el concepto de producción animal "limpia, verde y ética" (**"Clean, green and ethical"**) y se basa principalmente en tres estrategias: utilización de señales socio-sexuales para la inducción y sincronización de celos ("efecto macho"), suplementación nutricional durante periodos de tiempo muy críticos del proceso reproductivo ("focus feeding"), y gestión, nutrición y selección genética por comportamiento para maximizar la supervivencia de las crías (Martin y Kadokawa, 2006; Scaramuzzi y Martin, 2008). Se trata en todos los casos de estrategias interesantes para mejorar la eficiencia reproductiva, pero dadas las condiciones estructurales, sociales y de mercado de la producción ovina en España y en Aragón, no pueden ser utilizadas todavía de forma exclusiva sino complementaria.

Una buena estrategia de mejora es la **selección genética** de caracteres reproductivos, como la fertilidad y la prolificidad. Sin embrago, tiene el inconveniente de que dichos caracteres presentan una heredabilidad muy baja, generalmente entre 0,05 y 0,15 (Notter 2008, 2012), lo que conlleva lentitud en el proceso de selección poligénica. La aplicación de biotecnologías reproductivas, como la IA, MOET ó LOPU-PIV, puede contribuir a acelerar el progreso genético aumentando la intensidad de selección y reduciendo el intervalo intergeneracional, pero son técnicas que requieren, unas más que otras, una gran

tecnificación. Una alternativa más rápida para mejorar la prolificidad es el **cruce con razas prolíficas**, como la Romanov, estrategia que ya se inició en los años 80 en Aragón y que dio lugar a la creación de la raza sintética Salz (Rasa Aragonesa X Romanov). Sin embargo, en la Rasa Aragonesa estas prácticas van en contra de los intereses comerciales de los productores, impidiéndoles comercializar un producto diferenciado y con valor añadido, como es el "Ternasco de Aragón" (BOA, 2009), que exclusivamente contempla las razas autóctonas Rasa Aragonesa, Roya Bilbilitana y Ojinegra de Teruel.

Por estas razones, en los últimos años se están llevando a cabo en todo el mundo numerosos trabajos sobre producción animal centrados en la búsqueda de marcadores genéticos y endocrinos que permitan una selección de los mejores animales de una manera mucho más rápida que la selección clásica. En las condiciones de explotación de la Rasa Aragonesa, dichos marcadores, bien de prolificidad o de fertilidad, serían particularmente interesantes si se pudieran medir antes de los 3 meses de edad, para permitir la elección de las corderas de reposición antes de que alcancen la edad a la que se envían a matadero para comercializarse según las condiciones de la IGP "Ternasco de Aragón" (BOA, 2009).

1.3. Mejora de la prolificidad mediante Genes mayores

1.3.1. Alelo FecX^R del gen BMP15 en Rasa Aragonesa

1.3.1.1. Descubrimiento del alelo *FecX^R*

Dada la importancia económica demostrada de la prolificidad, en el año 1994 se inició un programa de selección poligénica por este carácter en la raza Rasa Aragonesa que continúa en la actualidad. El programa lo desarrolla UPRA-Carnes Oviaragón SCL, en colaboración con el Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón, el Centro de Mejora Ganadera (ATPSYRA) del Gobierno de Aragón, y el Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) de Madrid. En el marco del Programa de selección se observó que existían familias con un elevado valor genético en prolificidad, significativamente diferente al valor medio de la población, que no se podía explicar por la herencia poligénica clásica. Los estudios de segregación llevados a cabo en el CITA de Aragón en colaboración con el INIA demostraron la existencia de un gen mayor o un QTL de gran efecto sobre el carácter prolificidad (Jurado y Calvo, 2007). Dichas sospechas condujeron en 2007 al descubrimiento de un nuevo polimorfismo natural en el gen *BMP15*, al que se le denominó alelo *FecX^R* ó ROA® según su denominación comercial (Rasa Oviaragón), que producía un aumento de la prolificidad en las ovejas heterocigotas (R+) y esterilidad en las hembras homocigotas (RR; Martínez-Royo et al., 2008; Jurado et al., 2008; Martíez-Royo, 2011). Desde su descubrimiento en 2007, fue registrado en la Oficina Española de Patentes y Marcas como "Procedimiento de mejora de la productividad en ganado ovino" (ES2338960; Martínez-Royo et al., 2010), cuyos titulares son Carnes

Oviaragón SCL, CITA e INIA. El mismo polimorfismo se encontró también en un rebaño perteneciente a ANGRA, la otra Asociación de Productores gestora de la raza (Monteagudo et al., 2009).

Dicho polimorfismo constituía la sexta mutación descrita en el gen *BMP15* a nivel internacional, como veremos a continuación.

1.3.1.2. Plan de divulgación del alelo *FecX^R*

A diferencia de otras mutaciones en genes de prolificidad, una de las ventajas de las mutaciones en el gen *BMP15* es que se encuentran ligadas al cromosoma X, lo que permite una rápida divulgación mediante IA si se utilizan machos hemicigotos (R; Folch et al., 2010). El inconveniente más importante es la esterilidad de las ovejas que portan el polimorfismo *FecX^R* en homocigosis, de forma que si se cruzan machos hemicigotos con ovejas heterocigotas, el 50% de las hijas serán estériles (*Figura 5*). Por lo tanto, es fundamental hacer una buena programación de los cruzamientos en las ganaderías.

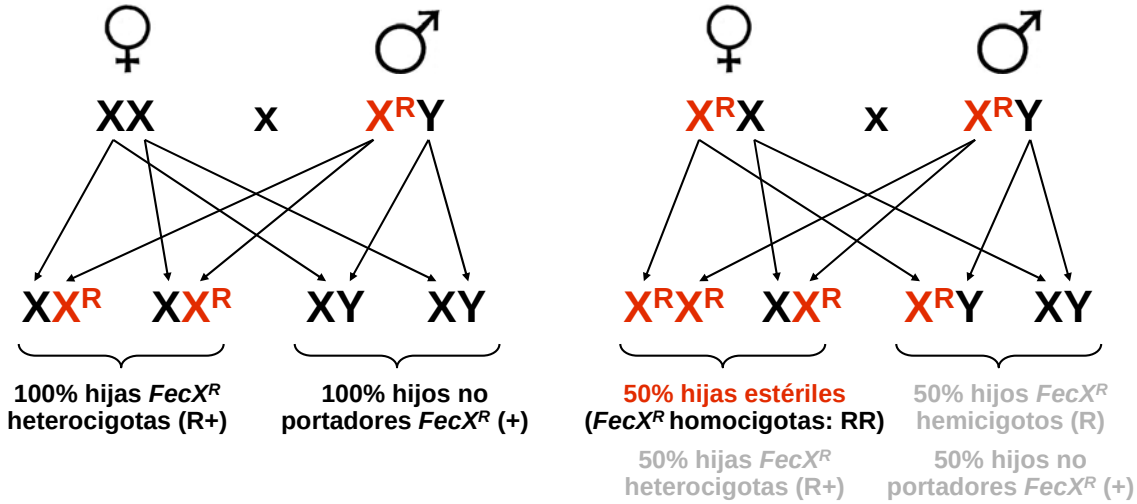


Figura 5. **Descendencia de los cruzamientos de machos hemicigotos para el alelo *FecX^R* (X^{R}Y) con hembras no portadoras (XX) o portadoras heterocigotas (X^{R}X)**

Desde su descubrimiento ha habido un creciente interés por parte de los ganaderos, no sólo de Rasa Aragonesa sino también de otras razas, en la introducción de esta nueva variante génica. Estudios recientes han demostrado su repercusión económica en las ganaderías de Rasa Aragonesa como herramienta para aumentar la prolificidad. Así, las explotaciones con más de un 5% de ovejas R+ presentan mayores ingresos por oveja que las explotaciones que sólo siguen el programa de selección poligénica o que no hacen ningún tipo de selección (5 y 21 €, respectivamente), aun teniendo mayores costes de alimentación y de manejo (Pardos et al., 2010).

A pesar de las ventajas que ofrece el alelo *FecX*^{R}, UPRA-Grupo Pastores consideró que, antes de realizar su divulgación a gran escala, era preciso conocer las características productivas y reproductivas de las ovejas Rasa Aragonesa portadoras heterocigotas y asegurar que este alelo no fuera asociado a caracteres no deseables. Para realizar estos estudios, el Ministerio de Ciencia e Innovación financió un Proyecto de colaboración entre UPRA-Grupo Pastores y el CITA de Aragón titulado "Mejora de la prolificidad de las ganaderías de Oviaragón SCL asociando la difusión de la variante génica *BMP15* (Alelo ROA) y la mejora poligénica". La finalidad última del proyecto era establecer las bases de utilización del alelo *FecX*^{R} en las ganaderías.

Para ello, en primer lugar era necesario medir su efecto sobre la tasa de ovulación y la prolificidad, y compararlo con los efectos observados en otras razas con mutaciones similares, así como evaluar sus posibles efectos adversos sobre otros parámetros. Los ganaderos utilizan de forma rutinaria tratamientos hormonales consistentes en esponjas de FGA y eCG, tanto para la inducción del celo en estación desfavorable, como para realizar IA a tiempo fijo a lo largo de todo el año. Por lo tanto, era preciso además conocer si en las ovejas heterocigotas se producía un efecto aditivo o sinérgico de la aplicación de la eCG y la presencia del alelo *FecX*^{R} sobre la tasa de ovulación y prolificidad, y en tal caso, cuantificarlo para evitar que se produjese un número indeseado de partos múltiples. En consecuencia, estos objetivos se abordaron en los primeros estudios de la presente Tesis Doctoral, y dieron lugar a la primera publicación.

Por otro lado, con vistas a la aplicación de la IA a tiempo fijo en las ganaderías, era preciso conocer si la presencia del alelo *FecX*^{R} modificaba el pico preovulatorio de LH, teniendo en cuenta de nuevo la posible interacción del genotipo con la aplicación de los tratamientos hormonales, lo que dio lugar a la segunda publicación de la Tesis.

1.3.2. Mutaciones que afectan a la tasa de ovulación y a la prolificidad en ovino

En la especie ovina se han descrito en diferentes razas diversas mutaciones en genes mayores que dan lugar a fenotipos con mayor tasa de ovulación y prolificidad (*Tabla 1*). La mayoría de ellas afectan a los productos de los genes y receptores asociados con la superfamilia del TGF-ß (Transforming growth factor-ß; Davis, 2005), localizándose principalmente en tres genes: *BMPR1B* (bone morphogenetic protein receptor tipo 1B o activin-like kinase 6; ALK6), *GDF-9* (growth differentiation factor 9), y *BMP15* (bone morphogenetic protein 15, también denominado *GDF9b*).

La primera de estas mutaciones que fue descubierta se localiza en el gen ***BMPR1B*** y se conoce como Booroola ó *FecB*^{B} (Piper y Bindon, 1980; Davis et al., 1982). Fue descrita en los años 80 en ovejas Merinas de Australia. Desde entonces y hasta hoy se ha realizado su introgresión en numerosas razas de todo el mundo para mejorar su prolificidad.

Actualmente, además de estar presente en la raza Merina, se ha encontrado en la raza india Garole, la Javanese de Indonesia, y en las razas chinas Hu y Han de cola corta (Davis et al., 2002, 2006a; Chu et al., 2007). En España, se realizó en los años 90 un intento de introducir el gen Booroola en la raza Segureña, pero el programa no llegó a concluirse con éxito (Serradilla, comunicación personal).

En el gen ***GDF9*** se han descrito tres mutaciones prolíficas. Las dos primeras son la mutación *FecG*^{H} en las razas Belclare y Cambridge de Irlanda y Reino Unido (Hanrahan et al., 2004) y la mutación *FecG*^{T} en la raza Thoka Icelandic (Nicol et al., 2009), y ambas producen esterilidad en homocigosis. La tercera mutación de este gen (*FecG*^{E}) ha sido descrita recientemente en la raza brasileña Santa Inés, y sorprendentemente no produce esterilidad en homocigosis sino que da lugar a un fenotipo hiperprolífico (Silva et al., 2011).

En el gen ***BMP15***, localizado en el cromosoma X, se encuentran las mutaciones *FecX*^{I} y *FecX*^{H} en la raza Romney de Nueva Zelanda (Galway et al., 2000), *FecX*^{B} y *FecX*^{G} en las razas Belclare y Cambridge (Hanrahan et al., 2004), *FecX*^{L} en la raza francesa Lacaune (Bodin et al., 2007) y *FecX*^{R} en la Rasa Aragonesa (Martínez-Royo et al., 2008). Todos estos polimorfismos presentan el mismo fenotipo: las ovejas heterocigotas tienen mayor tasa de ovulación y las mutantes homocigotas son estériles debido a un bloqueo del desarrollo folicular ovárico a nivel del estadio primario. Muy recientemente, se acaban de describir dos nuevos polimorfismos en este gen: El alelo *FecX*^{Gr} en la raza francesa Grivette y el alelo *FecX*^{O} en la raza polaca Olkuska (Demars et al., 2013). En contraposición con los fenotipos encontrados hasta 2008 en *BMP15*, las hembras homocigotas para *FecX*^{Gr} y *FecX*^{O} no son estériles sino hiperprolíficas. Estos resultados recientes inducen a considerar la existencia de una compleja acción conjunta de *BMP15* y *GDF9* en el control de la tasa de ovulación.

Además de estos fenotipos prolíficos, existen otros de los que todavía no se conoce toda la información referente a su localización en el genoma o a sus efectos fenotípicos. El alelo *FecL*^{L} (Lacaune) en la raza Lacaune se ha asociado a incrementos en TO y prolificidad tanto en heterocigosis como en homocigosis. Se ha localizado en el cromosoma 11, pero todavía no se ha podido identificar el gen responsable (Bodin et al., 2002), aunque estudios recientes proponen el gen *B4G4LNT2* como candidato (Mansanet et al., 2012). En la raza Coopworth, el alelo Woodlands (*FecX2*^{W}) se localiza en el cromosoma X, pero no se conoce el gen responsable. También da lugar a fenotipos prolíficos, tanto en heterocigosis como en homocigosis, pero posee ciertas particularidades en cuanto al modo de herencia (Davis et al., 2001). También hay evidencias de genes mayores en otras razas, como en las razas Belle-Ile (Malher y Le Chere, 1998), Wishart (Davis et al., 2006b), o Davisdale (Juengel et al., 2011).

Tabla 1. **Fenotipos descritos en ganado ovino con sus respectivos efectos sobre la tasa de ovulación y la prolificidad**

Gene	Chr	Name	Allele	Effects on OR and LS[1]	Breed	Reference
BMP15	X	Inverdale	***FecX^{I}***	**I+**: +1.0 (OR); +0.6 (LS) **II**: Infertile (streak ovaries)	Romney	Galloway et al. (2000)
		Hanna	***FecX^{H}***	**H+**: +1.0 (OR); +0.6 (LS) **HH**: Infertile (streak ovaries)	Romney	Galloway et al. (2000)
		Belclare	***FecX^{B}***	**B+**: +1.0 (OR) **BB**: Infertile (streak ovaries)	Belclare	Hanrahan et al. (2004)
		Galway	***FecX^{G}***	**G+**: +0.7 (OR)[2] **GG**: Infertile (streak ovaries)	Belclare, Cambridge	Hanrahan et al. (2004)
		Lac X-mutated	***FecX^{L}***	**L+**: +1.5 (OR) **LL**: Infertile (streak ovaries)	Lacaune	Bodin et al. (2007)
		ROA	***FecX^{R}***	**R+**: +0.32 (LS) **RR**: Infertile (streak ovaries)	Rasa Aragonesa	Martínez-Royo et al. (2008)
		Grivette	***FecX^{Gr}***	**Gr+**: +0.41 (OR); +0.10 (LS) **GrGr**: +2.05 (OR); +0.67 (LS)	Grivette	Demars et al. (2013)
		Olkuska	***FecX^{O}***	**O+**: +0.50 (OR); +0.62 (LS) **OO**: +1.76 (OR); +1.21 (LS)	Olkuska	Demars et al. (2013)
BMPR-1B	6	Booroola	***FecB^{B}***	**B+**: +1.5(OR); +1.0 (LS)[3] **BB**: +3.0 (OR); +1.5 (LS)	Merino, Garole, Javanese, Hu, Han	Mulsant et al. (2001) Souza et al. (2001) Wilson et al. (2001)
GDF9	5	High Fertility	***FecG^{H}***	**H+**: +1.8 (OR) **HH**: Infertile (streak ovaries)	Belclare, Cambridge	Hanrahan et al. (2004)
		Thoka	***FecG^{T}***	**I+**: +1.2 (OR); +0.7 (LS) **II**: Evidence of infertility	Icelandic	Nicol et al. (2009)
		Embrapa	***FecG^{E}***	**E+**: +0.12 (OR); +0.31(LS) **EE**: +1.0 (OR); +0.65 (LS)	Santa Inês	Silva et al. (2011)
?[4]	11	Lacaune	***FecL^{L}***	**L+**: +1.5 (OR) **LL**: +3.0 (OR)	Lacaune	Bodin et al. (2002)
?	X	Woodlands	***FecX2^{W}***	**W+**: +0.4 (OR); +0.25 (LS) **WW**: ≥W+ (OR&LS)	Coopworth	Davis et al. (2001)

[1] Increases on ovulation rate (OR) and litter size (LS) in relationship with non-mutated individuals
[2] Effect on Small Tailed Han: G+: +0.55 (LS); Chu et al. (2007)
[3] Effect on Small Tailed Han: B+: +1.11 (LS); BB: +1.40 (LS); Chu et al. (2007)
[4] *B4GALNT2* candidate gene (Mansanet et al., 2012)

1.3.3. Foliculogénesis y dinámica folicular en la oveja

1.3.3.1. Foliculogénesis basal y terminal

A nivel del ovario, se producen dos procesos de desarrollo que son indisociables y que van a determinar el número y la calidad de los oocitos producidos. Son la **ovogénesis**, o formación de los gametos femeninos capaces de ser fecundados, y la **foliculogénesis**, o formación de los folículos ováricos. Ambos se inician en el estadio fetal y van a continuar a lo largo de la toda la vida del animal. Desde un punto de vista funcional, la foliculogénesis se

puede dividir en dos etapas: la foliculogénesis basal, que es independiente de gonadotropinas y durante la cual se adquiere la competencia meiótica del oocito, y la foliculogénesis terminal, que es plenamente dependiente de las gonadotropinas *(Figuras 6 y 7)*.

Durante el periodo fetal se inicia el proceso de formación de los folículos ováricos, tan temprano como a los 35 días de la fecundación en la especie ovina. Las oogonias se unen a las células pregranulosas circundantes y comienzan la meiosis. Los oocitos están rodeados por una única capa de células escamosas, y se establece el reservorio de **folículos primordiales**, que en ovino oscila entre 40.000 y 300.000 (Driancourt et al., 1993). Desde la mitad de la gestación hasta el nacimiento se produce una apoptosis masiva de gran parte de estos folículos, aún por razones desconocidas, durante la cual al menos el 80% de las células germinales experimentan apoptosis (Sawyer et al., 2002). El crecimiento folicular se inicia antes de que se formen los últimos folículos primordiales y continúa a lo largo de toda la vida fetal, neonatal y adulta. Durante la edad fetal se completa toda la formación de folículos ováricos, y a partir de entonces van a ir declinando a lo largo de toda la vida del animal. En relación al primer folículo primordial, el primer folículo primario tarda en aparecer alrededor de 25 días en ovino. Estos folículos primordiales en la especie ovina ya expresan cientos de genes con diversas funciones, entre ellos algunos factores de crecimiento de la familia TGF-β, como *GDF9* y *BMPR1B* (McNatty et al., 2007). En el caso de *BMP15,* se empieza a expresar a partir del estadio de folículo primario en adelante. Por ello, diversos autores consideran que algunos de estos factores están involucrados, bien negativa o positivamente en el inicio del crecimiento folicular.

Los folículos van abandonando progresivamente el pool de folículos primordiales e inician una fase de crecimiento. Tanto los folículos primordiales como los reclutados son prácticamente independientes de la acción de las gonadotropinas, de manera que en esta fase el proceso está controlado principalmente por el oocito. Conforme va aumentando el número de células de la granulosa, que pasan a ser cuboidales, y aumenta el tamaño del oocito, pasan al estadio de **folículo primario,** en el que ya se han activado cientos de genes nuevos, como el gen *BMP15*.

En los **folículos secundarios** ya se observan 2 ó 3 capas de células de la granulosa. De aquí en adelante, los folículos progresivamente se vuelven más sensibles a las gonadotropinas, hasta volverse totalmente dependientes de ellas. Se pasará entonces de un proceso de crecimiento lineal en los estadios preantrales y antrales tempranos (foliculogénesis basal), a un proceso de crecimiento en ondas foliculares (foliculogénesis terminal), que en la oveja empieza en folículos de alrededor de 2 mm, como veremos posteriormente.

En los estadios **preantrales**, sigue aumentando el número de capas de células de la granulosa, aparece la zona pelúcida y la teca interna, ya con receptores de LH. En el estadio

preantral las células de la granulosa se diferencian en dos líneas distintas tanto anatómica como funcionalmente: las de la pared del folículo, con una función esteroidogénica, y las del cumulus. Estas últimas son las que rodean al oocito formando los COCs (cumulus-oocyte complexes), que desde un punto de vista práctico son las estructuras que recuperamos cuando puncionamos folículos para realizar la *producción in vitro de embriones* (PIV). Se van formando pequeñas cavidades que se llenan de fluido, y progresivamente se va formando el antro (Mc Natty et al., 2007). Es importante resaltar que el antro se forma bajo la influencia de la FSH, por lo que los folículos sensibles a las gonadotropinas pueden ser desde preantrales o antrales jóvenes, hasta completamente antrales. La aparición de los receptores de LH en las células la granulosa determina la madurez completa del folículo, y en la oveja ocurre cuando los folículos alcanzan alrededor de 3–3,5 mm de diámetro (Monniaux et al., 2009). Cuando el antro está formado completamente se denomina **folículo terciario o de Graaf**. Estos folículos son dependientes de las gonadotropinas y crecen hasta el estadio de folículo preovulatorio. Los **folículos preovulatorios** son aquellos capaces de ovular si se dan las condiciones endocrinas adecuadas de estradiol, baja progesterona y alta frecuencia de la pulsatilidad de la LH. Se ha estimado que el periodo de crecimiento folicular desde el estadio primordial hasta el preovulatorio excede los 6 meses (Cahill and Mauleon, 1980).

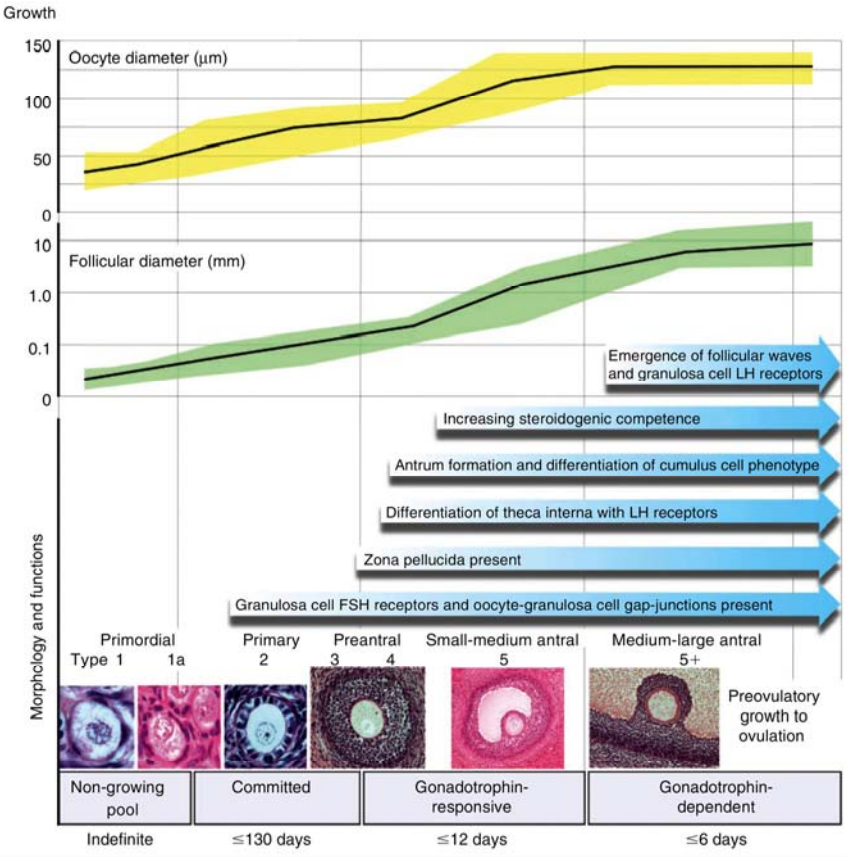


Figura 6. **Resumen de la foliculogénesis en la oveja desde un punto de vista morfológico y funcional. Adaptado de Scaramuzzi et al. (2011)**

Durante esta fase de foliculogénesis terminal, los folículos crecen y regresan en patrones de "**ondas de crecimiento**". En ovino, durante un ciclo estral ocurren de 2 a 4 ondas foliculares (Viñoles, 2000; Bartlewski et al., 2011). Este hecho, explica en parte la gran variación que se encuentra en respuestas ováricas entre animales y razas en esta especie. Dentro del pool de folículos sensibles a las gonadotropinas se encuentran folículos en diferentes estadios. Al incrementar la concentración de FSH, emergen los más avanzados para formar la cohorte de folículos dependientes de las gonadotropinas. Conforme van creciendo, uno o más folículos dominantes emergen y van a inhibir el reclutamiento y crecimiento de otros folículos (González-Bulnes et al., 2004).

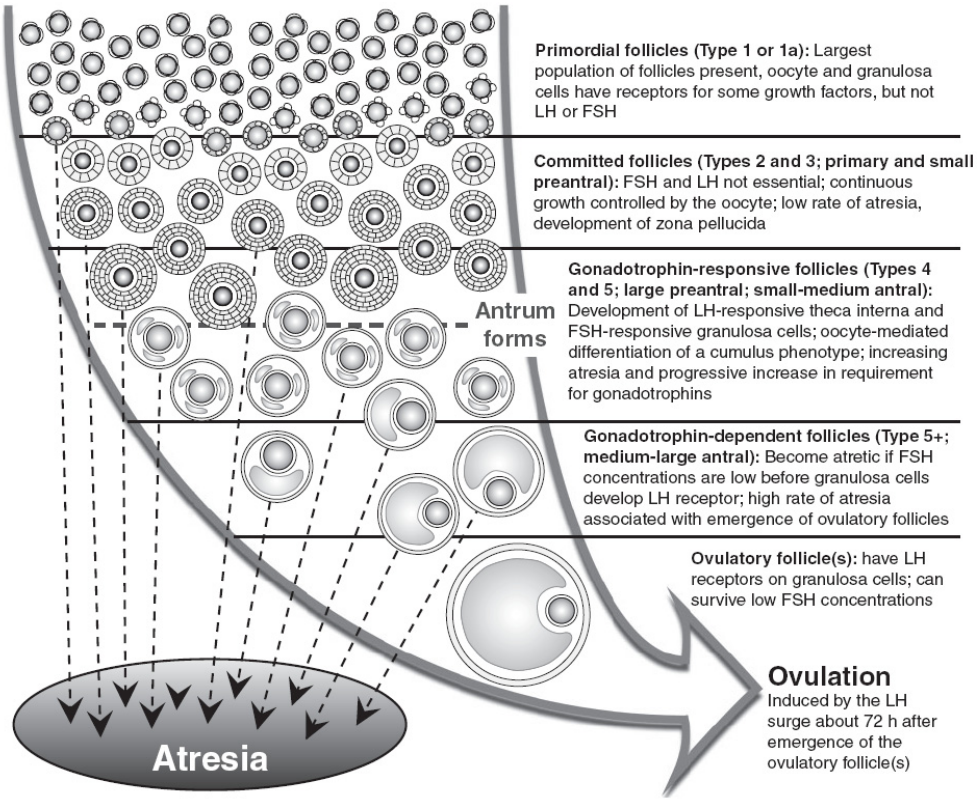


Figura 7. **Modelo de foliculogénesis en la oveja (Scaramuzzi et al., 1993, 2011)**

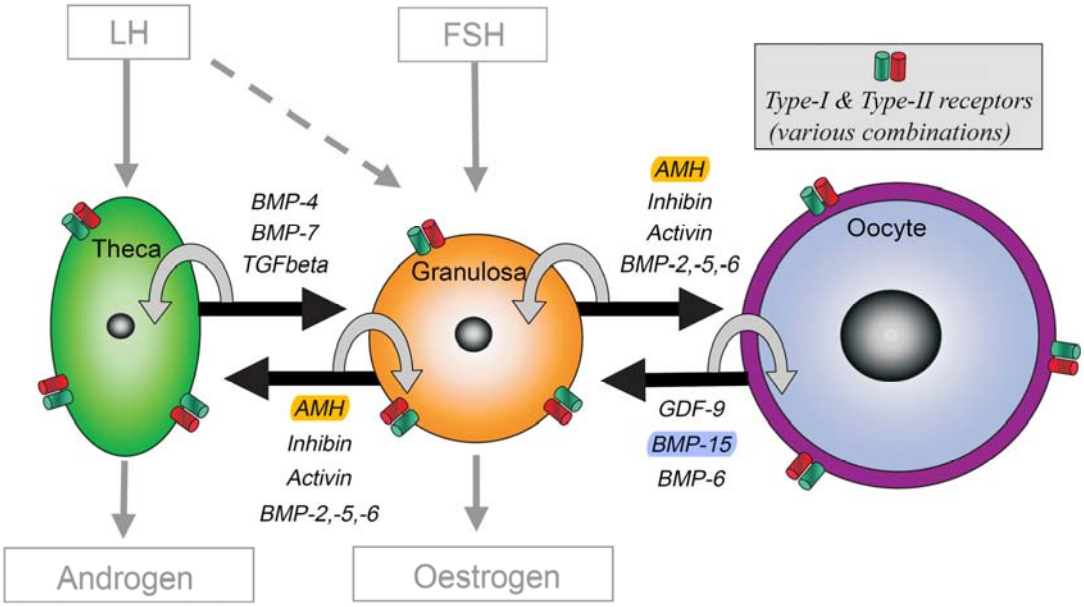
1.3.3.2. Interacciones folículo-oocito: secreción de *BMP15* y AMH

Durante la foliculogénesis existe una relación bidireccional entre el oocito y el folículo, de forma que el oocito crece y se desarrolla de manera dependiente de las células somáticas foliculares (Thibault, 1977), y promueve el desarrollo folicular. El oocito primero adquiere la competencia meiótica y después, gradualmente, la competencia para el desarrollo, es decir, un estado bioquímico y molecular que le permita fertilizarse normalmente, superar con éxito la preimplantación embrionaria, y desarrollarse a término (Hyttel et al., 1997; Duranthon y

Renard, 2001). En los estadios primordiales e independientes de gonadotropinas el oocito es incompetente, tanto meióticamente como en competencia para el desarrollo. Tras la formación del antro, el oocito adquiere la habilidad de reiniciar la meiosis y progresivamente va adquiriendo su competencia citoplasmática para el desarrollo (Sirard et al., 2006). Por lo tanto, la proporción de oocitos competentes aumenta conforme aumenta el tamaño folicular (Revisado por Mermillod et al., 2008). De esta manera, en trabajos recientes llevados a cabo en cabras se ha demostrado que aunque la eficiencia de la PIV en animales prepúberes es mucho menor que en los adultos, se pueden obtener los mismos resultados entre grupos de edad siempre y cuando se puncionen folículos de más de 3 mm en los animales más jóvenes (Romaguera et al., 2011). Este hecho se debe tener en cuenta cuando se obtienen oocitos para PIV de embriones, tanto por técnicas de aspiración folicular *in vivo* (LOPU), como a partir de ovarios de matadero.

El oocito segrega **factores de crecimiento** que juegan un papel esencial en la regulación de la foliculogénesis, diferenciación de las células del cumulus y de la granulosa, tasa de ovulación y prolificidad (McNatty et al., 2004). Dos de estos factores se expresan principalmente en el oocito: ***GDF9*** y ***BMP15***, y juegan un papel clave en promover el crecimiento folicular.

Ambos pertenecen a la **superfamilia del TGF-β**, que está formada por un grupo de al menos 35 proteínas con diversa funcionalidad pero similar estructura (Knight y Glister, 2006). Esta familia se subdivide en las subfamilias TGF-β, BMP (bone morphogenetic protein; a la que pertenece BMP15), GDF (growth and differentiation; a la que pertenece GDF9), activin/inhibin, GDNF (glial cell-derived neurotrophic factor), e incluye otros miembros como la AMH (anti-Müllerian hormone; revisión de Knight y Glister, 2006). Estos factores son expresados por las células somáticas del ovario y por los oocitos en diferentes momentos de su desarrollo, y actúan como reguladores ováricos de la foliculogénesis. Como se ha comentado previamente, *GDF9* y *BMP15* son expresados por el oocito desde etapas muy tempranas de su crecimiento (estadio de folículo primordial y primario, respectivamente), y ejercen su acción de manera paracrina sobre las células de la granulosa, y de manera autocrina sobre el mismo oocito (*Figura 8*). A partir del estadio de folículo primario también se empieza a expresar en las células de la granulosa la AMH, como veremos más adelante. Desde la formación del antro, GDF9 y BMP15 juegan un papel esencial en la prevención de la luteinización de las células de la granulosa. Durante la fase antral, continúan actuando ambos factores: promoviendo la actividad mitogénica e inhibiendo la luteinización en las células murales de la granulosa, y proveyendo al oocito con factores paracrinos y otras moléculas.



La mayoría de los miembros de la familia TGF-β ejercen sus efectos sobre las células diana mediante la unión a varios tipos de receptores (tipo I y II). Actualmente se conocen en los mamíferos siete receptores de tipo I y cinco de tipo II (Revisado por Knight and Glister, 2006). La activación del receptor conduce a la fosforilación de unas moléculas llamadas Smads, que modularán a nivel del núcleo la expresión de los genes diana. BMP15 se une al receptor BMPRII (receptor de tipo 2) que activa BMPR1B (receptor de tipo I), y que activa la ruta de las SMADs 1-5-8 (Juengel and McNatty 2005). Esta ruta es la misma que activa la AMH, que tiene un receptor específico de tipo II (AMHR2), pero también varios receptores de tipo I que comparte con otros miembros de la familia del TGF-β, como las BMPs (Josso and di Clemente, 2003).

Figura 8. **Características de BMP15, AMH y otros miembros de la familia TGF-β, como ligandos extracelulares implicados en la comunicación bidireccional entre las células de la teca y de la granulosa, y entre las células de la granulosa y el oocito. Acciones autocrinas (flechas grises gruesas) y paracrinas (flechas negras gruesas). Adaptado de Knight y Glister (2006)**

El folículo contiene otros muchos sistemas autocrinos y paracrinos, como por ejemplo el del IGF, muy importante en la foliculogénesis ya que aumenta la sensibilidad de los folículos pequeños a la estimulación con gonadotropinas y estimula su transición hacia folículos dependientes de gonadotropinas (Silva et al., 2009), pero no se tratarán en la presente Tesis.

1.3.3.3. Posibles mecanismos de acción de las mutaciones *Fec*

Como se ha descrito previamente, en ovino se han encontrado diversas mutaciones naturales en el gen *BMP15.* En la primera mutación que se descubrió (*FecB*^{B}) en el gen *BMPR1B* se observó que los folículos de las hembras homocigotas maduraban y ovulaban a diámetros foliculares menores y con menos células de la granulosa, sin alteraciones en la secreción de esteroides o inhibina. Sus oocitos reiniciaban la meiosis a un tamaño menor y alcanzaban su diámetro maduro en la fase preantral, mientras que las no portadoras lo hacían tras la formación del antro (Cognié et al., 1998). Por otro lado, los animales

fuertemente inmunizados contra BMP15 o GDF9 tienen inhibido el crecimiento folicular desde los estadios primarios y primordiales (McNatty et al., 2006).

Algunos autores consideran que el sistema BMP juega un papel principal en el control de la foliculogénesis ovárica y de la tasa de ovulación, modulando la proliferación y diferenciación de las células de la granulosa y de la teca en respuesta a la estimulación por gonadotropinas, existiendo numerosas variaciones a nivel intrafolicular (Campbell et al., 2003). Las mutaciones descritas en *BMP15* aparentemente ocasionan una interrupción de la síntesis de proteína BMP15 biológicamente activa por parte de ese alelo, por lo que la cantidad de proteína funcional en el ovario se encontraría disminuida, o bien afectada su unión a los receptores (Knight y Glister, 2006). Podría ser que las mutaciones en genes *Fec*, debido a esta disminución en la cantidad de proteína, induzcan una maduración precoz de los folículos ováricos y se altere su sensibilidad a las gonadotropinas. Algunos trabajos sugieren una mayor sensibilidad de las hembras portadoras heterocigotas tanto a la FSH como a la LH (Fabre et al., 2006), mientras que otros muestran tan solo una aparición precoz de los receptores de LH (McNatty et al., 2009), favoreciéndose en cualquier caso su selección. Como consecuencia, en las ovejas portadoras de la mutación se produciría la ovulación y luteinización de numerosos folículos antrales más pequeños, con menos células de la granulosa, mayor sensibilidad a la FSH y cuyos receptores de LH aparecen de una manera precoz, lo que conduce a una mayor tasa de ovulación (Fabre et al., 2006, *Figura 9*).

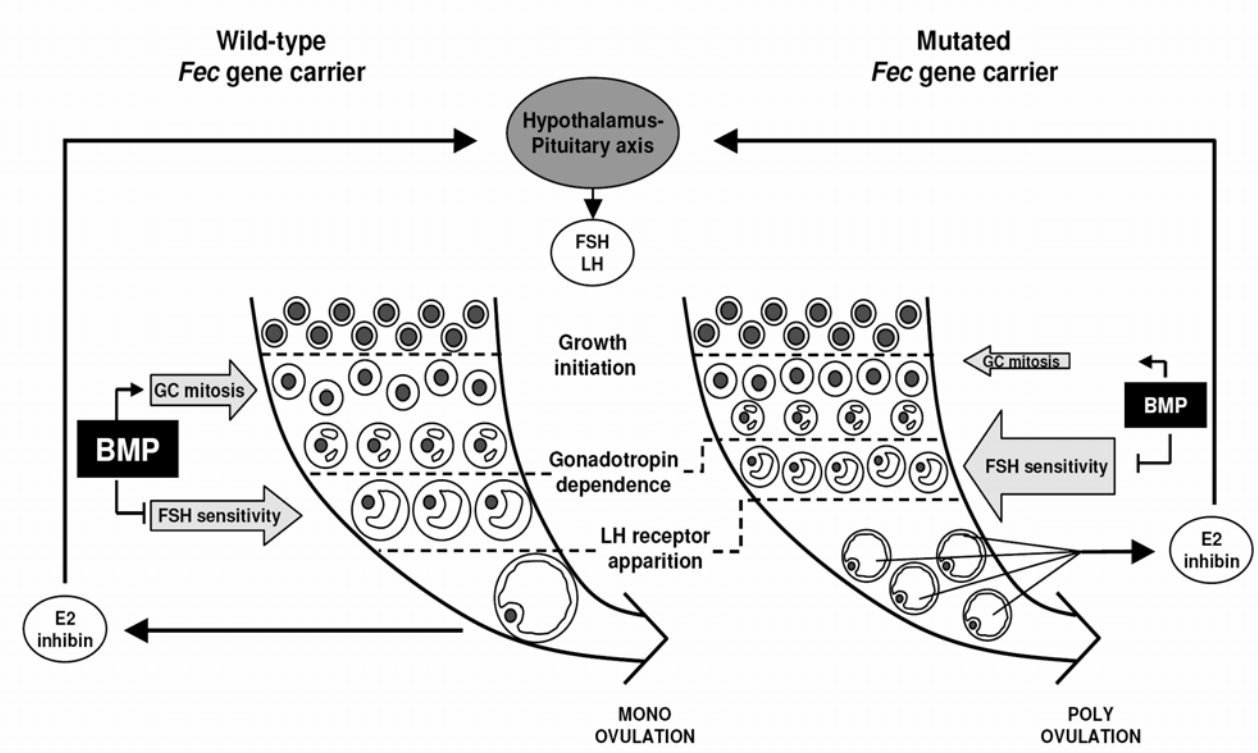


Figura 9. **Representación esquemática de los efectos de los alelos *Fec* de prolificidad sobre la foliculogénesis y tasa de ovulación (Fabre et al., 2006)**

Se han desarrollado algunos modelos para explicar la regulación de la tasa de ovulación. Según la teoría de la "ventana" propuesta por Scaramuzzi et al. (2011), en el caso de ovulaciones simples, un folículo ovulatorio emerge de un pool de folículos dependientes de las gonadotropinas bajo el control de la FSH. El periodo durante el cual el nivel de FSH permanece por encima de un umbral determinado abre algo parecido a una "ventana" que permite a un folículo maduro escapar de la atresia, pasar por esa "ventana" y alcanzar la ovulación (*Figura 10*). Las ovulaciones múltiples se podrían alcanzar mediante diferentes mecanismos. Por un lado, aumentando esa "ventana", ya sea disminuyendo el umbral de FSH o bien aumentado los niveles de FSH circulante. Por otro lado, incrementando el pool de folículos en crecimiento que pueden ser estimulados por las gonadotropinas. Estos mecanismos no serían excluyentes entre si (Scaramuzzi et al., 2011; Vinet et al., 2012).

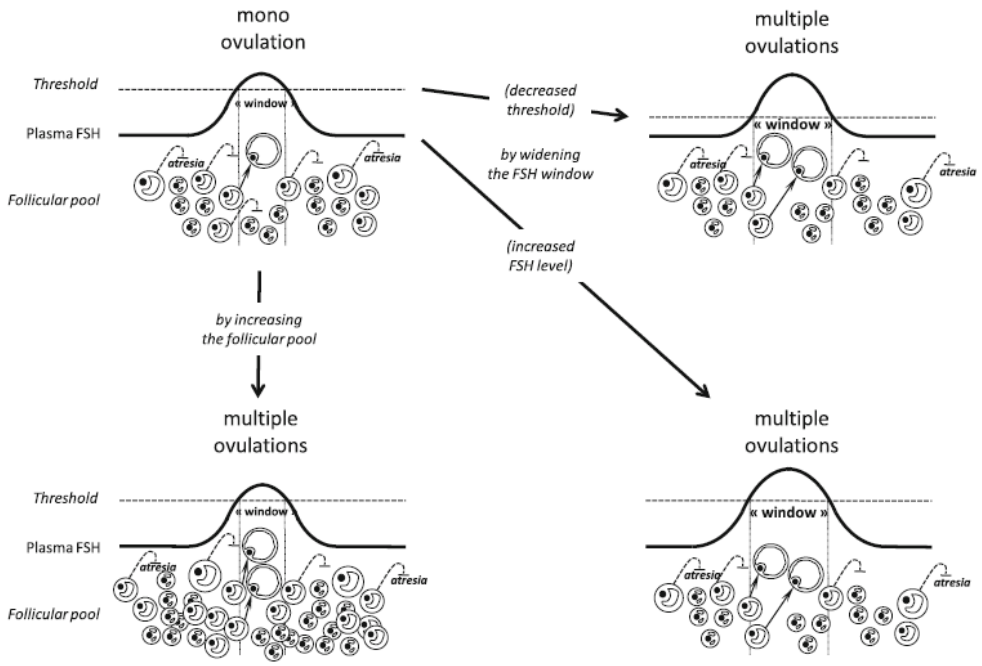


Figura 10. **"Teoría de la ventana" propuesta por Scaramuzzi et al. (2011) para explicar los posibles escenarios para el incremento de la tasa de ovulación (Vinet et al., 2012)**

Teniendo en cuenta todos estos aspectos, en la presente Tesis se quiso evaluar la influencia del alelo *FecX*^{R} del gen *BMP15* sobre la población folicular de las ovejas heterocigotas, y su repercusión sobre la competencia de sus oocitos. Los resultados de estos experimentos se presentan en la tercera publicación.

1.4. La AMH como marcador endocrino de la reserva folicular

La hormona anti-Mülleriana (AMH), también conocida como Müllerian inhibiting substance (MIS), es una glycoproteina de 140 kDa perteneciente a la familia del transforming growth factor beta (TGF-β), que se expresa únicamente en la gónadas (Cate et al., 1986). En los machos, es producida por las células de Sertoli y causa la regresión de los conductos de Müller durante el estadio fetal. En el ovario de las hembras, se ha visto que ejerce un acción inhibitoria sobre el reclutamiento de folículos primordiales hacia folículos en crecimiento, evitando el agotamiento temprano de la reserva folicular (Revisado por Monniaux et al., 2013)**.**

En los mamíferos la "**reserva folicular ovárica**", es decir, el número de folículos primordiales, es muy variable entre los individuos. Aunque no se ha encontrado un marcador directo de la reserva folicular ovárica, se considera que la "**reserva folicular funcional o activa**" es normalmente un buen indicador de la misma (Anderson et al., 2012; Grynnerup et al., 2012). Hoy en día, la AMH es el mejor marcador endocrino de los folículos en crecimiento, es decir, de la reserva folicular funcional o activa, en diversas especies. Actualmente se utiliza la AMH como predictor de la respuesta ovárica a las gonadotropinas en la edad adulta en el ser humano (Visser y Themmen, 2005; Visser et al., 2006; Lamarca et al., 2010), el ratón (Kevenaar et al., 2006), la vaca (Rico et al., 2009), y recientemente se ha demostrado también su utilidad en la yegua (Vernunft et al., 2011) y en la cabra (Monniaux et al., 2011). En la vaca, la AMH está altamente correlacionada con el número de folículos antrales de 3 a 7 mm de diámetro, que son la principal diana de los tratamientos superovulatorios (*Figura 11*). Por tanto, la medición de la concentración de AMH es un reflejo del la reserva folicular activa, y permite predecir el número de ovulaciones y de embriones producidos en respuesta a la estimulación ovárica con FSH en esta especie (Rico et al., 2009, 2011; Monniaux et al., 2010), que es un carácter repetible y posiblemente heredable. Es importante destacar que en la vaca, la concentración plasmática de AMH es característica de cada animal durante un largo periodo de vida, existiendo una alta variabilidad entre individuos.

Basándose en este descubrimiento, se empezó a utilizar la concentración plasmática de AMH como criterio de elección de vacas donadoras en programas comerciales o MOET (Multiple Ovulation and Embryo Transfer).

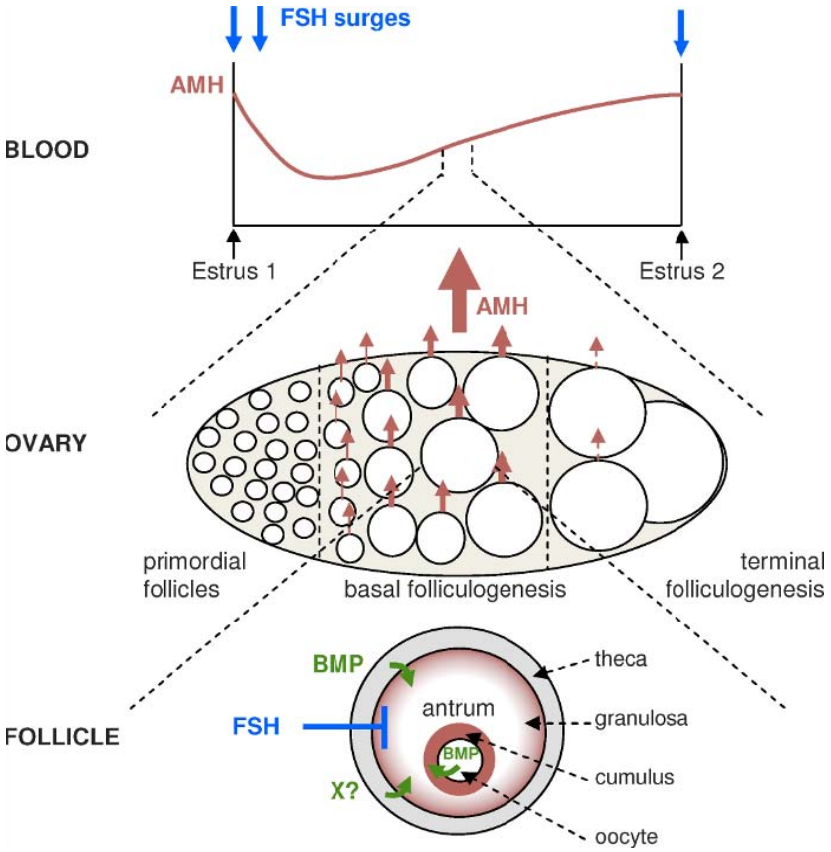


Figura 11. **Modelo de regulación de la producción de AMH a nivel celular, folicular, ovárico y endocrino en la vaca. Las flechas rojas muestran la expresión de la AMH (De Rico et al., 2011)**

Cuando se planteó esta Tesis la información sobre la AMH plasmática en ovino era inexistente, por lo que se creyó conveniente investigar las relaciones entre la AMH y la población folicular. De existir alguna relación entre ellas, podría derivarse un método para identificar las ovejas con mayor capacidad de respuesta ovárica, que tal vez pudiera ser aplicado a la selección.

En esta Tesis se planteó el interés de determinar la concentración de AMH tanto en ovejas adultas como en corderas. Si la AMH en la edad adulta era capaz de reflejar una determinada población de folículos sanos en crecimiento (foliculogénesis basal terminal), quizás en animales prepúberes pudiera reflejar lo mismo, lo cual tal vez podría tener implicaciones sobre la precocidad sexual. La existencia de trabajos previos donde se había demostrado que a las 12–14 semanas del nacimiento ya había un número importante de folículos sensibles a las gonadotropinas en el ovario de corderas (Bartlewski et al., 2006; Mahdi et al., 2008) nos animó a llevar a cabo estos experimentos. Los resultados de estos trabajos se presentan en la cuarta y quinta publicaciones de la Tesis.

Para realizar estos estudios el INIA de Madrid financió un proyecto titulado “Marcadores de productividad de la oveja medidos en edad prepúber”.

1.4.1. Relación entre BMP15 y AMH

Recientemente se ha demostrado que los factores BMP son capaces de estimular la expresión de la AMH en las células de la granulosa en condiciones *in vitro* (Vacuno: Rico et al., 2011; Ovino: Monniaux et al., 2013). Por lo tanto, una mutación que afecta al gen *BMP15*, como es el caso del alelo *FecX*^{R} donde existe una deleción de 17 pares de bases en la secuencia que codifica el gen que conlleva una disminución de su expresión a nivel del oocito, podría estar afectando también la producción de AMH de los folículos en crecimiento, y por lo tanto, afectar en último término a sus concentraciones plasmáticas.

Actualmente no se conocen los efectos que dichas mutaciones puedan tener sobre la AMH, por lo que deberían ser estudiadas. Por lo tanto, en la presente Tesis Doctoral se consideró de interés evaluar, además de la relación entre la AMH y la población folicular, el efecto del genotipo sobre la concentración plasmática de esta hormona.

A modo de conclusión, diremos que en los últimos años se está empezando a trabajar en la búsqueda de nuevas estrategias para mejorar la eficiencia reproductiva de las ovejas, como es el uso de variantes génicas o la búsqueda de compuestos fisiológicos del propio animal que estén asociados a mejores resultados reproductivos. Las dos estrategias que se presentan en la presente Tesis persiguen poder seleccionar los animales más eficientes desde el punto de vista reproductivo a una edad muy temprana, lo que permitiría acelerar el proceso de selección interna en los rebaños, y minimizar el uso de productos hormonales. El trabajo que se presenta en esta Tesis, enfoca el estudio de dos de estas estrategias que afectan a la población folicular en la oveja Rasa Aragonesa:

1) Aumento de la prolificidad a través del alelo *FecX*^{R} del gen *BMP15*

2) Utilización de hormona Anti-Mülleriana como marcador de la población folicular

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OBJETIVOS / OBJECTIVES

2.

Objetivos

Mediante la realización de la presente Tesis Doctoral se pretende avanzar en el conocimiento y la utilización de marcadores genéticos y endocrinos de la población folicular en ovejas Rasa Aragonesa para la mejora de su eficiencia reproductiva. Para ello se pretende evaluar el efecto del alelo *FecX*^{R} del gen *BMP15* sobre el rendimiento reproductivo, así como investigar otros posibles biomarcadores como la hormona anti-Mülleriana.

Este objetivo general se pretende abordar mediante los siguientes objetivos parciales:

1. Determinar el efecto del alelo *FecX*^{R} del gen *BMP15* sobre la tasa de ovulación, la prolificidad y el momento de ovulación en ovejas heterocigotas, así como sus interacciones con los tratamientos hormonales clásicos de sincronización de celos utilizados en las ganaderías.
2. Estudiar la influencia del alelo *FecX*^{R} del gen *BMP15* sobre la población folicular de ovejas heterocigotas y su repercusión sobre la calidad del oocito, evaluada mediante técnicas de producción *in vitro* y transferencia de embriones a partir de oocitos derivados de LOPU.
3. Estudiar la relación entre los niveles plasmáticos de hormona anti-Mülleriana (AMH) y la población folicular, en función del genotipo *BMP15* (heterocigotas o no portadoras del alelo *FecX*^{R}) y de la edad (corderas y adultas).
4. Investigar la relación entre los niveles plasmáticos de hormona anti-Mülleriana (AMH) en corderas prepúberes y su reserva folicular sensible a gonadotropinas, como posibles marcadores de fertilidad en la edad adulta.

Objectives

This Doctoral Thesis was designed to advance the knowledge and use of genetic and endocrine markers of follicle population in Rasa Aragonesa sheep, in order to improve their reproductive efficiency. This was accomplished by assessing the effect of the *FecX*^{R} allele in the *BMP15* gene on reproductive performance, as well as exploring other potential biomarkers such as anti-Müllerian hormone.

This general objective was addressed by the following partial objectives:

1. To study the effect of the *FecX*^{R} allele of the *BMP15* gene on ovulation rate, prolificacy and time of ovulation in heterozygous ewes, and their interactions with standard hormonal treatments used in farms for estrus synchronization.
2. To assess the influence of the *FecX*^{R} allele of the *BMP15* gene on follicle population in heterozygous ewes and its impact on oocyte quality, evaluated by *in vitro* embryo production techniques and embryo transfer from LOPU-derived oocytes.
3. To study the relationship between plasma anti-Müllerian hormone (AMH) concentrations and follicle population, depending on age (ewe lambs and adults) and *BMP15* genotype (carrying or not carrying the *FecX*^{R} allele).
4. To look into the relationship between plasma anti-Müllerian hormone (AMH) concentrations in prepubertal ewe lambs and the population of gonadotropin-responsive follicles as potential markers for fertility in adulthood.

PUBLICACIONES

3.

3.1. Presentación de las publicaciones

Presentación de las publicaciones

En el marco de la presente Tesis Doctoral se han realizado 5 publicaciones.

Los primeros estudios tuvieron como objetivo "Determinar el efecto del alelo *FecX*^{R} del gen *BMP15* sobre la tasa de ovulación, la prolificidad y el momento de ovulación en ovejas heterocigotas, así como sus interacciones con los tratamientos hormonales clásicos de sincronización de celos utilizados en las ganaderías" (**Objetivo 1** de la Tesis). Este objetivo general se abordó mediante cuatro experimentos. Los tres primeros dieron lugar a la primera publicación, y el cuarto experimento dio lugar a la segunda publicación.

<u>Publicación 1:</u> Effect of the *FecX*^{R} polymorphism in the bone morphogenetic protein 15 gene on natural or equine chorionic gonadotropin-induced ovulation rate and litter size in Rasa Aragonesa ewes and implications for on-farm application.

En esta primera publicación se determinó el aumento de tasa de ovulación y de prolificidad que produce la presencia del alelo *FecX*^{R} tanto en condiciones naturales como al aplicar diferentes dosis de eCG, y se evaluaron sus posibles efectos adversos sobre la supervivencia de los corderos.

Para ello, en un primer experimento se determinó mediante laparoscopia la tasa de ovulación natural en corderas y ovejas adultas de ambos genotipos (R+ y ++).

En un segundo experimento, se aplicaron diferentes dosis de eCG a ovejas adultas de ambos genotipos con el fin de evaluar una posible interacción entre el genotipo y la aplicación de gonadotropinas sobre la prolificidad. El objetivo fue determinar la dosis óptima a aplicar a las ovejas R+, así como evaluar la posible influencia del incremento en tasa de ovulación sobre las pérdidas embrionarias y el peso al nacimiento de los corderos.

En el último experimento, se quiso determinar el efecto del alelo *FecX*^{R} sobre la prolificidad en condiciones reales y sobre un número elevado de animales, teniendo en cuenta de nuevo el efecto de la aplicación de eCG. Para ello, se contó con los datos del control de producciones recogidos durante 10 años en las ganaderías que forman parte del programa de mejora genética por prolificidad de la UPRA-Grupo Pastores. En dichas ganaderías existía el problema de que había hembras R+ sin identificar. Con el fin de hacer una estimación de la frecuencia del polimorfismo en las explotaciones, evitar en un futuro emparejamientos que llevasen a producir ovejas homocigotas estériles, y calcular de manera fiable la prolificidad, era necesario identificar las hembras R+. Para ello se contrastó el método de asignación de genotipos por genealogía frente al genotipado por PCR. Una vez validado el método e identificadas correctamente las hembras R+, se calculó su prolificidad en relación a la de las hembras ++.

Los resultados de los tres primeros experimentos permitieron definir el incremento de la tasa de ovulación y prolificidad que produce esta mutación en corderas y adultas de Rasa Aragonesa, en condiciones experimentales y de rebaño. Se extrajeron conclusiones sobre los efectos de diferentes dosis de eCG, que permitirían tomar decisiones a nivel de explotación sobre la dosis a aplicar en función de la prolificidad y distribución de partos deseable y asumible por el ganadero. Por último, los resultados permitieron validar un método de identificación de animales portadores en la explotación que resultase viable a nivel práctico y económico.

<u>Publicación 2.</u> Caracterización del pico preovulatorio de LH en ovejas Rasa Aragonesa portadoras o no del alelo *FecX^R* (ROA®).

En la raza Rasa Aragonesa la IA es una técnica utilizada dentro del programa de selección para la conexión de rebaños y la difusión de la mejora genética. Previamente a la IA, las ovejas se sincronizan con un tratamiento clásico de esponja de FGA y eCG, y se realiza la IA a tiempo fijo. En los experimentos de la publicación 1 se demostró que la presencia del alelo *FecX^R* en heterocigosis producía un incremento de la tasa de ovulación y prolificidad, así como una mayor respuesta a la eCG. Estas diferencias podrían conllevar cambios endocrinos que podrían afectar a la descarga preovulatoria de LH. Por lo tanto, se decidió estudiar el momento de aparición del pico preovulatorio de LH con el fin de conocer si la presencia del alelo modificaba el momento de ovulación. En caso afirmativo, sería necesario modificar el momento de IA en este genotipo.

Con este objetivo se extrajeron muestras sanguíneas de ovejas adultas portadoras o no del alelo *FecX^R*, tratadas o no con eCG, y se determinaron las concentraciones de LH.

Los resultados del cuarto experimento permitieron establecer las recomendaciones para la realización de la IA en ambos genotipos.

<u>Publicación 3.</u> Influence of the *FecX^R* allele in heterozygous ewes on follicular population and outcomes of IVP and ET using LOPU-derived oocytes.

El siguiente estudio tuvo como objetivo "Estudiar la influencia del alelo *FecX^R* del gen *BMP15* sobre la población folicular de ovejas heterocigotas y su repercusión sobre la calidad del oocito, evaluada mediante técnicas de producción *in vitro* y transferencia de embriones a partir de oocitos derivados de LOPU" (**Objetivo 2** de la Tesis), y sus resultados se presentan en la tercera publicación.

Se buscó profundizar en los efectos del alelo *FecX^R* a nivel ovárico, y avanzar así en el conocimiento de los mecanismos de acción que conducen al incremento de la tasa de ovulación, así como estudiar sus implicaciones sobre la fertilidad. Para ello, se utilizó la

tecnología Laparoscopic Ovum Pick-Up (LOPU), que permitió contabilizar y medir los folículos ováricos, y obtener oocitos de ovejas de ambos genotipos (R+ y ++), tratadas o no con FSH. Los oocitos recuperados fueron sometidos a un proceso de maduración, fertilización y cultivo *in vitro*, y como último criterio de calidad oocitaria, aquellos que alcanzaron el estadio de blastocisto fueron transferidos a ovejas receptoras.

Los resultados de este trabajo permitieron sacar conclusiones sobre diferencias en la competencia para el desarrollo de los oocitos de hembras de ambos genotipos.

Publicación 4. Anti-Müllerian hormone (AMH) concentration in sheep and its dependence of age and independence of *BMP15* genotype: an endocrine predictor to select the best donors for embryo biotechnologies.

La disminución de la proteína BMP15 biológicamente activa que ocurre en las hembras R+ podría conllevar una disminución en la producción de AMH. Debido a las ventajas reproductivas del alelo *FecX*^{R}, las hembras R+ coexisten con las ++ en los rebaños de Rasa Aragonesa. Por lo tanto, las posibles diferencias en las concentraciones de AMH entre genotipos deberían investigarse para determinar si la capacidad predictiva de la AMH podría hacerse extensiva o no a ambos genotipos. Por ello, se realizó un experimento para investigar en primer lugar, la relación entre la AMH y el genotipo en función de la edad. En segundo lugar, se quiso investigar la relación entre la AMH y la población folicular en ovejas adultas, para lo cual fueron sometidas a varias sesiones de LOPU. Con estas experiencias se quisó abordar el **Objetivo 3** de la Tesis, y sus resultados se presentan en la cuarta publicación. Se consideró que si bien establecer las relaciones entre la AMH en la edad adulta y el rendimiento en LOPU sería de gran interés, todavía sería más interesante si se pudiese relacionar la AMH en la edad prepúber con el rendimiento en LOPU en la edad adulta, con el fin de poderla utilizar como un criterio de selección precoz de las corderas con una mayor población folicular.

Las concentraciones de AMH se determinaron en muestras sanguíneas que se obtuvieron antes de la pubertad y en la edad adulta en animales de ambos genotipos (R+ y ++). En la edad adulta fueron sometidas a LOPU.

Este trabajo permitió establecer los perfiles de AMH en función de la edad y relacionar las concentraciones de AMH con el rendimiento en LOPU para ser utilizada como criterio de selección de las mejores donantes. Por otro lado, se pudo conocer si se deberían establecer distinciones entre genotipos a la hora de utilizar la AMH como posible herramienta de selección.

<u>Publicación 5.</u> Anti-Müllerian hormone plasma concentration in prepubertal ewe lambs as a predictor of their fertility at a young age.

En la Rasa Aragonesa la edad media al primer parto es de 17–18 meses, a pesar de que podrían cubrirse con éxito a partir de los 7 meses, lo que conlleva importantes pérdidas económicas. Los resultados presentados en la publicación 4 mostraron una gran variación en las concentraciones de AMH entre animales y edades, lo que nos hizo pensar que quizás la AMH podría estar reflejando un desarrollo folicular más avanzado en unos animales que en otros. De ser cierto, se pensó que tal vez se podrían seleccionar a una edad muy temprana los animales más precoces, lo que podía repercutir positivamente en su vida productiva, y por lo tanto podría tener en un futuro una repercusión económica importante para la ganadería ovina. Por estas razones, se llevó a cabo el siguiente experimento, que dio lugar a la quinta publicación de la Tesis, y cuyo objetivo era: "Investigar la relación entre los niveles plasmáticos de hormona Anti-Mülleriana (AMH) en corderas prepúberes y su reserva folicular ovárica, como posibles marcadores de fertilidad en la edad adulta" (**Objetivo 4** de la Tesis).

Para ello se determinó la concentración de AMH en corderas prepúberes y se relacionó con su respuesta ovulatoria tras la aplicación de eCG, como posible indicador de la presencia de folículos sensibles a las gonadotropinas a esa edad. Los datos obtenidos se relacionaron con su fertilidad a la primera cubrición.

Se demostró la relación entre la AMH y la respuesta ovulatoria antes de la pubertad con la fertilidad temprana, y se evaluó la posibilidad de utilizarlos como un criterio de selección de las corderas con mejor fertilidad en su primera cubrición.

3.2. Publicación 1

Effect of the *FecX*^{R} polymorphism in the bone morphogenetic protein 15 gene on natural or equine chorionic gonadotropin-induced ovulation rate and litter size in Rasa Aragonesa ewes and implications for on-farm application.

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Journal of Animal Science 2011, 89:3522–3530

Effect of the *FecX*^{R} polymorphism in the bone morphogenetic protein 15 gene on natural or equine chorionic gonadotropin-induced ovulation rate and litter size in Rasa Aragonesa ewes and implications for on-farm application[1]

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ABSTRACT: A new mutation in the bone morphogenetic protein 15 (*BMP15*) gene (*FecX*^{R} allele) causing increased prolificacy in heterozygous (R+) and sterility in homozygous ewes has been recently described in Rasa Aragonesa, a low-prolificacy Mediterranean breed. The current study determined, first, the effect of this polymorphism on natural and eCG-induced ovulation rate (OR) and the effect of eCG dose on reproductive performance; and second, its effect on prolificacy and its interaction with progestagen + eCG treatment on farms, which have not been reported to date. The *FecX*^{R} allele increased OR by 0.44 and 0.63 ovulations in young (n = 91) and adult (n = 84) R+ ewes, respectively (both, $P < 0.01$), increments less than reported in prolific breeds carrying other mutations in *BMP15*. When the standard dose of eCG used on farms (480 IU) was applied to R+ ewes (n = 36), an extremely high OR (3.95) was recorded, which was accompanied by greater partial failure of multiple ovulations (PFMO). On the contrary, OR using 240 IU in R+ ewes (2.90; n = 35) was similar to 480 IU in wildtype (++) ewes (2.82; n = 48; both $P < 0.01$ when compared with 480 IU in R+ ewes). No differences were found in the birth weight of the offspring between R+ and ++ eCG-stimulated ewes within the same litter size. To validate the genealogy identification on farms, PCR genotyping was carried out in 1,667 ewes from 4 elite flocks, resulting in a negligible misclassification of R+ ewes, which demonstrated that identification by genealogy is a reliable tool to identify *FecX*^{R} ewes within the breeding program. In recorded farms, the natural litter size of ++ ewes (1.34, n = 599,160 lambing records) was increased due to the *FecX*^{R} allele by 0.35 lambs ($P < 0.0001$, n = 6,593 lambing records). A similar increase (0.30) was observed when comparing ++ and R+ ewes treated with 480 IU of eCG ($P < 0.0001$, n = 62,055 and n = 866, respectively). When applying 480 IU of eCG to R+ ewes, the increase in prolificacy was only due to increased percentages of triplets ($P < 0.001$) and quadruplets ($P < 0.0001$), but not of twin births. In conclusion, the favorable reproductive performance of R+ ewes, with 0.63 extra ovulations and 0.35 extra lambs per lambing ewe, is responsible for the increased interest in the use of this polymorphism. Nevertheless, care must be taken in the application of eCG to R+ ewes, with the current results showing that the standard dose increases prolificacy by only increasing triple and higher-order births.

Key words: *BMP15*, breeding program, ovulation rate, pregnant mare serum gonadotropin, prolificacy, sheep

J. Anim. Sci. 2011. 89:3522–3530
doi:10.2527/jas.2010-3828

[1]This work was supported by TRACE PET-2008-0076 project (Spain). B. Lahoz and A. Martínez-Royo were supported by an INIA fellowship. The authors also thank Pilar Sánchez and Elías Echegoyen [Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón, Unidad de Tecnología en Producción Animal, Zaragoza, Spain] for technical support and Nancy D'Cruz (ARAID Fundación Agencia Aragonesa para la Investigación y Desarrollo, Zaragoza, Spain, and CITA) for manuscript revision. All authors hold the patent related to the present research, titled "(ES)Procedimiento de mejora de la productividad en Ganado ovino," number ES2338960, and published on May 13, 2010.

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Received December 30, 2010.
Accepted May 20, 2011.

INTRODUCTION

Rasa Aragonesa is an autochthonous Mediterranean breed of sheep from the northeast of Spain, with about 500,000 animals recorded, mainly reared in extensive or semiextensive farming systems and oriented to meat production. A selection program to increase prolificacy has been carried out in this breed since 1994 because the number of lambs born per ewe has a key role in the efficiency and viability of these farms (Pardos et al., 2008). In Rasa Aragonesa, phenotypic prolificacy is 1.37 (lambs/birth; 16th catalog of the selection program 2009, unpublished). In 2007, some descendents of the tested rams presented an uneven increase in prolificacy unexplained by polygenic heredity, leading to the discovery of a new naturally occurring polymorphism in bone morphogenetic protein 15 (***BMP15***), a fecundity gene with a major effect on ovulation rate (**OR**) in sheep. The polymorphism ($FecX^R$ allele) consists of a deletion of 17 bp in the coding region of *BMP15*, located on the X chromosome, causing increased prolificacy in heterozygous (**R+**) and sterility in homozygous ewes (Martínez-Royo et al., 2008). Because the $FecX^R$ allele allows increased prolificacy while maintaining breed morphology, it is being used on farms. In fact, in 2008 the estimated population of R+ ewes was approximately 1,500, showing a clear trend toward increased numbers. In spite of its economic interest, the increase in OR, which led to this increase in litter size (**LS**), and the interactions with progestagen + eCG treatment (widely used to induce out-of-season breeding) still remain to be determined in this breed.

Therefore, this study was performed to determine the effect of this new polymorphism on natural OR in young and adult Rasa Aragonesa ewes, as well as OR and reproductive performance of adult ewes treated with fluorogestone acetate (**FGA**) and 2 different doses of eCG. In addition, we wished to determine the increase in prolificacy due to the presence of the $FecX^R$ allele and its type of birth distribution in eCG-treated or untreated ewes on farms.

MATERIALS AND METHODS

All experimental procedures were performed in accordance with the guidelines of the European Union (2003/65/CE) and Spanish regulations (RD 1201/2005, BOE 252/34367–91) for the use and care of animals in research.

Exp. 1. Natural OR of Young and Adult $FecX^R$ Heterozygous Ewes

This experiment was carried out from November to December (the natural breeding season) in the facilities of Centro de Investigación y Tecnología Agroalimentaria (**CITA**), a research center located in Zaragoza (Spain). Ovulation rate was recorded in 91 heterozygous $FecX^R$ carriers (**R+**) and 20 wild-type (++) young ewes, aged 311 ± 11 d (mean ± SD), and in 84 R+ and 19 ++ adult ewes, aged 683 ± 37 d (mean ± SD), previously identified by PCR genotyping as described by Martínez-Royo et al. (2009). Within each age group, R+ and ++ groups were similar in BW and BCS. Animals were kept at a constant feeding level and treated with 30-mg FGA sponges (Sincropart 30 mg, CEVA Salud Animal S.A., Barcelona, Spain) for 14 d to synchronize estrus. No eCG was used at withdrawal and no males were used for heat detection. Ovulation rate was recorded by laparoscopy during 3 consecutive cycles for each animal, always performed by the same observer, starting 6 d after sponge withdrawal and repeating 17 and 34 d later. Ewes were fasted for 24 h. Tranquilization was carried out using acepromazine, and local anesthesia was given by a subcutaneous injection of lidocaine in the place of insertion of the trocars as described by Cognié et al. (2007). Ovulation rate of each animal was the mean of the observations recorded in the ovulating cycles. To calculate the distribution of ovulations, a total of 257 and 292 ovulation records in young and adult ewes, respectively, were considered.

Exp. 2. OR, Partial Failure of Multiple Ovulations, and Offspring Birth Weight of Adult $FecX^R$ Heterozygous Ewes Treated with 2 Different Doses of eCG

This experiment was also carried out in the facilities of CITA. A total of 71 R+ and 48 ++ ewes were treated for 14 d with vaginal sponges containing 30 mg of FGA (Sincropart 30 mg, CEVA Salud Animal S.A.). At sponge withdrawal, ++ ewes received 480 IU of eCG intramuscularly (**++480**; Sincropart PMSG 6,000 UI, CEVA Salud Animal S.A.), the standard dose used for AI in Rasa Aragonesa ewes on farms, whereas R+ received either 240 IU (n = 35; **R+240**) or 480 IU (n = 36; **R+480**). Cervical insemination was carried out 44.5 ± 1 h after sponge withdrawal with semen of proven fertility diluted in skim milk and refrigerated at 15°C. Each ewe received 400×10^6 spermatozoa. Ovulation rate was recorded once in 75 ewes (R+240: n = 21; R+480: n = 21; ++480: n = 33 ewes) 8 d after sponge withdrawal, following the procedures described in Exp. 1.

One week before lambing, ewes were placed in individual pens. At lambing, offspring were immediately identified and weighed using a digital balance with a precision of 0.1 kg. Partial failure of multiple ovulations (**PFMO**) was calculated for each of the 3 groups as the difference between OR and LS of lambing ewes with ≥2 ovulations.

Exp. 3. Prolificacy of $FecX^R$ Heterozygous Ewes Identified by Genealogy, With and Without eCG Stimulation, in Flocks Enrolled in the Selection Program

The Use of PCR to Validate Genotype by Genealogy. To assess the suitability of the identifica-

tion method of *FecX*^{R} genotype by genealogy within the selection program, genotype assignment by genealogy was compared with PCR genotyping. For this purpose, only adult ewes from 4 elite flocks (n = 1,667 ewes) were genotyped for the presence of *FecX*^{R} allele (++, R+, RR). Blood samples were collected by jugular puncture using 5-mL vacuum tubes with EDTA. Genotypes for *BMP15* were determined by PCR using primers flanking the *FecX*^{R} polymorphism, as described previously by Martínez-Royo et al. (2009). Amplification by PCR was carried out under standard conditions, with PCR products separated by standard electrophoresis in a 3.5% TBE (0.045 *M* Tris-borate, 0.001 *M* EDTA) agarose gel. Amplification of the *FecX*^{R} and *FecX*$^{+}$ alleles by PCR produced fragments of 101 and 118 bp, respectively. On farms belonging to the selection program from 1985, all maternal ancestry is known, and from 1994 paternal ancestry is known when derived from AI or controlled mating. Since the discovery of *FecX*^{R} polymorphism in 2007, all rams were genotyped for *FecX*^{R} allele presence (Martínez-Royo et al., 2009), and since 2008, all *FecX*^{R} hemizygous sires have been under the control of the insemination center. For genotype identification by genealogy, daughters of *FecX*^{R}-genotyped hemizygous rams were assigned as R+, whereas daughters of wild-type or unknown rams were considered as ++ ewes due to the presumably low frequency of the *FecX*^{R} allele in the population. Daughters of known R+ ewes were not taken into account. Prolificacy estimates of R+ and ++ genotypes identified by PCR on these farms, treated or untreated with FGA + eCG, were compared with prolificacy estimates when genotype assignment was made only by genealogic information, to validate further estimates of prolificacy of both genotypes assigned by genealogy in flocks.

Prolificacy of FecX^{R} Heterozygous Ewes, With and Without eCG Stimulation, in Flocks Enrolled in the Selection Program. Prolificacy of *FecX*^{R} heterozygous adult ewes, treated or untreated with eCG, was investigated in all the flocks in the selection program. Genotype assignation by genealogy was made as described above. Because the first lambing of a known heterozygous *FecX*^{R} ewe was recorded in 1998, only data from this year onward were analyzed. A total of 668,674 lambing records, collected from 1998 to 2008 from these Rasa Aragonesa controlled flocks, were analyzed to calculate LS for heterozygous *FecX*^{R} and noncarrier ewes. Within each genotype, lambing records were divided into 2 groups, depending on whether mating was carried out without hormonal treatments (++: n = 599,160; R+: n = 6,593) or after the application of FGA sponges and 480 IU of eCG (++: n = 62,055; R+: n = 866).

Statistical Analysis

Differences among lots in OR, prolificacy, PFMO, and variables expressed as percentages were analyzed by GLM for categorical variables using the CATMOD procedure (SAS Inst. Inc., Cary, NC). Differences among lots in lamb birth weight were assessed by 1-way ANOVA using the GLM procedure of SAS and the Bonferroni-adjusted LSD test. Because significant differences were found among genotypes in prolificacy, a full factorial fixed model including genotype and prolificacy was built to test for differences in lamb birth weight attributable only to genotype. The level of significance was set at $P \leq 0.05$, whereas P-values > 0.05 but ≤ 0.10 were considered trends.

RESULTS

Natural OR of Young and Adult FecX^{R} Heterozygous Ewes (Exp. 1)

The mean OR and the distribution of the number of ovulations in young and adult R+ and ++ ewes are shown in Table 1. The percentage of animals ovulating in at least 1 of the 3 cycles was close to 100%, in both young and adult ewes, with no differences among genotypes ($P \geq 0.91$). In young ewes, OR was 0.44 points greater in R+ than in ++ ewes, mainly due to an increase in twin ovulations (+50.5%; $P < 0.01$), because triple ovulations were not increased (+1.9%; $P = 0.78$). In adult ewes, OR was +0.63 points greater in R+ than in ++ ewes because of the increase in double (+36.4%, $P < 0.01$) as well as in triple plus quadruple ovulations (+12.8%; $P < 0.05$).

OR, PFMO, and Offspring Birth Weight of Adult FecX^{R} Heterozygous Ewes Treated with 2 Different Doses of eCG (Exp. 2)

Ovulation rate and PFMO of R+ ewes treated with 480 or 240 IU of eCG are shown in Table 2. Ovulation rate after eCG stimulation was greater in the R+480 ewes, presenting 1.13 and 1.05 extra ovulations when compared with ++480 and R+240 ewes, respectively ($P < 0.01$ for both). In R+480 ewes, the predominant type of ovulation was quadruple or higher-order ovulation (57.1%), greater than in the ++480 (+32.9%; $P < 0.05$) and R+240 (+33.3%; $P < 0.05$) groups. No differences in OR were found between R+240 and ++480 groups ($P = 0.80$), even though triple ovulation, the predominant type in the R+240 group (47.6%), was greater in the R+240 than in the ++480 group (+26.4%; $P < 0.05$). The different ranges in OR in both groups (1 to 4 in R+240 vs. 1 to 10 in ++480 groups; Table 2) could explain this difference. In this way, ewes ovulating more than 4 ova did increase the mean OR of the ++480 group up to a value similar to that of R+240 group, despite its decreased percentage of triple ovulations ($P < 0.05$). In ++480 ewes, the predominant type of ovulation was double ovulation (42.4%), greater than in the R+480 group (+33.4%; $P < 0.01$).

Partial failure of multiple ovulations was 1.85 in R+480, greater than in the R+240 and ++480 groups

Table 1. Natural ovulation rate (OR) in young (311 ± 11 d) and adult (683 ± 37 d) Rasa Aragonesa ewes heterozygous for the $FecX^R$ allele or homozygous for the wild-type allele (least squares means ± SEM, range, and distribution of the number of ovulations, %)[1]

	Genotype × age			
Item	R+ young[2]	++ young[3]	R+ adult[4]	++ adult[5]
No. of ewes	91	20	84	19
Ewes ovulating, %	91.2[a]	95.0[a]	98.8[a]	100.0[a]
OR[6]	1.60 ± 0.04[a]	1.16 ± 0.11[b]	1.99 ± 0.04[a]	1.36 ± 0.09[b]
OR range	1 to 3	1 to 2	1 to 4	1 to 3
Type of ovulation, %				
Single	38.9[a]	91.3[b]	15.5[a]	64.8[b]
Double	59.2[a]	8.7[b]	69.7[a]	33.3[b]
≥Triple	1.9[a]	0.0[a]	14.7[c]	1.9[d]

In a row, within each age group, values without a common superscript differ at [a,b]$P < 0.01$; [c,d]$P < 0.05$.

[1]The means of 3 cycles are presented.

[2]$FecX^R$ heterozygous young ewes.

[3]Wild-type young ewes.

[4]$FecX^R$ heterozygous adult ewes.

[5]Wild-type adult ewes.

[6]Corrected means for a BW of 42.5 kg in young and 47.4 kg in adult ewes.

(1.15 and 0.77; $P = 0.13$ and $P < 0.05$, respectively). No differences in PFMO were found between the R+240 and ++480 groups ($P = 0.29$).

Birth weights of lambs born from adult ewes are shown in Table 3. In the R+480 group, birth weight was lighter when compared with the ++480 (−0.57 kg; $P < 0.05$) or R+240 group (−0.42 kg; $P = 0.08$). Nevertheless, when lambs of the same type of birth were compared, no differences between groups were observed ($P \geq 0.10$).

Prolificacy of $FecX^R$ Heterozygous Ewes Identified by Genealogy, With and Without eCG Stimulation, in Flocks Enrolled in the Selection Program (Exp. 3)

The Use of PCR to Validate Genotype Assignation by Genealogy. When comparing the genotype results assigned by genealogy with those determined by PCR genotyping, 97.8% (83.2% ++ and 14.6% R+) of ewes were correctly classified by geneal-

Table 2. Ovulation rate (OR; means ± SEM, range, and distribution, %) and partial failure of multiple ovulations (PFMO; means ± SEM) in adult ewes, heterozygous for $FecX^R$ allele or homozygous for the wild-type allele, treated with fluorogestone acetate and 480 or 240 IU of eCG[1,2]

	Genotype × treatment		
Item	R+480[3]	R+240[4]	++480[5]
No. of ewes	21	21	33
OR	3.95 ± 0.30[a]	2.90 ± 0.18[b]	2.82 ± 0.29[b]
OR range	2 to 7	1 to 4	1 to 10
Type of ovulation, %			
Single	0.0[c]	4.8[cd]	12.1[d]
Double	9.5[b]	23.8[ab]	42.4[a]
Triple	33.3[cd]	47.6[c]	21.2[d]
≥Quadruple	57.1[c]	23.8[d]	24.2[d]
PFMO[6]	1.85 ± 0.41 (13)[d]	1.15 ± 0.25 (13)[cd]	0.77 ± 0.28 (13)[c]

Within a row, values differ at [a,b]$P < 0.01$; [c,d]$P < 0.05$.

[1]Fluorogestone acetate: Sincropart 30 mg, CEVA Salud Animal S.A., Barcelona, Spain.

[2]Equine chorionic gonadotropin: Sincropart PMSG 6,000 IU, CEVA Salud Animal S.A.

[3]$FecX^R$ heterozygous adult ewes treated with 480 IU of eCG.

[4]$FecX^R$ heterozygous adult ewes treated with 240 IU of eCG.

[5]Wild-type adult ewes treated with 480 IU of eCG.

[6]PFMO = difference between OR and litter size of lambing ewes with ≥2 ovulations (number of ewes is shown in parentheses).

Table 3. Birth weight (kg) of lambs born from adult ewes, heterozygous for the *FecX*^{R} allele or homozygous for the wild-type allele, treated with fluorogestone acetate and 480 or 240 IU of eCG (least squares means ± SEM)[1,2,3]

	Genotype × treatment		
Type of birth	R+480[4]	R+240[5]	++480[6]
Overall	3.09 ± 0.17 (53)[bd]	3.51 ± 0.19 (35)[c]	3.66 ± 0.14 (33)[a]
Singletons	4.27 ± 0.29 (3)[a]	4.21 ± 0.16 (10)[a]	4.33 ± 0.20 (6)[a]
Twins	3.26 ± 0.15 (22)[a]	3.01 ± 0.18 (16)[a]	3.40 ± 0.16 (20)[a]
≥Triplets	2.40 ± 0.13 (28)[a]	2.54 ± 0.12 (9)[a]	2.90 ± 0.23 (7)[a]

Within a row, least squares means without a common superscript differ at [a,b]$P < 0.05$; [c,d]$P < 0.1$.

[1]Number of lambs is shown in parentheses.

[2]Fluorogestone acetate: Sincropart 30 mg, CEVA Salud Animal S.A., Barcelona, Spain.

[3]Equine chorionic gonadotropin: Sincropart PMSG 6,000 IU, CEVA Salud Animal S.A.

[4]*FecX*^{R} heterozygous adult ewes treated with 480 IU of eCG.

[5]*FecX*^{R} heterozygous adult ewes treated with 240 IU of eCG.

[6]Wild-type adult ewes treated with 480 IU of eCG.

ogy (Table 4). The percentage of R+ ewes in these farms, determined by PCR, was 16.3% (n = 272), from which 10.3% (n = 28) were misclassified as ++ ewes based on progeny data. Likewise, 0.57% of ++ ewes (8 out of 1,395) were erroneously assigned as R+ by genealogy. Similar percentages ($P = 0.86$) were obtained when expressed in terms of lambing records, with 97.9% (85.6% ++ and 12.3% R+) of lambing records coming from correctly classified ewes.

To assess the effect of genealogy-induced misclassification on prolificacy data, the mean LS of R+ and ++ ewes was calculated taking into account only genealogy assignation or based on PCR genotyping. Very close mean LS estimates of *FecX*^{R} carrier and wild-type ewes, with or without eCG treatment, were obtained ($P \geq 0.64$). In fact, only prolificacy of the untreated ++ group was 0.01 lambs born per lambing ewe less based on genealogy records (data not shown).

Prolificacy of FecX^R Heterozygous Ewes, With and Without eCG Stimulation, in Flocks Enrolled in the Selection Program. Prolificacy and LS distribution of *FecX*^{R} heterozygous Rasa Aragonesa adult ewes, treated or untreated with eCG, are shown in Table 5. On farms, prolificacy recorded in untreated R+ ewes was greater than in ++ ewes ($P < 0.0001$), resulting in 0.35 extra lambs per lambing ewe. This greater prolificacy was mainly due to an increase in twin births (+15.9%; $P < 0.0001$), in addition to triplet or higher-order births (+9.2%; $P < 0.0001$).

When ewes were treated with FGA + 480 IU eCG, prolificacy was also greater in R+ than in ++ ewes, resulting in 0.30 extra lambs per lambing ewe ($P < 0.0001$) because of the increase in twin (+6.0%; $P < 0.001$) and in triplet and higher-order births (+10.4%; triplets and quadruplets: $P < 0.0001$; quintuplets: $P < 0.01$).

Heterozygous ewes for *FecX*^{R} allele and ++ ewes displayed different increases in prolificacy after eCG stimulation (+0.16 and +0.21 extra lambs born, respectively; $P < 0.0001$ for both).

DISCUSSION

The polymorphism described in the *BMP15* gene of the Rasa Aragonesa sheep breed (*FecX*^{R}; Martínez-Royo et al., 2008) produces an increase in OR of +0.44 and +0.63 in young and adult ewes, respectively. This increase is less than those found for similar mutations

Table 4. Classification table comparing the identification of the *FecX*^{R} heterozygous ewes and wild-type ewes by genealogy or by PCR genotyping[1,2,3]

Genotype			
Genealogy	PCR	Ewes,[4] %	Lambing records,[4] %
++	++	83.2 (1,387)	85.6 (9,364)
R+	R+	14.6 (244)	12.3 (1,352)
++	R+	1.7 (28)	1.5 (163)
R+	++	0.5 (8)	0.6 (66)

[1]R+: *FecX*^{R} heterozygous ewes.

[2]++: wild-type ewes.

[3]Daughters of *FecX*^{R} genotyped hemizygous rams were assigned as R+ and daughters of wild-type or unknown rams were considered as ++.

[4]Within each row, percentages of the total of ewes (1,667) or lambing records (10,945) and number of ewes or lambing records (in parentheses) are shown.

Table 5. Mean prolificacy and litter size distribution (%) of adult Rasa Aragonesa ewes heterozygous for $FecX^R$ or homozygous for the wild-type allele, assigned by genealogy, untreated or treated with fluorogestone acetate and 480 IU of eCG[1,2,3,4]

	Genotype × treatment			
Item	++0[5]	R+0[6]	++480[7]	R+480[8]
Lambing records	599,160	6,593	62,055	866
Prolificacy	1.34[a]	1.69[b]	1.55[c]	1.85[d]
LS distribution, %				
Singletons	67.7[a]	42.5[b]	52.2[c]	35.7[d]
Twins	31.0[a]	46.9[b]	41.1[c]	47.1[b,d]
Triplets	1.3[a]	9.7[c]	6.0[b]	14.1[d]
Quadruplets	0.054[a]	0.77[b]	0.63[b]	2.8[c]
Quintuplets	0.0042[b]	0.091[c,d]	0.066[c]	0.35[d]

[a–d]Within a row, values without a common superscript differ at prolificacy, singletons, quadruplets: $P < 0.0001$. Twins, triplets: $P < 0.0001$; except [c,d]$P < 0.001$. Quintuplets: $P < 0.0001$; except [c,d]$P < 0.01$.

[1]Data recorded in flocks involved in the selection program during 12 yr (1998 to 2010).

[2]Daughters of $FecX^R$ genotyped hemizygous rams were assigned as R+ and daughters of wild-type or unknown rams were considered as ++.

[3]Fluorogestone acetate: Sincropart 30 mg, CEVA Salud Animal S.A., Barcelona, Spain.

[4]Equine chorionic gonadotropin: Sincropart PMSG 6,000 IU, CEVA Salud Animal S.A.

[5]Wild-type ewes untreated with eCG.

[6]$FecX^R$ heterozygous ewes untreated with eCG.

[7]Wild-type ewes treated with 480 IU of eCG.

[8]$FecX^R$ heterozygous ewes treated with 480 IU of eCG.

in the *BMP15* gene in other sheep breeds. The Galway mutation ($FecX^G$) in F700-Belclare and Cambridge breeds increases OR by +0.62 in ewe lambs and +0.72 in adults (Hanrahan et al., 2004). Conversely, the Belclare ($FecX^B$) mutation in the F700-Belclare breed, and Inverdale ($FecX^I$) and Hanna ($FecX^H$) mutations in Romney, all increase OR by 1.0 (Galloway et al., 2000; Hanrahan et al., 2004; Davis, 2005). The $FecX^L$ mutation produces about 1.5 extra ovulations, likely because of an additive effect of the phenotypic background of the Lacaune breed (Bodin et al., 2007). Other polymorphisms in *GDF9* and *BMP15* genes have been described recently in the Moghani and Ghezel breeds (Barzegari et al., 2010), as well as in the Barbarine breed (Vacca et al., 2010), though their effects still are not well known. In both young and adult Rasa Aragonesa ewes, this increase in OR is mainly due to an increase in double ovulations, making the use of the $FecX^R$ polymorphism suitable for application in commercial farms. This is the first mutation in the *BMP15* gene described in a Mediterranean breed, whose natural OR and prolificacy are between 15 to 40% less than that of other breeds in which mutations in *BMP15* have been found. This decreased natural OR could explain the smaller increase in OR and prolificacy produced by the $FecX^R$ mutation in this breed. This fact makes the introgression of this mutation into other Mediterranean breeds with similarly reduced OR and which are exploited under similar conditions of interest, such as some breeds from the north of Africa or from other countries where sheep are reared under semiextensive conditions.

One of the main objectives of this study was to assess the response of R+ ewes to eCG for later application on farms because hormonal treatments are widely used in systems such as 3 lambings/2 yr to induce out-of-season reproduction in breeds oriented to meat production. Although it has been demonstrated that prolific sheep are more sensitive to PMSG (Bindon et al., 1986), it appears that there is no previous literature dealing with the response of *BMP15*-mutated sheep to this hormone. In mutated *Fec* gene ewes, many authors have described increased FSH sensitivity and precocious LH receptor expression in antral follicles from these ewes (Fabre et al., 2006), whereas others have demonstrated only an earlier acquisition of LH responsiveness by granulosa cells, but not an increase in FSH sensitivity (McNatty et al., 2009). In our study, the OR of untreated R+ ewes was 0.63 ova greater than untreated ++ ewes. This advantage increased to 1.13 ova when ewes received the standard dose of 480 IU eCG. This difference in the response to eCG could not be explained by the present work. Possible explanations could be either a greater in vivo sensitivity to gonadotropins or a greater population of gonadotropin-dependent follicles because it was demonstrated in sheep that PMSG-induced OR is significantly correlated with the number of healthy follicles from 0.8 to 2.0 mm in diameter (Driancourt, 1987). When 480 IU eCG was used, this extremely high OR was also accompanied by a greater PFMO. On the contrary, when the dose was halved (240 IU), a more suitable OR and a reduced PFMO were achieved. In fact, OR and PFMO of R+ ewes stimulated with 240 IU were very close to those recorded in the wild-type ewes stimulated with 480 IU of eCG. Whereas this is the first report of *BMP15*-mutant animal response to eCG stimulation, our results demonstrate a similar response to that reported with the Booroola genotype, a mutation in the type 1B receptor of BMP (*BMPR-*

1B), where the OR response to doses ≤750 IU of eCG was significantly different between heterozygous carrier and homozygous noncarrier ewes (Kelly et al., 1983; Gootwine et al., 1993). We found similar results in nulliparous Rasa Aragonesa ewes at the beginning of the anestrus season (February; Lahoz et al., 2009), in agreement with previous studies showing that prepubertal ewe lambs carrying the Booroola mutation presented increased sensitivity to PMSG when compared with noncarrier ewe lambs (Bindon et al., 1986). In conclusion, our results confirm that *FecX*^{R} heterozygous ewes present a greater response to eCG. Therefore, it may be beneficial to halve the standard dose used on farms in this breed to avoid increased PFMO. However, AI trials on farms would be necessary before recommending halving of the eCG dose in R+ ewes.

No differences were observed in birth weight among lambs of the same birth type between heterozygous or wild-type ewes. Hence, the lighter BW observed in lambs born from R+ ewes were only due to their greater prolificacy. Differences between genotypes in birth weight is of concern as lighter birth weights contribute to reduced perinatal survival (Hinch et al., 1985; Owens et al., 1985). It appears that no published data concerning birth weight of lambs from ewes carrying any mutation in *BMP15* are available. Several studies concerning the effects of the Booroola mutation (*FecB*) on the BW of lambs at birth have been performed, with discrepant results (Fogarty, 2009); in this way, in Garole (*FecB* fixed) × Malpura crossbred sheep, the birth weight of BB and B+ lambs was less than that of noncarriers (Kumar et al., 2008). In Booroola-Assaf crosses, BB ewe lambs were significantly lighter at birth than ++, but also lighter than B+ ewe lambs. Moreover, a lighter birth weight was observed in ewe lambs born to BB compared with B+ or ++ dams (Gootwine et al., 2006). Conversely, there was no significant effect of maternal genotype on birth weight in the Mérinos d'Arles breed (Abella et al., 2005). In these 3 reports, LS differences among genotypes were taken into account when analyzing the effect of the genotype on the BW at birth. Hence, the effect of the Booroola mutation on birth weight, corrected for LS, may depend on the breed where it is introgressed. It remains to be seen whether similar results will be discovered with the *BMP15* mutations where homozygous carrier ewes are sterile.

In the present work, PCR validation was used with 2 main objectives: to validate prolificacy data recorded in flocks and to validate the suitability of identification of *FecX*^{R} heterozygous ewes on farms by genealogy assignation. We have shown that misclassification induced by genealogy assignation was rare. Genotyping demonstrated that 3.3% of ewes classified as R+ by genealogy were in fact ++, and 2.0% of ewes classified as ++ were confirmed as R+ after genotyping. On these farms, because only a small percentage of ewes are inseminated while the remainder (the vast majority) are naturally mated to rams at the same time, once an error of parental assignation occurs, the probability of assigning lambs from inseminated ewes to naturally mated ewes is very great, whereas the probability of assigning lambs from naturally mated ewes to inseminated ewes is negligible. Under these circumstances, the 3.3% of lambs misclassified as R+ would arise from paternity error after inseminations with R sires, whereas these 2% unidentified R+ ewes would mainly reflect the R+ ewes born to R rams present on farms, or to hidden R+ ewes, because the daughters of known R+ ewes have been discarded in this work. Regardless, misclassification due to incorrect genotype assignation by genealogy did not affect mean prolificacy estimates in a population with 16.3% of *FecX*^{R} heterozygous ewes. When considering all of the flocks, where the frequency of *FecX*^{R} ewes may be close to 2.96% (data not shown), the error induced by misclassification would presumably be even less. Thus, the prolificacy estimates of eCG-treated or untreated ewes of both genotypes reported here, obtained on farms in which genotypes were assigned by genealogy, can be considered reliable. Furthermore, in a recent study carried out in this breed, no significant differences in prolificacy estimates were found between noncarrier ewes born to *FecX*^{R} genotyped rams and ewes assumed as noncarriers born to unknown rams (−0.031 lambs/lambing ewe), which indicates that the percentage of unknown R+ ewes in flocks is minimal (Jurado et al., 2008). This fact could be explained by the small percentage of R rams serving in flocks (<7%) detected by PCR genotyping in 2007 (Martínez-Royo et al., 2009). The frequency of R rams serving on farms is low because farmers keep sires from AI as replacement animals. Because *FecX*^{R} is located in the X chromosome, these rams do not receive the mutated allele unless the mother was heterozygous for *FecX*^{R}. As stated above, the percentage of unknown R+ ewes is negligible. Thus, assignation of *FecX*^{R} heterozygotes through genealogy on farms has proven to be reliable, as demonstrated by similar results found after PCR identification. This technique is simpler and less expensive for on-farm application and allows the spread of the polymorphism in a controlled way due to the fact that *FecX*^{R} polymorphism is X-linked, and therefore, it could be spread from *FecX*^{R} hemizygous rams to their daughters. In conclusion, identification of heterozygous ewes on farms by genealogy assignation may provide an effective and simple method of identification once *FecX*^{R} hemizygous rams and heterozygous ewes have been previously identified.

Prolificacy recorded on farms in heterozygous *FecX*^{R} ewes, treated or not with eCG, reflects the increase in OR observed at our experimental center. In untreated ewes, the presence of the *FecX*^{R} allele increases prolificacy by 0.35 extra lambs per lambing ewe, similar to the previous estimation (+0.32) reported by Jurado et al. (2008) in this breed. These results are slightly less than those described for similar polymorphisms in *BMP15*.

One copy of the Inverdale ($FecX^I$) or Hanna ($FecX^H$) allele increases LS by about 0.6 lambs per lambing ewe in the Romney breed (Davis, 2005), and the Galway ($FecX^G$) allele increases prolificacy by 0.55 in the Small Tailed Han breed (Chu et al., 2007). A similar difference in prolificacy (0.30) was observed between $FecX^R$ and wild-type ewes under eCG stimulation. The stimulation with eCG produced 0.21 extra lambs born in wild-type ewes, compared with only 0.16 in carrier ewes. Concerning the distribution of the type of lambing, R+ ewes receiving eCG treatment did not demonstrate an increase in the percentage of twin births. The observed increment in prolificacy was only due to increased percentages of triple and higher-order births. Our results confirm those of Bodin and Elsen (1989) showing that a common pattern of distribution occurs independently of breed. According to this pattern, the percentage of twin births increases with prolificacy reaching a threshold, and from this point forward starts decreasing. The increased percentages of triplet and higher-order births under eCG treatment observed on farms reinforces the results obtained at our experimental center and should be taken into account. Triple and higher-order births are problematic because of their reduced lamb survival rates. In a work carried out in New Zealand it was reported that lamb survival in intensive conditions was 0.90 for single, 0.85 for twins, 0.65 for triplets, and 0.55 for quadruplets (Amer et al., 1999). In Australia, the poorer lamb survival from Booroola × Merino compared with control Merino ewes was attributed to reduced survival of triple and higher-order births because survival rates were similar for singles and twins. Industry exploitation of the advantages of the *FecB* carrier ewes is dependent upon reducing lamb losses, especially among higher-order births (Fogarty, 2009). Rasa Aragonesa is an autochthonous sheep breed oriented to meat production. In normal conditions, the breed displays a low prolificacy (1.34) accompanied by <1.5% triplet births as shown in this study. Therefore, to avoid increased lamb mortality in $FecX^R$ heterozygous ewes, either eCG dose should be reduced or their management conditions should be improved (e.g., increasing maternal nutrition during pregnancy or implementing supplemental supply of milk to the lambs).

A recent study carried out in the Rasa Aragonesa breed highlighted the importance of the number of lambs born per ewe on the efficiency and viability of these sheep farms (Pardos et al., 2008). In this way, it has been demonstrated that Rasa Aragonesa farms with >5% of $FecX^R$ ewes present better reproductive and economic results, selling 0.34 extra lambs per ewe per year when compared with farms using breeding programs without $FecX^R$ ewes (Pardos et al., 2010). Commercial use of the Inverdale mutation ($FecX^I$) in New Zealand was also shown to be highly beneficial (Amer et al., 1998). The observed trend to increase the proportion of carrier ewes in the population of Rasa Aragonesa ewes demonstrates the interest of farmers in the use of this allele. In fact, in 2009, 120 out of 203 flocks of Rasa Aragonesa ewes enrolled in the breeding program had $FecX^R$ ewes, with varying percentages ranging from 0.1 to 24.8% (J. L. Alabart, B. Lahoz, J. H. Calvo, E. Fantova, J. J. Jurado, A Martínez-Royo, and J. Folch, unpublished data). This variation in percentages of R+ ewes within flocks could be explained by an unintentional dissemination of the mutation when selecting dams and sires based on their breeding value, before the discovery of this polymorphism. In 2009, the percentage of $FecX^R$ heterozygous ewes in relation to the total population of Rasa Aragonesa ewes was 2.20%, reaching 2.96% when only considering flocks with both genotypes present. This percentage is expected to increase considerably, as shown by the fact that in 2009, 6,218 out of 10,776 (58%), and in 2010, 7,002 out of 10,833 (65%) total AI performed in flocks enrolled in the selection program were performed with semen from $FecX^R$ hemizygous rams (J. L. Alabart, B. Lahoz, J. H. Calvo, E. Fantova, J. J. Jurado, A Martínez-Royo, and J. Folch, unpublished data).

As has been described by Notter (2008), the optimal fecundity in most situations is well below the maximum attainable level, and can be targeted by combining selection within breeds using an expanding array of single-gene mutations affecting OR and LS. Our results suggest that it is possible to quickly improve prolificacy on farms by correctly using the $FecX^R$ allele, with a minor use of hormonal treatments. In the case of mutations in the *BMP15* gene, carrier animals must be maintained in a crossbreeding system to avoid the appearance of sterile ewes, which makes the use of these genes slightly more complex when compared with other major genes that do not produce sterility in homozygous ewes. On the other hand, because these mutations are linked to the X chromosome, the control of carrier ewes coming from a carrier male makes possible their identification and control by genealogy, as has been demonstrated in this work. The use of this marker-assisted selection using *BMP15* genotypic information allows the accurate identification of valuable young animals, which is of great economic interest for farmers. However, traditional polygenic selection also remains necessary while the possible existence of associated genes, undesirable characteristics, or inbreeding problems are being studied, as well as to preserve genetic variability.

In conclusion, in the present study the effect of the new $FecX^R$ allele in the ovine *BMP15* gene was quantified for the first time. Heterozygous $FecX^R$ ewes present 0.63 extra ovulations and 0.35 additional lambs per lambing adult ewe, increments less than those previously reported in more prolific breeds carrying other polymorphisms in the *BMP15* gene. These reproductive advantages are behind the increasing interest in the use of this polymorphism. Nevertheless, care must be taken in the application of eCG to ewes carrying this polymorphism, with the current results showing that a standard dose increases their prolificacy by only increasing triple and higher-order births.

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3.3. Publicación 2

Caracterización del pico preovulatorio de LH en ovejas Rasa Aragonesa portadoras o no del alelo *FecX*^{R} (ROA®) / Preovulatory LH surge of Rasa Aragonesa ewes carrying or not the *FecX*^{R} allele (ROA®).

Lahoz B., Folch J., Sánchez P., Alabart J.L.
ITEA-Información Técnica Económica Agraria 2013,
doi:http://dx.doi.org/10.12706/itea.2013.025

Caracterización del pico preovulatorio de LH en ovejas Rasa Aragonesa portadoras o no del alelo *FecX^R* (ROA®)

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Resumen

La presencia del alelo *FecX^R* en heterocigosis produce un incremento de la tasa de ovulación y prolificidad, así como una mayor respuesta a la eCG. Sin embargo, todavía se desconocen sus posibles efectos sobre la descarga preovulatoria de LH, lo cual es esencial para realizar la inseminación artificial en el momento óptimo. Con este objetivo, se extrajeron muestras sanguíneas a intervalos de 4-6 horas durante las 72 horas siguientes a la retirada de una esponja (FGA, 30 mg), de 50 ovejas adultas agrupadas en 5 lotes en función de su genotipo (R+ y ++; portadoras y no portadoras) y tratamiento hormonal (sin eCG, 240 ó 480 UI). Las concentraciones de LH se analizaron mediante un kit comercial ELISA. No se observó ninguna diferencia significativa en el pico preovulatorio de LH entre ovejas R+ y ++. La eCG adelantó la descarga preovulatoria de LH en ambos genotipos, sin diferencias entre ovejas R+ tratadas con 240 ó 480 UI. Estos resultados demuestran que la presencia de *FecX^R* no afecta al pico preovulatorio de LH, independientemente de la dosis utilizada. Por lo tanto, la dosis de eCG que se aplique a las ovejas R+ estará en función de las necesidades y posibilidades de la explotación para asumir una mayor prolificidad y multiplicidad de partos. En las ovejas ++ tratadas con eCG se recomienda seguir inseminando a las 54 ± 1 horas de retirar las esponjas, como se hace habitualmente, ya que se observó una buena sincronización del pico preovulatorio de LH a las 37 horas.

Palabras clave: BMP15, eCG, inseminación artificial, ovulación, PMSG, prolificidad.

Abstract
Preovulatory LH surge of Rasa Aragonesa ewes carrying or not the *FecX^R* allele (ROA®)

The presence of the *FecX^R* allele in heterozygosity (R+) increases ovulation rate and prolificacy in Rasa Aragonesa ewes, and leads to a greater response to eCG. However, their potential effects on preovulatory LH surge are still unknown, which is essential to establish the optimum time for artificial insemination. With this aim, blood samples were taken at 4-6 hours intervals during 72 hours after the removal of a 30 mg FGA sponge, from 50 adult ewes divided in 5 groups depending on their genotype (R+ and ++; carriers and non-carriers) and hormonal treatment (without eCG, 240 or 480 IU). The LH concentrations were analyzed using a commercial ELISA kit. There was no significant difference on the time of the preovulatory LH surge between R+ and ++ ewes. Treatment with eCG significantly advanced the preovulatory LH discharge in both genotypes, with no difference between R+ ewes treated with 240 or 480 IU. These results demonstrate that the presence of the *FecX^R* allele did not affect the preovulatory LH surge, regardless of eCG dose. Therefore, the dose of eCG that should be applied to R+ ewes could be a decision of the farmer depending on the needs and possibilities of the farm to manage

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DOI: http://dx.doi.org/10.12706/itea.2013.025

greater prolificacy and multiple births. In ++ ewes treated with eCG it is recommended to continue with the established protocol and inseminate 54 ± 1 hours after sponge withdrawal, as it is usually done, since a good synchronization of the preovulatory LH peak was observed at 37 hours.

Key words: Artificial insemination, BMP15, eCG, ovulation, PMSG, prolificacy.

Introducción

La Rasa Aragonesa es una raza autóctona mediterránea principalmente explotada en sistemas semi-extensivos orientados a la producción de cordero ligero. En las condiciones estructurales de Aragón, los resultados reproductivos son de vital importancia para la rentabilidad y viabilidad de estas explotaciones, siendo esencial el número de corderos vendidos por oveja (Pardos *et al*., 2008). Por ello, desde el año 1994, se está llevando a cabo un programa de selección por prolificidad en las ganaderías de esta raza. En 2007, se observó que determinados animales del programa presentaban una prolificidad muy elevada que no se correspondía con la esperada por una herencia poligénica clásica, lo que dio lugar al descubrimiento de un nuevo polimorfismo en el gen *BMP15* (alelo $FecX^R$), localizado en el cromosoma X, que produce un aumento de la prolificidad en portadoras heterocigotas (R+) y esterilidad en homocigotas (RR; Martínez-Royo *et al*., 2008). Este aumento de la prolificidad de las ovejas R+ (+0,35 corderos/parto) se debe a un aumento de su tasa de ovulación (+0,63 cuerpos lúteos/oveja; Lahoz *et al*., 2011). Dichas ventajas productivas han suscitado un creciente interés en las ganaderías por la introducción de esta variante génica, explotándose con el nombre comercial de ROA® (Rasa Oviaragón), cuyas ventajas económicas han sido recientemente cuantificadas (Pardos *et al*., 2010).

La Rasa Aragonesa es una raza con un anestro estacional moderado, presentando celos entre un 20 y 40% de las ovejas entre finales de invierno y principios de verano, siendo algo mayor el porcentaje de animales cíclicos determinado por progesterona (Sierra, 1969; Alonso de Miguel, 1979; Forcada *et al*., 1992; Folch *et al*., 1999). Como consecuencia, la producción de corderos y sus precios sufren fluctuaciones a lo largo del año, traduciéndose en menores precios en el primer semestre del año y mayores en el segundo. El porcentaje de corderos vendidos en el segundo semestre ha demostrado ser uno de los factores que más inciden en el balance económico de las explotaciones (Pardos *et al*., 2008; Fantova *et al*., 2011). Para mejorar la fertilidad y prolificidad durante la época de anestro estacional, así como para la utilización de la inseminación artificial (IA) a tiempo fijo durante todo el año dentro del programa de mejora genética, el uso de tratamientos hormonales, principalmente de esponjas intravaginales y eCG, es una práctica habitual en las ganaderías.

Se sabe que la aplicación de la dosis habitual de eCG (480 UI) a las ovejas portadoras de la mutación en heterocigosis produce un importante aumento en la tasa de ovulación que conlleva un aumento importante en los partos triples o de orden superior. Por el contrario, se ha demostrado que aplicando 240 UI a ovejas R+ se obtiene una tasa de ovulación similar a cuando se aplica 480 UI a ovejas no portadoras (++), lo que debe tenerse en cuenta por parte del ganadero en relación a su capacidad de gestión de los partos múltiples (Lahoz *et al*., 2011). Sin embargo, se desconocen los posibles efectos que puedan tener tanto el genotipo como la utilización de diferentes dosis de eCG sobre la aparición y agrupación de celos y, por tanto, si debe modificarse el momento de aplicar la IA. Según Jabbour y Evans (1991), la causa más común de fallo de la IA sería la asincronía entre la IA

y la ovulación. Es por tanto necesario estudiar si la presencia del alelo *FecX*^{R} afecta al momento y concentración de las ovulaciones, lo cual constituye el objetivo del presente trabajo. Para ello, se determinó la aparición del pico preovulatorio de LH en ovejas portadoras heterocigotas y no portadoras, tratadas o no con dos dosis diferentes de eCG.

Material y métodos

Todos los experimentos se llevaron a cabo de acuerdo a las recomendaciones de la Unión Europea (2003/65/CE) y normativa española (RD 1201/2005, BOE 252/34367-91) para el uso y cuidado de animales en experimentación. El procedimiento fue aprobado por el comité de ética del Centro de Investigación y Tecnología Agroalimentaria de Aragón (Número de registro: CITA 2011-08).

Animales y diseño experimental

El experimento se llevó a cabo en las instalaciones del CITA (Zaragoza, España) en noviembre de 2011 (estación sexual). Se utilizaron 50 ovejas adultas, multíparas y secas, con un intervalo al parto anterior superior a tres meses, de raza Rasa Aragonesa, siendo 30 de ellas portadoras heterocigotas del alelo *FecX*^{R} (R+), y 20 ovejas no portadoras (++). Los animales estaban mantenidos en el mismo rebaño en idénticas condiciones de explotación. Previamente al inicio del experimento los animales se dividieron en 5 lotes similares en peso y condición corporal (Russel *et al*., 1969), dependiendo del genotipo de *BMP15* y del tipo de tratamiento hormonal, tal y como se muestra en la tabla 1. Las diferencias en peso y condición corporal entre lotes no fueron estadísticamente significativas.

Tabla 1. Características de los lotes experimentales: número de ovejas, genotipo, tratamiento hormonal, peso y condición corporal
Table 1. Characteristics of the experimental groups: number of ewes, genotype, hormonal treatment, weight and body condition score

Lote	Nº ovejas	Genotipo	Dosis eCG[1]	Peso (kg)[2]	CC[2]
Sin eCG					
++ 0	10	++	–	52,3 ± 2,4	2,9 ± 0,1
R+ 0	10	R+	–	58,3 ± 2,0	2,8 ± 0,1
Con eCG					
++ 480	10	++	480 UI	51,5 ± 1,9	2,9 ± 0,1
R+ 480	10	R+	480 UI	58,0 ± 3,1	2,9 ± 0,1
R+ 240	10	R+	240 UI	56,9 ± 1,7	2,7 ± 0,1

++: Ovejas de genotipo salvaje.

R+: Ovejas heterocigotas para el alelo *FecX*^{R}.

[1] Sincropart® PMSG 6000 UI, CEVA Salud Animal SA (Barcelona, España). Todas las ovejas recibieron previamente una esponja de 30 mg de FGA (Sincropart® 30 mg; CEVA Salud Animal SA; Barcelona, España).

[2] medias ± error estándar.

CC: Condición corporal.

Tratamiento hormonal

Se insertó a las 50 ovejas el mismo día una esponja vaginal de 30 mg de FGA (Sincropart® 30 mg; CEVA Salud Animal SA; Barcelona, España), que permaneció durante 14 días. En el momento de la retirada, 10 ovejas ++ y 10 ovejas R+ no recibieron ningún tratamiento hormonal (lotes "++0" y "R+0", respectivamente). Las 30 ovejas restantes recibieron en ese mismo momento una única dosis de eCG (Sincropart® PMSG 6000 UI, CEVA Salud Animal SA; Barcelona, España) de la siguiente manera: 10 ovejas ++ y 10 ovejas R+ recibieron 480 UI de eCG (dosis estándar utilizada en IA en las ganaderías de esta raza; lotes "++480" y "R+480", respectivamente), y 10 ovejas R+ recibieron 240 UI de eCG (lote "R+240").

No se utilizaron machos para detección de celos con el fin de reproducir las condiciones de IA de las ganaderías, así como para evitar un posible adelanto de la descarga preovulatoria de LH y de la ovulación (Signoret y Cognié, 1975; Maxwell 1986; Romano *et al*., 2001).

Toma de muestras sanguíneas

Se tomaron muestras de sangre de todas las ovejas mediante punción yugular utilizando tubos con heparina de litio. La toma de muestras se inició en el momento de la retirada de la esponja de FGA (0 h), a intervalos de 6 horas durante las 24 horas siguientes y cada 4 horas desde las 24 hasta las 72 horas (17 muestras por animal). Inmediatamente tras la obtención, las muestras fueron centrifugadas durante 25 minutos a 2100 x g para obtener el plasma que fue congelado a -20ºC y almacenado hasta su posterior análisis de LH.

Determinación de la tasa de ovulación

Con el objetivo de verificar la ocurrencia de la ovulación, ésta se determinó en todos los animales por endoscopia siete días después de la retirada de la esponja, siguiendo la metodología descrita previamente (Lahoz *et al*., 2011).

Análisis hormonales

Las muestras se analizaron mediante un kit ELISA comercial (LH-DETECT, ReproPharm, INRA Tours, Nouzilly, France), siguiendo las instrucciones del fabricante. El límite de detección fue 0,3 ng/ml. Los coeficientes de variación intraanálisis e interanálisis fueron los siguientes: para un pool de 28,2 ng/ml, 7,4 y 10,9%; para un pool de 45,5 ng/ml, 6,7 y 10,4%, respectivamente. Las muestras con valores sospechosamente anómalos se volvieron a analizar.

Análisis estadístico

Para la caracterización de la descarga preovulatoria de LH se consideraron las siguientes variables: Su concentración máxima (ng/ml), tiempo al pico preovulatorio de LH, comienzo, final y duración del pico preovulatorio de LH (en horas tras la retirada de la esponja) y área del pico preovulatorio de LH [(ng/ml)•h].

El tiempo al pico preovulatorio de LH se calculó como las horas transcurridas desde la retirada de la esponja hasta el momento de máxima concentración de LH. La hora de comienzo del pico preovulatorio de LH se determinó como la de la toma anterior al pico de LH con una concentración por debajo del límite de detección más cercana al pico preovulatorio. De igual modo, la hora del final del pico preovulatorio de LH se determinó como la de la toma posterior al pico de LH con una concentración por debajo del límite de detección más cercana al pico preovulatorio de LH. La duración del pico preovulatorio de LH se calculó como la diferencia entre ambas horas. El área bajo el pico de LH se calculó mediante la regla trapezoidal.

Dichas variables, junto con el peso y la condición corporal, se analizaron mediante ANOVA,

utilizando el procedimiento MIXED del paquete estadístico SAS (SAS Institute Inc., 2011). El modelo incluyó el genotipo y la dosis de eCG jerarquizada al genotipo como efectos fijos. La normalidad de los residuos se comprobó con el test de Jarque-Bera (Procedimiento AUTOREG de SAS), aplicando la corrección para muestras finitas (Lawford, 2005). La falta de normalidad sólo se observó en la concentración máxima de LH. Mediante el procedimiento TRANSREG de SAS, se determinó que la transformación más conveniente para esta variable era la transformación logarítmica. La homogeneidad de las varianzas se comprobó mediante el test de Levene. La falta de homogeneidad se observó en las siguientes variables: horas al pico preovulatorio de LH, y comienzo y final del mismo. En dichas variables, la significación corregida por comparaciones múltiples de las diferencias entre las medias de mínimos cuadrados (LSMEANS) se determinó mediante el método MaxT con simulación y corrección de Satterthwaite para los grados de libertad (Westfall *et al*., 2011a). Para el resto de variables, se aplicó el ajuste por etapas de Shaffer con simulación (Shaffer, 1986; Westfall, 1997), incluyendo restricciones lógicas. En ambos casos se utilizó el comando LSMESTIMATE (Westfall *et al*., 2011b).

Las correlaciones se analizaron mediante el procedimiento CORR de SAS.

Resultados

Observación de las ovulaciones y valores anómalos de LH

Todas las ovejas ovularon, aunque en dos de ellas no se pudo determinar exactamente el número de cuerpos lúteos por la presencia de adherencias a nivel del ovario. Una oveja del lote ++0 fue eliminada del estudio ya que no presentó pico preovulatorio de LH, presentando una concentración máxima de 0,4 ng/ml a las 6 horas de la retirada de la esponja. Dicha oveja presentó dos cuerpos lúteos de aspecto más reciente comparado con el resto de ovejas. Una oveja del lote ++480 fue eliminada por presentar un pico débil de LH anormalmente retrasado (concentración máxima a las 60 horas de 8,76 ng/ml). En el momento de la endoscopia presentó dos cuerpos lúteos. Mientras que en la mayoría de ovejas las concentraciones basales de LH estuvieron por debajo del límite de detección (datos no presentados), una oveja del lote R+240 presentó una LH basal extremadamente alta (5,9 ± 0,6 ng/ml; media ± DS), y cinco cuerpos lúteos, por lo que también fue excluida del estudio (Figura 2). Todas las ovejas presentaron el pico preovulatorio de LH a partir de las 24 horas tras la retirada de la esponja.

Efecto del alelo $FecX^R$

No se observó ninguna diferencia significativa en ninguna de las variables estudiadas entre ovejas de los genotipos R+ y ++, independientemente de si fueron tratadas o no con eCG. Las concentraciones medias de LH no fueron significativamente diferentes entre los dos genotipos, y el pico preovulatorio de LH apareció en tiempos muy similares (Tabla 2, Figura 1).

Efecto del tratamiento con eCG

El tratamiento con eCG adelantó significativamente el pico preovulatorio de LH, su comienzo y finalización, en ambos genotipos, mientras que no tuvo ningún efecto sobre el resto de variables. Cuando no se aplicó eCG, el pico preovulatorio de LH se produjo a las 48,4 ± 2,7 h en las ovejas ++ y a las 52,8 ± 2,6 h en las ovejas R+ (NS).

En las ovejas ++, el tiempo transcurrido desde la retirada de la esponja hasta el pico preovulatorio de LH fue 11,1 horas menor en las ovejas tratadas con eCG ($P < 0,01$), comenzando y

Tabla 2. Características del pico preovulatorio de LH en ovejas portadoras heterocigotas del alelo *FecX*^{R} y no portadoras, tratadas o no con dos dosis diferentes de eCG (240 y 480 UI). Medias de mínimos cuadrados (LSMEANS) ± error estándar
Table 2. Characteristics of preovulatory LH surge in FecX^{R} *heterozygous carrier and non-carrier ewes, treated or not with two different doses of eCG (240 and 480 IU). Least squares means (LSMEANS) ± standard error*

Genotipo	++		R+			Significación (P <)	
Dosis de eCG (UI)	0	480	0	480	240	Genotipo	Dosis eCG*
Numero de ovejas	9	9	10	10	9	–	–
Tiempo al pico (h)	48,4 ± 2,7^{a}	37,3 ± 0,7^{b}	52,8 ± 2,6^{ac}	40,8 ± 2,2^{d}	40,9 ± 1,6^{b}	NS	0,001
Comienzo del pico (h)	43,6 ± 2,9^{a}	31,6 ± 0,8^{b}	45,6 ± 2,7^{c}	34,0 ± 1,8^{d}	35,6 ± 1,4^{d}	NS	0,001
Final del pico (h)	59,1 ± 2,8^{a}	46,2 ± 1,0^{b}	64,0 ± 2,6^{a}	50,4 ± 2,4^{b}	51,1 ± 1,6^{b}	NS	0,001
Duración del pico (h)	15,6 ± 0,4	14,7 ± 0,9	18,4 ± 0,9	16,4 ± 1,3	15,6 ± 0,8	0,1	NS
Conc. máxima (ng/ml)	51,0 ± 8,4	49,8 ± 5,9	40,3 ± 4,3	33,0 ± 4,5	45,5 ± 5,8	0,1	NS
Área del pico (ng/ml)·h	271,0 ± 31,0	265,4 ± 31,0	228,4 ± 29,4	204,9 ± 29,4	271,9 ± 31,0	NS	NS

Diferencias entre dosis de eCG sólo dentro de cada genotipo: Dentro de una misma fila, $^{a,b}P < 0,01$; $^{c,d}P < 0,05$.

++: Ovejas de genotipo salvaje.

R+: Ovejas heterocigotas para el alelo *FecX*^{R}.

* Dosis dentro de Genotipo. Sincropart® PMSG 6000 UI, CEVA Salud Animal SA (Barcelona, España).

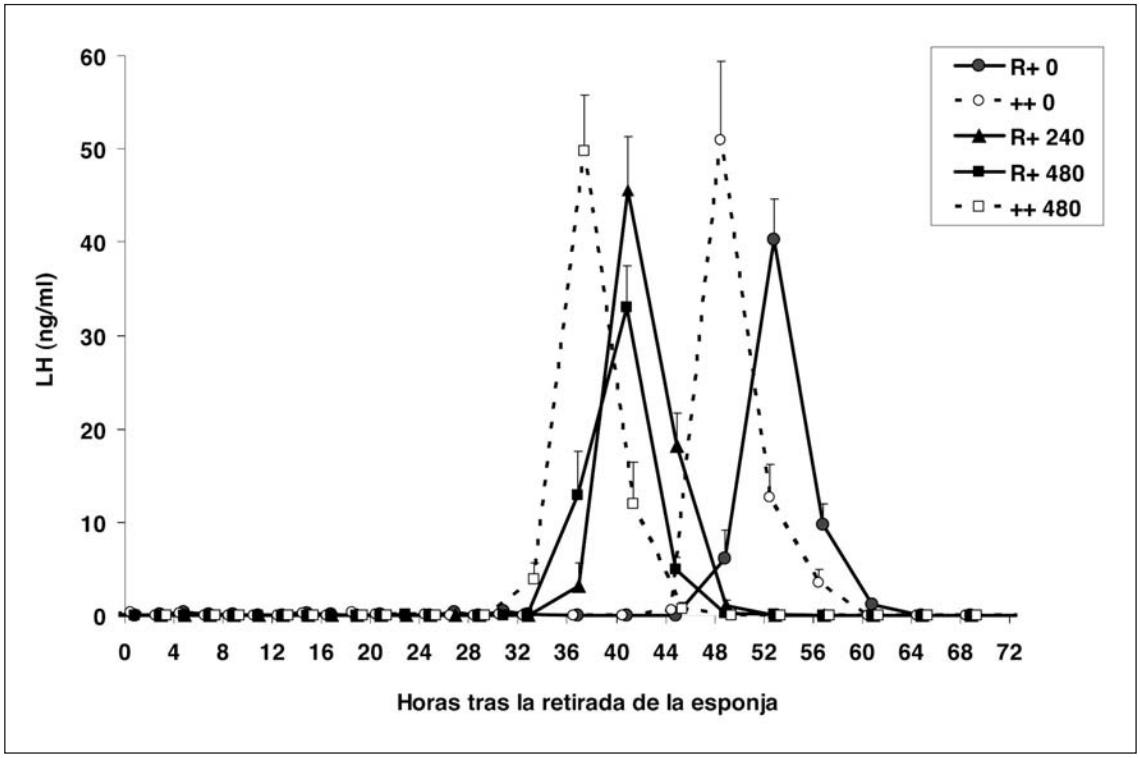

Figura 1. Pico preovulatorio de LH en ovejas portadoras heterocigotas del alelo *FecX*^{R} y no portadoras, tratadas o no con dos dosis diferentes de eCG (240 y 480 UI). Se han representado las medias de cada grupo.

++: Ovejas de genotipo salvaje.

R+: Ovejas heterocigotas para el alelo *FecX*^{R}.

Figure 1. Preovulatory LH surge in FecX^{R} *heterozygous carrier and non-carrier ewes, treated or not with two different doses of eCG (240 and 480 IU). Means of each group are shown.*

finalizando 12 y 12,9 horas antes, respectivamente (ambos P < 0,01), sin afectar a la duración, concentración máxima o área del mismo.

En las ovejas R+, la eCG adelantó el pico preovulatorio de LH respecto a las ovejas R+ no tratadas, tanto cuando se trataron con 480 UI (-12 h, P < 0,05), como con 240 UI (-11,9 h, P < 0,01). Tanto en las ovejas R+480 como en las R+240, la descarga preovulatoria de LH comenzó antes (-11,6 h y -10 h; ambos P < 0,05), y finalizó antes (-13,6 h y -12,9 h; ambos P < 0,01). No se observó ninguna diferencia significativa en ninguna de las variables estudiadas entre las ovejas R+ tratadas con 480 UI o con 240 UI de eCG.

Aunque la eCG adelantó la aparición del pico preovulatorio de LH en ambos genotipos, los picos de LH mostraron rangos diferentes, como puede verse en la figura 2. Cuando se aplicó la dosis estándar de 480 UI a las ovejas ++, se obtuvo una gran sincronización en el pico preovulatorio de LH, con un 90% de animales que lo presentaron entre las 36 y 40 horas tras la retirada. Cuando se aplicó la

eCG a las ovejas R+, se observó una mayor dispersión en la presentación de los picos preovulatorios de LH en comparación con las ovejas ++. No hubo diferencias entre aplicar 240 o 480 UI de eCG.

En todos los grupos se observó una correlación muy significativa entre la concentración máxima de LH y el área del pico preovulatorio de LH (+0,87; P < 0,001). En las ovejas R+ sin tratamiento, se observó una correlación positiva entre el área del pico preovulatorio de LH y la tasa de ovulación posterior (+0,83; P < 0,01), y una tendencia a la significación entre la concentración máxima de LH y la tasa de ovulación (+0,57; P < 0,1); Figura 3. No se observó ninguna correlación significativa entre la tasa de ovulación y ambas variables en ovejas ++, o en ambos genotipos cuando fueron tratados con eCG.

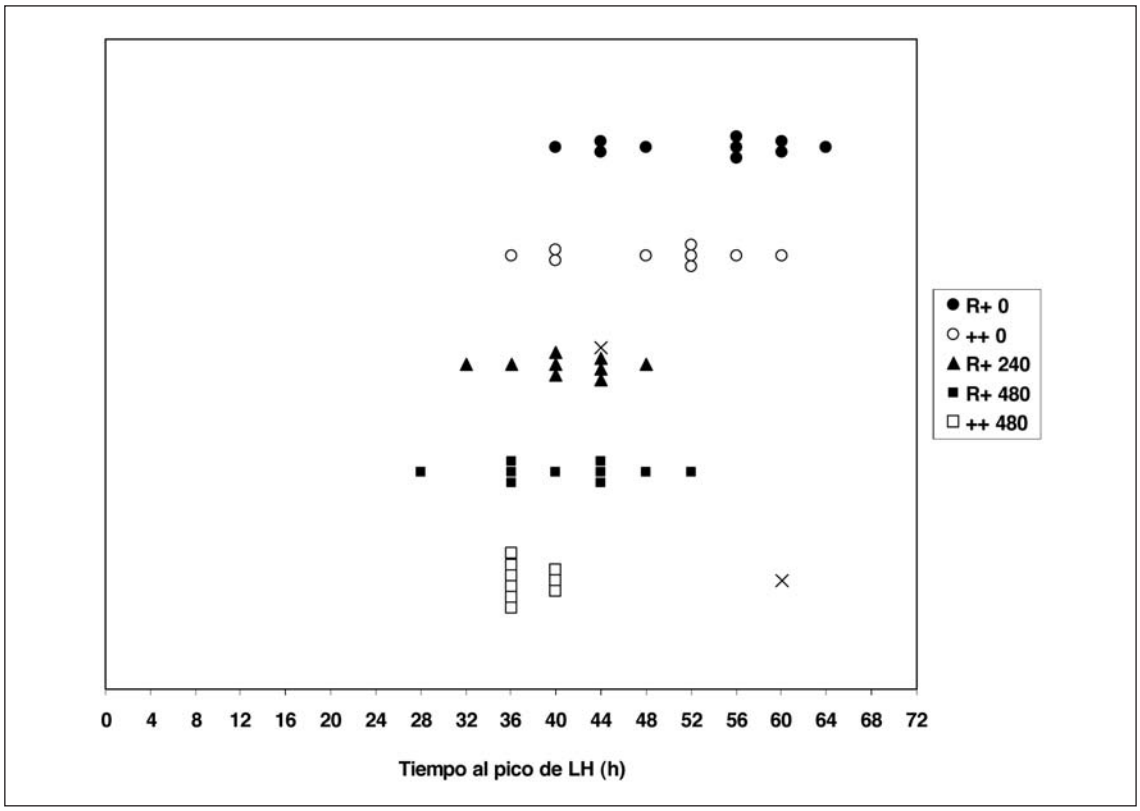

Figura 2. Sincronización de los picos preovulatorios de LH en cada uno de los lotes de ovejas portadoras heterocigotas del alelo *FecX*^{R} y no portadoras, tratadas o no con dos dosis diferentes de eCG (240 y 480 UI). Se han representado los valores individuales de cada animal (Dotplot).

El tiempo al pico de LH corresponde con las horas desde la retirada de las esponjas de 30 mg de FGA hasta el momento de máxima concentración de LH. Las ovejas excluidas del estudio por presentar valores anómalos se han representado con el símbolo "x".

Figure 2. Synchronization of preovulatory LH surges in experimental groups of FecX^{R} *heterozygous carrier and non-carrier ewes, treated or not with two different doses of eCG (240 and 480 IU). Individual values for each ewe are shown (Dotplot).*

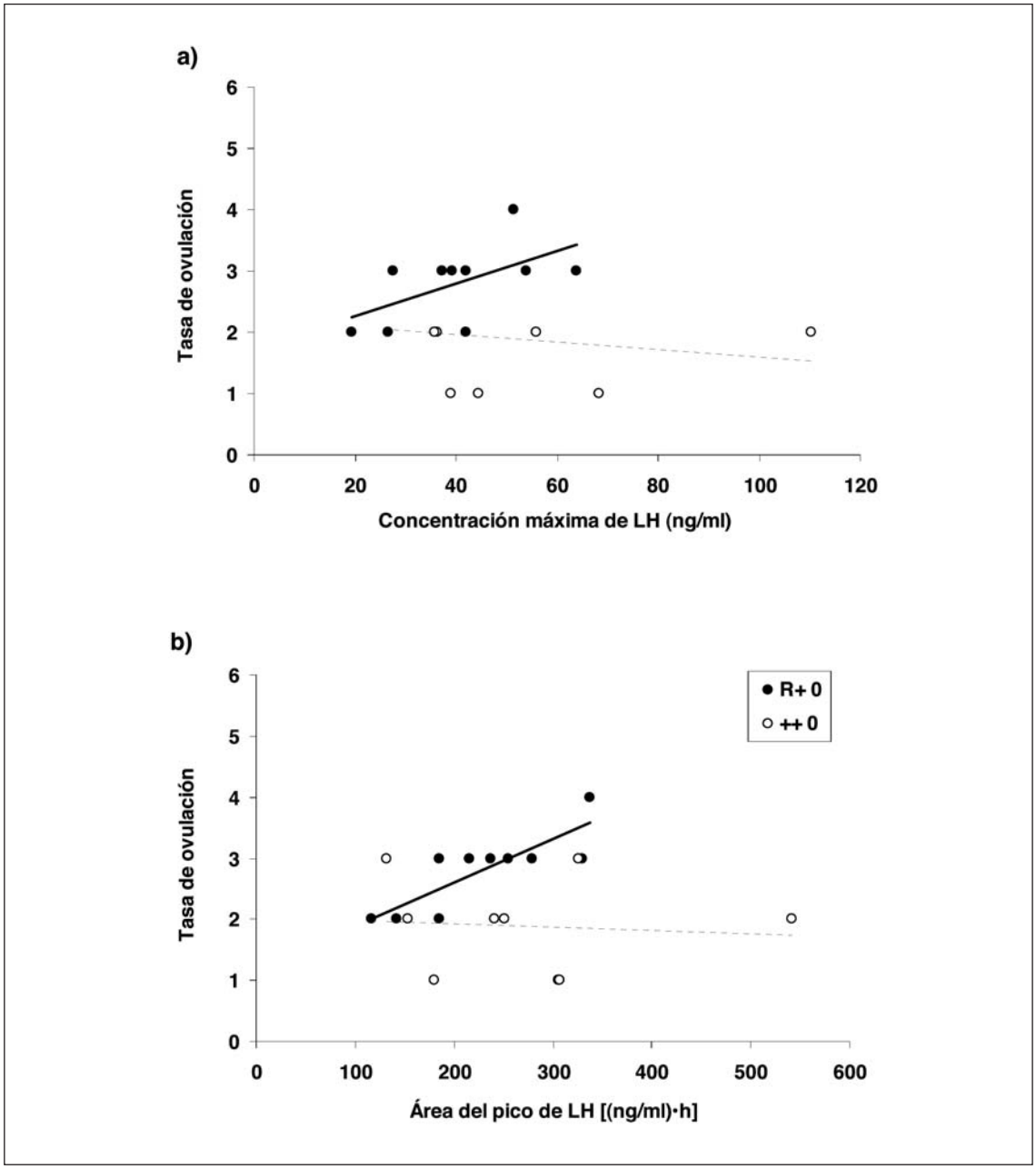

Figura 3. Correlación entre: a) la concentración máxima de LH y la tasa de ovulación; b) el área del pico preovulatorio de LH y la tasa de ovulación; en ovejas portadoras heterocigotas del alelo *FecX*^{R} (R+) y no portadoras (++) no tratadas hormonalmente.

Figure 3. Correlation between: a) the maximum concentration of LH and ovulation rate, b) the area of the preovulatory LH surge and ovulation rate, in FecX^{R} *heterozygous carrier and non-carrier ewes, hormonally untreated.*

Discusión

Nuestros resultados muestran que la presencia del alelo *FecX*^{R} no afecta al pico preovulatorio de LH en ovejas Rasa Aragonesa. Estos resultados concurren con los observados por otros autores que han estudiado mutaciones similares localizadas en genes pertenecientes a la familia del TGF-β, como son *BMP15* y su receptor *BMPR-1B*. Así, Shackell *et al*. (1993) observaron que las ovejas de raza Inverdale heterocigotas portadoras de una mutación similar (*FecX*^{I}) en el mismo gen (*BMP15)* no presentaban diferencias en relación a los niveles plasmáticos de P4, inhibina, o de gonadotropinas pituitarias en relación a ovejas no portadoras. De igual modo, Campbell *et al*. (2003) tampoco observaron diferencias endocrinas en ovejas Booroola homocigotas para esta mutación.

Por el contrario, en ovejas de raza Lacaune homocigotas para la mutación *FecL*^{L}, se han encontrado importantes diferencias endocrinas entre ovejas silvestres (++) y portadoras (LL). En concreto, se ha observado que el pico preovulatorio de LH estaba adelantado en las ovejas portadoras de la mutación (Drouilhet *et al*., 2010). Hay que tener en cuenta que, aunque esta mutación también incrementa la tasa de ovulación, no se trata de una mutación en el gen *BMP15*, como es el caso del polimorfismo hiperprolífico *FecX*^{L} también descrito en esta raza (Bodin *et al*., 2007), sino en otro gen recientemente identificado en el cromosoma 11. Además, estudios recientes parecen indicar que el incremento de la tasa de ovulación debido a *FecL*^{L} se produciría siguiendo una ruta fisiológica diferente a la que siguen los miembros de la superfamilia del TGF-β (Mansanet *et al*., 2012), por lo que no puede compararse directamente.

El tratamiento con esponjas de FGA y eCG en ovejas se aplica en Aragón (España) desde hace años con fines genéticos o comerciales (Folch *et al.*, 1979). Actualmente, es una práctica muy habitual. Sirva a modo de ejemplo que durante el año 2012, dentro del programa de selección de la UPRA-Grupo Pastores constituido por 204.360 ovejas (a 30/12/2012), se realizaron 9.835 tratamientos con esponja + eCG para realizar IA (datos cedidos por la Asociación). Ese mismo año, de las más de 400.000 ovejas que forman parte de la Cooperativa Carnes Oviaragón S.C.L., 35.588 (alrededor del 10%) recibieron un tratamiento de esponja + eCG para realizar monta natural. A pesar de ello, hasta donde nosotros conocemos no existen datos publicados sobre el pico preovulatorio de LH en estas condiciones, lo cual es importante de cara a futuras decisiones, especialmente a la hora de planificar la IA. En los estudios previos realizados en ovejas de esta raza se determinó la concentración del pico de LH cuando fueron tratadas sólo con una esponja de FGA (42,5 ng/ml; Alabart *et al*., 1988). En estudios posteriores se observó que aplicando un tratamiento de superestimulación con FSH/LH, o FSH/LH más GH, el tiempo al pico así como la concentración máxima fueron 32,0 h y 34,3 ng/ml, y 34,6 h y 27,1 ng/ml, respectivamente, algo inferiores a los observados en el presente estudio (Folch *et al*., 2001). En estudios anteriores realizados en condiciones similares (esponjas intravaginales de FGA y 500 UI de eCG) pero en ovejas de raza Manchega, se observó que el pico de LH apareció de media a las 37,1 h y las ovulaciones ocurrieron a las 64,3 horas de media (entre las 61 y 66 horas tras la retirada de la esponja), con un intervalo constante del celo al primer incremento de la concentración de LH (3,4 horas; López-Sebastian, 1991). Cumming *et al*. (1973), también observaron que en ovejas sincronizadas con progestágenos el intervalo entre el pico preovulatorio de LH y la ovulación era constante (entre 21 y 26 horas). Por lo tanto, a partir de nuestros datos podríamos estimar que el momento de ovulación en las ovejas Rasa Aragonesa tratadas con eCG estaría en torno a las 61-66 horas. Teniendo en cuenta

que la IA a tiempo fijo en las ganaderías se realiza a las 54 ± 1 h, y que el tiempo estimado de migración de los espermatozoides desde la vagina hasta el oviducto es de 8-10 horas (Hunter *et al*., 1980; Druart *et al*., 2009), nuestros resultados parecen indicar que el momento de IA que se viene utilizando en las ganaderías es adecuado.

La eCG adelantó la descarga preovulatoria de LH en todas las ovejas, tal y como ya describieron Cognié *et al*. (1970). Sin embargo, los patrones de agrupación de los picos preovulatorios de LH obtenidos en ambos genotipos fueron distintos. En las ovejas R+ tratadas con ambas dosis de eCG se obtuvo una menor sincronización que en las ovejas ++ tratadas. Las causas de dichas diferencias no pueden ser dilucidadas en el presente trabajo; tal vez pudieran estar relacionadas con diferencias en su foliculogénesis y tasa de ovulación.

En resumen, la respuesta de LH a la aplicación de eCG fue similar en ambos genotipos, ya que no se observó ninguna diferencia significativa en ninguna de las variables estudiadas relacionadas con la LH entre ovejas portadoras o no. Por lo tanto, en el caso de realizar IA a ovejas R+, se harían extensivas las recomendaciones sobre el momento de IA de las ovejas ++. Tampoco se encontraron diferencias en relación a la dosis de eCG aplicada cuando se compararon los resultados entre las ovejas R+ tratadas con 480 UI o con 240 UI de eCG. Estos resultados son importantes porque posibilitan la elección de la dosis de eCG a aplicar a las ovejas R+ en función de la capacidad de gestión de los partos múltiples del ganadero, sin afectar a la descarga preovulatoria de LH, y por tanto, a los resultados de la IA. Con una dosis reducida de eCG en las ovejas R+ (240 UI), se obtendrá una tasa de ovulación similar a la de ovejas ++ tratadas con una dosis estándar de 480 UI, sin que esto afecte a su pico preovulatorio de LH. Si se aplicase una dosis estándar de 480 UI de eCG a las R+, se lograría una mayor prolificidad debido a un aumento del porcentaje de partos triples y de orden superior, pero sin que se viese afectado el pico preovulatorio de LH.

Estudios previos llevados a cabo en ganaderías de Oviaragón-Grupo Pastores sobre resultados de fertilidad de más de 16.000 ovejas inseminadas a tiempo fijo utilizando 480 UI de eCG mostraron que las hembras R+ tuvieron una fertilidad igual o superior a la de las ovejas no portadoras (Alabart *et al*., 2009). Resultados similares se obtuvieron en trabajos experimentales realizados con un número más reducido de animales en las instalaciones del CITA (Zaragoza, España). Se observó que la fertilidad del lote ++480 fue del 40%, mientras que la de los lotes R+480 y R+240 fue del 61% y 58%, respectivamente (Datos no publicados). Con los resultados del presente estudio, se demostraría que no se debieron a diferencias en la descarga preovulatoria de LH. Al tratarse de fertilidad al parto, podrían estar relacionados con la mayor tasa de ovulación de la ovejas R+, o bien a otros parámetros relacionados con la fertilidad que no han sido estudiados todavía en este genotipo.

En las ovejas portadoras del alelo *FecX*^{R} se observó una correlación significativa entre el área total del pico de LH, su concentración máxima y la tasa de ovulación, como puede verse en la figura 3. Hasta donde nosotros conocemos, no existen trabajos similares al respecto. En trabajos previos llevados a cabo por nuestro equipo, se observó que las ovejas R+ tratadas con FSH presentaban un mayor número de folículos ováricos de un menor diámetro respecto a las ovejas ++ (Lahoz *et al*., 2013). Estos resultados apoyarían la hipótesis según la cual las ovejas *Fec* heterocigotas tendrían un mayor número de folículos de menor diámetro susceptibles de ovular (Fabre *et al*., 2006).

En conclusión, el tratamiento actualmente comercializado de esponjas vaginales de 30 mg de FGA y 480 UI de eCG induce una buena sin-

cronización del pico preovulatorio de LH en ovejas ++ Rasa Aragonesa adultas en otoño. La presencia del alelo *FecX*^{R} no tuvo ningún efecto sobre el pico preovulatorio de LH, independientemente de la dosis de eCG utilizada. Estos datos refuerzan la recomendación de que la dosis de eCG aplicada a las ovejas R+ deberá adaptarse a las necesidades y posibilidades de la explotación, sin que afecte a los resultados de la IA. No es recomendable modificar el momento de realizar la IA cervical a tiempo fijo en ninguno de los dos genotipos.

Agradecimientos

Este trabajo se ha realizado con fondos del Proyecto TRACE PET-2008-0076. B. Lahoz disfruta de una beca predoctoral INIA. Los autores quieren agradecer a E. Echegoyen y a R. Mozo su colaboración en la toma de muestras, y a los compañeros de UPRA-Grupo Pastores la cesión de los datos sobre tratamientos hormonales.

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(Aceptado para publicación el 25 de abril de 2013)

3.4. Publicación 3

Influence of the *FecX*^{R} allele in heterozygous ewes on follicular population and outcomes of IVP and ET using LOPU-derived oocytes.

Lahoz B., Alabart J.L., Folch J., Sánchez P., Echegoyen E., Cocero M.J.
Reproduction in Domestic Animals 2013, 48:717–723

Reproduction in Domestic Animals

Reprod Dom Anim **48**, 717–723 (2013); doi: 10.1111/rda.12150
ISSN 0936–6768

Influence of the *FecX*^{R} Allele in Heterozygous Ewes on Follicular Population and Outcomes of IVP and ET using LOPU-Derived Oocytes

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Contents

Ewes heterozygous for the *FecX*^{R} allele (R+) in the *bone morphogenetic protein 15* (*BMP15*) gene display increased ovulation rate and prolificacy. Besides this phenotypic advantage, the influence of the *FecX*^{R} allele on follicle number and size, oocyte competence and *in vitro* production (IVP) remains undefined. With these aims, 8 R+ and 8 wild-type (++) ewes were subjected to 2 laparoscopic ovum pick-up (LOPU) trials (four sessions per trial; two with and two without FSH) and subsequent IVP and fresh embryo transfer. All follicles >3 mm were punctured (n = 1673). Genotype did not significantly affect the number of punctured follicles per ewe and session (10.4 and 10.2 in R+ and ++ untreated ewes, 17.4 and 14.3 in R+ and ++ FSH-treated ewes, respectively), but follicular diameter of R+ ewes was significantly reduced compared with ++ ewes (−0.2 mm in untreated and −0.8 mm in FSH-treated ewes; $p < 0.01$). R+ ewes showed higher recovery rate and increased numbers of total and suitable cumulus–oocyte complexes for *in vitro* maturation (IVM). Similar rates of day 8 blastocysts were observed in R+ (36.1%, 147/407) and ++ (32.6%, 100/307) ewes, but the final output of day 8 blastocysts per ewe and session was higher in R+ ewes (+0.75; $p < 0.005$), without differences in survival rate at birth of the transferred embryos (40.4%, 21/52 vs 36.4%, 16/44, respectively). In conclusion, a higher number of oocytes proven to be competent for *in vitro* development and embryo survival after transfer are recovered from R+ ewes, despite the lower mean size of their follicles at puncture.

Introduction

In sheep, several breeds have been identified with different mutations in the *bone morphogenetic protein 15* (*BMP15*) gene, which display a higher ovulation rate in heterozygous ewes when compared with wild-type animals (Galloway et al. 2000; Hanrahan et al. 2004; Bodin et al. 2007; Martínez-Royo et al. 2008). One of these polymorphisms corresponds to the *FecX*^{R} allele in the Rasa Aragonesa breed and was discovered in 2008 (Martínez-Royo et al. 2008). In a previous study, we described phenotypic effects of 0.63 extra ovulations and 0.35 extra lambs per *FecX*^{R} heterozygous (R+) lambing ewe (Lahoz et al. 2011). However, the influence of the *FecX*^{R} allele on follicle number and size, oocyte competence and how it could further affect *in vitro* embryo production and viability remains to be assessed.

The exact mechanism by which these mutations increase ovulation rate is still unclear, although reduced activity of the BMP signalling system in heterozygous ewes may cause smaller antral follicles with fewer granulosa cells and altered sensitivity to gonadotropins (revised by Fabre et al. 2006). Thereby, folliculogenesis in heterozygous ewes is expected to be altered, which potentially affects the proportion of developmentally competent oocytes, that is, with the ability to mature, be fertilized and give rise to normal and fertile offspring after normal gestation (Duranthon and Renard 2001). The proportion of competent oocytes is widely accepted to increase along with follicular size (Mermillod et al. 2008); thus, follicle size undoubtedly affects the overall efficiency of ovum pick-up, and increased productivity is associated with large and medium follicles (Rodríguez et al. 2006).

On the other hand, BMP15 is an important oocyte-secreted factor (OSF), which plays key roles in folliculogenesis, follicular development and ovulation, as well as in oocyte quality, in mammalian ovaries. Recent studies highlight the importance of OSFs as regulators of cumulus cell function and the quality of the oocyte (Gilchrist et al. 2008). Supplementing *in vitro* oocyte maturation media with exogenous OSFs [BMP15 or growth differentiation factor (GDF) 9] enhances oocyte developmental competence in cattle (Hussein et al. 2006), and the lower developmental competence of calf oocytes compared with those of adult cows has been proposed to be due to different expression of BMP15 and/or GDF9 in oocytes and cumulus cells between calves and cows (Hosoe et al. 2011). In humans, oocytes retrieved from follicles with a significantly higher follicular fluid level of BMP15 had a higher fertilization rate and superior cleavage and embryo quality (Wu et al. 2007). A very recent study in Booroola ewes has confirmed lower oocyte-derived BMP15 mRNA levels in ewes homozygous for a mutation in BMPR-1B (Crawford et al. 2011), and lower overall concentration or biological activity of BMP15 has been proposed in Inverdale (*FecX*^{I}) or Hana (*FecX*^{H}) ewes heterozygous for a mutation in *BMP15* (Galloway et al. 2000). Because analogous reductions are expected in R+ ewes, a hypothetical deleterious effect on oocyte competence should be discarded in this genotype.

Moreover, the overall efficiency of certain embryo biotechnologies in ovines, such as laparoscopic ovum pick-up (LOPU)–*in vitro* production (IVP) programmes, is still low. Therefore, protocols including exogenous gonadotropins are commonly used to increase oocyte number. Considering that gonadotropins may affect R+ ewes differently, in the present study we aimed to investigate the suitability of R+ ewes for embryo biotechnologies. To our knowledge, the only reported study on IVP of embryos in *Fec*-mutation carrier ewes involve the Booroola mutation (Cognié et al. 1998), but there are no reports on the results of LOPU procedures in any of these genotypes. It must be taken into account that the *FecB*^{B} allele is located in a different gene, corresponding to a single mutation in the coding sequence of the bone morphogenetic protein receptor type 1B (revised by Fabre et al. 2006), a receptor of

BMP15 as well as other BMPs and other members of the transforming growth factor-beta (TGF-β) superfamily, such as anti-Müllerian hormone (Kaivo-oja et al. 2006).

Thus, the aims of the present study were to evaluate the influence of the $FecX^R$ allele, with or without *in vivo* FSH treatment, on (i) the quantity and size of follicles available for aspiration in a LOPU-IVP programme and (ii) the competence of LOPU-derived oocytes subjected to *in vitro* production of embryos and their further ability to sustain pregnancy to term.

Materials and Methods

All experimental procedures were performed in accordance with the guidelines of the European Union (2003/65/CE) and Spanish regulations (RD 1201/2005, BOE 252/34367-91) for the use and care of animals in research. All chemicals used for IVP were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise specified. All FSH used in this study was highly purified porcine FSH provided by J.F. Beckers (Laboratory of Endocrinology of the Faculty of Veterinary Medicine, University of Liege, Belgium). Quantities are expressed in mg of the NIH-FSH-P1 standard (1 mg NIH-FSH-P1 = 0.038 mg NIADDK-oFSH-17).

Animals and experimental design

The experiment was performed in January and November 2010 (breeding season) at the facilities of the research centre (CITA). The live donors included 16 healthy adult Rasa Aragonesa ewes, similar in weight and body condition score. Half the ewes (n = 8) were $FecX^R$ heterozygous (R+) and the other half (n = 8) were wild-type ewes (++). Two LOPU-IVP trials, 10 months apart, were performed. In each trial, four LOPU sessions were conducted at 7-day intervals, the first two without and the last two with FSH treatment. Collected cumulus–oocyte complexes (COCs) were subjected to *in vitro* maturation, fertilization and culture procedures. Day 7 and 8 blastocysts were fresh-transferred to assess their viability by their ability to sustain pregnancy to term.

Hormonal treatment

Prior to each LOPU trial, all animals received two doses of 125 μg cloprostenol (Estrumate®; Schering-Plough Animal Health, Madrid, Spain) 8 days apart, and 5 days after the second injection, a 30-mg fluorogestone acetate (FGA) sponge (Sincropart® 30 mg; CEVA Animal Health SA, Barcelona, Spain) was inserted and replaced by a new one in each LOPU session. The first LOPU session was performed 12 days after the insertion of the sponge and the second session 7 days later. For the third and fourth LOPU sessions, ewes received a total of 32 mg FSH administered in decreasing doses: 8 mg (60 h), 8 mg (48 h), 6 mg (36 h), 6 mg (24 h) and 4 mg (12 h) prior to each LOPU session. Coinciding with the first injection of FSH, 125 μg cloprostenol was administered.

Recovery of oocytes by LOPU

Donor ewes were fasted for 12 h prior to each LOPU session. Ewes were anaesthetized with 3 mg/kg propofol (Propofol ®Lipuro 1%; Braun, Barcelona, Spain) intravenously (i.v.) followed by inhalation maintenance anaesthesia with isofluorane (IsoFlo®; Veterinary Esteve, Barcelona, Spain). Follicular puncture was performed by a modified LOPU procedure based on the technique previously described by Alberio et al. (2002). An endoscope was inserted through a 1-cm incision, approximately 10 cm cranial to the udder, into the abdominal cavity, and an atraumatic grasping forceps was introduced through a contralateral incision to immobilize the ovary. By a third 1-cm incision adjacent to the midline, a 23-G needle connected to a vacuum pump (V-MAR 5100; Cook Ltd, Eight Mile Plains, Australia) adjusted to 25 mmHg was introduced to aspirate all ovarian follicles >3 mm in diameter. The number and diameter of punctured follicles in each donor and session were recorded, using a 2-mm scale located on the needle. COCs were collected into 15-ml falcon tubes, which contained 2 ml collection medium [TCM 199 with 10 mM/ml HEPES supplemented with 100 IU/ml heparin, 4 μl/ml gentamicin and 0.2% bovine serum albumin (BSA)]. Falcon tubes were maintained in a water bath at 35°C until processing. After oocyte collection, the ovaries were flushed with sterile physiological saline solution (0.9% NaCl) supplemented with 5 UI/ml heparin. The incisions were closed with staples and sprayed with topical chlortetracycline hydrochloride (Pederol® spray; Syva, León, Spain), and 200 mg/10 kg oxytetracycline dihydrate (Oxycen-200 L.A., s.p.® veterinaria, s.a., Tarragona, Spain) was administered by intramuscular injection to each ewe. During each session, ewes of both genotypes were subjected to LOPU in alternate order and the practitioner was blinded to the genotype.

In vitro maturation (IVM)

The recovered COCs were washed four times in TCM 199 + 10 mM HEPES + 0.04 mg/ml gentamicin and morphologically scored into five categories (I to V) as previously described by Stangl et al. (1999). Only those surrounded by continuous multilayer unexpanded cumulus cells and showing a homogeneous cytoplasm (categories I, II and III) were used for IVM. Until placed in the maturation medium, all the COCs remained in TCM 199 + 0.04 mg/ml gentamicin. For maturation, 30–40 COCs were placed in 500 μl maturation medium, containing TCM 199 + 132 μg/ml pFSH (equivalent to 5 μg/ml NIADDK-oFSH-17) + 10% (v/v) follicular fluid + 40 μg/ml gentamicin sulphate, using 4-well plates (Nunclon®; Fisher Bioblock, Madrid, Spain). Follicular fluid was prepared as described previously (Cocero et al. 2011). Plates were incubated at 38.5°C in 5% CO_2 in humidified air for 24 h.

In vitro fertilization (IVF)

Oocytes were denuded by gentle pipetting with a small-bore pipette and washed three times in fertilization medium [synthetic oviductal fluid (SOF) + 4 μl/ml gentamicin + 20% heat-inactivated oestrous ewe serum]. Ten to 15 oocytes were placed into 49-μl droplets of IVF

medium on polystyrene Petri dishes under mineral oil. Fresh semen from a single Rasa Aragonesa ram of proved *in vitro* fertility was used in all sessions. For capacitation, semen was maintained at room temperature in the dark for 2 h after collection, subsequently diluted 1:10 (v/v) in SOF + 0.3% BSA + 40 μg/ml gentamicin sulphate and then centrifuged at 200 ***g*** for 5 min. After counting and evaluating progressive individual subjective motility, the pellet was diluted with fertilization medium, and 1 μl was introduced into each 49-μl oocyte-containing drop, yielding a final concentration in the drop of 1×10^6 spz/ml. Incubation was carried out for 20–24 h at 38.5°C in 5% CO_2 humidified air.

In vitro culture (IVC)

After IVF, presumptive zygotes were washed four times in culture medium, containing SOF + 0.3% BSA (fatty acid free) + 40 μg/ml gentamicin sulphate. Once spermatozoa were removed, presumptive zygotes were transferred in 30-μl droplets containing culture medium overlaid with mineral oil on Petri dishes (10–15 presumptive zygotes/droplet) and incubated for 8 days at 38.5°C in a 5% CO_2/5% O_2/90% N_2 humidified atmosphere. At days 3 and 5 after IVF, half of the media (15 μl) was replaced with 15-μl SOF supplemented with 20% and 10% (v/v) foetal calf serum (FCS), respectively, to obtain from day 3 onwards a FCS concentration of 10% (v/v). Cleavage and blastocyst rates were assessed at 48 h and 7 and 8 days after IVF.

Embryo transfer

Blastocysts produced *in vitro* were functionally assessed by their ability to sustain pregnancy to term. For this purpose, a random sample of either day 7 or 8 blastocysts showing good morphology, from all the experimental groups and LOPU-IVP trials, were directly transferred. A total of 48 adult wild-type Rasa Aragonesa ewes were used as recipients. Prior to the embryo transfer, ewes received 2 doses of 125 μg cloprostenol 7 days apart, and 4 days after the second injection, a 30-mg FGA sponge was inserted for 12 days. At sponge withdrawal, 400 IU eCG (Sincropart® PMSG 6000 IU; CEVA Animal Health SA) was administered. Eight or 9 days after withdrawal, depending on whether day 7 or 8 blastocysts were transferred, respectively, ovulation rate was assessed by laparoscopy and embryo transfer was carried out.

Sedation was performed using propofol (8 ml/ewe i.v.). Two blastocysts were placed preferably into the uterine horn ipsilateral to the ovary showing a morphologically healthy corpus luteum using a tomcat catheter.

In vivo embryo viability

For early pregnancy diagnosis, a blood sample was collected from each recipient by jugular puncture 25 days after embryo transfer using 5-ml vacuum tubes containing lithium heparin for plasmatic pregnancy-associated glycoprotein (PAG) determination. Ewes with a plasma PAG concentration ≥ 1.3 ng/ml were considered pregnant (Alabart et al. 2010). Additionally, abdominal ultrasound pregnancy diagnosis was performed 30 days after embryo transfer. One week before lambing, ewes were placed in individual pens and the offspring were immediately identified at lambing.

Statistical analysis

The effects of genotype and FSH treatment on the following LOPU-related variables, which were measured per ewe and session, were analysed by repeated-measures analysis of variance (ANOVA) using the MIXED procedure of SAS Institute Inc., 2004 (SAS OnlineDoc® 9.1.3; SAS Institute Inc., Cary, NC, USA): the number of punctured follicles of each size, the mean follicle size, the number of recovered COCs of each quality score and individual recovery rate. The following fixed effects were included in the models: genotype, treatment and session, as well as all the possible interactions among them. Treatment and session within each treatment were treated as repeated factors. Normality of residuals was tested by the Jarque–Bera test, applying a correction for finite samples (Lawford 2005). A Box–Cox transformation was applied to variables failing to accomplish the requirement of residual normality.

The effects of genotype, FSH treatment and their interaction on the variables related either to *in vitro* embryo production, survival of the transferred embryos, or fertility of the recipient ewes were analysed by ANOVA for categorical variables using the categorical modelling procedure (PROC CATMOD) of SAS (SAS Institute Inc. 2004).

Results

Follicular puncture and recovery of LOPU-derived oocytes

A total of 1673 follicles were aspirated in 8 LOPU sessions, yielding 995 recovered oocytes (mean collection rate, 59.5%). Genotype did not affect the total number of aspirated follicles per ewe and session (10.4 and 10.2 in R+ and ++ untreated ewes, 17.4 and 14.3 in R+ and ++ FSH-treated ewes, respectively) but significantly affected mean follicular size (Table 1). The $FecX^R$ heterozygous ewes showed smaller follicular diameter, lower number of >6 mm follicles ($p < 0.01$, both with and without FSH treatment) and higher number of 4-mm follicles ($p < 0.02$, only with FSH treatment), compared with ++ ewes. Genotype significantly affected the recovery rate, favouring R+ over ++ ewes. The number of collected COCs as well as the number of suitable COCs for IVM was higher in R+ than in ++ treated ewes (+3.8 and +3.6, respectively; $p < 0.01$ for both). The number of quality I COCs was similar in R+ and ++ ewes, but the number of quality II–III COCs was higher in R+ ewes when treated with FSH (7.0 in R+ vs. 3.3 in ++; $p < 0.01$).

Independent of genotype, FSH treatment increased all the LOPU-related variables, except the number of 3-mm follicles, significantly (Table 1). FSH treatment affected

Table 1. Follicle population and COC recovery depending on genotype and treatment

	Follicles						COCs				RR
	3 mm	4 mm	5 mm	6 mm	>6 mm	Total	MFS	Total	MIV	I	II+III	IV+V	%
Untreated													
R+	5.8 ± 0.7	2.0 ± 0.3	1.2 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	10.4 ± 1.1	4.1 ± 0.1	5.9 ± 0.6	5.0 ± 0.5	2.8 ± 0.4	2.3 ± 0.3	0.9 ± 0.2	59.2 ± 3.7
++	4.9 ± 0.7	2.2 ± 0.3	0.9 ± 0.2	0.9 ± 0.2	1.3 ± 0.2	10.2 ± 1.1	4.3 ± 0.1	5.4 ± 0.6	4.7 ± 0.5	2.6 ± 0.4	2.0 ± 0.3	0.7 ± 0.2	52.0 ± 3.7
FSH treated													
R+	5.6 ± 0.6	5.3 ± 0.7	3.8 ± 0.4	1.7 ± 0.3	1.0 ± 0.2	17.4 ± 1.3	4.3 ± 0.1	11.8 ± 0.8	11.0 ± 0.7	4.0 ± 0.7	7.0 ± 0.6	0.8 ± 0.3	68.2 ± 2.5
++	3.5 ± 0.6	2.7 ± 0.4	3.2 ± 0.4	1.9 ± 0.3	3.0 ± 0.4	14.3 ± 1.3	5.1 ± 0.1	8.0 ± 0.9	7.4 ± 0.7	4.1 ± 0.7	3.3 ± 0.6	0.6 ± 0.3	56.5 ± 2.6
Significance (p<)													
Treatment (T)	0.23	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.01**	**0.03**	**0.01**	0.76	**0.03**
Genotype (G)	0.06	**0.02**	0.17	0.47	**0.01**	0.23	**0.01**	**0.01**	**0.01**	0.95	**0.01**	0.45	**0.01**
T × G	0.19	**0.01**	0.62	0.97	0.13	0.16	0.31	**0.01**	**0.01**	0.95	**0.01**	0.94	0.47

Least squares means per ewe and LOPU session ± SEM. R+: heterozygous *FecX*^{R} ewes. ++: wild-type ewes. Follicles: number of follicles of each diameter (mm). MFS: mean follicle size (mm). COCs: cumulus–oocyte complexes. MIV: COCs of quality I, II and III according to the classification of Stangl et al. (1999). RR: recovery rate (100 × COCs total/follicles total) on a per ewe basis. Within each column, bold values indicate significant differences due to treatment, genotype and/or their interaction ($p < 0.05$).

R+ ewes differently from ++ ewes with regard to the numbers of 4-mm follicles, total COCs, IVM COCs, and quality II–III COCs ($p < 0.01$ for all) and the proportion of quality I COCs over IVM COCs ($p < 0.02$), as shown by the significant interactions found between genotype and treatment.

IVM, IVF and IVC

Cleavage rate ranged from 84.0% to 87.8%, with no differences between oocytes originating from R+ or ++ ewes, either with or without FSH treatment (Table 2). No significant differences were observed in day 7 and 8 blastocysts, as well as the *in vitro* final yield, between R+ and ++ groups (average differences, 1.6, 3.9 and 3.6 percentage points, respectively). The effect of R+ genotype on day 7 and 8 blastocysts, and on *in vitro* final yield, was slightly higher in untreated ewes, although the interaction between genotype and treatment was not significant.

The percentage of day 7 and 8 blastocysts, as well as *in vitro* final yield, was significantly higher in both R+ and ++ groups treated with FSH when compared with untreated groups (average increases, 8.3, 8.2 and 7.6 percentage points, respectively). The increases in day 7 and 8 blastocysts due to FSH treatment were slightly higher in ++ than in R+ ewes, although significance was not achieved. In fact, there was no significant interaction between treatment and genotype in any of the studied variables.

As the number of recovered and suitable COCs for IVM was higher in R+ donors, and no significant differences in blastocyst rate were observed between genotypes, R+ ewes demonstrated a better yielding of competent oocytes than ++ ewes in the whole. Thereby, R+ ewes produced 0.75 extra day 8 blastocysts per session compared with ++ ewes, and FSH-treated ewes produced 1.6 extra day 8 blastocysts compared with untreated ewes (average increases). Therefore, the global output of IVP blastocysts obtained at day 8 per ewe and session was significantly affected by both genotype and FSH treatment ($p < 0.005$ and $p < 0.0001$, respectively; Table 2).

Embryo transfer and viability

Fertility of recipients and survival rate of transferred embryos are shown in Table 3. No differences were observed in any of these variables, neither between genotypes nor between treatment groups. Overall fertility was 77.1% (37/48 recipients) at 25 days post-transfer determined by PAG, 66.7% (32/48 recipients) at 30 days determined by ultrasound diagnosis and 56.3% (27/48) at lambing. Overall *in vivo* survival rate of transferred embryos was 38.5% (37/96).

Discussion

In the present study, the *FecX*^{R} allele did not affect the number of follicles ≥ 3 mm, but reduced the mean follicle size in R+ ewes. In particular, R+ showed a decreased number of follicles larger than 6 mm in diameter when compared with ++ ewes. These results

Table 2. Embryo IVP depending on genotype and treatment

	Cleavage rate (%)	Day 7 blastocyst rate (%)	Day 8 blastocyst rate (%)	*In vitro* final yield (%)	Day 8 blastocysts per ewe and session[a]
Untreated					
R+	84.0 (126/150)	27.0 (34/126)	32.5 (41/126)	27.3 (41/150)	1.3 ± 0.3
++	85.4 (117/137)	21.4 (25/117)	25.6 (30/117)	21.9 (30/137)	0.9 ± 0.3
FSH treated					
R+	87.8 (281/320)	31.3 (88/281)	37.7 (106/281)	33.1 (106/320)	3.3 ± 0.1
++	84.8 (190/224)	33.7 (64/190)	36.8 (70/190)	31.3 (70/224)	2.2 ± 0.2
Significance (p<)					
Treatment (T)	0.53	**0.02**	**0.03**	**0.02**	**0.0001**
Genotype (G)	0.76	0.64	0.29	0.26	**0.005**
T × G	0.40	0.26	0.41	0.58	0.13

R+: heterozygous $FecX^R$ ewes. ++: wild-type ewes.
[a]Means ± SEM per ewe and LOPU session. Within each column, bold values indicate significant differences due to treatment, genotype and/or their interaction ($p < 0.05$).

Table 3. Fertility of recipients and embryo survival depending on genotype and treatment

	Fertility (%)[a]			Survival rate (%)[b]
	At day 25 (PAG)	At day 30 (ultrasound)	At lambing	At lambing
Untreated				
R+	83.3 (5/6)	66.7 (4/6)	66.7 (4/6)	41.7 (5/12)
++	66.7 (4/6)	50.0 (3/6)	50.0 (3/6)	50.0 (6/12)
FSH treated				
R+	75.0 (15/20)	70.0 (14/20)	60.0 (12/20)	40.0 (16/40)
++	81.2 (13/16)	68.7 (11/16)	50.0 (8/16)	31.3 (10/32)
Significance (p<)				
Treatment (T)	0.82	0.49	0.84	0.38
Genotype (G)	0.71	0.58	0.41	0.99
T × G	0.41	0.63	0.84	0.46

R+: heterozygous $FecX^R$ ewes. ++: wild-type ewes.
[a]Pregnant/transferred recipients.
[b]Lambs born/transferred embryos.

therefore concur with those previously reported in Belclare ewes carrying a similar mutation on *BMP15* gene (Reynaud et al. 1999), as well as in Booroola (*FecB*) ewes carrying a mutation in the BMP type 1B receptor (Driancourt et al. 1985; McNatty et al. 1986). In both studies, ovulatory follicles from carrier ewes were also found to be smaller. Similar differences in follicular population were found between Inverdale ewes carrying a similar mutation in *BMP15* ($FecX^I$) and wild-type ewes, exhibiting more small follicles (1–2.5 mm) than ++ ewes, with no differences in larger follicles (Shackell et al. 1993). The increased ovulation rate and prolificacy observed in *FecX* and *FecB* carrier ewes are thought to be due to follicles having a lower number of granulosa cells, with precocious LH receptor expression, which results in a higher number of smaller follicles susceptible for selection and ovulation, which explains such differences in follicle size between genotypes (revised by Fabre et al. 2006).

FSH treatment increased the mean follicle diameter, the number of punctured follicles per ewe and session, the recovery rate, and the numbers of recovered COCs and suitable COCs for IVM per ewe and session in both genotypes. This fact highlights the usefulness of stimulation treatment with FSH to increase the performance of LOPU-IVP procedures in sheep. Regarding to follicle population, FSH treatment increased the numbers of follicles from 4 mm onwards in both genotypes. However, the observed increase in the number of 4-mm follicles due to FSH treatment was significantly higher (6.6 times) in R+ than in ++ ewes. Although the cause of this difference between genotypes could not be elucidated with the present work, it is reasonable to assume that R+ ewes could have had a greater proportion of non-atretic follicles smaller than 4 mm able to respond to gonadotropins the days before LOPU, when FSH was administered. If so, this would agree with recent results in Inverdale ewes ($FecX^I$), which yielded a greater proportion of non-atretic follicles >2.5 mm with granulosa cells able to respond to hCG (McNatty et al. 2009). Conversely, the increase in the number of follicles ≥ 6 mm due to FSH treatment was higher in ++ than in R+ ewes (4.25 times), although significance was not achieved. The reduced size of the preovulatory follicles is a characteristic of ewes carrying the $FecX^I$ (Inverdale), $FecB^B$ (Booroola) and $FecL^L$ (Lacaune) mutations, as well as in wild-type prolific Romanov breed, whose control of ovulation rate is polygenic (Drouilhet et al. 2010).

The FSH treatment also increased the proportion of grade II–III COCs over the suitable COCs for IVM in R+ but not in ++ ewes. In our opinion, this observation may be due to the fact that R+ ewes showed smaller follicle diameter and therefore their follicles are assumed to have fewer granulosa cell layers. As explained before, similar ovarian phenotypes with reduced number of granulosa cells were found in ewes carrying similar *FecX* mutations (Belclare: Reynaud et al. 1999; Inverdale: Shackell et al. 1993), as well as in $FecB^B$ mutated Booroola ewes (McNatty et al. 1986). As a result, the morphological evaluation method used in this study for assessing COC quality (Stangl et al. 1999), widely accepted and used by many groups, could have disadvantaged the $FecX^R$ heterozygous ewes when classifying their COCs. Nevertheless, it should be noted that, while follicles from R+ ewes were smaller and their COCs apparently had fewer granulosa cell layers, IVP did not show differences in developmental competence. In our opinion, this is an indication of advanced developmental competence for smaller follicle size in R+ ewes. Nevertheless, this

should be further confirmed by comparing the developmental competence of oocytes of both genotypes for each follicle size.

In addition, these differences in follicular diameters between genotypes could partially be responsible for the higher recovery rate found in R+ ewes. Some authors have reported in sheep a tendency for higher rates of recovery for 1- to 5-mm follicles compared with follicles >5 mm, as large follicles have increased density of follicular fluid and aspiration may be negatively affected by low flow rates and small needle diameters (Baldassarre et al. 1994). Others have found no influence of follicular diameter on recovery rate (Rodríguez et al. 2006). Discrepancy between authors exists regarding the effect of follicle size on recovery rate, although in our work, the potential influence of an advanced follicle structure on recovery rate should not be discarded.

In the present work, no differences were observed between genotypes for *in vitro* embryo production and *in vivo* survival rates regardless of FSH treatment, demonstrating their ability to yield healthy offspring after the LOPU-IVP process. However we would expect lower *in vitro* development of oocytes from R+ ewes as they are supposed to have lower BMP15 protein levels, and in our work, a greater number of grade II and III oocytes from R+ ewes were included in IVP. Therefore, based on our findings, it is reasonable to assume that oocytes from R+ ewes acquire competence at earlier stages of antral follicular development, as there was a higher number of suitable oocytes for IVM recovered from R+ ewes, which came from smaller follicles, but which displayed similar percentages of day 7 and 8 blastocysts when compared with those from ++ ewes. This would be possibly due to an altered folliculogenesis, resulting in a mismatch between follicular diameter and maturation status, when comparing R+ and ++ ewes. Although there are no similar works on *BMP15* mutated ewes, oocytes from heterozygous Booroola ewes are able to resume meiosis and develop to the blastocyst stage at a smaller size compared with wild-type oocytes (Cognié et al. 1998). These *in vitro* results in Rasa Aragonesa ewes concur with what is observed *in vivo*, as we did not find differences on fertility between R+ and ++ ewes using artificial insemination (Alabart et al. 2009). Likewise, oocytes from mature follicles in homozygous Booroola ($FecB^B$) ewes appear fully competent and produce viable offspring with no apparent differences in fertility or embryo viability among genotypes (Montgomery et al. 2001). Moreover, in short-term immunizations against BMP15 protein, which results in an increment in ovulation rate similar to that observed in *BMP15*-mutated ewes, no adverse affects on fertilization, embryo survival or ability of ewes to maintain their pregnancy were observed (McNatty et al. 2004).

Regarding FSH treatment of LOPU donor ewes, it enhanced day 7 and 8 blastocyst rates as well as *in vitro* final yield, independently of genotype, without affecting embryo quality. From a productive point of view, FSH treatment still plays an important role in increasing the output of embryo biotechnology programmes in sheep.

In conclusion, the $FecX^R$ allele did not affect the number of punctured follicles per ewe and session, but reduced follicular size. In addition, R+ ewes displayed higher performance in the number of competent oocytes available after LOPU, demonstrating to be as competent as those from wild-type ewes for IVP, with no differences between genotypes in survival rate at birth of the transferred embryos, despite their follicles being smaller. Collectively, the increased number of IVP blastocysts per R+ donor and session demonstrated the higher efficiency of R+ ewes as donors in a LOPU-IVP programme when compared with ++ ewes.

Acknowledgements

This work was supported by Mº Ciencia e Innovación (TRACE PET-2008-0076 project) and INIA (grant for B. Lahoz).

Conflict of interest

None of the authors have any conflict of interest to declare.

Author contribution

B. Lahoz, J.L. Alabart, J. Folch and M.J. Cocero carried out the experimental design. B. Lahoz, J. Folch and E. Echegoyen performed LOPU and embryo transfer. B. Lahoz, P. Sánchez and M.J. Cocero carried out *in vitro* embryo production. J.L. Alabart performed statistical analysis. B. Lahoz, J.L. Alabart, J. Folch and M.J. Cocero wrote the manuscript. All authors have read and approved the final version of the manuscript.

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Submitted: 2 Mar 2012; Accepted: 4 Jan 2013

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3.5. Publicación 4

Anti-Müllerian hormone (AMH) concentration in sheep and its dependence of age and independence of *BMP15* genotype: an endocrine predictor to select the best donors for embryo biotechnologies.

Lahoz B., Alabart J.L., Cocero M.J., Monniaux D., Echegoyen E., Sánchez P., Folch J.
Theriogenology 2013, doi:10.1016/j.theriogenology.2013.09.033

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Anti-Müllerian hormone (AMH) concentration in sheep and its dependence of age and independence of *BMP15* genotype: an endocrine predictor to select the best donors for embryo biotechnologies

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Received 26 June 2013; received in revised form 16 September 2013; accepted 30 September 2013

Abstract

Embryo biotechnologies contribute significantly to the genetic enhancement of livestock, although their efficiency is still limited in sheep, mainly due to variable ovarian responses to gonadotropins. At present, anti-Müllerian hormone (AMH), which is produced by the granulosa cells of the small antral follicles, is a reliable endocrine marker of the ovarian follicle reserve in many species. The expression of AMH in granulosa cells was shown to be stimulated by bone morphogenetic proteins (BMPs) in vitro, so a mutation affecting the *BMP15* gene might modulate AMH production in vivo. The present study aimed to assess plasma AMH concentrations before puberty in two groups of Rasa Aragonesa ewes that were carrying (R+) or not carrying (++) the prolific $FecX^R$ allele and to relate them with their AMH concentrations at adulthood. Additionally, we sought to establish in both genotypes whether AMH measurements during a LOPU programme could be predictive of the number of ovarian follicles (≥ 3 mm) and recovered cumulus-oocyte complexes (COCs). No differences in AMH were found between the R+ and ++ ewes before puberty or during the adult age. Before puberty, the AMH concentration tended to increase from 3 to 4.5 months and to decline at 6 months to levels similar to those observed later in adults (333.8 ± 73.3, 483.2 ± 135.5 and 184.1 ± 38.2 pg/mL, respectively; $P < 0.1$), showing a large variability between individuals and between ages. A relationship between the

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doi:10.1016/j.theriogenology.2013.09.033

AMH concentrations before puberty and during adulthood was not found, likely reflecting different follicular growth dynamics. In adults, the AMH concentration at the beginning of the FSH treatment was strongly correlated with the number of punctured follicles at LOPU in R+ and ++ ewes (r = 0.75 and 0.78, respectively; P < 0.001), and it was possible to accurate determine AMH cut-off values for both genotypes to identify high-responding ewes. On average, 5.1 extra follicles and 2.7 extra COCs were expected per each 100 pg/mL increase in AMH (P < 0.0001 and P < 0.01, respectively). The repeatability of AMH concentration from session to session was 0.70 (P < 0.0001). Our results demonstrated that, regardless of age, the presence of the *FecX*^{R} allele did not affect plasma AMH levels. During adulthood, AMH proved to be a good predictor of the ovarian response to FSH stimulation. Such an indicator could therefore be used to improve the performance of embryo biotechnologies in sheep.

Keywords: COCs; *FecX;* follicle; FSH; ovarian reserve; ovum pick-up

1. Introduction

Reproductive efficiency in sheep is of great importance to optimise labour, improve the income of farmers and the viability of farms, and contribute to optimising the use of resources. The use of embryo biotechnologies contributes to an increase in the selection intensity and shortens the generation interval, allowing for the production of a large number of descendants from ewes with high genetic merit. In sheep, embryo biotechnologies have developed considerably in recent years due to a higher demand and an increased efficiency of the protocols and techniques. Apart from artificial insemination (AI) and multiple ovulation and embryo transfer (MOET), laparoscopic-ovum pick up (LOPU) and in vitro production of embryos (IVP) represent the next generation of techniques to improve the number of offspring in small ruminants [1], presenting many advantages over traditional MOET. Using OPU-IVP, more embryos can be produced in the same period of time per donor animal, as the procedure can be performed over a long period, including pregnancy, prepuberty, or anestrus, without detrimental effects on the fertility of the donor. LOPU-IVP is a versatile technique with a much greater potential for the future compared with MOET, and it could therefore significantly contribute to enhancing genetic improvement and preserving endangered breeds.

However, the overall efficiency of LOPU-IVP in sheep is still far from the yields reached in cattle [2]. Apart from the aspects related to IVP and the conservation of the produced embryos, a major limiting factor is the large between-individual variability in the ovarian response to stimulation treatments using exogenous FSH [3], resulting in high variability in the final number of transferable embryos in sheep. Variations in the ovarian response to FSH reflect the variability of the follicular population present at the beginning of the treatment in small ruminants [4–6].

Anti-Müllerian hormone is a glycoprotein belonging to the transforming growth factor (TGF)-β family that is only expressed in the gonads [7,8]. In women and female animals, AMH is a reliable marker of the size of the ovarian pool of growing follicles. At present, AMH is the best predictive endocrine marker of the number of available follicles in Assisted Reproductive Technologies (ART) in response to ovarian stimulatory treatment in humans [9], cows [10], goats [11], and mares [12]. In the sheep ovary, AMH has recently been found to be highly produced by the antral follicles from 1 to 3 mm in diameter [13], but its ability

to predict the number of follicles available for puncture in a LOPU programme has not been investigated yet. While the use of AMH in adulthood is of great interest, it would be even more interesting if it could be determined earlier in life. In a previous work, we investigated the ability of plasma AMH to predict the ovarian follicle reserve before puberty and to correlate it with early fertility at the first lambing for the early selection of replacement ewe lambs. Our results suggested that AMH could be a marker of the ovarian follicle reserve before puberty related to sexual precocity [14]. Thus, in the present work, we aimed to assess the usefulness of AMH determination at an early age for the precocious selection of the best future donors for embryo biotechnologies.

In recent years, several sheep breeds have been identified with different mutations in the bone morphogenetic protein 15 (*BMP15*) gene, which are responsible for an increase in the ovulation rate and prolificacy in heterozygous ewes [15–19]. One of the mentioned polymorphisms corresponds to the *FecX*^{R} allele in the Rasa Aragonesa breed [18]. We recently found that the presence of the *FecX*^{R} allele in heterozygosity led to a decrease in follicle diameter, with no effects on the developmental competence of their oocytes [20]. The *BMP15* gene, like AMH, belongs to the TGF-β superfamily [21]. Other BMPs belonging to this superfamily have recently been shown to stimulate *AMH* mRNA expression in granulosa cells in vitro, both in cattle (BMP4 and BMP6) [22] and sheep (BMP4) [13]. These recent results strongly suggest an important role for BMPs in enhancing AMH production in small antral follicles in vivo. Many questions remain to be addressed regarding regulation of AMH expression, including the potential effects of other members of the BMP family, such as BMP15 or GDF9. For this reason, the potential influence of a decrease in BMP15 protein expression in the oocyte due to a mutation affecting the *BMP15* gene, on AMH production in follicles and therefore, on its endocrine level, should not be disregarded. Due to the reproductive advantages of the *FecX*^{R} allele, ewes heterozygous for *FecX*^{R} coexist with wild-type ewes in Rasa Aragonesa flocks. Therefore, potential differences in plasma AMH concentrations between genotypes should be assessed to determine whether the predictive ability of AMH could be extended or not to both genotypes and therefore if distinctions between the genotypes when using AMH as a selection tool should be made or not.

Therefore, the aims of this study were the following: 1) to assess whether the presence of the *FecX*^{R} mutation in the *BMP15* gene could affect the AMH plasma concentrations; 2) to characterise the plasma AMH concentrations before puberty and to establish the relationship between AMH measured during this period of life and AMH and LOPU performance at the adult age; and 3) to establish whether plasma measurements of AMH during adulthood could be used to determine the number of ovarian follicles and cumulus-oocyte complexes (COCs) that a given sheep can produce during repeated LOPU sessions, using FSH or not.

2. Materials and methods

All experimental procedures were performed in accordance with the guidelines of the European Union (2003/65/CE) and Spanish regulations (RD 1201/2005, BOE 252/34367-91) for the use and care of animals in research. The experiments were carried out at the facilities of the research centre (CITA de Aragón, Spain). The *BMP15* genotype was determined in all animals by PCR genotyping, as previously described by Martínez-Royo et al. [23].

2.1. Experiment 1: Plasma AMH concentration before puberty

Twelve ewe lambs of the Rasa Aragonesa breed, including six heterozygous for the *FecX*^{R} allele (R+) and six wild-type ewe lambs (++), were used. The animals were born in April, with a maximum age difference between animals of 3 days. They were provided feed and water ad libitum and reared under the same conditions. Ewe lambs were submitted to blood sampling at 3, 4.5, and 6 months. The experimental protocol aimed to compare the plasma AMH concentrations depending on age and genotype.

2.1.1. Blood sampling

For AMH determination, three blood samples were obtained from each ewe lamb before puberty as follows: first sampling at 90 days of age (July), second sampling at 135 days of age (August), and third sampling at 180 days of age (October). All samples were taken at 9 a.m., with a maximum interval from the first to the last sampling of 10-15 minutes. In order to determine whether puberty had occurred by the time of AMH sampling, two blood samples at nine days interval were taken at 3, 4.5 and 6 months of age for P4 determination. The samples were collected by jugular puncture using 5-mL vacuum tubes with lithium heparin that were immediately centrifuged at 2100 X g for 25 min. The plasma was stored at -20 ºC until it was assayed for AMH and P4. After that, the ewe lambs were kept in irrigated pastures with ad libitum access to water and mineral supplements.

2.2. Experiment 2: Plasma AMH concentration at LOPU

When the ewe lambs from experiment 1 were adults (19 months), ten of them were submitted to four LOPU sessions, together with six additional ewes of similar age (8 R+ and 8 ++ in total), and blood samples were taken during each session for AMH measurement. The AMH samples were taken in the second LOPU trial of a previous work, consisting in two LOPU-IVP trials performed to evaluate the effects of the *FecX*^{R} allele and FSH on follicle population and oocyte quality [20]. The present experiment aimed to assess the ability of the plasma AMH concentrations to predict the performance of an individual ewe submitted to a LOPU program, taking into account the potential effect of the R+ genotype and LOPU session on the AMH concentration. In addition, the relationships between the AMH concentrations of the ewe lambs before puberty with the AMH concentrations and performance (number of punctured follicles and COCs) of adult ewes submitted to LOPU were investigated.

2.2.1. Hormonal treatment and blood sampling

Four LOPU sessions were carried out 7 days apart, as follows (Fig. 1). The first and second sessions were carried out without FSH treatment, and the third and fourth sessions took place following FSH treatment. Prior to the first session, the ewes received two doses of 125 μg of cloprostenol (Estrumate, Schering-Plough Animal Health, Madrid, Spain) 8 days apart. Five days after the second injection, 30 mg fluorogestone acetate sponges (FGA; Sincropart 30 mg, CEVA Animal Health SA, Barcelona, Spain) were inserted and replaced by new ones at each LOPU session. The first LOPU session took place 12 days after the

insertion of the first sponge, and the second took place one week after. Prior to the third and forth LOPU sessions, the ewes received a total of 32 mg of FSH (ULG, Liège, Belgium) administered in decreasing doses as follows: 8 mg (60 h), 8 mg (48 h), 6 mg (36 h), 6 mg (24 h), and 4 mg (12 h). Coinciding with the first injection of FSH, 125 µg of cloprostenol was injected. All FSH used in this study was highly purified porcine FSH, supplied by the Laboratory of Endocrinology of the Faculty of Veterinary Medicine, University of Liege, Belgium. The quantities are expressed in mg of the NIH-FSH-P1 standard.

A total of four blood samples for plasma AMH determination were taken from each ewe in different moments to establish the relationship between follicle and COC numbers with AMH. In sessions 1 and 2, samples were taken at the time of LOPU, and in sessions 3 and 4, the samples were taken coinciding with the first FSH injection in each session (60 h prior to LOPU). Blood sampling and storing was performed as in experiment 1.

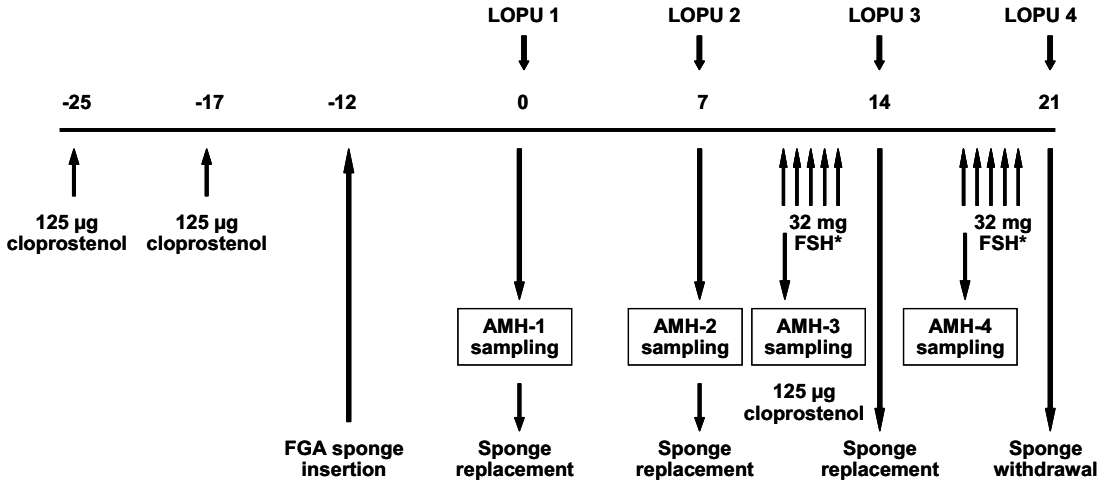

Fig. 1. Experimental design of laparoscopic ovum pick-up (LOPU). Hormonal treatment and blood sampling for AMH determination performed in 16 adult ewes, carrying or not carrying the $FecX^R$ allele (R+; n = 8, ++; n = 8).

2.2.2. Laparoscopic Ovum Pick Up (LOPU) and recovery of oocytes

Follicular aspiration was carried out as previously described [20]. Briefly, the donors were fasted for 12 hours before the LOPU session, anesthetised with 3 mg / kg body weight of propofol (Propofol ®Lipuro 1%, Braun, Spain) and submitted to inhalation anaesthesia with isofluorane (IsoFlo®, Veterinary Esteve, Spain). An endoscope and an atraumatic grasping forceps were inserted into the abdominal cavity, and a 23G needle was introduced by a third incision to aspirate the contents of all the antral follicles ≥ 3 mm in diameter present on the ovaries, using a vacuum pump adjusted to -25 mm Hg (V-MAR 5100; Cook Ltd, Eight Mile Plains, Australia). The number and diameter of the punctured follicles was recorded for each donor and session. After oocyte collection, the ovaries were washed with sterile physiological saline solution (0.9% NaCl) supplemented with 5 IU heparin / mL. A staple and topical spraying with chlortetracycline hydrochloride (Pederol® spray, Syva, León, Spain) were applied to each of the three 1-cm incisions, and 1 mL/10 kg im of oxytetracycline (Oxycen-200 L.A. ®, S.P.veterinaria, s.a., Tarragona, Spain) was administered to each ewe. The Falcon tubes were brought to the laboratory and poured into a petri dish to recover the COCs, that were morphologically scored into five categories as previously described by Stangl et al. [24]. Only those surrounded by continuous multilayer unexpanded cumulus cells and showing a homogeneous cytoplasm were considered as

suitable for in vitro maturation (IVM, categories I, II and III).

2.3. Assay for anti-Müllerian hormone

The plasma concentrations of AMH were measured with the AMH GenII ELISA Kit (Beckman Coulter France, Roissy CDG, France). This kit is an enzymatically amplified two-site immunoassay, providing materials for the quantitative measurement of AMH in human serum. For validation of the assay in sheep, serial dilutions of different ovine plasma and follicular fluid samples in steer plasma were analysed using the kit. The results showed that the follicular fluid dilution curves were linear and parallel to the standard curve (data not shown). Just before the assay, the frozen plasma samples were thawed in a warm water bath, vortexed and centrifuged (3200 g, 10 min, 4 °C) to remove any cell fragments that could interfere with the reagents of the assay. The anti-Müllerian hormone concentrations were determined in 50-µL undiluted plasma samples. The samples were incubated overnight at 4 °C in the presence of the primary antibody and then for 1.5 h at room temperature in the presence of the secondary antibody. Dilutions of the points of the standard curve were made in steer plasma. With these conditions, the limit of detection of the assay was found to be 15 pg/mL. The intra-assay coefficients of variation were 9.9, 6.0, and 2.7% for the three quality control blood samples that were tested, containing 80, 160, and 400 pg/mL of AMH, respectively.

2.4. Assay for progesterone

Progesterone was analyzed by a direct competitive ELISA kit intended for ovine plasma (Ridgeway Science, St. Briavels, Gloucestershire, UK), following the manufacturer's instructions. The sensitivity was 0.28 ng/mL. All samples were run in the same assay. Intra-assay coefficients of variation for plasma pools of 0.5 and 1 ng/mL were 11.5 and 15.0%, respectively. The threshold considered for luteal activity was 0.5 ng/mL.

2.5. Statistical analyses

The effects of *FecX*^{R} genotype and age on AMH concentrations at a prepubertal age were tested by repeated-measures analysis of variance (ANOVA) using the MIXED procedure of SAS [25]. The following terms were included in the model: genotype, age, and their interaction as fixed effects and the donor ewe within genotype as random.

Correlations between the variables were assessed by the Pearson's correlation coefficient. The repeatability of the AMH concentrations at different times was calculated as the ratio of the between-animal variance to the sum of the between-animal and the within-animals variance. The variance components were estimated by the method of restricted maximum likelihood using the VARCOMP procedure of SAS. The statistical significance from zero of the repeatability estimates were assessed using confidence intervals [26].

The effects of *FecX*^{R} genotype and FSH treatment on the total number of punctured follicles (≥ 3 mm), recovered COCS and COCs suitable for IVM per ewe and session were analysed by repeated-measures ANOVA using the MIXED procedure of SAS. The following fixed effects were included in the models: genotype, treatment, and session, as

well as all the possible interactions among them. The treatment and the session within each treatment were treated as repeated factors. The donor ewe within the genotype group was included as a random term.

The effects of the *FecX*^{R} genotype and the number of sessions on the plasma AMH concentrations, which were measured per ewe and per session, were analysed by repeated-measures ANOVA using the MIXED procedure of SAS. The following fixed effects were included in the model: genotype, session, and their interaction. Session was treated as the repeated factor. The donor ewe within the genotype group was included as a random term.

Between-groups comparisons were performed by pairwise contrasts and the raw probabilities were adjusted for multiple comparisons by the step-down Shaffer-simulated method incorporating logical constraints [27,28] using the LSMESTIMATE statement [29].

The effect of the *FecX*^{R} genotype on the linear relationship between the variables recorded in the LOPU sessions (number of punctured follicles, recovery rate, numbers of recovered COCs and COCs suitable for IVM per ewe and per session) and AMH was analysed by repeated-measures analysis of the variance-covariance (ANCOVA). Separate models were fitted for untreated sessions (first and second) and treated sessions (third and fourth). The terms included in the models were the following: *FecX*^{R} genotype (allowing for different intercepts for each genotype), AMH (included as covariate), and their interaction (allowing for different slopes for each genotype), as fixed effects; and the donor ewe within the genotype as random. If the interaction between genotype and AMH was not significant, a common slope could be assumed (two parallel lines were fitted, one for each genotype). In addition, if the genotype effect was not significant, a common intercept could be assumed (a common line was fitted for both genotypes) [30].

The performance of the plasma AMH concentration prior to FSH treatment to predict donor ewes with higher numbers of punctured follicles (≥ 3 mm) at the day of LOPU was tested by receiver-operating characteristic (ROC) analysis, using the LOGISTIC procedure of SAS. Donors with more than 18 punctured follicles per session were considered as "high-responding" and those with 18 follicles or less as "low-responding" ewes, as the median number in all ewes (both genotypes pooled) was 18.5 follicles per session in treated sessions. Sensitivity (Se) was defined as the ratio between the donors correctly predicted as "high-responding" and the total number of "high-responding" donors. Specificity (Sp) was defined as the ratio between the donors correctly predicted as "low-responding" and the total number of "low-responding" donors. The ROC curve is a plot of sensitivity (the true positive rate) versus one minus specificity (the false positive rate). The area under the ROC curve (AUC) was used as a measure of the predicting ability of AMH. As it is known that the values of AUC are too higher when obtained from the data set used to fit the model, AUC values obtained by the leave-one-out cross-validation method are also reported. Between-genotypes comparisons of the AUC values were carried out using the method described by Greiner et al. [31]. The maximum value of the Youden index (J = Se + Sp - 1) was used to determine the optimal value of plasma AMH (cut-off value) to discriminate between "high" and "low responding" donors to FSH.

The normality of the ANOVA and ANCOVA residuals were tested by the Jarque–Bera test, applying a correction for finite samples [32]. A Box–Cox transformation was applied to variables failing to meet the requirement of residual normality. The significance level was set at $P < 0.05$. Otherwise indicated, results are reported as means ± standard errors.

3. Results

3.1. Experiment 1: Plasma AMH concentration before puberty

Progesterone was lower than 0.5 ng/mL in the two samples taken from all ewe lambs at 3, 4.5 or 6 months, so all animals were assumed to have not reached puberty at these ages. Plasma AMH was detected in all the ewe lambs, although one of them showed a non-detectable value at 6 months, preceded by concentrations higher than 250 pg/mL at 3 and 4.5 months, and with AMH values higher than 100 pg/mL at the adult age. Therefore, the value at 6 months was not taken into account in this ewe lamb.

Effect of the $FecX^R$ genotype on plasma AMH concentration before puberty

No significant differences in plasma AMH concentrations were found between R+ and ++ ewes at 3 months (355.3 ± 118.0 vs. 312.2 ± 86.4 pg/mL), at 4.5 months (490.5 ± 205.4 vs. 476.0 ± 176.6 pg/mL), or at 6 months (145.1 ± 56.5 vs. 223.1 ± 65.5 pg/mL), respectively (Fig. 2). The interaction between genotype and age was not significant, indicating that variations in AMH with age were similar in both genotypes.

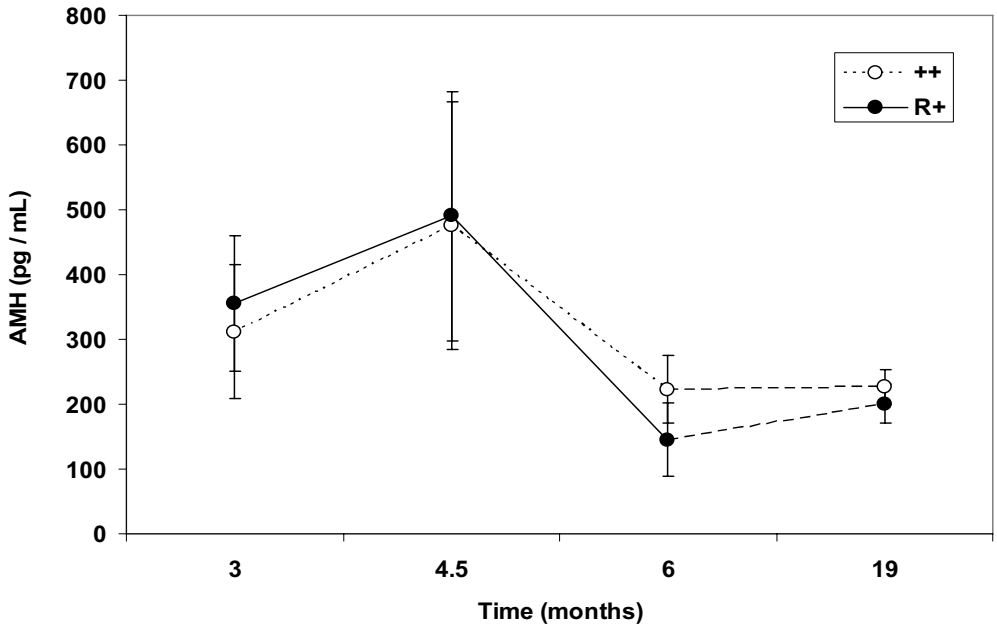

Fig. 2. Changes in the plasma anti-Müllerian hormone (AMH) concentration before puberty and the AMH concentrations at the adult age in ewes carrying or not carrying the $FecX^R$ allele. Blood samples were taken at 3, 4.5, 6, and 19 months of age (Mean ± S.E.M.) in 12 ewe lambs (R+; n = 6, ++; n = 6) and in 16 adult ewes (R+; n = 8, ++; n = 8).

Effect of age on plasma AMH concentration before puberty

Plasma AMH levels at 3, 4.5 and 6 months were 333.8 ± 73.3, 483.2 ± 135.5 and 184.1 ± 38.2 pg/mL, respectively. Plasma AMH concentrations at 6 months were lower than those found at 3 or 4.5 months ($P < 0.05$ for both), although significance was not attained when probabilities were adjusted for multiple comparisons ($P < 0.1$ for both). The AMH profile throughout this period was different between animals, so the maximum AMH values were

found at different times in different ewe lambs at 3 months (n = 4), at 4.5 months (n = 6), or at 6 months (n = 2). The plasma AMH concentrations ranged from 20.4 to 829.1 pg/mL at 3 months, from 69.3 to 1370.8 pg/mL at 4.5 months, and from 69.3 to 471.4 pg/mL at 6 months, demonstrating high between-individual variability. Overall, the within-animal repeatability in the plasma AMH concentration at 3, 4.5, and 6 months was found to be low (0.17; NS). When only those ewe lambs that presented the AMH peak at the same time (4.5 months) were considered, the overall within-animal repeatability at 3, 4.5, and 6 months was higher, although significance was not attained (0.39; $P < 0.1$).

3.2. Experiment 2: Plasma AMH concentration at LOPU

Plasma AMH was detected in all the animals and sessions, ranging from 19.6 to 446.0 pg/mL.

Effect of the $FecX^R$ genotype and LOPU session on plasma AMH concentration at LOPU

No significant differences in the plasma AMH concentration were found between R+ and ++ ewes (Table 1). The interaction between genotype and session was not significant, indicating that variations in AMH within a session were similar in both genotypes. The interaction between genotype and treatment was not significant.

Table 1. Plasma AMH concentrations (pg/mL) in adult ewes that were carrying (R+) or not carrying (++) the *$FecX^R$* allele of the *BMP15* gene, submitted to four LOPU sessions, with or without FSH treatment, one week apart.

Effect			AMH (pg/mL)	P-value
Genotype (G)				
	R+		166.2 ± 14.9	0.71
	++		179.8 ± 17.8	
Session (S)				
		1	213.8 ± 19.9 [a]	0.0001
		2	213.6 ± 25.8 [a]	
		3	154.4 ± 21.0 [b]	
		4	109.9 ± 14.9 [b]	
G X S				
	R+	1	200.9 ± 30.5	0.88
		2	214.9 ± 28.3	
		3	149.8 ± 26.5	
		4	99.1 ± 17.0	
	++	1	227.3 ± 26.7	
		2	212.3 ± 45.4	
		3	158.9 ± 34.5	
		4	120.8 ± 25.1	

a,b: $P < 0.02$

In sessions 1 and 2, the plasma AMH concentrations were similar (213.8 ± 19.9 vs. 213.6 ± 25.8 pg/mL), and both were higher than those in the subsequent sessions 3 and 4 ($P < 0.02$). The plasma AMH concentrations were slightly higher in the third than in the fourth session, although significance was not attained (154.4 ± 21.0 vs. 109.9 ± 14.9; $P < 0.1$). A trend to significance was found for the interaction between session and FSH treatment ($P < 0.1$).

Repeatability of AMH concentrations during LOPU sessions

The individual repeatability of plasma AMH concentration during repeated sessions of LOPU was 0.57 ($P < 0.0001$), increasing up to 0.70 when only considering sessions 3 and 4 (with FSH; $P < 0.0001$ for both).

Representative ewes of both genotypes are shown in Fig. 3.

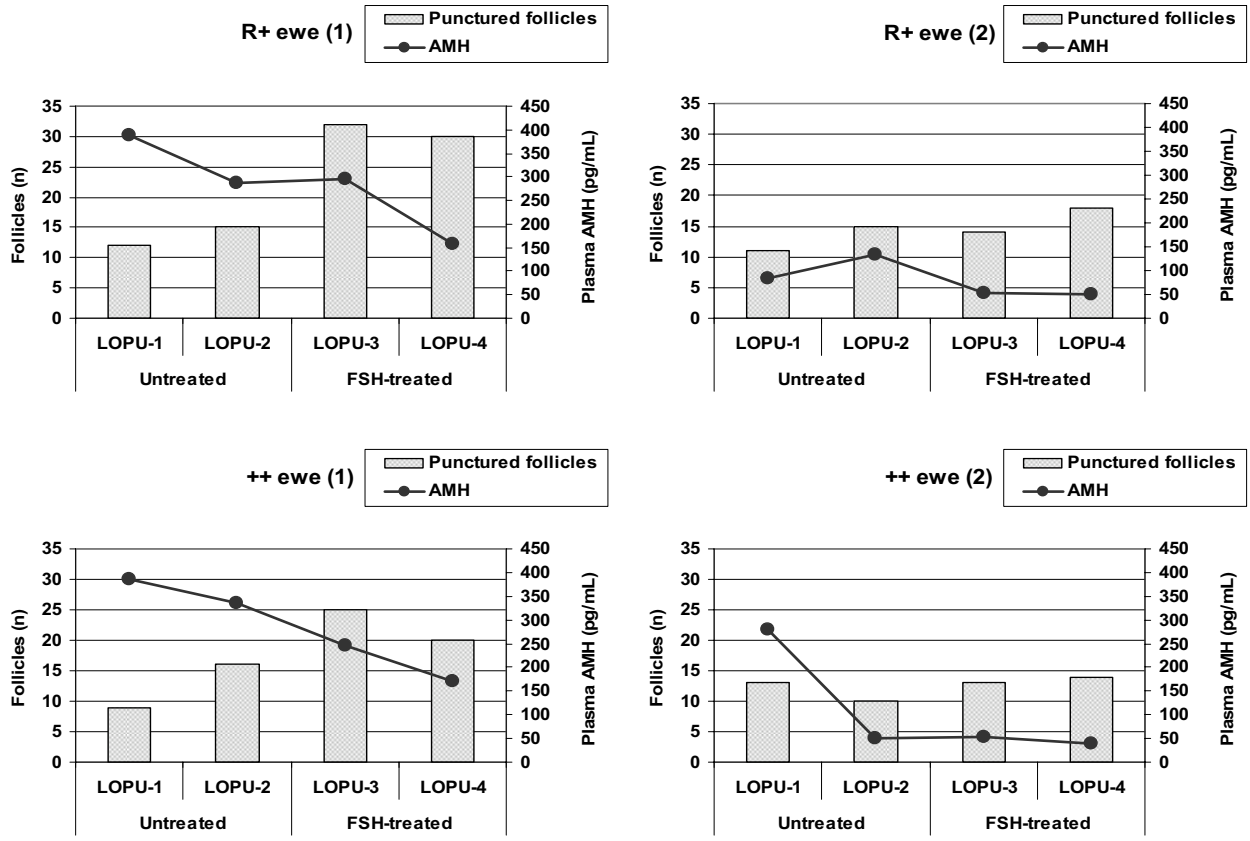

Fig. 3. Plasma AMH concentrations and the number of punctured follicles (≥ 3 mm) during each LOPU session. Representative profiles with high and low AMH (above and below the determined cutt-off point to detect "high-responding" ewes) and follicle numbers are shown for two ewes of each genotype; with (R+) and without (++) the *FecX*^{R} allele.

Relationships between plasma AMH concentrations and LOPU yields

No significant differences were found between R+ and ++ ewes in the number of punctured follicles (16.9 ± 1.1 vs. 14.8 ± 0.9), total COCs (10.2 ± 0.8 vs. 8.3 ± 0.6) and COCs suitable for IVM (8.8 ± 0.8 vs. 7.2 ± 0.7; respectively).

In sessions 1 and 2 (without FSH), the number of punctured follicles was lower than in sessions 3 and 4, with FSH (12.9 ± 0.9 vs. 18.8 ± 1.1; $P < 0.001$). We found a weak correlation between the AMH concentrations and the number of punctured follicles (≥ 3 mm) at LOPUs 1 and 2 ($r = 0.30$; $P < 0.1$). As shown in Fig. 4, a common regression line for both genotypes could be assumed. The correlations between AMH and recovery rate, total COCs recovered, or COCs suitable for IVM were not significant ($r = -0.08$; $r = 0.09$ and $r = -0.08$; respectively). For each of these variables, a common regression line for both genotypes could be assumed (not shown).

Conversely, in sessions 3 and 4 (with FSH), the number of punctured follicles (≥ 3 mm) at LOPU was strongly correlated with the AMH concentrations measured at the beginning of the FSH treatment in both R+ and ++ ewes ($r = 0.75$ and $r = 0.78$, respectively; $P < 0.001$ for both). In this way, 5.1 ± 1.0 extra follicles were expected to be punctured per 100 pg/mL increase in AMH in both genotypes ($P < 0.0001$). In this case, two parallel regression lines, one for each genotype, were fitted (Fig. 4). For the same AMH concentrations, 4.5 ± 1.6 extra follicles were expected to be punctured in the R+ genotype than in ++ genotype ($P < 0.05$). As in sessions 1 and 2, the recovery rate of COCs was not correlated with plasma AMH measured at the beginning of FSH treatment ($r = -0.13$; $P < 0.49$) and a common regression line could be assumed for both genotypes (not shown). Conversely, correlations with plasma AMH were also found for total COCs recovered or COCs suitable for IVM ($r = 0.41$ and 0.39, respectively; both each $P < 0.05$). For these variables two parallel regression lines, one for each genotype, were fitted (not shown). In this way, 2.7 ± 0.7 extra COCs were expected to be recovered per 100 pg/mL increase in AMH in both genotypes ($P < 0.01$). In addition, for the same AMH concentrations, 4.0 ± 1.2 extra COCs were expected to be recovered in R+ than in ++ genotype ($P < 0.01$). Likewise, 2.3 ± 0.8 extra COCs suitable for IMV were expected to be recovered per 100 pg/mL increase in AMH in both genotypes ($P < 0.01$). For the same AMH concentrations, 3.7 ± 1.2 extra COCs suitable for IVM were expected to be recovered in R+ than in ++ genotype ($P < 0.01$).

Determination and predicting performance of AMH cut-off values to detect "high-responding" ewes

The AUC values were 0.867 ± 0.107 and 0.883 ± 0.086 for the R+ and ++ genotypes, respectively. These values were different from 0.5, the AUC value of a test without discriminatory capacity (both $P < 0.001$). After cross-validation, these were lowered to 0.783 ± 0.129 and 0.767 ± 0.132, respectively, also different from 0.5 (both $P < 0.05$). The AUC values were similar for both genotypes, either prior to ($P < 0.90$) or after cross-validation ($P < 0.93$). The optimal cut-offs for selecting "high-responding" ewes were 112 pg/mL for the R+ genotype (corresponding to 80.0% sensitivity and 83.3% specificity) and 169 pg/mL for the ++ genotype (corresponding to 66.7% sensitivity and 100% specificity; Fig. 5).

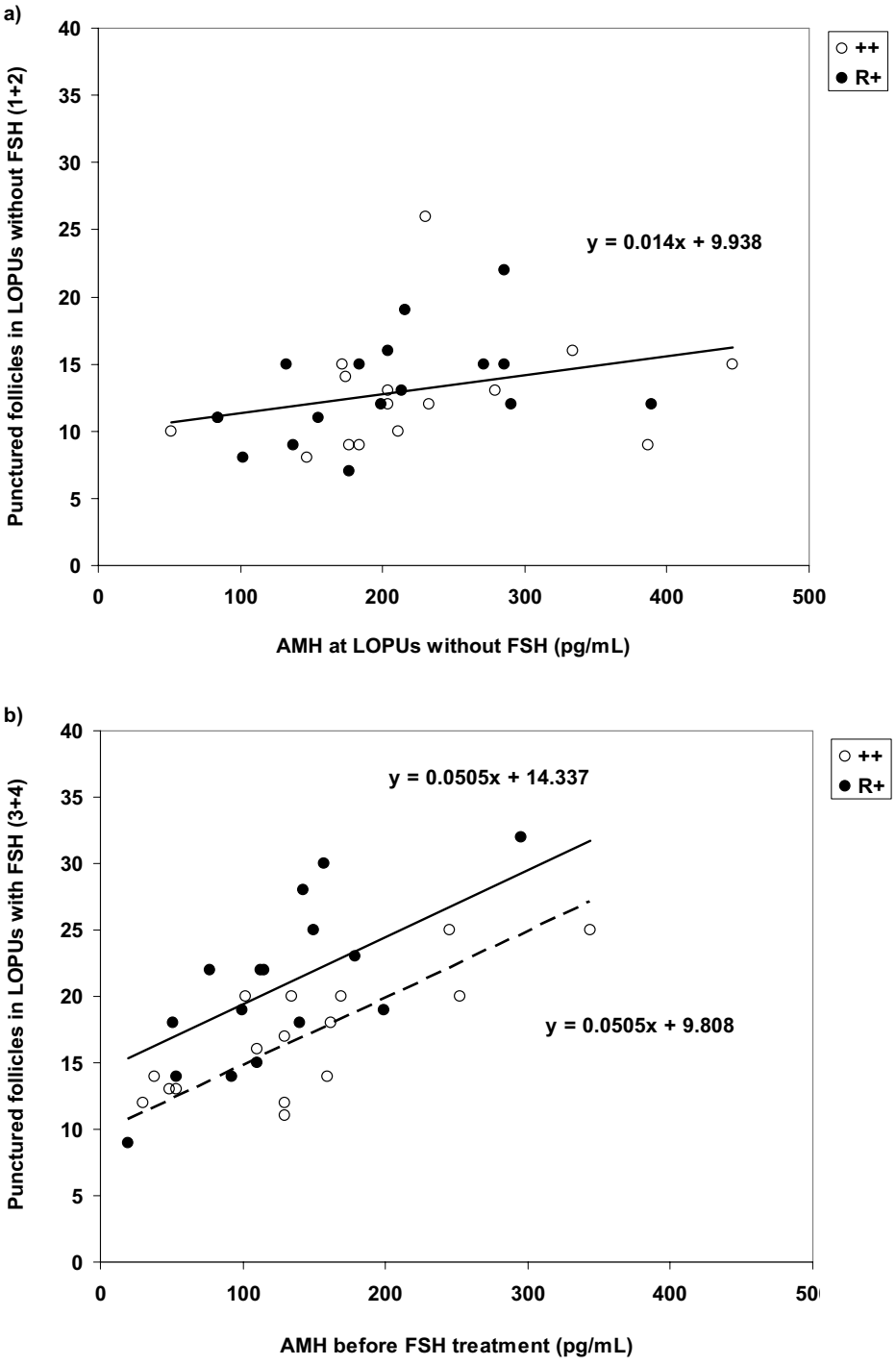

Fig. 4. Relationship between plasma AMH concentration and the number of punctured follicles (≥ 3 mm) at LOPU. Plasma AMH was measured in 16 adult ewes carrying or not carrying the *FecX*^{R} allele (R+; n = 8, ++; n = 8) at the time of LOPU in non-stimulated ewes (a; sessions 1+2), or at the beginning of the FSH treatment in LOPUs using FSH (b; sessions 3+4).

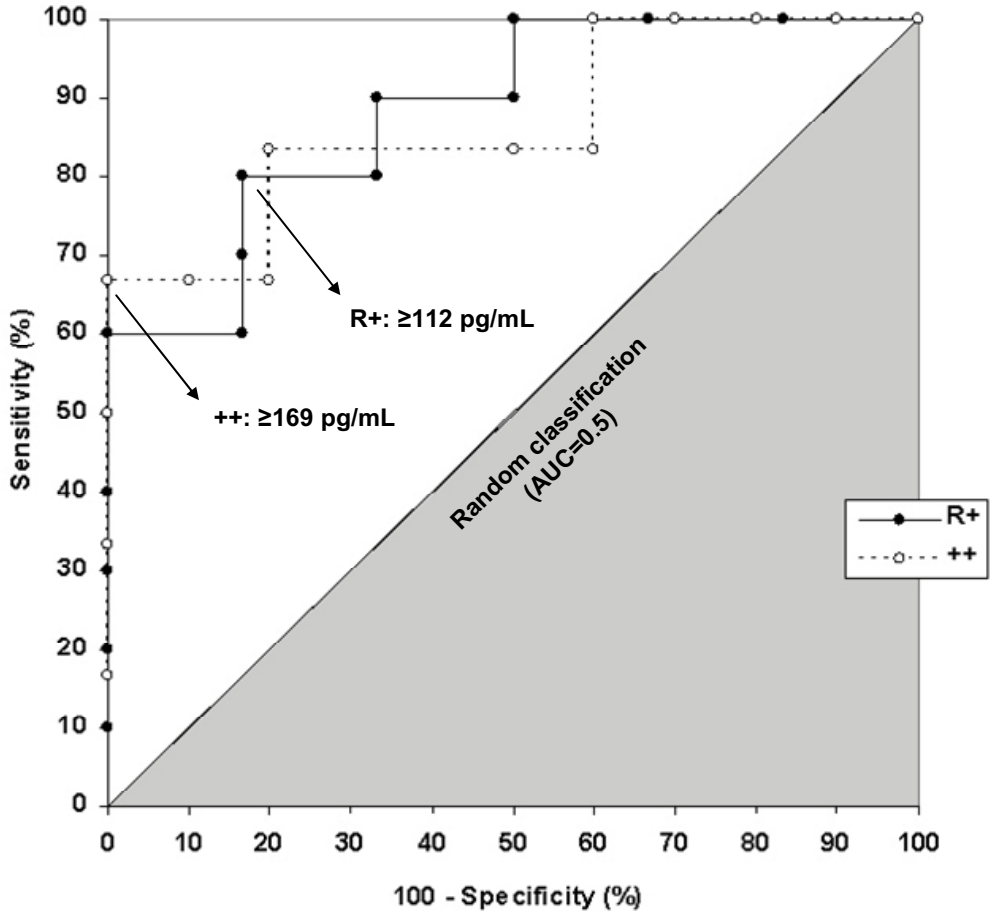

Fig. 5. Receiver-operating characteristic (ROC) curves for predicting high (≥18 follicles) and low (<18 follicles) responding R+ and ++ ewes at LOPU. The arrows show the cut-off points for both genotypes that maximize the Youden index and their corresponding AMH values. A test without capacity of discrimination (random test) is represented (diagonal).

Relationship between plasma AMH concentration at puberty and LOPU performance at the adult age

There was no significant relationship between the plasma AMH concentrations before puberty (at 3, 4.5 and 6 months) and the plasma AMH at the adult age (at the first, second, third, and fourth LOPU sessions) for any genotype. In the same way, the relationships between plasma AMH concentrations at 3, 4.5, and 6 months and the follicle yield at the LOPU sessions at the adult age were not significant (Fig. 6). The within-animal repeatability of AMH at 3, 4.5, or 6 months with AMH at the adult age was close to zero.

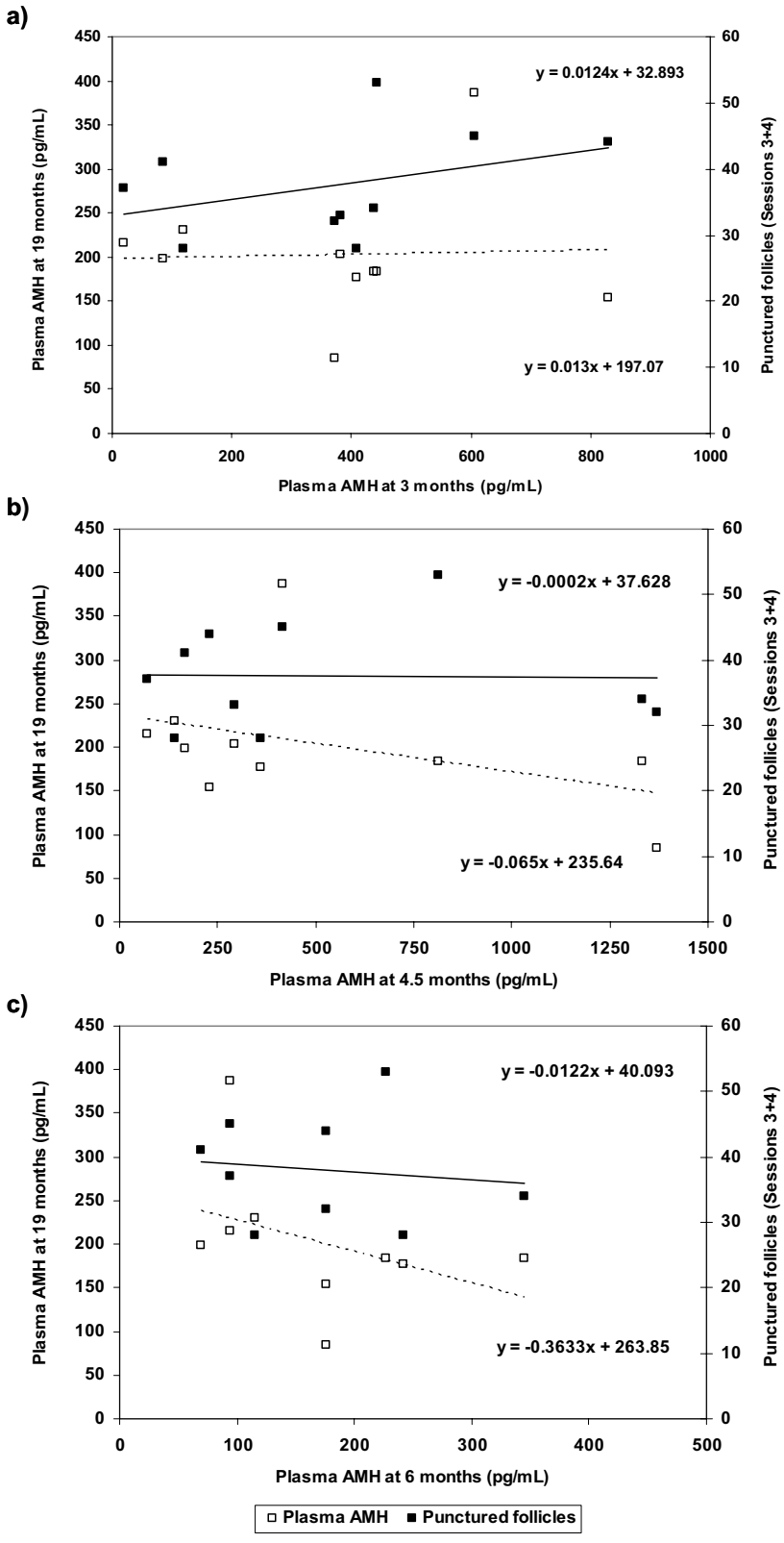

Fig. 6. Relationships between the plasma AMH concentrations before puberty and during adulthood. Plasma AMH at 3 (a), 4.5 (b), and 6 (c) months in relationship to the plasma AMH concentration at 19 months (open squares, dotted line) and the total number of follicles ≥ 3 mm to be punctured after FSH treatment at 19 months (closed squares, solid line).

In (c), nine points are represented both for AMH and punctured follicles, as there was one ewe lamb with non-detectable plasma AMH concentration at 6 months not taken into account.

4. Discussion

The present study shows that: 1) the presence of the *FecX*^{R} mutation in the *BMP15* gene did not affect the plasma AMH concentrations; 2) the plasma AMH concentration in the prepubertal age was demonstrated to be highly variable between individuals and months, and it did not demonstrate a relationship with the plasma AMH or follicle number at the adult age; 3) In adults, the plasma AMH proved to be a good predictor of the response to FSH stimulation, and therefore it could be used to improve the performance of the LOPU-IVP procedures in both R+ and ++ ewes.

In the present study, we aimed to investigate whether the presence of the *FecX*^{R} allele, with a deletion in the sequence encoding the *BMP15* gene, influences the plasma AMH concentrations, as other BMP factors (BMP4 and BMP6) have been shown to promote in vitro AMH expression in granulosa cells in cattle [22] and sheep [13]. We found no differences in the average plasma AMH concentrations between R+ and ++ ewes, neither before puberty, nor at the adult age. Nevertheless, when ewes with similar AMH concentrations at the time of FSH administration were compared, we found that R+ ewes developed a higher number of follicles at LOPU in response to FSH than ++ ewes. In fact, we proposed a different AMH cut-off value for each genotype to select the best-responding ewes. We found similar indications of an increased response of R+ ewes to exogenous gonadotropins in previous works. First, the advantage on the ovulation rate of the R+ ewes with respect to the ++ ewes was doubled when applying a standard dose of eCG [33]. Second, in LOPU experiments, the punctured follicles from R+ ewes were smaller than those from ++ ewes, and the application of FSH leads to approximately three extra follicles in R+ ewes, while similar numbers of follicles were punctured without FSH in both genotypes [20]. Third, we found a positive correlation in the untreated R+ ewes, but not in ++ ewes, between the LH peak area and ovulation rate, suggesting that the same LH concentration can trigger the ovulation of an increased number of follicles in R+ ewes [34]. This increased ovulatory response of R+ ewes could be explained by the "window" theory proposed by Scaramuzzi et al. [35]. According to that theory, the multiple ovulations could be due to an increase in the pool of gonadotropin-dependent follicles and/or to a lowering of the FSH threshold, or an increase in circulating FSH levels. The most accepted model for ewes carrying the Booroola mutation or other mutations in *BMP15* or *GDF9* relies on an increased size of the pool of gonadotropin-dependent follicles of smaller size, without apparent changes in endocrine patterns [36,37]. We can therefore hypothesise that R+ ewes could have an increased number of small growing follicles able to respond to gonadotropins, contributing to the secretion of AMH, that we have not taken into account in the present study. In addition, in other prolific breeds (Finn ewes) the increased ovulation rate was found to be achieved by rescuing some follicles from atresia, which came from the preceding follicular wave [38]. The rescue of some follicles undergoing atresia would be another hypothesis to explain the increased ovulatory response of R+ ewes. Our results are a first and gross analysis at the plasma level of differences in AMH between animals carrying or not carrying a deletion in the *BMP15* gene. Therefore, further studies are needed to address whether R+ ewes could have an increased number of small antral follicles able to respond to gonadotropins, as well as the AMH secretory capacity of R+ follicles in relationship with their diameter.

Studies on plasma AMH at the prepubertal age in domestic animals are scarce. In beef heifers, plasma AMH concentrations were found to increase markedly between 1 and 3 months of age, remaining high at 6 months, and then declining slowly until 12 months,

which corresponds with their age of puberty [13]. In the present study, using Rasa Aragonesa ewes in which puberty occurs at approximately 7 to 8 months [39], we found a similar profile for AMH, showing an increase at 4.5 months, and low AMH concentrations at 6 months, similar to those observed later in adulthood. This increase in AMH concentrations at 4.5 months would be coincident with a period where a peak in the total number of follicles ≥ 3 mm has been observed in other sheep breeds at approximately 16 weeks [40]. However, in the present study the maximum AMH concentrations were not found at the same time in all the ewe lambs, so individuals were not consistently ranked, which could explain the low repeatability found at these ages. In Sarda sheep, the plasma AMH concentrations determined at 40 and 110 days have been related to the total antral follicle count (AFC) [41]. It therefore appears that AMH at these early ages reflects changes in the ovarian follicle status; however, future studies are needed to assess the causes of these AMH variations before puberty and to evaluate whether these observed changes in AMH reflect changes in the number of small antral follicles or are due to changes in the amount of AMH secreted by each follicle.

In the present study, the AMH concentration in the plasma before puberty was not related with AMH concentration or the number of follicles that could be punctured after FSH treatment at the adult age. In a previous study, we found that plasma AMH measured at three months in prepubertal ewe lambs was a marker of the number of ovarian follicles able to respond to gonadotropins at these early ages and was also positively correlated with fertility at their first mating [14]. According to both studies, it appears that AMH is a quantitative marker of the number of ovarian follicles at any age, although its implications before puberty may be different than later in life. The large between-animal variations observed in the pattern of plasma AMH concentrations before puberty appear to reflect that ovarian maturity has occurred at an earlier age in some ewe lambs than in others. During the prepubertal phase, the AMH levels have been found to be negatively correlated with the FSH levels in cattle [13]. In our opinion, this relationship should be further investigated with a larger number of animals, a higher sampling frequency, and determination of other hormones related to puberty.

In the present study, the plasma AMH concentration at LOPUs without FSH were not fully predictive of the number of follicles ≥ 3 mm present in the ovary at these time points, although a tendency was found. In follicles from sheep, AMH concentrations are the highest in small antral follicles and decrease markedly when follicles increase to their preovulatory size. In Romanov ewes, the highest intrafollicular AMH concentrations were found in follicles from 1 to 3 mm, where it was approximately three-fold higher than the intrafollicular AMH found in 3 to 5-mm follicles, while it was almost negligible in follicles greater than 5 mm [13]. This pattern of production, together with the fact that the population of follicles from 1 to 3 mm is usually larger than the other populations, could explain the trend observed in the present study: when puncturing follicles from 3 mm onwards, we are only accounting for one small part of the AMH-producing follicles.

On the other hand, AMH prior to FSH treatment was highly predictive of the ovarian response to FSH treatment at LOPU, being correlated with the number of punctured follicles, recovered COCs and COCs suitable for IVM. As expected, correlations between AMH and punctured follicles were stronger than AMH and COCs, as the number of retrieved oocytes is affected by the recovery rate, which usually varies among animals and sessions, and which can be affected by several technical aspects external to the animal itself. Regarding the performance of AMH determination to detect high or low-responding ewes, the AUC values were greater than 0.7 in both genotypes, even after cross-validation.

According to an arbitrary rule that classifies tests as non-informative (AUC = 0.5), less accurate (0.5 < AUC < 0.7), moderately accurate (0.7 < AUC < 0.9), highly accurate (0.9 < AUC < 1) and perfect tests (AUC = 1) [31], we can rate this AMH test as "moderately accurate". Moreover, AMH was demonstrated to be highly repeatable between the sessions. Our results are in agreement with those previously found by Rico et al. [42] in cows undergoing repeated OPUs. Variations in the ovarian responses to FSH treatments are known to reflect the ovarian follicular population present at the beginning of treatment in small ruminants [5,43], so according to our results in sheep, it appears that the follicle population that produces the highest amounts of AMH is the same population able to be stimulated to grow by FSH. In conclusion, the use of AMH as a predictive method to select the best-responding donor ewes for embryo biotechnologies could improve the poor performance of the technique, and the cost of the analyses are expected to be largely offset by the reduced spending on stimulatory treatments, labour, or the optimised use of recipients.

In goats, the administration of exogenous FSH induces a decrease in the AMH concentrations, which occurs 3 to 4 days after treatment, apparently due to the temporary depletion of gonadotropin-responsive follicles, which return to the initial values before 3 weeks [11]. In agreement with this work, in the present study we found a diminution of plasma AMH after the administration of FSH. In the conditions of our work, this occurred seven days after the last FSH injection, in the last session. The diminution of AMH observed in the third session, which was not preceded by FSH treatment, is likely due to the short interval between follicle puncture in session 2 and sampling in session 3, separated only by 4.5 days, suggesting that the population of AMH-producing follicles was not fully restored after puncture. During the first two sessions, when FSH was not used, the plasma AMH concentrations remained constant, despite the AMH sampling in session 2 was preceded by ovarian puncture in session 1.

5. Conclusions

In conclusion, regardless of age, the presence of the *FecX*^{R} allele did not affect plasma AMH concentrations. Therefore, for its application on farms as a selection tool for early fertility, distinction between genotypes would not be needed. However, when applied to select the best-responding ewes to FSH for LOPU-IVP technologies, the *FecX*^{R} genotype should be taken into account. At the adult age, we propose that the determination of the plasma AMH concentration in a single blood sample prior to FSH treatment is a suitable method for selecting the best oocyte donors for embryo biotechnologies. However, the precocious selection of such animals by AMH sampling at their prepubertal age would not currently be feasible, as the plasma AMH concentrations before puberty varied significantly between months and between individuals.

Acknowledgements

This work was supported by INIA (RTA2011-128 project and fellowship for B. Lahoz).

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3.6. Publicación 5

Anti-Müllerian hormone plasma concentration in prepubertal ewe lambs as a predictor of their fertility at a young age.

Lahoz B., Alabart J.L., Monniaux D., Mermillod P., Folch J.
BMC Veterinary Research 2012, 8:118

Lahoz et al. BMC Veterinary Research 2012, 8:118
http://www.biomedcentral.com/1746-6148/8/118

BMC Veterinary Research

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Anti-Müllerian hormone plasma concentration in prepubertal ewe lambs as a predictor of their fertility at a young age

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Abstract

Background: In mammals, the ovarian follicular reserve is highly variable between individuals and impacts strongly on ovarian function and fertility. Nowadays, the best endocrine marker of this reserve in human, mouse and cattle is the anti-Müllerian hormone (AMH). The objectives of this work were to determine whether AMH could be detected in the plasma of prepubertal ewe lambs and to assess its relationship with their fertility at a young age.

Results: Plasma was taken from 76 Rasa Aragonesa ewe lambs at 3.6 months of age for AMH determination. Simultaneously, 600 IU equine chorionic gonadotropin (eCG) was administered and the number of ovulations recorded 6 days later. AMH was detected in 93% of the lambs, and the concentrations were about 3–4-fold higher in ovulating than in non-ovulating lambs ($P < 0.004$). Ewes aged around 10 months were mated, giving an overall fertility of 29%, and those failing to conceive were mated again 4 months later. Fertility at first mating was significantly correlated with plasma AMH concentration at 3.6 months (Spearman's $\rho = 0.34$; $P < 0.01$). To use plasma AMH concentration as a screening test, a value of 97 pg/mL was determined as the optimum cutoff value to predict fertility at first mating (sensitivity = 68.2%; specificity = 72.2%). Fertility at first mating was 34.8 percentage points higher in ewe lambs with an AMH ≥ 97 pg/mL than in those with lower AMH concentrations (50% vs. 15%; $P < 0.001$).

Conclusions: Plasma AMH concentration might be a reliable marker of the ovarian status of prepubertal ewe lambs, reflecting their ability to respond to eCG stimulation. A single AMH measurement performed on ewe lambs early in age could be useful to select for replacement ewes with a higher predicted fertility at first mating.

Background

The successful mating of ewe lambs within their first year of age reduces the effective cost of replacement ewes, increases the lifetime productivity of ewes in the flock, reduces the generation interval [1] and reduces the culling risks [2]. Moreover, an earlier first lambing brings about a higher productivity of the ewe throughout its life [3]. In some breeds such as the Rasa Aragonesa, even though females reach an adequate live weight at 12–13 months, the mean age at first lambing is 17–18 months [3], leading to extended unproductive periods. Therefore, it is challenging to find endocrine markers that could help to select the most precocious ewes, particularly if they could be used very early in life.

Sheep, as other mammals, are born with a limited and highly variable number of ovarian germ cells, which decreases drastically with age [4]. At birth, the oocytes are mostly contained in resting primordial and early growing follicles that constitute the so-called follicular reserve. Genetic factors and maternal nutrition during gestation both seem to contribute to this inherently high variation in the follicular reserve in sheep [4-8]. At 12–14 weeks after birth, an important population of gonadotropin-sensitive follicles is already present in the ovary [9,10]. Moreover, recent results suggest that the number of growing antral follicles present in the ovaries of young adult cattle may be related to their fertility [11]. Therefore, the follicular populations present in the

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ovaries of young ruminants seem to have a great impact on their adult reproductive life.

Presently, anti-Müllerian hormone (AMH) is the best endocrine marker of the ovarian follicular reserve of growing follicles, and it is used as a predictor of the ovarian response to gonadotropins in the human [12,13], mouse [14] and bovine species [15]. In the cow, AMH concentration in plasma is highly correlated with the number of antral follicles of 3 to 7 mm in diameter, which are the main targets of superovulatory treatments [15]. Also, AMH measurement can help to predict the aptitude of individuals to produce high or low numbers of embryos after superovulation, a repeatable and possibly heritable trait in the cow [16]. Recently, similar results were reported in mares, indicating that AMH could be a predictor of the number of follicles available in a follicle aspiration program [17]. Although a direct relationship between AMH and fertility has not been reported to date in domestic animals, some evidence supports this idea. On one hand, in young adult cattle, a high degree of correlation among AMH, antral follicle count (AFC) and the number of healthy follicles and oocytes in ovaries has been demonstrated; and on the other hand, lower numbers of antral growing follicles (AFC) have been related with suboptimal fertility [11]. Up to now, there are no data on plasma AMH concentration in sheep, though it would be of great interest for the future selection of animals for embryo production, as well as of young animals with a high fertility in the adult age.

Therefore, the aims of the present study were, first, to determine if AMH can be detected in the plasma of prepubertal Rasa Aragonesa ewes and to evaluate its relationship with the ovarian response to a gonadotropin treatment, and second, to relate AMH with fertility at first and second mating opportunities. With this aim, the plasma AMH concentration of ewe lambs aged around 3.6 months was assessed as well as the ovarian response to an equine chorionic gonadotropin (eCG) challenge, and their fertility was established after mating at 10 and 14 months.

Results

Plasma AMH concentration and its relationship with eCG-induced ovulation in prepubertal ewe lambs

AMH was detected in the plasma of 71 out of 76 ewe lambs (94%). The concentrations were highly variable between animals, ranging from 0 ($n = 5$) to 590 pg/mL. The age and weight of the ewe lambs with nondetectable AMH concentrations ranged from 88 to 126 days and from 23.5 to 28 kg. AMH was detected in the youngest lamb of this study (68 days) as well as in the lightest lamb (19 kg), reaching 362.5 and 53.7 pg/mL, respectively.

AMH concentration in plasma was not correlated with age ($\rho = -0.19$; NS) or live weight ($\rho = -0.08$; NS). Plasma AMH concentration was correlated with the occurrence of ovulation in response to eCG ($\rho = 0.42$; $P < 0.0001$) but not with the number of ovulations when only considering ovulating ewes ($\rho = 0.08$; NS). Neither the occurrence of ovulation nor the number of ovulations of ovulating ewes were correlated with age ($\rho = 0.13$ and -0.27, respectively; both NS) or live weight ($\rho = 0.04$ and 0.02; both NS). As expected, age and live weight were highly correlated ($\rho = 0.38$; $P < 0.001$).

Non-ovulating ewe lambs had lower plasma AMH concentrations than ovulating ewe lambs with 1, 2 or ≥ 3 ovulations (all $P < 0.004$; Table 1). However, no significant differences in AMH were found between the different groups of ovulating ewe lambs. No significant differences in live weight or age were found between any of the groups.

Relationship between plasma AMH concentration of prepubertal ewe lambs and their fertility at the first and second service periods

Ultrasound pregnancy diagnosis was coincident with fertility results both in the first (28.9%, n = 22 pregnant ewes) and in the second mating (77.8%, n = 42 pregnant ewes), at 10 and 14 months, respectively, revealing the absence of abortions and of mistakes in the lambing records. The overall fertility after the two consecutive mating opportunities was 84.2%.

The fertility of adult ewes at first, at second and after both consecutive service periods was correlated with their AMH concentration in the prepubertal phase ($\rho = 0.34$, $P < 0.01$; $\rho = 0.33$, $P < 0.05$; $\rho = 0.36$, $P < 0.01$, respectively). At the first mating, neither age nor live weight was significantly different between pregnant and non-pregnant ewes (315 ± 5 vs. 311 ± 3 days and 33.4 ± 0.6 vs. 32.6 ± 0.5 kg, respectively; both NS). Prepubertal plasma AMH concentration was higher in ewes that became pregnant at the first mating than in those which failed to conceive at first but became pregnant at the second mating ($P < 0.05$) or in non-pregnant ewes

Table 1 Plasma anti-Müllerian hormone (AMH) concentration (pg/mL), age (d) and live weight (kg) at the moment of ovarian stimulation with 600 IU eCG, for each ovulation group (0, 1, 2 and ≥3 ovulations) of prepubertal ewe lambs (means ± SEM)

No. of ovulations	No. of ewes	Age	Live weight	AMH
0	19	108 ± 2^{a}	25.0 ± 0.4^{a}	43.0 ± 14.6^{a}
1	17	118 ± 4^{a}	25.5 ± 0.6^{a}	126.9 ± 34.0^{b}
2	21	104 ± 5^{a}	24.7 ± 0.4^{a}	99.8 ± 18.3^{b}
≥3	19	108 ± 4^{a}	24.7 ± 0.8^{a}	163.1 ± 41.0^{b}

Within columns, means with different superscripts differ significantly: a,b: $P < 0.004$.

after two service periods ($P < 0.01$). It was also higher in ewes which became pregnant at the second mating than in non-pregnant ewes ($P < 0.05$; Figure 1).

The performance of AMH concentration at prepubertal age for predicting the fertility of adult ewes at first mating is shown in Figure 2. The receiver-operating characteristic (ROC) curve represents the relationship between the sensitivity of the diagnostic test based on AMH concentration at prepubertal age to predict the occurrence of pregnancy in adults (true-positive percentage) and 100 minus the specificity of the test to predict the failure of pregnancy (false-positive percentage) at first mating.

The predicting performance of plasma AMH concentration, assessed by the AUC (area under the ROC curve), was 0.698 ± 0.063, significantly different from 0.5 (the AUC value corresponding to a non-informative or "random test") ($P < 0.01$). The AUC values estimated by the leave-one-out validation and 10-fold cross-validation methods were somewhat lower (0.660 ± 0.066 and 0.650 ± 0.013, respectively), although also different from 0.5 ($P < 0.02$ and $P < 0.001$, respectively).

The cutoff point of plasma AMH concentration that could be used for a screening test of the future ability of ewes to get pregnant at first mating was found to be 97 pg/mL. This AMH concentration corresponded to a sensitivity value to predict the occurrence of pregnancy of 68.2% (15/22) and a specificity to predict the failure of pregnancy of 72.2% (39/54). The positive and negative predictive values (PPV and NPV) were 50.0% and 84.4%, respectively (Table 2). The fertility at first mating of ewes with AMH concentrations equal or higher than 97 pg/mL before puberty was 34.8 percentage points higher than that of ewes with lower AMH concentrations ($P < 0.001$).

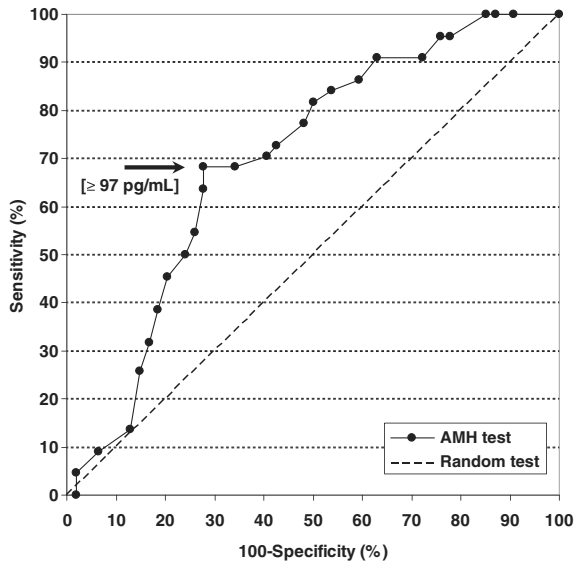

Figure 2 Receiver-operating characteristic (ROC) curve for predicting fertility at first mating through AMH in prepubertal age. The arrow shows the cutoff point maximizing the Youden index, and its corresponding AMH concentration is shown within brackets. A test without capacity of discrimination (random test) is represented as a dashed line.

The fertility at second mating or after both consecutive service periods was also higher in ewes with AMH

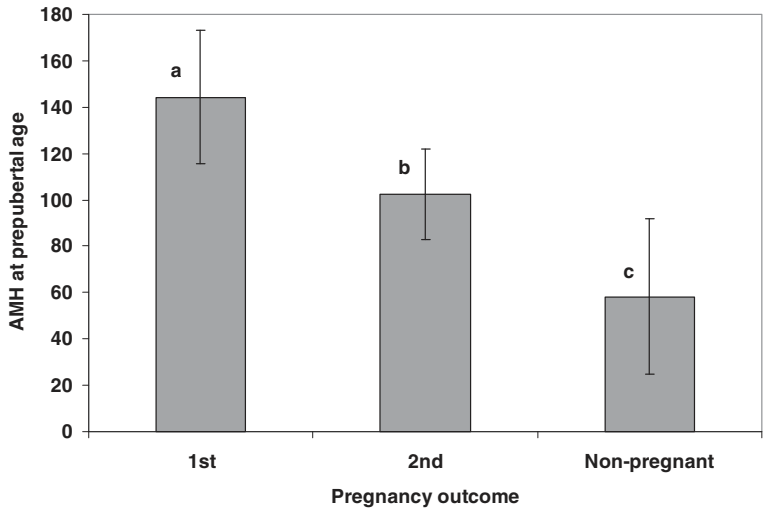

Figure 1 Relationship between AMH at prepubertal age and pregnancy outcome. AMH: Anti-Müllerian hormone plasma concentration (pg/mL) at prepubertal age (means ± SEM). Pregnancy outcome: 1st, ewes pregnant at first service (n = 22); 2nd, ewes pregnant at second service (n = 42); Non-pregnant, ewes failing to conceive after being mated twice four months apart (n = 12). Means with different superscripts differ: a,c: $P < 0.01$; a,b or b,c: $P < 0.05$.

Table 2 Number and percentages (between brackets) of lambing and non-lambing ewes at first mating predicted through their plasma AMH concentration at prepubertal age using a cutoff value of 97 pg/mL

Selection criterion	AMH group	Lambing	Non-lambing	No. of ewes
AMH	≥ 97 pg/mL	15 (50.0) [a]	15 (50.0)	30
	< 97 pg/mL	7 (15.2) [b]	39 (84.8)	46
No selection	—	22 (28.9)	54 (71.1)	76

Within columns, percentages with different superscripts differ: a,b: $P < 0.001$.

concentration equal or higher than 97 pg/mL before puberty (93.3 vs. 71.8%; $P < 0.03$ and 96.7 vs. 76.1%; $P < 0.004$, respectively).

In the present work, the benefit of selecting replacement ewes by AMH (calculated as the difference between PPV, 50% and overall flock fertility at first mating, 28.9%), was 21.1 percentage points. As PPV (as well as NPV) depends on the prevalence of the character in the population [18] (herein, the overall flock fertility), PPV for assumed flock fertility values at first mating from 5 to 95% has been depicted in Figure 3. It is shown that the benefit obtained selecting replacement ewes by prepubertal AMH would be higher for intermediate than for either high or low flock fertility values.

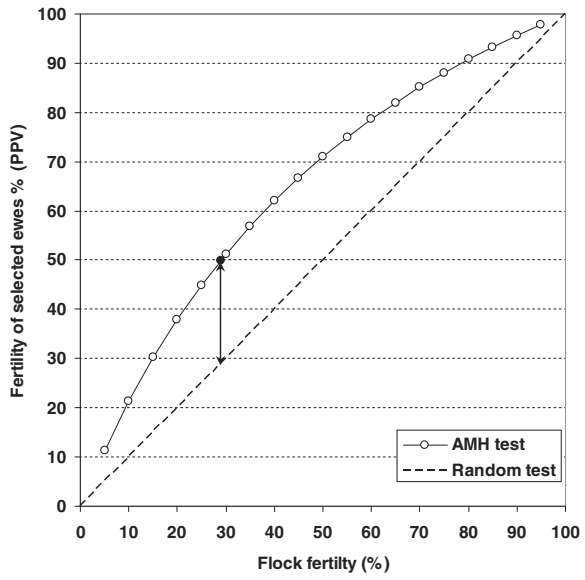

Figure 3 Fertility of the selected ewes as a function of the flock fertility at first mating. Data represent the predictive positive values (PPV, open circles). PPV values were calculated using the sensitivity and specificity corresponding to the cutoff point (plasma AMH ≥ 97 pg/mL; Se: 68.2%; Sp: 72.2%). The closed circle shows the PPV value corresponding to flock fertility at the first mating in the present study (28.9%). A test without capacity of discrimination (random test) is represented as a dashed line. The arrow shows the increase in fertility obtained in the present work by selecting ewes with AMH ≥ 97 pg/mL.

Discussion

This study shows for the first time that: (1) AMH is present in the plasma of prepubertal ewe lambs; (2) plasma AMH concentration is related to the occurrence of ovulation in ewe lambs after administration of an ovarian stimulation treatment, possibly reflecting that a population of gonadotropin-responsive follicles is already present at this age in most ewes; and (3) plasma AMH concentration before puberty could be used as a predictor of the fertility of adult ewes at first mating. This test would allow farmers to perform a precocious selection of replacement ewe lambs with the highest expected fertility at first mating. In the conditions of the present study, plasma AMH concentrations did not depend on age and weight, being detectable in most ewe lambs. Nevertheless, longitudinal studies would be necessary to accurately determine the age and weight at which AMH becomes detectable as well as the precise relationships of AMH with age and live weight.

In ewe lambs at around 3.6 months of age, plasma AMH concentration was about 3–4-fold higher in ovulating than in non-ovulating ewe lambs after eCG treatment. This difference could be due to the presence of a different numbers of antral gonadotropin-responsive follicles between animals at this age. In this way, the mean age of the ewe lambs of this study (15.6 weeks) would be coincident with a period where a peak in the total number of follicles ≥ 3 mm has been observed in other breeds of sheep, varying between 14 weeks [13] and 16 weeks [19]. Therefore, the relationship found between the ovulatory response to eCG and plasma AMH concentrations could be explained by the fact that they likely both reflect the presence of antral gonadotropin-responsive follicles in the ovaries of ewe lambs. In adult cows, a high correlation of plasma AMH concentrations with the number of corpora lutea after superovulation was found [15]. However, in the present study a relationship between AMH and the number of ovulations in ovulating ewe lambs was not found. This discrepancy could be attributed to physiological differences between prepubertal and adult females and/or to differences in the ovarian stimulation treatments. In this way, the ovarian response to a single eCG treatment without previous progestogen priming might be different to the response to a superovulation protocol consisting in multiple FSH decreasing doses with previous progestogen synchronization.

In the present work, neither plasma AMH concentration before puberty nor fertility at first mating was related with age or live weight. Moreover, all ewes received the same feeding plane during the whole experiment. Therefore, the observed differences in plasma AMH concentrations amongst ewes of similar age and similar live weight may be due to inherent variations in the follicular population, possibly influencing fertility at

first mating. Genetic factors or maternal nutrition during pregnancy could therefore be responsible for this asynchronous follicular development; as in sheep, alterations in the maternal diet have been demonstrated to affect fetal ovarian development [5,7]. Nevertheless, further studies are needed to elucidate the causes of this asynchronous development of the ovary between the same aged and weighted ewes, as well as to determine whether it could affect ovarian function and reproductive performance during the adult life.

Apart from age, live weight and nutritional status, it is widely known that successful pregnancy is affected by many other factors, such as season, endocrine status or uterine conditions [20]. In the present work, healthy ewe lambs were reared under the same conditions to minimize the impact of such factors, although possible unknown individual disorders can not be discarded. The results of the present study showed that ewes with higher prepubertal plasma AMH concentrations displayed a higher probability of becoming pregnant at first mating than those with lower plasma AMH concentrations. Moreover, those ewes which failed to conceive after two consecutive mating opportunities had the lowest prepubertal plasma AMH concentrations. As far as we know, this is the first time that a relationship between AMH and early fertility has been established in a domestic species. Our study reinforces the prognostic value of AMH, even when the physiological status is not fully known. Both in the human and bovine, AMH levels are almost independent of the phase of the ovarian cycle, explaining why a single AMH measurement is usually sufficient [21,22]. This could be due to the fact that AMH is not involved in feed-back mechanisms of the hypothalamo-pituitary-gonadal axis [21]. Previous studies in the human have demonstrated a relationship between AMH and fertility. Very low concentrations of AMH are associated with infertility in the case of premenopause and premature ovarian failure, and very high AMH concentrations with an absence of ovulation in women with polycystic ovarian syndrome. Low or undetectable levels of AMH is explained by decrease or absence of follicular growth due to depletion of the ovarian reserve of primordial follicles. Abnormally high levels of AMH are explained by an excess of antral follicles whose growth is blocked prior to ovulation [21]. Recent results have also evidenced that AMH seems to be an excellent marker of ovarian function in girls and adolescents. In this way, elevated serum AMH levels have been found in girls with a precocious maturation of the adrenal cortex, suggesting that follicular development has proceeded to a more advanced developmental stage than expected for chronological age [23]. It has been demonstrated in adolescent patients with inherited reproductive problems (Turner syndrome) that their AMH concentrations correlate significantly with ovarian function at time of AMH measurement, showing an excellent sensitivity and specificity as a screening test of premature ovarian failure. Furthermore, it has been suggested that variations in AMH concentrations during childhood may theoretically predict the duration of any given girl's reproductive lifespan [24], as AMH seems to reflect the continuous decline of the follicle pool with age [25].

In the case of ewe lambs, the observed AMH values would be rather a reflection of the beginning of follicular growth in prepubertal animals, showing an ovarian activity quantitatively different between animals of the same age. It is possible that ewe lambs in our study with the highest ovarian activity at 3.6 months were also the most precocious in terms of early puberty, resulting in the improved fertility observed when testing fertility of different ewes at the same age. The lambs with the earliest follicular development would be fertile at an early age. All these findings highlight the importance of individual differences in chronological follicular development and in the follicular reserve as being responsible for future reproductive life.

In sheep, recent in vitro studies have demonstrated that prepubertal oocytes isolated from ovaries with a high number of follicles larger than 2 mm are more competent for in vitro development, presenting a higher cleavage rate, developmental kinetics and cell number at the blastocyst stage [26]. As it has been established that the granulosa cells of large preantral and small antral healthy follicles produce the highest AMH amounts in rat [27] and cow [15,22], prepubertal ewe lambs with higher plasma AMH concentrations are supposed to have a higher number of healthy follicles in these stages, suggesting that the quality of the oocytes and embryos coming from these ewes may be higher. Although further studies are needed to confirm this hypothesis, it could partially explain the increased fertility at first mating of ewe lambs with the higher concentrations in plasma AMH during their prepubertal phase.

Concerning the ability of AMH to be used as a screening test to select the most precocious ewes in terms of fertility at first mating, in the present study the AUC value for AMH (0.698) was very close to 0.7, the limit value to classify tests as "moderately accurate" according to an arbitrary guideline. This guideline distinguishes between non-informative ($AUC = 0.5$), less accurate ($0.5 < AUC < 0.7$), moderately accurate ($0.7 < AUC < 0.9$), highly accurate ($0.9 < AUC < 1$) and perfect tests ($AUC = 1$) [28]. The values obtained by cross-validation methods were somewhat lower and would correspond to a "less accurate" test. This is not strange as it its well known that the establishment and maintenance of pregnancy depends upon several genetic and environmental factors. Previous reports in the human have shown that while high AMH levels prior to

the initiation of IVF treatments is a good predictor of the ovarian response, it has not proven to be as predictive of successful pregnancy. This indicates that factors other than the quantitative aspects of ovarian reserve may influence the fate of pregnancy [29]. Nevertheless, although not being highly accurate, we have shown that fertility of replacement ewes at first mating can be increased up to about 20 percentage points by using plasma AMH concentration as a screening test. It remains to be assessed at which age and weight the diagnostic test based on the measurement of AMH concentrations on ewe lambs is the most efficient for accurate prediction. Concerning fertility at the second mating and fertility after two consecutive service periods, we have shown that the differences between ewes with AMH concentration above and below the cutoff point are also significant and of great practical importance (+21.5 and +20.6 percentage points, respectively). This would indicate that AMH could also be a predictor of fertility in adulthood, but this needs further confirmation.

Conclusions

In conclusion, a single AMH measurement in the plasma of prepubertal ewe lambs may be useful to select, in a very early age, the replacement ewes with a high predicted fertility at first mating.

Methods

All experimental procedures were performed in accordance with the guidelines of the European Union (2003/65/CE) and Spanish regulations (RD 1201/2005, BOE 252/34367-91) for the use and care of animals in research.

Experimental design

Prepubertal ewe lambs (aged around 3.6 months) were sampled for plasma AMH determination. In order to test the presence of gonadotropin-sensitive follicles at this age, an injection of eCG was applied, and 6 days after, the induced ovulation (number of ovulations) was recorded by laparoscopy. The relationship between AMH concentration and the ovarian response to eCG was investigated. Seven months later, the same young ewes were first joined to rams twice, 4 months apart. The relationship between fertility at these mating opportunities and plasma AMH concentration before puberty was studied. A ROC curve for AMH was analyzed to find the best cutoff value to predict fertility at first mating.

Plasma sampling and determination of eCG-induced ovulation in prepubertal ewe lambs

The experiment started in July (beginning of the breeding season) in the facilities of the research center (CITA). A total of 76 Rasa Aragonesa prepubertal ewe lambs aged 109 ± 18 days (mean ± SD) and weighing of 25.0 ± 2.4 kg (mean ± SD) were used. Lamb ewes were weaned at 50 days postpartum and fed at libitum with concentrate until the beginning of the experiment. Ewe lambs received eCG (600 IU im; Sincropart PMSG, CEVA Salud Animal S.A., Barcelona, Spain), and simultaneously, blood samples were collected by jugular puncture using 5 mL vacuum tubes with lithium heparin that were immediately centrifuged at 2100 × g for 25 min. Plasma was stored at −20°C until analyzed for AMH. Six days after eCG treatment, ovulation was recorded by laparoscopy as follows. Ewe lambs were sedated with propofol (6 mL iv; Propofol Lipuro 1%, Braun, Spain) and local anesthesia with lidocaine was applied. An endoscope was inserted into the abdominal cavity through a 1-cm incision, approximately 10 cm cranial to the udder, and an atraumatic grasping forceps was introduced through a contralateral 1-cm incision. After total visualization of both ovaries, the number of healthy corpora lutea was recorded. The incisions were closed with staples and sprayed with topical chlortetracycline hydrochloride, and 1 mL/10 kg oxytetracyclinedihydrate (200 mg/mL; Oxycen-200 L.A., s.p.® veterinaria, s.a., Tarragona, Spain) was administered intramuscularly. After that, animals were fed at libitum under grazing conditions.

Evaluation of the fertility of the ewes at first and second mating

In this study, fertility was defined as the ability of a ewe to become pregnant in a given mating period. In January (end of the breeding season), when ewes were 312 ± 18 days old (mean ± SD) and weighing 33.1 ± 3.1 kg (mean ± SD), they were first joined to four adult Rasa Aragonesa rams of proven fertility. The minimum ram-ewe lamb ratio was around 5% (first mating: 4/76; second mating: 4/54). This ratio was similar or slightly greater than those proposed by other authors [30,31]. Males were removed after 34 days. Fertility at first mating was calculated as the percentage of ewes lambing (and thus successfully mated) over ewes exposed to rams (n = 76) in this first service period. Four months later (in May), the non-pregnant ewes were mated again for 34 days, according to an accelerated lambing program of three lambings in 2 years. Fertility at second mating was calculated as the percentage of ewes lambing (and thus successfully mated) over ewes exposed to rams (not considering pregnant ewes at first mating) in this second service period. Overall fertility after the two consecutive mating periods was calculated as the percentage of ewes lambing after either first or second mating periods, over all the ewes exposed to rams (n = 76). Ten and 30 days after the rams' removal, ultrasound diagnosis of pregnancy was performed. Lambing took place in individual pens and offspring were immediately identified.

AMH assay

Plasma concentrations of AMH were measured with the Active MIS/AMH ELISA Kit (Beckman Coulter France, Roissy CDG, France), as described previously [15,32]. Just before the assay, the frozen plasma samples were thawed in a warm water bath, vortexed and centrifuged (3200 g, 10 min, 4°C) to remove any cell fragments that could interfere with the reagents of the assay. AMH concentrations were determined in 50-μL undiluted plasma samples. The samples were incubated overnight at 4°C in the presence of the primary antibody, then for 1.5 h at room temperature in the presence of the secondary antibody. Dilutions of the points of the standard curve were made in steer plasma. With these conditions, the limit of detection of the assay was found to be 15 pg/mL. All the plasma samples were analyzed in the same assay. The intra-assay coefficients of variation were all lower than 5 % for the four quality control plasma samples that were tested, containing 27, 112, 195 and 274 pg/mL of AMH. For further validation of the assay, serial dilutions of different ovine plasma and follicular fluid samples in steer plasma were analyzed. Results showed that dilution curves were linear and parallel to the standard curve (Figure 4).

Evaluation of plasma AMH concentration in prepubertal age as a predictor of fertility of the ewe at first and second mating

An ROC analysis was performed to evaluate the predicting ability of plasma AMH concentration to detect the ewes with a high fertility at first mating. The sensitivity was defined as the ratio between the ewes correctly predicted as pregnant above an AMH value and all the pregnant ewes. The specificity was the ratio between the ewes correctly predicted as non-pregnant below an AMH value and all the non-pregnant ewes. The PPV was the ratio between the ewes correctly classified as pregnant and those predicted as pregnant (in other words, the fertility of the selected ewes above an AMH value). The NPV was the ratio of ewes correctly classified as non-pregnant and those predicted as non-pregnant (100 minus fertility of non-selected ewes, below an AMH value).

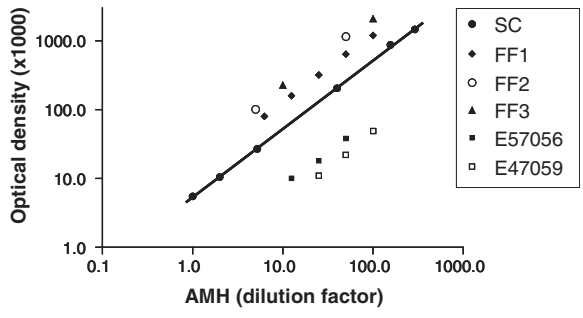

Figure 4 Parallelism of dilution curves of ovine plasma and follicular fluids with the human AMH standard curve in the Active MIS/AMH ELISA kit. The figure depicts the results of the serial dilutions of ovine follicular fluid samples (FF1, FF2 and FF3), plasma from 2 ewes (E57056 and E47059) and the standard curve (SC) of the AMH ELISA. Dilutions of the standard points and the ovine samples were all made in steer plasma. The absorbance (optical density) measured at 450 nm at the end of the test is directly proportional to the concentration of AMH in the samples.

Statistical analysis

Correlations among the variables: occurrence of ovulation (1 = ovulating; 0 = non-ovulating), number of ovulations in ovulating ewes, plasma AMH concentration, age, live weight and fertility (1 = pregnant; 0 = non-pregnant) were assessed by the Spearman correlation coefficient (ρ). Continuous variables (age, live weight, plasma AMH concentration) were tested by general linear models (ANOVA). Normality of residuals was tested by the Jarque-Bera test, applying a correction for finite samples [33]. Homogeneity of within-groups variances (homoscedasticity) was tested by the Brown-Forsythe test. As plasma AMH concentration failed to accomplish the requirements of residuals normality and homoscedasticity, its values (expressed in pg/mL) were transformed to decimal logarithms ($\log_{10}[AMH + 1]$) prior to ANOVA tests. Pairwise comparisons between means were carried out by the least significant difference test, applying the "false discovery rate" approach [34] to adjust probabilities for multiple comparisons. For plasma AMH concentrations, although significance shown in tables was obtained through the transformed data, arithmetic means and SEM of the original, non-transformed data are presented. Dichotomous variables, presented as percentages in text and tables, were analyzed by chi-square tests. When the number of ovulations was used as independent variable in ANOVA, ewes with numbers of ovulations higher than two (from 3 to 7) were grouped into one category owing to the small number of ewes in these categories. In order to evaluate plasma AMH concentration as a predictor test of the fertility at first mating, the ROC curve was analyzed. The AUC was used as an indicator of the global performance of the test. The values of AUC were compared with 0.5 (AUC for a "random test"), using the non-parametric approach for testing statistical significance [28]. As AUC values computed from the data set used for model fitting tend to indicate better accuracy than the actual model allows in practice, AUC values estimated by the leave-one-out validation method and by 10-fold cross-validation method are also presented [35]. The 10-fold cross-validation was repeated 10 times and the average AUC of these 10 replicates ± SD was calculated. The cutoff value of AMH to discern between fertile and non-fertile ewes at first

mating was chosen as the corresponding point of the ROC curve that maximized the Youden index, a prevalence-independent criterion [28].

All statistical analyses were performed using SAS software [36]. Otherwise specified, all data presented in text are means ± standard errors.

Authors' contributions

BL, JLA, PM and JF designed research. BL and JF administered treatments, performed endoscopy and collected samples and data. DM and PM analyzed samples. JLA carried out statistical analyses. BL, JLA, DM and JF have written the manuscript. All authors have contributed to the discussion. All authors read and approved the final manuscript.

Acknowledgments

This work was supported by INIA (Ministerio de Ciencia e Innovación, Spain; RTA-2011-128 and RZP-2010-002 projects and fellowship for B. Lahoz). The authors would also like to thank Pilar Sánchez and Elías Echegoyen for their technical support.

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Received: 24 November 2011 Accepted: 28 June 2012
Published: 23 July 2012

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doi:10.1186/1746-6148-8-118
Cite this article as: Lahoz *et al.*: Anti-Müllerian hormone plasma concentration in prepubertal ewe lambs as a predictor of their fertility at a young age. *BMC Veterinary Research* 2012 **8**:118.

RESUMEN / SUMMARY

4.

Resumen

Esta Tesis Doctoral se planteó para avanzar en el conocimiento y la utilización de marcadores genéticos y endocrinos de la población folicular en ovejas para ser seleccionadas por su eficiencia reproductiva. Para ello se evaluó el efecto del alelo *FecX*^{R} del gen *BMP15* sobre el rendimiento reproductivo (publicaciones 1, 2 y 3), y se investigó la capacidad de la hormona Anti-Mülleriana como otro posible biomarcador (publicaciones 4 y 5). Todos los experimentos se llevaron a cabo en el Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón (Coordenadas: 41º43'19''N-0º48'32''W), o bien a partir de datos obtenidos en las ganaderías que integran el programa de mejora genética por prolificidad de la UPRA-Grupo Pastores. Los análisis de LH, PAG y P4 se llevaron a cabo en el CITA y los de AMH en el Institut National de la Recherche Agronomique (INRA) de Nouzilly (Francia). Todos los estudios se llevaron a cabo en animales de la raza Rasa Aragonesa, siendo los lotes similares en peso y condición corporal dentro de cada experimento. Todos los procedimientos experimentales se realizaron de conformidad con las directrices de la Unión Europea (2003/65/CE) y la normativa española (RD 1201/2005, BOE 252/34367-91) para el uso y cuidado de los animales en la investigación.

<u>Publicación 1.</u> Effect of the *FecX*^{R} polymorphism in the bone morphogenetic protein 15 gene on natural or equine chorionic gonadotropin-induced ovulation rate and litter size in Rasa Aragonesa ewes and implications for on-farm application.

En 2007, se descubrió en la Rasa Aragonesa una nueva mutación en el gen *BMP15*, un Gen Mayor con gran efecto sobre la tasa de ovulación y la prolificidad. Cuando está presente en heterocigosis produce un incremento de la prolificidad y en homocigosis da lugar a fenotipos estériles. Mediante los experimentos de la presente publicación se trataron de caracterizar sus efectos sobre dichos caracteres reproductivos (objetivo 1 de la Tesis). Para ello se realizaron tres experimentos:

En el **primer experimento** se comparó la tasa de ovulación natural de corderas y ovejas adultas portadoras del alelo *FecX*^{R} en heterocigosis (R+) con la de las ovejas no portadoras (++). Este experimento se llevó a cabo entre noviembre y diciembre (estación sexual). Se utilizaron 111 corderas (91 R+ y 20 ++) con una edad media de 311±11 días, y 103 ovejas adultas (84 R+ y 19 ++) con una edad media de 683±37 días (medias±SD; grupos 'R+ young', '++ young', 'R+ adult' y '++ adult', respectivamente). Todos los animales se trataron con esponjas de 30 mg de acetato de fluorogestona (FGA) durante 14 días con el objetivo de sincronizar el ciclo sexual. No se aplicó eCG a la retirada de las esponjas para no interferir con su tasa de ovulación. La tasa de ovulación se determinó durante tres ciclos consecutivos en cada animal mediante observación laparoscópica, realizando la primera observación el sexto día tras la retirada de la esponja, y repitiéndola a los 17 y 34 días. La

tasa de ovulación de cada animal fue la media de las observaciones registradas en los ciclos ovulatorios.

Se observó que las hembras R+ presentaron una tasa de ovulación superior a las ++, tanto en corderas como en adultas (+0,44 y +0,63 ovulaciones, respectivamente; ambas P<0,01). En cuanto a la distribución de las ovulaciones, las corderas R+ presentaron un mayor porcentaje de ovulaciones dobles que las corderas ++ (+50,5%; P<0,01). Las ovejas adultas R+, además de un incremento de las ovulaciones dobles (+36,4%; P<0,01), también presentaron un mayor porcentaje de ovulaciones triples o de orden superior respecto a las ovejas ++ (+12,8%; P<0,05).

En el **segundo experimento** se trató de evaluar la posible interacción entre el genotipo y la aplicación de gonadotropinas, comúnmente utilizadas en los tratamientos comerciales de sincronización de celos. Para ello se determinó la tasa de ovulación de las ovejas adultas R+ en respuesta a dos dosis diferentes de eCG. Se estudió la influencia del incremento en la tasa de ovulación sobre la mortalidad embrionaria y el peso al nacimiento de los corderos, con el fin de determinar la dosis óptima a aplicar a las ovejas R+ en condiciones de campo.

El experimento se llevó a cabo en septiembre (estación sexual). Se utilizaron 119 ovejas (71 R+ y 48 ++), que se trataron con esponjas vaginales de 30 mg de FGA durante 14 días. En el momento de la retirada de las esponjas, las ovejas ++ recibieron 480 UI de eCG im (grupo "++480"), la dosis estándar que se utiliza en las ganaderías para la IA en ovejas Rasa Aragonesa, mientras que las ovejas R+ recibieron 240 UI (grupo "R+240"; n=35) ó 480 UI (grupo "R+480"; n=36) de eCG. A las 54±1 h de la retirada de las esponjas se realizó la IA cervical con semen refrigerado a 15°C (400×10^6 espermatozoides/oveja). La tasa de ovulación se determinó 8 días después de la retirada de las esponjas, durante un único ciclo, en 75 de las 119 ovejas ("R+240": n=21; "R+480": n=21; "++480": n=33 ovejas), siguiendo la metodología del experimento 1. Una semana antes del parto, las ovejas se individualizaron en jaulas de parición y los corderos se identificaron y pesaron. Se determinaron las pérdidas embrionarias (PFMO: Partial Failure of Multiple Ovulation) calculando la diferencia entre la tasa de ovulación y la prolificidad en aquellas ovejas que parieron y cuya tasa de ovulación fue igual o superior a 2.

La mayor tasa de ovulación se observó en las ovejas R+ tratadas con 480 UI de eCG (3,95; P<0,01), mostrando casi un 60% de ovulaciones cuádruples o de mayor orden. Cuando se redujo la dosis a 240 UI, la tasa de ovulación de las ovejas R+ fue similar a la de las ovejas ++ que recibieron 480 UI (2,90 *vs.* 2,82). En el grupo "R+240" predominaron las ovulaciones triples (47,6%), mientras que en el grupo "++480" predominaron las dobles (42,4%). El mayor PFMO se observó en el grupo "R+480" (1,85; P<0,05), mientras que no hubo diferencias significativas entre los grupos "R+240" y "++480". Cuando se compararon las tasas de ovulación de los experimentos 1 y 2 para determinar el efecto de la eCG dentro de

cada genotipo, se observó una mayor respuesta en las hembras R+ (+0,50 ovulaciones adicionales).

Los resultados de este trabajo indican que, desde un punto de vista aplicativo, sería recomendable reducir la dosis estándar de eCG a la mitad cuando se aplique a ovejas R+ en las ganaderías para evitar porcentajes muy elevados de ovulaciones múltiples. Los registros de partos mostraron que no hubo diferencias en el peso al nacimiento de los corderos debidas al genotipo cuando se compararon los corderos del mismo tipo de parto.

En el **tercer experimento** se determinó la prolificidad de las ovejas adultas R+ tratadas o no con eCG en las ganaderías que forman parte del programa de mejora genética por prolificidad de la UPRA-Grupo Pastores. Para ello fue necesario en primer lugar, validar la asignación por genealogía como un método de identificación de las hembras R+ nacidas en los rebaños antes del descubrimiento de la mutación en *BMP15*. Para ello se genotiparon todas las ovejas de cuatro rebaños élite pertenecientes al programa de selección (1.667 ovejas con 10.945 partos), en las que se suponía una alta proporción de animales portadores. Se determinó el genotipo *FecX*^{R} mediante PCR en muestras sanguíneas utilizando tubos con EDTA. Para la asignación del genotipo por genealogía se consideró que todas las hijas de machos hemicigotos para el alelo *FecX*^{R} (R) eran portadoras (R+), mientras que las hijas de machos no portadores (+) o de padre desconocido se consideraron como ++. Las hijas de madre R+ conocida se eliminaron del estudio. La asignación del genotipo por genealogía fue consistente con la identificación por PCR (98% de hembras correctamente clasificadas). Esta validación nos permitió estimar la prolificidad de las poblaciones de ovejas R+ y ++, tratadas o no con eCG, en todos los rebaños del programa de selección.

Se analizaron un total de 668.674 registros de partos, recogidos en el control de producciones desde 1998 hasta 2008. Dentro de cada genotipo, los partos se dividieron en 2 grupos dependiendo de si la cubrición se llevó a cabo en condiciones naturales (++: n=599.160; R+: n=6.593) o tras la aplicación de un tratamiento de esponja de FGA y 480 UI de eCG (++: n=62.055; R+: n=866). Se observó que en ovejas no tratadas la presencia del alelo *FecX*^{R} aumenta la prolificidad en 0,35 corderos por oveja y parto (P<0,0001). Cuando se aplica eCG, esta diferencia de prolificidad entre genotipos es de 0,30 corderos más por oveja R+ y parto (P<0,0001). Este incremento de prolificidad observado en las ovejas R+ tratadas se debió únicamente a un incremento de los porcentajes de partos triples (+4,4%; P<0,001) y cuádruples (+2,0%; P<0,0001).

En conclusión, la variante génica *FecX*^{R} en la Rasa Aragonesa va asociada a un incremento moderado de la prolificidad en condiciones normales de reproducción, algo menor que los incrementos descritos en otras razas con mutaciones similares en *BMP15*. Por el contrario, si se emplea eCG, el incremento de prolificidad que se consigue es debido a un incremento en los partos triples o de orden superior, lo que debe tenerse en cuenta por

parte de los ganaderos y técnicos. La asignación del genotipo *FecX*^{R} por genealogía en las ganaderías es un método efectivo y viable, siempre que se lleve a cabo un control estricto de las cubriciones y de la identificación de los animales, realizando el genotipado por PCR en los casos que se estime necesario.

<u>Publicación 2.</u> Caracterización del pico preovulatorio de LH en ovejas Rasa Aragonesa portadoras o no del alelo *FecX*^{R} (ROA®).

En las ganaderías de la UPRA-Grupo Pastores se realizan de forma habitual unas 10.000 inseminaciones artificiales al año con machos mejorantes para el carácter prolificidad, tanto poligénicos como portadores de *FecX*^{R}. Las ovejas son sincronizadas previamente con esponjas de FGA y 480 UI de eCG para poder llevar a cabo la IA a tiempo fijo. Dado que existe un número creciente de hembras R+ en las ganaderías, y que en los experimentos anteriores se demostró que el alelo *FecX*^{R} tenía un efecto sobre la tasa de ovulación, la prolificidad, y la respuesta a la eCG, en el presente experimento se estudió el pico preovulatorio de LH con el fin de determinar posibles diferencias endocrinas entre genotipos. Los resultados de este estudio completan el primer objetivo de la Tesis. El experimento se llevó a cabo en noviembre (estación sexual). Se utilizaron 50 ovejas, que se dividieron en 5 lotes de 10 ovejas dependiendo de su genotipo y del tratamiento hormonal (mismos grupos que en los estudios de tasa de ovulación; publicación 1): lote “R+” y lote “++” sin tratamiento hormonal, lote “R+” con 240 UI de eCG, lote “R+” con 480 UI de eCG y lote “++” con 480 UI de eCG. Se insertó una esponja de FGA de 30 mg a todas las ovejas, que permaneció durante 14 días. Se tomaron muestras sanguíneas mediante punción yugular utilizando tubos de 5 ml con heparina de litio de la siguiente manera: durante las primeras 24 horas tras la retirada de la esponja, 1 muestra cada 6 horas, y de las 24 a las 72 horas siguientes 1 muestra cada 4 horas. Las muestras se centrifugaron a 2100 x g durante 25 minutos y el plasma se almacenó a -20ºC para el posterior análisis de LH. Las muestras fueron analizadas mediante un kit comercial ELISA (LH-DETECT, ReproPharm, Tours INRA, Nouzilly, Francia).

No se observaron diferencias significativas en el pico de LH entre ovejas R+ y ++. La eCG adelantó el pico de LH en ambos genotipos, sin diferencias entre ovejas R+ tratadas con 240 ó 480 UI. Cuando se aplicó la dosis estándar de 480 UI a las ovejas ++, se logró una gran sincronización del pico de LH, de forma que el 90% de los animales lo presentó entre las 36 y 40 h. Sin embargo, se observó una mayor dispersión en las ovejas R+ tratadas. Estos resultados demostraron que la presencia del alelo *FecX*^{R} no afecta al pico preovulatorio de LH, independientemente de la dosis de eCG utilizada. Nuestros resultados coinciden con los encontrados en otras razas con polimorfismos similares en *BMP15* o en su receptor (*BMPR1B*), donde no se han encontraron diferencias a nivel endocrino entre los genotipos.

Teniendo en cuenta también los resultados del primer artículo, **se concluye** que la dosis de eCG que se aplique a las ovejas R+ en las ganaderías podrá elegirse en función de las necesidades y posibilidades del ganadero para asumir una mayor prolificidad y multiplicidad de partos. Se recomienda seguir inseminando en ambos genotipos a las 54±1 horas, como se viene haciendo habitualmente, ya que se observó una buena sincronización del pico de LH en torno a las 37 horas de la retirada de las esponjas y aplicación de la eCG.

<u>Publicación 3.</u> Influence of the *FecX^R* allele in heterozygous ewes on follicular population and outcomes of IVP and ET using LOPU-derived oocytes.

Los efectos observados del alelo *FecX^R* sobre la tasa de ovulación podían conllevar cambios a nivel de la población folicular que afectasen a la calidad del oocito y a la fertilidad, por lo que se vio la conveniencia de profundizar en el conocimiento de sus efectos a ese nivel. Para ello se evaluó la influencia del alelo *FecX^R* sobre el número y tamaño de los folículos ováricos y su repercusión sobre la calidad del oocito, utilizando técnicas de producción *in vitro* y transferencia de embriones (TE) a partir de oocitos derivados de LOPU, lo cual constituyó el segundo objetivo de la Tesis.

Se llevaron a cabo dos ensayos de LOPU-PIV (Laparoscopic Ovum Pick Up-Producción *In Vitro*), ambos en estación sexual (enero y noviembre), utilizando como donantes de oocitos 8 ovejas R+ y 8 ++. En cada ensayo se realizaron 4 sesiones de LOPU separadas 7 días, las dos primeras sin aplicar FSH y las 2 últimas aplicando un tratamiento de estimulación ovárica con 32 mg de FSH porcina. Las donantes se mantuvieron permanentemente con esponjas (FGA, 30 mg) que se reemplazaron en cada sesión de LOPU. Se aspiraron todos los folículos ováricos mayores o iguales de 3 mm. Los COCs (complejo cumulus-oocito) recuperados se transportaron al laboratorio en tubos con medio de recolección (TCM199 + 10 mM HEPES suplementado con 10 UI/ml de heparina, 40 µg/ml de gentamicina, y 0,2% BSA), para su posterior maduración (MIV), fertilización (FIV) y cultivo (CIV) *in vitro*. El medio de MIV consistió en TCM199 + 132 µg/ml pFSH + 10% FF + 40 µg/ml de gentamicina. La FIV se llevó a cabo en medio SOF + 40 µg/ml gentamicina + 20% de SOC. El CIV se realizó en SOF + 0,3% BSA + 40 µg/ml gentamicina, suplementado con 10% de FCS a partir del tercer día de cultivo. Las tasas de división y de blastocistos se evaluaron a las 48 horas y a los 7 y 8 días de la FIV. La viabilidad de los blastocistos producidos *in vitro* se evaluó por su capacidad para llevar a término la gestación. Para ello se transfirieron blastocistos de todos los grupos experimentales y de ambos ensayos, tanto de día 7 como de día 8, a un total de 48 ovejas receptoras. Para sincronizar el ciclo ovárico de las donantes y receptoras, estas últimas recibieron 2 dosis de 125 µg de cloprostenol con 7 días de diferencia, y 4 días después de la segunda inyección se les insertó una esponja de 30 mg de FGA que permaneció durante 12 días. En la retirada se aplicaron 400 UI de eCG. Ocho o nueve días después de la retirada, dependiendo de si se transfirieron blastocistos de día 7 u 8,

respectivamente, se confirmó la tasa de ovulación por laparoscopia y se transfirieron 2 blastocistos por receptora. Se realizó diagnóstico de gestación mediante determinación de PAG (Glicoproteína asociada a la gestación) en plasma a los 25 días de la TE, y mediante ecografía abdominal 30 días después de la transferencia. Una semana antes del parto, las ovejas fueron individualizadas en jaulas para la correcta identificación de la descendencia.

El genotipo no tuvo ningún efecto sobre el número de folículos aspirados por oveja y sesión (10,4 y 10,2 en R+ y ++ no tratadas; 17,4 y 14,3 en R+ y ++ tratadas con FSH). Sin embargo, el diámetro folicular fue significativamente menor en las ovejas R+ (-0,2 mm en ovejas no tratadas y -0,8 mm en ovejas tratadas con FSH, P<0,01). El tratamiento con FSH aumentó el tamaño folicular en ambos genotipos, aunque de diferente manera: produjo un mayor incremento del número de folículos de 4 mm en las ovejas R+, mientras que a la inversa, incrementó más el número de folículos ≥6 mm en las ovejas ++ (P<0,01 para ambos). En las ovejas R+ se obtuvo una mayor tasa de recuperación, que conllevó un aumento del número total de COCs así como del número de COCs aptos para MIV. Aunque los folículos de las ovejas R+ fueron menores, se observaron tasas similares de blastocistos de día 8 en ovejas R+ (36,1%, 147/407) y ++ (32,6%, 100/307). El resultado global de blastocistos de día 8 por oveja y por sesión fue mayor en las ovejas R+ (+0,75; P<0,005), sin observarse ninguna diferencia en la tasa de supervivencia al nacimiento de los embriones transferidos (40,4% en R+ *vs.* 36.4% en ++).

En resumen, la presencia del alelo *FecX*^{R} no afectó al número de folículos ováricos ≥3 mm, pero sí a su tamaño folicular medio. A pesar del menor tamaño de los folículos de las hembras R+, sus oocitos demostraron ser tan competentes como los de las hembras ++ para el desarrollo *in vitro* y la supervivencia embrionaria tras la transferencia, lo que sugiere que podrían adquirir antes la competencia para el desarrollo. El rendimiento global de la LOPU-FIV fue mejor en las hembras R+, debido principalmente a una mayor respuesta a la FSH y una mayor tasa de recuperación. Estos resultados de viabilidad embrionaria concuerdan con los observados en las ganaderías, donde no se han encontrado efectos adversos de la presencia de *FecX*^{R} sobre la fertilidad.

<u>Publicación 4.</u> Anti-Müllerian hormone (AMH) concentration in sheep and its dependence of age and independence of BMP15 genotype: an endocrine predictor to select the best donors for embryo biotechnologies.

La AMH es en la actualidad uno de los mejores marcadores endocrinos de la reserva folicular ovárica en varias especies. Su uso en ovino podría contribuir a mejorar la eficiencia de las biotecnologías embrionarias. Actualmente, en los rebaños de Rasa Aragonesa las hembras *FecX*^{R} heterocigotas conviven con las no portadoras. Basándonos en estudios previos, trabajamos sobre la hipótesis de que la disminución de la proteína BMP15 biológicamente activa a nivel del ovario que ocurre en las ovejas R+ podría afectar la

producción de AMH de los folículos en crecimiento y por lo tanto sus concentraciones plasmáticas. Con este objetivo, en la publicación 4 se investigó la relación entre la concentración plasmática de AMH en función de la edad, la presencia de alelo *FecX*^{R} del gen *BMP15* y la población folicular, para abordar así el tercer objetivo de la Tesis.

El experimento se inició con doce corderas, 6 R+ y 6 ++, nacidas en abril, con una diferencia de edad máxima entre ellas de 3 días. Se obtuvieron tres muestras sanguíneas para la determinación de AMH de cada cordera a los 90 (julio), 135 (agosto) y 180 (octubre) días de edad. Con el fin de determinar si habían iniciado la pubertad se analizó la concentración de progesterona en dos muestras de sangre tomadas con un intervalo de 9 días en esos periodos. Las muestras se obtuvieron por punción yugular utilizando tubos de vacío de 5 ml con heparina de litio que se centrifugaron inmediatamente a 2100 x g durante 25 min. El plasma se almacenó a -20ºC hasta su análisis. Las corderas se mantuvieron en pastos de regadío con acceso *ad libitum* a agua y suplementos minerales hasta que alcanzaron la edad adulta. Para relacionar la AMH con la población folicular, en noviembre del año siguiente (estación sexual), diez de ellas fueron sometidas junto con otras seis ovejas similares en edad (total 8 R+ y 8 ++), a cuatro sesiones de LOPU siguiendo el protocolo descrito en la publicación 3. Se obtuvieron cuatro muestras sanguíneas de cada oveja para la medición de AMH de la siguiente manera: en las sesiones 1 y 2, en las que no se aplicó FSH, coincidiendo con la punción folicular, y en las sesiones 3 y 4, en las que se aplicó FSH antes de la LOPU, coincidiendo con la primera inyección de FSH (60 h antes de la LOPU). Las muestras se procesaron tal y como se ha descrito anteriormente para las corderas. Las concentraciones plasmáticas de AMH se analizaron con el kit AMH GenII Kit ELISA (Beckman Coulter, Francia) en los laboratorios del INRA-Nouzilly (Francia). Las concentraciones de progesterona se analizaron con un kit comercial ELISA (Ridgeway Science, St. Briavels, Gloucestershire, UK) en los laboratorios del CITA.

No se observaron diferencias en la concentración plasmática de AMH entre ovejas R+ y ++, ni antes de la pubertad, ni en la edad adulta. Antes de la pubertad, la concentración de AMH aumentó de los 3 a los 4,5 meses, y luego disminuyó a los 6 meses hasta niveles similares a los observados en la edad adulta (333,8±73,3, 483,2±135,5 y 184,1±38,2 pg/ml, respectivamente, P<0,1), demostrando una gran variabilidad interindividual. Aunque el pico de AMH se observó de media a los 4,5 meses, hubo animales que lo presentaron antes, y otros después, lo que parecería indicar que la AMH estaría reflejando un desarrollo folicular más avanzado en unas corderas respecto a otras. Como consecuencia, no se encontró una relación entre la AMH en la edad prepúber y la AMH o el número de folículos aspirados en la edad adulta. En la LOPU, la concentración de AMH al inicio del tratamiento con FSH estuvo muy correlacionada con el número de folículos aspirados, tanto en R+ como en ++ (r=0,75 y 0,78, respectivamente, P<0,001). Según nuestros resultados, por cada 100 pg/ml adicionales de AMH se podría esperar una media de 5,1 folículos adicionales (P<0,0001). Se pudo determinar de manera precisa un cut-off o “punto de corte” óptimo para cada

genotipo que permitiría identificar y seleccionar las ovejas adultas con una mayor respuesta a la FSH (>18 folículos puncionados por sesión). Además, la repetibilidad de la concentración de AMH entre sesiones fue elevada (0,70; P<0,0001).

Este experimento nos permitió llegar a tres **conclusiones principales**. En primer lugar, se observó que la presencia de *FecX*^{R} independientemente de la edad no tenía ningún efecto sobre las concentraciones plasmáticas de AMH, por lo que a efectos prácticos, en el caso de que se pudiese utilizar como predictor endocrino de los caracteres reproductivos, no habría que establecer diferencias entre genotipos. No obstante, cuando se comparó la respuesta ovulatoria de ovejas que tenían la misma concentración de AMH en el momento de administrar la FSH, se encontró que las hembras R+ desarrollaban un mayor número de folículos en la LOPU. En segundo lugar, las concentraciones de AMH fueron muy variables antes de la pubertad, tanto entre edades como entre individuos, lo que hizo sospechar que la AMH podría estar reflejando una dinámica de crecimiento folicular diferente que requiere ser estudiada en profundidad. En tercer lugar, se demostró que al igual que ocurría en la vaca, la concentración de AMH en la edad adulta estaba relacionada con la respuesta a los tratamientos estimulatorios del ovario, por lo que podría ser utilizado con éxito para seleccionar las mejores donantes para programas de LOPU-PIV y previsiblemente también de MOET.

<u>Publicación 5.</u> Anti-Müllerian hormone plasma concentration in prepubertal ewe lambs as a predictor of their fertility at a young age.

En la Rasa Aragonesa existe un retraso en la edad media al primer parto que conlleva importantes pérdidas económicas, por lo que tiene especial interés investigar nuevas estrategias de mejora. Como se había visto que la concentración de AMH era capaz de reflejar de manera fiable la población folicular en ovejas adultas, nos planteamos como objetivo estudiar la posible relación entre los niveles plasmáticos de AMH y la población folicular sensible a gonadotropinas en corderas prepúberes, como posibles marcadores de precocidad sexual y fertilidad en la edad adulta, lo que constituyó el cuarto objetivo de la Tesis.

El experimento comenzó en julio con 76 corderas prepúberes de alrededor de 3,6 meses de edad y de peso similar. Con el fin de determinar la presencia de folículos sensibles a gonadotropinas a esta edad, se les aplicó 600 UI de eCG im, inmediatamente después de tomar muestras sanguíneas mediante punción yugular con tubos de heparina de litio. Seis días después se determinó la ovulación mediante laparoscopia. El plasma se almacenó a -20°C hasta el análisis de AMH, que se llevó a cabo en el INRA-Nouzilly (Francia), utilizando el Kit de activo MIS/AMH ELISA (Beckman Coulter Francia, Roissy Charles de Gaulle, Francia).

Con el fin de estudiar la relación entre la AMH y la fertilidad temprana, cuando las ovejas tuvieron 312±18 días de edad (enero), se pusieron a cubrir con cuatro machos adultos durante 34 días. Cuatro meses más tarde (mayo), las ovejas que no quedaron gestantes se cubrieron de nuevo durante 34 días, siguiendo un programa típico de cubriciones en esta raza de 3 partos en 2 años. A los 10 y 30 días de la retirada de los machos se hizo diagnóstico ecográfico de gestación. Los partos tuvieron lugar en jaulas individuales de parición. Se determinó un punto de corte (cut-off) óptimo de los valores de AMH en la edad prepúber para identificar a las ovejas con mayor fertilidad en la primera cubrición, para lo que se realizó un análisis ROC.

La AMH se detectó en la mayor parte de las corderas (93%). Las concentraciones de AMH en las corderas prepúberes se relacionaron con la presencia de ovulación tras la administración de eCG. De esta manera, las concentraciones de AMH fueron alrededor de 3–4 veces mayores en las corderas que ovularon que en las que no ($P<0,004$), lo que sugirió que la AMH estaba reflejando la existencia de una población de folículos capaces de responder a las gonadotropinas en la mayoría de las corderas a esa edad. La fertilidad de las ovejas adultas en su primera cubrición (10 meses) estuvo correlacionada positivamente con su concentración de AMH en la edad prepúber ($\rho=0,34$, $p<0,01$). En este trabajo se determinó un cut-off o punto de corte de 97 pg/ml de AMH a los 3,6 meses de edad como el más adecuado para predecir la fertilidad en la primera cubrición. Así, la fertilidad de la primera cubrición fue 34,8 puntos porcentuales mayor en las ovejas con AMH≥97 pg/ml en la edad prepúber que en las que presentaron valores inferiores ($P<0,001$).

De este trabajo **se concluyó** que las concentraciones de AMH antes de la pubertad son capaces de reflejar la capacidad de respuesta ovárica a la estimulación con eCG, y podrían reflejar diferencias individuales en la población de folículos sensibles a las gonadotropinas entre animales de la misma edad. La concentración de AMH a los 3,6 meses de edad serviría para seleccionar las corderas de reposición con una mayor fertilidad predicha en su primera cubrición a los 10 meses de edad. Es importante destacar la precocidad del test, que permite al ganadero elegir las corderas de reposición antes de que sean enviadas a sacrificio para su comercialización según los requisitos de la IGP “Ternasco de Aragón”.

En resumen, los trabajos presentados en esta Tesis se enmarcan en la búsqueda de alternativas para aumentar la eficiencia reproductiva de las explotaciones ovinas con el objetivo de mantener en el rebaño únicamente a las ovejas más productivas. Para ello se han estudiado en la raza Rasa Aragonesa dos marcadores relacionados con su población folicular, que actúan a través de mecanismos diferentes. Ambos indicadores se pueden determinar antes de la pubertad, en el momento en que el ganadero elige la reposición, y son indicadores de la capacidad reproductiva de las ovejas en su estado adulto. El primero de ellos, el polimorfismo *FecX*^{R} en el gen *BMP15*, produce en heterocigosis un aumento de

la tasa de ovulación que conlleva un aumento de la prolificidad del 26% (0,35 corderos por parto), sin efectos adversos a nivel de mortalidad embrionaria, peso de los corderos, perfil de LH o fertilidad. El segundo marcador es la AMH, que ha demostrado ser un indicador de la precocidad sexual en las corderas, y que es un marcador de la población folicular sensible a las gonadotropinas en las ovejas adultas. Se puede alcanzar un incremento de 21,1 puntos en la fertilidad en la primera cubrición seleccionando las corderas con mayor AMH. No se ha encontrado una interacción entre la concentración de AMH y la presencia del alelo *FecX*^{R} en heterocigosis, por lo que se pueden utilizar de manera complementaria para elegir las ovejas más eficientes.

Summary

This Doctoral Thesis was designed to deepen the knowledge and use of genetic and endocrine markers of follicular population in ewes, to assist selection of ewes by their reproductive efficiency. With this aim, the effect of the *FecX*^{R} allele of the *BMP15* gene on reproductive performance was assessed (manuscripts 1, 2 and 3), and the utility of anti-Müllerian hormone (AMH) as another potential biomarker was also investigated (manuscripts 4 and 5). All the experiments were carried out at the facilities of the Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragón (Spain; Coordinates: 41º43'19''N-0º48'32''W), and field data were obtained from farms belonging to the genetic selection scheme for prolificacy of UPRA-Grupo Pastores. The assays for LH, PAG and P4 determination were carried out at CITA, and the AMH assays were carried out at the Institut Nationale de la Recherche Agronomique (INRA) in Nouzilly (Tours, France). All the studies were performed in the Rasa Aragonesa sheep breed, the lots being similar in bodyweight and body condition score within each experiment. All experimental procedures were performed in accordance with the guidelines of the European Union (2003/65/CE) and Spanish regulations (RD 1201/2005, BOE 252/34367-91) for the use and care of animals in research.

<u>Manuscript 1.</u> Effect of the *FecX*^{R} polymorphism in the bone morphogenetic protein 15 gene on natural or equine chorionic gonadotropin-induced ovulation rate and litter size in Rasa Aragonesa ewes and implications for on-farm application.

In 2007, a new mutation in the *BMP15* gene, a Major Gene associated with increased ovulation and prolificacy in heterozygosity and sterility in homozygosity was found in the Rasa Aragonesa breed. The experiments in the present publication were carried out to characterize its effects on these reproductive traits and to address any potential adverse effects on other productive traits (objective 1 of the Thesis). With this purpose in mind, three experiments were carried out.

In the **first experiment**, the natural ovulation rates of ewe lambs and adult ewes carrying the *FecX*^{R} allele in heterozygosity (R+) were compared with those of wild-type counterparts (++). The experiment was conducted during the breeding season (November-December). Ovulation rate was recorded in 111 ewe lambs (91 R+ and 20 ++) aged 311 ± 11 days, and in 103 adult ewes (84 R+ and 19 ++) aged 683 ± 37 days (means ± SD; 'R+ young', '++ young', 'R+ adult' and '++ adult' groups, respectively). All animals were treated with 30 mg fluorogestone acetate (FGA) sponges for 14 days to synchronize estrus. No eCG was applied at the time of sponge withdrawal so as not to affect the ovulation rate. The ovulation rate was recorded by laparoscopy during 3 consecutive cycles in each animal, at 6 days after sponge withdrawal and 17 and 34 days later. The ovulation rate of each animal was the mean of the observations recorded in the ovulating cycles.

The ovulation rate was higher in R+ than in ++ ewes, in both ewe lambs and adults (+0.44 and +0.63 ovulations, respectively; P<0.01 for both). Concerning the distribution of ovulations, R+ ewe lambs showed an increase in twin ovulations with respect to ++ ewe lambs (+50.5%; P<0.01). In adult ewes, in addition to the increase in double ovulations (+36.4%, P<0.01), a higher rate of triple plus quadruple ovulations was found when compared to R+ ewes (+12.8%; P<0.05).

In the **second experiment** we sought to evaluate the potential interaction between genotype and the application of gonadotropins commonly used in commercial treatments for estrus synchronization. With this aim in mind, the ovulation rate was recorded in R+ adult ewes in response to two different doses of eCG. The influence of the increased ovulation rate on embryo mortality and lamb weight at birth was studied to determine the optimal eCG dose to be applied to R+ ewes under field conditions.

The experiment was carried out during the breeding season (September). A total of 119 adult ewes (71 R+ and 48 ++) were treated with 30 mg FGA vaginal sponges for 14 days. At sponge removal, ++ ewes received 480 IU im of eCG ("++480" group), the standard dose used on farms for AI in Rasa Aragonesa, whereas R+ ewes received either 240 IU ("R+240" group; n=35), or 480 IU ("R+480" group; n=36). Cervical insemination was carried out 54±1 hours after sponge withdrawal with semen of proven fertility diluted in skim milk and refrigerated at 15ºC (400×10^6 spermatozoa/ewe). The ovulation rate was recorded 8 days after sponge withdrawal during only one cycle in 75 out of 119 ewes ("R+240": n=21; "R+480": n=21; "++480": n=33 ewes), following the procedures described in experiment one. One week before lambing, ewes were placed in individual pens and offspring were identified and weighed. Embryo losses were calculated as "Partial failure of multiple ovulations (PFMO)", being the difference between OR and litter size of lambing ewes with ≥ 2 ovulations.

The highest ovulation rate was observed in R+ ewes treated with 480 IU of eCG (3.95; P<0.01), presenting nearly 60% of quadruple or higher-order ovulations. When the eCG dose was reduced to 240 IU, the ovulation rate of R+ ewes was similar to that recorded in ++ ewes treated with 480 IU (2.90 *vs.* 2.82). In the "R+240" group, triple ovulations were predominant (47.6%), while in the "++480" group double ovulations predominated (42.4%). The greatest PFMO was recorded in the "R+480" group (1.85; P<0.05), whereas no significant differences were found between the "R+240" and "++480" groups. When the ovulation records of experiments 1 and 2 were compared to assess the effect of the eCG within each genotype, we found that R+ ewes displayed an increased response (+0.50 additional ovulations).

The results of the present experiment demonstrate that, from an application point of view, it would be advisable to reduce the standard eCG dose when applied to R+ ewes on farms in order to avoid an excessive percentage of multiple ovulations. The lambing records showed

that there were no differences due to genotype in the weight at birth when lambs of the same type of birth were compared.

In the **third experiment** we aimed to assess the prolificacy of R+ adult ewes, treated or not with eCG, in farms belonging to the genetic selection scheme for prolificacy carried out by UPRA-Grupo Pastores. For this purpose, first of all it was mandatory to validate genealogy assignation as a method to identify the R+ ewes born in flocks before the mutation in the *BMP15* gene was discovered. For this purpose, all ewes from four elite flocks were genotyped (1,667 ewes with 10,945 lambing records). These flocks belonged to the genetic selection scheme and were suspected of having a high proportion of carrier animals. The *FecX*^{R} genotype was determined by PCR in blood samples collected using EDTA vacuum tubes. For genotype assignation by genealogy, daughters from *FecX*^{R} genotyped hemizygous rams were considered as R+, whereas daughters of wild-type or unknown rams were considered as ++ ewes. Daughters of known R+ ewes were discarded. Genotype assignation by genealogy was consistent with PCR identification (98% correctly classified ewes). This validation allowed us to estimate the prolificacy of R+ and ++ populations, treated or not with eCG, in all flocks constituting the genetic selection scheme.

A total of 668,674 lambing records, collected from 1998 to 2008, were analysed. Within each genotype group, lambing records were divided into two groups, depending on whether mating took place under natural conditions (++: n=599,160; R+: n=6,593), or after being treated with FGA sponges and 480 IU of eCG (++: n=62,055; R+: n=866). In untreated ewes, the presence of the *FecX*^{R} allele increased prolificacy in 0.35 lambs per lambing ewe ($P<0.0001$). When eCG was applied, the difference in prolificacy between genotypes was 0.30 additional lambs per lambing ewe ($P<0.0001$). The increase in prolificacy observed when R+ ewes were treated with eCG was due solely to an increase in the percentage of triplets (+4.4%; $P<0.001$) and quadruple births (+2.0%; $P<0.0001$).

In conclusion, the *FecX*^{R} allele in Rasa Aragonesa sheep is associated with a moderate increase in prolificacy under normal reproduction conditions; however, this is somewhat less than increases previously described in other breeds with similar mutations in the *BMP15* gene. On the contrary, when eCG is used, the increase in prolificacy is exclusively achieved by an increase in triplets and higher order births, which should be taken into account by farmers and technicians. Assignment of *FecX*^{R} genotype by genealogy on farms is an effective and viable method, provided that breeding and animal identification are carried out under strict controls and PCR genotyping is performed when necessary.

<u>Manuscript 2.</u> Preovulatory LH surge of Rasa Aragonesa ewes carrying or not the *FecX*^{R} allele (ROA®).

Around 10,000 AI per year are usually performed on farms belonging to UPRA-Grupo Pastores using prolific males, either polygenic (from the selection scheme) and/or carriers of the *FecX*^{R} allele. To perform AI at a fixed time, sheep are previously synchronized with FGA sponges and 480 IU of eCG. Since there are increasing numbers of R+ ewes on farms and previous experiments showed the *FecX*^{R} allele had an effect on ovulation rate, litter size, and the ovarian response to eCG, in the present experiment we aimed to study the preovulatory LH discharge in both genotypes to identify potential endocrine differences between them. On the other hand, R+ ewes still have an adequate ovulatory response when the eCG dose is halved (240 instead of 480 IU). So it was necessary to determine if reducing the eCG dose affects synchronization of ovulations before recommend its use in AI programs. The results of this study complete the first objective of this Doctoral Thesis.

The experiment was carried out during the breeding season (November). Fifty adult ewes were divided in 5 groups (10 ewes each) depending on their genotype and hormonal treatment (the same groups as in the ovulation rate studies; first manuscript): “R+” and “++” groups without eCG treatment, an “R+” group with 240 IU of eCG, an “R+” group with 480 IU of eCG and a “++” group with 480 IU of eCG. A 30 mg FGA sponge was inserted in all ewes, remaining for 14 days. Blood samples were taken by jugular puncture using 5 mL vacuum tubes with lithium heparin as follows: during the first 24 hours after sponge removal, at 6 hour interval, and from the following 24 to 72 hours at 4 hour interval. Samples were centrifuged at 2100 x g for 25 minutes and plasma was stored at -20ºC until assayed for LH. The assay was performed using a commercial ELISA kit (LH-DETECT, ReproPharm, Tours INRA, Nouzilly, France).

There was no significant difference between R+ and ++ ewes in the timing of the preovulatory LH surge. Treatment with eCG advanced the preovulatory LH discharge in both genotypes, with no differences between R+ ewes treated with 240 or 480 IU. When the standard dose of 480 IU was applied to ++ ewes, a tight synchronization of the preovulatory LH peak was observed, with 90% of the ewes presenting the LH peak between 36 and 40 hours. However, greater dispersion was found in R+ treated ewes. These results demonstrate that the presence of the *FecX*^{R} allele did not significantly affect the preovulatory LH surge regardless of eCG dose. Our results concur with results found in other breeds carrying similar polymorphisms in *BMP15* or in its receptor (*BMPR1B*), in which no differences at the endocrine level were found between genotypes.

Also taking into account the results of manuscript 1, **we concluded** that the eCG dose that should be applied to R+ ewes on farms could be chosen depending on the needs and capabilities of farmers to manage greater prolificacy and multiple births. It is recommended to

perform the AI at 54±1 hours after sponge removal in both genotypes, as is usually done at present, since we observed good synchronization of the LH peak around 37 hours after sponge removal and eCG application.

<u>Manuscript 3.</u> Influence of the *FecX^R* allele in heterozygous ewes on follicular population and outcomes of IVP and ET using LOPU-derived oocytes.

The observed effects of the *FecX^R* allele on ovulation rate could involve changes in follicle population, which may affect oocyte quality and fertility. Therefore, we considered it prudent to deepen our understanding of its effects at that level. For this purpose, the influence of the *FecX^R* allele on the number and size of ovarian follicles and on oocyte competence was evaluated using IVP and embryo transfer (ET) techniques from LOPU-derived oocytes, which constitutes the second objective of the present Thesis.

Two LOPU-IVP (Laparoscopic Ovum Pick Up-*In Vitro* Production) experiments, both during the breeding season (January and November), were performed using 8 R+ and 8 ++ adult ewes as oocyte donors. In each trial, 4 LOPU sessions were conducted at 7 day intervals, the first 2 sessions without FSH and the last 2 sessions using previous ovarian stimulation treatment (32 mg of porcine FSH). Prior to each LOPU trial, all animals received two doses of 125 µg cloprostenol 8 days apart, and 5 days after the second injection, a 30 mg FGA sponge was inserted. Sponges remained in place throughout the experiment, being replaced by new ones at each LOPU session. All follicles greater than or equal to 3 mm that were present on the ovarian surface were punctured at LOPU. Collected cumulus–oocyte complexes (COCs) were transported to the laboratory in Falcon tubes containing collection medium (TCM199 + 10 mM HEPES supplemented with 10 IU/mL heparin, 40 µg/mL gentamicin, and 0.2% BSA), where they were further subjected to *in vitro* maturation (IVM), fertilisation (IVF), and culture (IVC) procedures. The IVM medium consisted of TCM199 + 132 µg/mL pFSH + 10% FF + 40 µg/mL gentamicin. IVF was carried out in SOF medium + 40 µg/mL gentamicin + 20% oestrous ewe serum. The IVC took place in SOF medium + 0.3% BSA + 40 µg/mL gentamicin, supplemented with 10% FCS from day 3 onwards. The fertilization and blastocyst rates were evaluated morphologically after 48 hours and again on days 7 and 8 after IVF. The viability of the IVP blastocysts was assessed by their ability to sustain pregnancy and give rise to a healthy offspring. For this purpose, blastocysts from all the experimental groups and trials, from either day 7 or 8, were freshly transferred to 48 recipients. Prior to ET, recipient ewes received 2 doses of 125 µg cloprostenol 7 days apart. Four days after the second injection, a 30 mg FGA sponge was inserted for 12 days. Upon sponge withdrawal, 400 IU eCG was administered. Eight or 9 days after withdrawal, depending on whether day-7 or day-8 blastocysts were respectively transferred, the ovulation rate was confirmed by laparoscopy and 2 blastocysts per recipient were transferred. A pregnancy diagnosis was carried out at 25 days after ET by plasmatic pregnancy-associated

glycoprotein (PAG) determination and at 30 days after ET by abdominal ultrasound. One week before lambing, ewes were placed in individual pens to correctly identify the offspring.

Genotype did not affect the total number of aspirated follicles per ewe and session (10.4 and 10.2 in R+ and ++ untreated ewes, 17.4 and 14.3 in R+ and ++ FSH-treated ewes, respectively). However, mean follicle size was significantly lower in R+ ewes (-0.2 mm in untreated and -0.8 mm in FSH-treated ewes, $P<0.01$). The FSH treatment increased follicle size in both genotypes, although in a different way: it produced a greater increase in the number of 4 mm follicles in R+ ewes while, on the contrary, it produced a greater increase in the number of ≥6 mm follicles in ++ ewes ($P<0.01$ for both). The recovery rate was higher in R+ ewes, which led to an increase in the total number of collected COCs and in the number of COCs suitable for IVM in this genotype. Whereas follicles from R+ ewes were smaller, similar blastocyst rates were observed in the R+ (day 8: 36.1%, 147/407) and ++ (32.6%, 100/307) groups. The overall yield of day-8 blastocysts per ewe and session was higher in R+ ewes (+0.75; $P<0.005$), with no significant difference in the survival rate at lambing of the transferred embryos (40.4% in R+ *vs.* 36.4% in ++ groups).

To summarize, the presence of the *FecX*^{R} allele did not affect the number of ≥3 mm ovarian follicles, but did affect the mean follicle size. Despite the reduced size of follicles from R+ ewes, their oocytes were demonstrated to be as competent as those from ++ ewes for embryo IVP and transfer, which might suggest an advanced acquisition of developmental oocyte competence in this genotype. The overall yield of LOPU-IVP was greater in R+ ewes, mainly due to an increased response to FSH and a higher recovery rate. The embryo viability results found in this experiment agree with those observed in flocks, where no adverse effects of the *FecX*^{R} allele on fertility have been found.

Manuscript 4. Anti-Müllerian hormone (AMH) concentration in sheep and its dependence of age an independence of BMP15 genotype: an endocrine predictor to select the best donors for embryo biotechnologies.

Anti-Müllerian hormone is, at present, one of the best endocrine markers of the ovarian follicular reserve in several species. Its use in sheep could contribute to improving the still low efficiency of embryo biotechnologies. At present, in Rasa Aragonesa flocks wild-type ewes coexist with *FecX*^{R} heterozygous carrier ewes. Based on previous studies, we hypothesize that the decrease in biologically active BMP15 protein at the ovarian level that occurs in R+ ewes might affect AMH production in growing follicles, and thereby alter its endocrine level. With this purpose in mind, this manuscript aimed to assess the relationship between plasma AMH concentration depending on age, the presence of the *FecX*^{R} allele, and follicular population, to accomplish the third objective of this Thesis.

In this experiment, twelve ewe lambs (6 R+ and 6 ++) born in April with a maximum age difference between them of 3 days were used. Three blood samples were obtained for AMH determination from the ewe lambs at 90 days (July), 135 days (August), and 180 days (October) of age. In order to determine whether puberty had occurred by the time of AMH sampling, two blood samples at nine days interval were at these time points for P4 determination. Samples were obtained by jugular puncture using 5 mL vacuum tubes with lithium heparin, which were immediately centrifuged at 2100 x g for 25 min. The plasma was stored at -20 ºC until assayed for AMH. The ewe lambs were kept in irrigated pastures with *ad libitum* access to water and mineral supplements until they reach adulthood. With the aim of relating AMH and follicular population, in November of the following year (breeding season) ten of them were submitted to four LOPU sessions, together with six additional ewes of similar age (8 R+ and 8 ++ in total), following the same protocol previously described in manuscript 3. Four blood samples were taken from each ewe for AMH measurement as follows: in the first and second sessions (untreated sessions) at follicle puncture; and in the third and fourth sessions (FSH-treated sessions) coinciding with the first FSH injection in each session (60 hours before LOPU). Samples were processed as previously described for ewe lambs. The plasma AMH concentrations were measured by the AMH GenII ELISA Kit (Beckman Coulter, France) in the laboratories of INRA-Nouzilly (France). Progesterone was analyzed by a direct competitive ELISA kit intended for ovine plasma (Ridgeway Science, St. Briavels, Gloucestershire, UK) in the laboratories of CITA.

There were no differences in plasma AMH concentrations between R+ and ++ ewes, neither before puberty nor at adulthood. Before puberty, the AMH concentration increased from 3 to 4.5 months and then declined at 6 months to levels similar to those observed later in adults (333.8±73.3, 483.2±135.5 and 184.1±38.2 pg/mL, respectively; $P<0.1$), also demonstrating high inter-individual variability. Although the AMH peak was observed on average at 4.5 months, some ewe lambs presented such a peak earlier and others later in time, which would indicate that AMH could be reflecting a more advanced follicular development in some ewe lambs over others. As a result, we did not find any relationship between prepubertal AMH levels and AMH or the number of punctured follicles at the adult age. During adulthood, plasma AMH concentration at the beginning of the FSH treatment was highly correlated with the number of punctured follicles at LOPU, both in R+ and ++ ewes ($r=0.75$ and 0.78, respectively, $P<0.001$). According to our results, an average of 5.1 extra follicles ($P<0.0001$) per each 100 pg/mL increase in AMH could be expected. It was possible to accurate determine an optimal cut-off point for each genotype able to identify and select those adult ewes with a greater response to FSH (>18 punctured follicles per session). In addition, the session-to-session repeatability of the AMH concentration was high (0.70; $P<0.0001$).

This experiment allowed us to reach three **main conclusions**. First, the presence of the *FecX*^{R} allele does not have any effect on plasma AMH concentrations at any age, so in the case of using AMH as an endocrine predictive marker, distinctions between genotypes is not

needed. Nevertheless, R+ ewes developed an increased number of follicles at LOPU for the same AMH concentration. Second, plasma AMH concentrations before puberty varied significantly, both between ages and between individuals, which led to the suspicion that AMH could be reflecting differences in follicular growth dynamics that need to be further studied in depth. Third, as reported in cows, plasma AMH concentrations in adult sheep were related to the ovarian response to stimulatory treatments, so it could be used successfully to select the best donors for LOPU-IVP and likely for MOET programs.

Manuscript 5. Anti-Müllerian hormone plasma concentration in prepubertal ewe lambs as a predictor of their fertility at a young age.

In the Rasa Aragonesa breed the age at first lambing is delayed, leading to important economic losses. For this reason, it is of special interest to search for new strategies to improve this parameter. As reported in manuscript 4, plasma AMH concentration reliably reflects the follicular population in adult sheep. These findings encouraged us to investigate the possible relationship between plasma AMH levels and the responsive ovarian follicle population of prepubertal ewe lambs as potential markers of their sexual precocity and fertility in adulthood. This subject constituted the fourth objective of this Thesis.

The experiment began in July, using 76 ewe lambs aged around 3.6 months and with similar body weight. In order to determine the presence of gonadotropin-responsive follicles at this age, 600 IU of eCG were administered im. Just prior to this, blood samples were taken by jugular puncture using lithium heparin tubes. Six days afterwards, the occurrence of ovulation was determined by laparoscopy. Plasma was stored at -20°C until assayed. The AMH assay was carried out in the laboratories of INRA-Nouzilly (France), using the MIS / AMH ELISA Kit (Beckman Coulter France, Roissy Charles de Gaulle, France).

When ewe lambs were 312±18 days old (January), they were first joined to four adult Rasa Aragonesa rams of proven fertility for 34 days to investigate the relationship between AMH and early fertility. Four months later (May), non-pregnant ewes at first mating were mated again for 34 days, according to an accelerated lambing program of three lambings in 2 years. Ten and 30 days after male removal, pregnancy diagnosis was performed by abdominal ultrasound. Lambing took place in individual pens. We determined an optimal cut-off point of prepubertal AMH to detect ewes with higher fertility at first mating by means of an ROC analysis.

Anti-Müllerian hormone was detected in almost all ewe lambs (93%). Plasma AMH concentration in prepubertal ewe lambs was related to the occurrence of ovulation after eCG administration. Thus, plasma AMH levels were about 3–4 times higher in ovulating than in non-ovulating ewe lambs ($P<0.004$), suggesting that AMH was reflecting the presence of gonadotropin-responsive follicles in most ewe lambs at these early ages.

The fertility of adult ewes at first service was positively correlated with their AMH concentration in the prepubertal age (ρ=0.34, P<0.01). In the present work, a cut-off point of 97 pg/mL of plasma AMH at 3.6 months was determined as the most adequate to predict fertility at first mating. The fertility at first mating was 34.8 percentage points higher in ewes with AMH ≥97 pg/mL in their prepubertal age than in those with lower concentrations (P<0.001).

From the current results, **we conclude** that AMH concentrations before puberty are able to reflect the ovaries' ability to respond to eCG stimulation, and could reflect individual differences in gonadotropin-sensitive follicle population among ewe lambs of the same age. The determination of plasma AMH concentrations before puberty (at 3.6 months) would be useful for selecting replacement ewe lambs with the highest predicted fertility when first mated at 10 months. The precocity of the test should be highlighted, as it allows farmers to choose replacement ewe lambs before they are sent to the slaughterhouse for commercialization.

In summary, the studies presented in this Doctoral Thesis are in the context of searching for tools to increase reproductive efficiency on sheep farms, keeping only the best performing animals in the flock. For this purpose, two markers related to follicular population have been studied in the Rasa Aragonesa breed that act through different mechanisms. Both markers can be determined before puberty, when the farmer selects replacement ewes, and are indicators of reproductive performance during adulthood. The first marker is the *FecX*^{R} polymorphism in the *BMP15* gene. When present in heterozygosity, it produces an increase in ovulation rate which leads to a 26 % increase in prolificacy (0.35 lambs per lambing ewe), with no adverse effects on embryo mortality, lamb weight, preovulatory LH surge, or fertility. The second marker is anti-Müllerian Hormone, which is related to sexual precocity in ewe lambs, and is a marker of gonadotropin-responsive follicles in adult sheep. An improvement of 21.1 percentage points in fertility at first mating could be achieved by selecting ewe lambs with higher AMH concentrations. No interaction was found between the presence of the *FecX*^{R} allele in heterozygosity and plasma AMH concentrations, so they could be used as complementary markers to select the most efficient ewes.

CONCLUSIONES / CONCLUSIONS

5.

Conclusiones

1. El alelo *FecX*^{R} en heterocigosis produce un incremento de la tasa de ovulación de +0,44 ovulaciones en corderas y de +0,63 ovulaciones en ovejas adultas, que se traducen en un incremento de la prolificidad de 0,35 corderos por parto.

2. Las ovejas heterocigotas para *FecX*^{R} presentan una mayor respuesta a la eCG, de manera que la dosis estándar de 480 UI da lugar a una tasa de ovulación excesivamente elevada que conlleva un porcentaje elevado de ovulaciones cuádruples y de mayor orden, así como elevadas pérdidas embrionarias. En las ganaderías, esto origina un incremento extra de la prolificidad debido exclusivamente a un incremento de los partos triples y de mayor orden. Cuando se reduce a la mitad la dosis de eCG que se aplica a las ovejas heterocigotas para *FecX*^{R}, se consiguen tasas de ovulación y pérdidas embrionarias similares a las obtenidas en las ovejas no portadoras que reciben la dosis estándar.

3. La presencia del alelo *FecX*^{R} en heterocigosis no afecta al pico preovulatorio de LH. Cuando se aplica eCG, el pico preovulatorio se adelanta en ambos genotipos, pero sin diferencias entre ellos, independientemente de aplicar a las hembras heterocigotas 480 ó 240 UI. Por ello, se puede realizar la inseminación artificial a tiempo fijo en el mismo momento en ambos genotipos.

4. La presencia del alelo *FecX*^{R} en heterocigosis da lugar a una disminución en el tamaño medio de los folículos ováricos, principalmente por una disminución en el número de folículos >6 mm. A pesar de ello, sus oocitos han demostrado ser tan competentes como los de las no portadoras, lo que concuerda con los resultados de fertilidad observados en las ganaderías. Se observa una mayor eficiencia de este genotipo en el rendimiento de producción *in vitro*, debido principalmente a una mayor respuesta a la FSH.

5. No existen diferencias en la concentración plasmática de la hormona anti-Mülleriana entre las ovejas heterocigotas del alelo *FecX*^{R} y las no portadoras, por lo que la capacidad predictiva de la AMH puede hacerse extensiva a ambos genotipos.

6. En la edad adulta, la hormona anti-Mülleriana ha demostrado ser un buen predictor del número de folículos ováricos capaces de responder a las gonadotropinas, por lo que puede utilizarse como método de selección de las mejores donantes de oocitos para mejorar la eficiencia de las biotecnologías embrionarias en ovino.

7. La hormona anti-Mülleriana está presente en el plasma sanguíneo de corderas prepúberes, mostrando una gran variabilidad entre individuos y edades. A esta edad, se relacionó con la aparición de la ovulación tras la administración de eCG, siendo mucho mayor en las corderas que ovularon que en las que no. Se relacionó positivamente con la fertilidad en la primera cubrición, pero no con las concentraciones de AMH ni con el rendimiento en LOPU en la edad adulta.

Implicaciones y consideraciones finales

El alelo *FecX*^{R} y la AMH son dos herramientas a disposición del ganadero para controlar la prolificidad y fertilidad al primer parto, que se deberían de utilizar de acuerdo con la disponibilidad de comida y las condiciones de manejo e infraestructura disponibles, con el objetivo de optimizar más que maximizar el potencial reproductivo.

Mediante la utilización del alelo *FecX*^{R} se obtiene un incremento muy rápido de la prolificidad y de la fecundidad en las ganaderías, sin efectos adversos sobre otros parámetros productivos. La implementación de la utilización de *BMP15* permite cubrir las necesidades de mejora en prolificidad, si bien es necesario no abandonar la selección poligénica clásica para evitar la excesiva consanguinidad y para mejorar otros parámetros inherentes al aumento de la prolificidad como pueda ser el crecimiento de los corderos o la capacidad maternal. La asignación del genotipo *FecX*^{R} por genealogía en las ganaderías es un método eficaz y viable, siempre y cuando se lleve un estricto control técnico de la identificación y de los cruzamientos, y se recurra al genotipado por PCR cuando sea necesario. La mayor respuesta a las gonadotropinas de las hembras *FecX*^{R} heterocigotas hace pensar en diferencias a nivel de foliculogénesis basal, que deberían ser estudiadas en profundidad.

La hormona anti-Mülleriana ha demostrado ser un buen predictor de la respuesta ovárica a las gonadotropinas y de la fertilidad temprana, que puede utilizarse de manera complementaria al polimorfismo *FecX*^{R} para aumentar la eficiencia reproductiva. Al parecer, a una misma edad nos estaría mostrando un desarrollo folicular cuantitativamente diferente en unos animales que en otros, posiblemente con implicaciones diferentes en la pubertad y en la edad adulta. Para ser utilizada como test de selección en las ganaderías es necesario establecer un punto de corte en los valores de AMH que se podrá establecer en función de la disponibilidad de animales y objetivos de la selección: seleccionar las mejores o eliminar las peores.

Conclusions

1. The *FecX*^{R} mutation, when present in heterozygosity, produces 0.44 extra ovulations per cycle in ewe lambs and 0.63 extra ovulations in adult ewes. These increases in ovulation rate lead to an increase in prolificacy of 0.35 lambs per lambing ewe.

2. Heterzygous *FecX*^{R} ewes display a greater response to eCG, so that the standard dose of 480 IU gives rise to an extremely high ovulation rate resulting in a high percentage of quadruple or higher order ovulations and a rise in embryo mortality. On farms, it results in an increased prolificacy due solely to the increased rates of triplet and higher order births. When the eCG dose applied to *FecX*^{R} heterozygous ewes is halved, ovulation and embryo mortality rates are similar to those obtained in non-carrier ewes receiving the standard eCG dose.

3. The presence of the *FecX*^{R} allele in heterozygosity does not affect the preovulatory LH surge. When eCG is applied, treatment advances the preovulatory LH discharge in both genotypes, with no difference between them, regardless of whether 480 or 240 IU of eCG is applied to heterozygous ewes. Therefore, artificial insemination at a fixed time can be performed at the same time in both genotypes.

4. The presence of the *FecX*^{R} allele in heterozygosity reduces the mean follicle diameter of ovarian follicles, mainly due to a reduction in the number of follicles >6 mm. However, these oocytes were demonstrated to be as competent as those from non-carrier ewes, which is consistent with the fertility results observed on farms. A higher efficiency of the *FecX*^{R} genotype was found in terms of *in vitro* production yields, mostly due to a greater response to FSH.

5. There are no differences in plasma anti-Müllerian hormone concentrations between *FecX*^{R} heterozygous and non-carrier ewes, so the predictive ability of AMH could be extended to both genotypes.

6. At adulthood, anti-Müllerian hormone proved to be a good predictor of the number of ovarian follicles able to respond to gonadotropins, so it can be used as a tool to select the best oocyte donors and thereby improve the efficiency of embryo biotechnologies in sheep.

7. Anti-Müllerian hormone is present in blood plasma from prepubertal ewe lambs, showing great variability between individuals and ages. At this age, it was related to the occurrence of ovulation after eCG administration, being higher in ovulating than in non-ovulating ewe lambs. Anti-Müllerian hormone concentration was positively related with

fertility at first mating, but was not related to AMH concentration or LOPU yields in the adult stage.

Implications and final considerations

The *FecX*^{R} allele and AMH are two tools available to farmers to control prolificacy and fertility at first lambing; these should be used wisely according to food availability, infrastructure and management conditions, with the aim of optimizing, rather than maximizing, reproductive potential.

Using the *FecX*^{R} allele allows for a rapid increase in prolificacy and fecundity on farms, with no adverse effects on other productive parameters. Implementing the use of the *BMP15* genotype meets the need for increased prolificacy, although it is essential not to abandon classical polygenic selection in order to prevent excessive inbreeding and to improve other parameters associated with high prolificacy, such as lamb growth or maternal ability. *FecX*^{R} genotype assignment by genealogy on farms is an effective and viable method, provided that breeding and animal identification are carried out with strict controls and PCR genotyping is performed when necessary. The greater response to gonadotropins found in *FecX*^{R} heterozygous ewes suggests differences in basal folliculogenesis, which should be studied in depth.

Anti-Müllerian hormone has been shown to be a good predictor of the ovarian response to gonadotropins and early fertility, which may be used in a complementary manner to the *FecX*^{R} polymorphism to increase reproductive efficiency. Apparently, we should be observing quantitative differences between animals in follicular development, with different implications at puberty and at adulthood. In order to be used as a screening test on farms, it is necessary to set a cut-off point for AMH values, which could be established depending on the objectives of the selection and the replacement strategy: selecting the best or eliminating the worst.

APÉNDICE

6.

6.1. Características de las revistas científicas

	Revista	ISSN	Año	FI*	Área/s temática/s (cuartil)	Estado
Publicación 1	Journal of Animal Science	0021-8812	2011	**2,096**	Agriculture, dairy and Animal Science (Q1)	Publicado
Publicación 2	ITEA-Información Técnica Económica Agraria	1699-6887	2013	**0,349**	Agricultural Economics & Policy (Q4)	En prensa (doi)
Publicación 3	Reproduction in Domestic Animals	0936-6768	2013	**1,392**	Agriculture, dairy and Animal Science (Q1) Veterinary Sciences (Q2) Reproductive Biology (Q4)	Publicado
Publicación 4	Theriogenology	0093-691X	2013	**2,082**	Veterinary Sciences (Q1) Reproductive Biology (Q3)	Aceptado (doi)
Publicación 5	BMC Veterinary Research	1746-6148	2012	**1,861**	Veterinary Sciences (Q1)	Publicado

** Factor de impacto del año de publicación, o el último disponible en caso de artículos en prensa o aceptados*

6.2. Acreditación de aceptación de las publicación 2 y 4

Publicación 2

Estimada Belén Lahoz Crespo,

Hemos tomado una decisión sobre su presentación a ITEA-Información
Técnica Económica Agraria, "Caracterización del pico preovulatorio
de LH en ovejas Rasa Aragonesa portadoras o no del alelo FecXR (ROA®)".

Su artículo ha sido ACEPTADO para su publicación en la revista ITEA.
Dicho artículo se incluirá en el volumen 109-4, correspondiente al mes de diciembre 2013.

Reciba un cordial saludo,

Albina Sanz Pascua
Editor asociado de la revista ITEA (Información Técnica Económica Agraria)
Asociación Interprofesional para el Desarrollo Agrario (AIDA)
Avda. Montañana 930 (CITA de Aragón), 50059 Zaragoza, España
Tel.: 976713435
Fax: 976716335
Email: asanz@aragon.es
http://www.aida-itea.org/index.php/revista/revista-intro

Publicación 4

Asunto: Your Submission THERIO-D-13-00372R1
De: Daniel Sharp <sharp@ufl.edu>
Fecha: 30/09/2013 22:25
Para: <blahozc@aragon.es>

Ms. Ref. No.: THERIO-D-13-00372R1
Title: Anti-Müllerian hormone (AMH) concentration in sheep and its dependence of age and independence of BMP15 genotype: an endocrine predictor to select the best donors for embryo biotechnologies
Theriogenology

Dear Ms. Lahoz,

I am pleased to confirm that your manuscript, "Anti-Müllerian hormone (AMH) concentration in sheep and its dependence of age and independence of BMP15 genotype: an endocrine predictor to select the best donors for embryo biotechnologies, has been accepted for publication in Theriogenology.

The files have been forwarded to the publisher and you will be contacted in due course regarding galley proofs.

Thank you for submitting your work to this journal.

Sincerely,

Daniel C. Sharp, Ph.D.
Associate Editor
Theriogenology

6.3. Contribución de la doctoranda

D. **José Luis Alabart Álvarez**, Doctor en Ciencias Químicas, y D. **José Folch Pera**, Doctor en Veterinaria, ambos investigadores de la Unidad de Tecnología en Producción Animal del Centro de Investigador y Tecnología Agroalimentaria (CITA) del Gobierno de Aragón, como Directores de la presente memoria presentada por Dña. **Belén Lahoz Crespo** para optar al grado de Doctora,

HACEN CONSTAR

Que Dña. **Belén Lahoz Crespo**, ha participado activamente como ejecutora principal y en colaboración con otros investigadores en todas las publicaciones incluidas en la presente Tesis Doctoral titulada "**Mejora de la eficiencia reproductiva en ovejas Rasa Aragonesa mediante el uso del alelo *FecX*^{R} del gen *BMP15* y de la hormona anti-Mülleriana (AMH) como marcadores de su población folicular**". La contribución de Dña. Belén Lahoz Crespo ha consistido en la realización mayoritaria de los experimentos, el análisis de los resultados, la interpretación de los datos, la elaboración de las conclusiones y la escritura de los manuscritos. Los experimentos de LOPU y FIV se han realizado en colaboración con la Dra. Mª Jesús Cocero Oviedo. Los análisis de AMH se han realizado en colaboración con la Dra. Danielle Monniaux. Las determinaciones laparoscópicas se han realizado en colaboración con el Dr. José Folch Pera. Los análisis estadísticos se han realizado en colaboración con el Dr. José Luis Alabart Álvarez.

Y para que conste firmamos el presente documento, en Zaragoza, a uno de julio de dos mil trece.

Fdo. José Luis Alabart Álvarez

Fdo. José Folch Pera

6.4. Renuncia de los coautores no doctores

D. **José Luis Alabart Álvarez**, Doctor en Ciencias Químicas, y D. **José Folch Pera**, Doctor en Veterinaria, ambos investigadores de la Unidad de Tecnología en Producción Animal del Centro de Investigación y Tecnología Agroalimentaria (CITA) del Gobierno de Aragón, como Directores de la presente memoria presentada por Dña. **Belén Lahoz Crespo** para optar al grado de Doctora,

HACEN CONSTAR

Que todos los coautores de los manuscritos presentados en dicha memoria son Doctores, a excepción de D. Elías Echegoyen Pérez, D. Enrique Fantova Puyalto, y Dña. Pilar Sánchez Ruiz, quienes renuncian a presentar los manuscritos de los que son coautores como parte de otra Tesis Doctoral, como consta en los documentos adjuntos.

Firmado en Zaragoza, a uno de julio de dos mil trece.

GOBIERNO DE ARAGON
DPTO. DE INDUSTRIA E INNOVACIÓN
CENTRO DE INVESTIGACIÓN Y TECNOLOGÍA AGROALIMENTARIA DE ARAGÓN

Fdo. José Luis Alabart Álvarez

Fdo. José Folch Pera

D. **Enrique Fantova Puyalto**, con D.N.I. 18.014.129-T

HACE CONSTAR

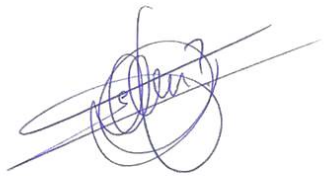
Que no estando en posesión del título de Doctor, y como coautor del artículo:

Lahoz B., Alabart J.L., Jurado J.J., Calvo J.H., Martínez-Royo A., **Fantova E.**, Folch J. 2011. Effect of the *FecX*^{R} polymorphism in the bone morphogenetic protein 15 gene on natural or equine chorionic gonadotropin-induced ovulation rate and litter size in Rasa Aragonesa ewes and implications for on-farm application. Journal of Animal Science.

Incluido en la Tesis Doctoral presentada como compendio de publicaciones por la Doctoranda Dña. Belén Lahoz Crespo, titulada "Mejora de la eficiencia reproductiva en ovejas Rasa Aragonesa mediante el uso del alelo *FecX*^{R} del gen *BMP15* y de la hormona anti-Mülleriana (AMH) como marcadores de su población folicular", dirigida por los Doctores D. José Luis Alabart Álvarez y D. José Folch Pera,

RENUNCIA a la inclusión de los mismos en otra Tesis Doctoral.

En Zaragoza, a uno de abril de dos mil trece.



Fdo. Enrique Fantova Puyalto

Dña. **Pilar Sánchez Ruiz**, con D.N.I. 25.139.440-A

HACE CONSTAR

Que no estando en posesión del título de Doctora, y como coautora de los artículos:

Lahoz B., Folch J., **Sánchez P.**, Alabart J.L. 2013. Caracterización del pico preovulatorio de LH en ovejas Rasa Aragonesa portadoras o no del alelo *FecX*^{R} (ROA®) / Preovulatory LH surge of Rasa Aragonesa ewes carrying or not the *FecX*^{R} allele (ROA®). ITEA-Información Técnica Económica Agraria.

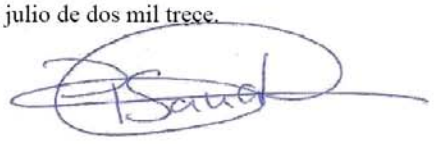
Lahoz B., Alabart J.L., Folch J., **Sánchez P.**, Echegoyen E., Cocero M.J. 2013. Influence of the *FecX*^{R} allele in heterozygous ewes on follicular population and outcomes of IVP and ET using LOPU-derived oocytes. Reproduction in Domestic Animals.

Lahoz B., Alabart J.L., Cocero M.J., Monniaux D., Echegoyen E., **Sánchez P.**, Folch J. 2013. Anti-Müllerian hormone (AMH) concentration in sheep and its dependence of age and independence of BMP15 genotype: an endocrine predictor to select the best donors for embryo biotechnologies. Theriogenology.

Incluidos en la Tesis Doctoral presentada como compendio de publicaciones por la Doctoranda Dña. Belén Lahoz Crespo, titulada "Mejora de la eficiencia reproductiva en ovejas Rasa Aragonesa mediante el uso del alelo *FecX*^{R} del gen *BMP15* y de la hormona anti-Mülleriana (AMH) como marcadores de su población folicular", dirigida por los Doctores D. José Luis Alabart Álvarez y D. José Folch Pera,

RENUNCIA a la inclusión de los mismos en otra Tesis Doctoral.

En Zaragoza, a uno de julio de dos mil trece.



Fdo. Pilar Sánchez Ruiz

D. **Elías Echegoyen Pérez**, con D.N.I. 15.809.699-M

HACE CONSTAR

Que no estando en posesión del título de Doctor, y como coautor de los artículos:

Lahoz B., Alabart J.L., Folch J., Sánchez P., **Echegoyen E.**, Cocero M.J. 2013. Influence of the *FecX*^{R} allele in heterozygous ewes on follicular population and outcomes of IVP and ET using LOPU-derived oocytes. Reproduction in Domestic Animals.

Lahoz B., Alabart J.L., Cocero M.J., Monniaux D., **Echegoyen E.**, Sánchez P., Folch J. 2013. Anti-Müllerian hormone (AMH) concentration in sheep and its dependence of age and independence of BMP15 genotype: an endocrine predictor to select the best donors for embryo biotechnologies. Theriogenology.

Incluidos en la Tesis Doctoral presentada como compendio de publicaciones por la Doctoranda Dña. Belén Lahoz Crespo, titulada "Mejora de la eficiencia reproductiva en ovejas Rasa Aragonesa mediante el uso del alelo *FecX*^{R} del gen *BMP15* y de la hormona anti-Mülleriana (AMH) como marcadores de su población folicular", dirigida por los Doctores D. José Luis Alabart Álvarez y D. José Folch Pera,

RENUNCIA a la inclusión de los mismos en otra Tesis Doctoral.

En Zaragoza, a uno de julio de dos mil trece.

Fdo. Elías Echegoyen Pérez

6.5. Certificados de estancias

Unité Mixte de Recherche de Physiologie de la Reproduction et des Comportements

ATTESTATION de STAGE

Je soussigné, **Thierry MAGALLON**, Directeur-Adjoint de l'Unité Mixte de Recherche de **P**hysiologie de la **R**eproduction et des **C**omportements (U.M.R. P.R.C.) à l'**I**nstitut **N**ational de la **R**echerche **A**gronomique (I.N.R.A.) - 37380 NOUZILLY, certifie que **Mlle LAHOZ CRESPO Belén,** de nationalité espagnole**,** a effectué un stage doctoral du 1er mars au 30 juin 2010, sous la responsabilité de Pascal MERMILLOD, Equie "Intéractions Cellulaires et Fertilité".

Fait à Nouzilly,
le 30 juin 2010

T. MAGALLON
Directeur-Adjoint de l'UMR P.R.C.

I.N.R.A. Centre de Tours
Unité Mixte de Recherches
INRA-CNRS-UNIVERSITE de TOURS
HARAS NATIONAUX
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et des Comportements - UMR 6175
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www.inra.fr




Unité Mixte de Recherche de Physiologie de la Reproduction et des Comportements

ATTESTATION de STAGE

Je soussigné, **Florian GUILLOU**, Directeur de l'Unité Mixte de Recherche de **P**hysiologie de la **R**eproduction et des **C**omportements (U.M.R. P.R.C.) à l'**I**nstitut **N**ational de la **R**echerche **A**gronomique (I.N.R.A.) - 37380 NOUZILLY, certifie que **Mlle LAHOZ CRESPO Belén,** de nationalité espagnole, a effectué un stage doctoral du 4 au 15 avril 2011, sous la responsabilité d'Alice FATET, Ingénieur de Recherche au sein de l'Equipe "Intéractions Cellulaires et Fertilité". Le thème de son stage portait sur la "Formation au suivi ovarien par échographie transrectale chez les petits ruminants".

Fait à Nouzilly,
le 15 avril 2011

I.N.R.A. Centre de Tours
Unité Mixte de Recherches
INRA-CNRS-UNIVERSITE de TOURS-
HARAS NATIONAUX
Physiologie de La Reproduction
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www.inra.fr

Unité Mixte de Recherche de Physiologie de la Reproduction et des Comportements

ATTESTATION de STAGE

Je soussigné, **Florian GUILLOU**, Directeur de l'Unité Mixte de Recherche de **P**hysiologie de la **R**eproduction et des **C**omportements (U.M.R. P.R.C.) à l'**I**nstitut **N**ational de la **R**echerche **A**gronomique (I.N.R.A.) - 37380 NOUZILLY, certifie que **Mlle LAHOZ CRESPO Belén,** de nationalité espagnole**,** du CITA Zaragoza, a effectué un stage au sein de l'UMR PRC du 2 mai au 1er juin 2012, sous la responsabilité de **Juliette COGNIE,** Chirurgien-vétérinaire de l'Unité.

Fait à Nouzilly,
le 11 juillet 2012

I.N.R.A. Centre de Tours
Unité Mixte de Recherches
INRA-CNRS-UNIV. de TOURS-HARAS
Physiologie de la Reproduction
et des Comportements
37380 NOUZILLY - France

F. GUILLOU
Directeur de l'UMR P.R.C.

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www.inra.fr

6.6. Otras contribuciones científicas

Publicaciones incluidas en el Journal Citation Reports (ISI)

Martínez-Royo A., **Lahoz B.**, Alabart J.L., Folch J., Calvo J.H. 2012. Characterisation of the Melatonin Receptor 1A (MTNR1A) gene in the Rasa Aragonesa sheep breed: association with reproductive seasonality. Animal Reproduction Science 133, 169–175.

Cocero M.J., Alabart J.L., Hammami S., Martí J.I., **Lahoz B.**, Sánchez P., Echegoyen E., Beckers J.F., Folch J. 2011. The Efficiency of *In vitro* Ovine Embryo Production Using an Undefined or a Defined Maturation Medium is Determined by the Source of the Oocyte. Reproduction in Domestic Animals 46, 463–470.

Publicaciones no incluidas en el Journal Citation Reports (ISI): Divulgación

Folch J., Sevilla E., Jurado J.J., Pardos L., **Lahoz B.**, Quintín F., Martínez-Royo A., Vijil,E., Alabart, J.L., Calvo J.H., Fantova E., Equipo veterinario de UPRA-Grupo Pastores. 2012. La Rasa Oviaragón (ROA). Una alternativa para aumentar la rentabilidad de las ganaderías de ovino. Revista Feagas 37, 142–147.

Pardos L., Fantova E., Folch J., Alabart J.L., **Lahoz B.**, y Equipo veterinario de UPRA Grupo Pastores. 2012. Influencia económica de la prolificidad en ovino de carme. Revista Albéitar 157, 16–17.

Folch J., Sevilla E., Jurado J.J., Pardos L., **Lahoz B.**, Quintín F., Martínez-Royo A., Vijil E., Alabart J.L., Calvo J.H., Fantova E., Equipo veterinario de UPRA Grupo Pastores. 2012. Resultados productivos y condiciones de utilización de las ovejas ROA. Revista Albéitar 156, 14–15.

Lahoz B., Alabart J.L., Folch J., Calvo J.H., Martínez-Royo A., Fantova E., Equipo de veterinarios de UPRA Grupo Pastores. 2010. Genes mayores para el incremento de la prolificidad. Revista Albéitar 136, 20–21.

Folch J., Jurado J.J., Calvo J.H., Martínez-Royo A., Alabart J.L., **Lahoz B.**, Fantova E., Equipo de veterinarios de UPRA Grupo Pastores. 2010. Cómo mejorar la prolificidad de las ganaderías de ovino de carne mediante el programa de divulgación de la variante génica Rasa Oviaragón (ROA). Revista Pequeños Rumiantes 11, 17–20.

Contribuciones a congresos internacionales

Estienne A., Fabre S., **Lahoz B.**, Alabart J.L., Folch J., Jarrier P., Monniaux D. 2013. Involvement of the Bone Morphogenetic Protein system in the regulation of the Anti-Müllerian Hormone and its type 2 specific receptor in the sheep ovary. L'Ovarian Club. Paris (France), November 14-16, 2013.

Lahoz B., Alabart J.L., Monniaux D., Cocero M.J., Folch J. 2013. Relationship between plasma AMH at prepuberty and adulthood in sheep. 29th Scientific Meeting of the E.E.T.A. Istanbul (Turkey), September 6-7, 2013.

Lahoz B., Alabart J.L., Folch J., Sánchez P., Echegoyen E., Cocero M.J. 2013. Effect of the *FecX*^{R} allele on follicle size and oocyte competence assessed by LOPU and IVP of sheep embryos. 29th Scientific Meeting of the E.E.T.A. Istanbul (Turkey), September 6-7, 2013.

Martínez-Royo A., Alabart J.L., **Lahoz B.**, Folch J., Fantova E., Calvo J.H. 2013. Whole genome association study for reproductive seasonality trait in the Rasa Aragonesa sheep breed. 64th Annual Meeting of the European Association for Animal Production. Nantes (France), August 26-30, 2013.

Folch J., **Lahoz B.**, Alabart J.L., Calvo J.H., Fantova E., Pardos L. 2013. Productivity improvement in sheep associating polygenic selection and diffusion of the *FecX*^{R} allele. 64th Annual Meeting of the European Association for Animal Production. Nantes (France), August 26-30, 2013. Presenting author (poster).

Mermillod P., Panneau B., Fatet A., Souza J.M., **Lahoz B.**, Locatelli Y. 2012. Production in vitro d'embryons de ruminants. 25ème Colloque Biotechnocentre. Seillac (France), October 11-12, 2012.

Calvo J.H., Folch J., Jurado J.J., **Lahoz B.**, Martínez Royo A., Alabart J.L., Sevilla E., Quintín F., Vijil E., Blasco M.E., Fantova E. y equipo técnico UPRA-Grupo Pastores. 2012. Uso del alelo *FecX*^{R}/ROA en el esquema de mejora genética para incrementar la prolificidad de UPRA-Grupo Pastores en la raza ovina Rasa aragonesa. VIII Congreso Ibérico sobre Recursos Genéticos Animales. Évora (Portugal), September 13-15, 2012.

Lahoz B., Alabart J.L. Monniaux D., Fabre S., Jarrier P., Cocero M.J., Folch J. 2012. Determination of Anti-Müllerian hormone plasma concentration can help to select donor sheep for laparoscopic ovum pick-up (LOPU). 17th International Congress on Animal Reproduction (ICAR). Vancouver (Canadá), July 29 - August 2, 2012. Proceedings, Rep Dom Anim, Vol.47(4):492. Presenting author (poster).

Fatet A., **Lahoz B.**, Poulin N., Guignot F., Alabart J.L., Folch J., Mermillod P. 2011. Effect of a modified WOW-system and embryo density during in vitro culture on developmental rate of caprine embryos. 27th Scientific Meeting of the E.E.T.A. Chester (England), September 9-10, 2011. Proceedings, p.142.

Lahoz B., Alabart J.L., Monniaux D., Mermillod P., Folch J. 2011. Anti-Müllerian hormone (AMH) plasma concentration and eCG-induced ovulation rate in prepubertal ewe lambs as predictors of fertility at the first mating. 62th Annual Meeting of the European Association for Animal Production. Stavanger (Norwey), August 29 - September 1, 2011. Proceedings, p.199. Presenting author (oral).

- ✓ Premio mejor comunicación oral “Sheep and goat commission”.

Martínez-Royo A., **Lahoz B.**, Alabart J.L., Folch J., Calvo J.H. 2011. Characterization of the gene Melatonin Receptor 1A (MTNR1A) in the Rasa Aragonesa sheep breed: association with reproductive seasonality. 62th Annual Meeting of the European Association for Animal Production. Stavanger (Norwey), August 29 - September 1, 2011. Proceedings, p.189.

Martínez-Royo A., **Lahoz B.**, Folch J., Alabart J.L., Jurado J.J., Calvo J.H. 2010. MTNR1A is a potential candidate gene for seasonal reproductive activity and prolificacy in Rasa Aragonesa sheep breed. 32nd Conference for the International Society for Animal Genetics. Edinburgh (Scotland), July 26-30, 2010. Proceedings, p.110.

Folch J., **Lahoz B.**, Alabart J.L. 2010. Researches in progress in reproductive physiology to increase the efficiency of sheep production. 61th Annual Meeting of the European Association for Animal Production. Heraklion (Crete), August 23-27, 2010. Proceedings, p.111.

Alabart J.L., **Lahoz B.**, Folch J., Sánchez P., Echegoyen E., Cocero M.J. 2010. Effect of a defined or an undefined in vitro maturation medium on cleavage rate and embryo development in sheep. 26th Scientific Meeting of the E.E.T.A. Kuopio (Finland), September 10-11, 2010. Proceedings, p.124. Presenting author (poster).

Lahoz B., Blasco M.E., Sevilla E., Folch J., Roche A., Quintín F.J., Martínez-Royo A., Galeote A.I., Calvo J.H., Fantova E., Jurado J.J., Alabart J.L. 2009. Fertility of select Rasa aragonesa rams carrying or not the *FecX*^{R} allele of *BMP15* gene when used in artificial insemination. 60th Annual Meeting of the European Association for Animal Production. Barcelona (Spain), August 24-27, 2009. Proceedings, p.278. Presenting author (poster).

Folch J., Cocero M.J., Martí J.I., **Lahoz B.**, Olivera J., Ramón J., Roche A., Dervishi E., Calvo J., Echegoyen E., Sánchez P., Alabart J.L. 2009. Ten years of embryo transfer applied to a selection Program for prolificacy in sheep. 60th Annual Meeting of the European Association for Animal Production. Barcelona (Spain), August 24-27, 2009. Proceedings, p.275.

Alabart J.L., Roche A., Olivera J., Cocero M.J., Martí J.I., **Lahoz B.**, Folch J. 2009. Repeatability and performance trend in successive laparoscopic ovum pick-up sessions in sheep. 25th Scientific Meeting of the E.E.T.A. Poznan (Poland), September 11-12, 2009. Proceedings, p.130.

Contribuciones a congresos nacionales

Cocero M.J., Sánchez P., Folch J., **Lahoz B.**, Calvo J.H., Quintín F., Sevilla E., Alabart J.L. 2013. La biopsia embrionaria con láser permite el diagnóstico genético preimplantacional ovino sin reducir la supervivencia a término. XV Jornadas sobre Producción Animal AIDA. Zaragoza, 14-15 de mayo de 2013. pp. 371–373.

Lahoz B., Alabart J.L., Echegoyen E., Sánchez P., Folch J. 2012. La hormona Anti-Mülleriana (AMH) y la respuesta ovulatoria a la eCG en corderas prepúberes como posibles predictores de su fertilidad en la primera cubrición. XXXVII Jornadas de la Sociedad Española de Ovinotecnia y Caprinotecnia. Ciudad Real, 19-21 de septiembre de 2012. pp. 433–436. Autor-presentador (oral).

Lahoz B., Folch J., Cocero M.J., Echegoyen E., Sánchez P., Alabart J.L. 2011. Laparoscopic ovum pick-up (LOPU) en ovejas Rasa Aragonesa portadoras o no de alelo *FecX*^{R} del gen *BMP15:* rendimiento, diámetro folicular y efecto del tratamiento estimulatorio con FSH. XIV Jornadas sobre Producción Animal AIDA. M. Joy et al. (Eds.). Zaragoza, 17-18 de mayo de 2011. pp. 347–349. Autor-presentador (oral).

Martínez-Royo A., **Lahoz B.**, Alabart J.L., Folch J., Calvo J.H. 2011. Caracterización del gen receptor de la melatonina 1A (MTNR1A) en la raza ovina Rasa Aragonesa: asociación con la estacionalidad reproductiva. XIV Jornadas sobre Producción Animal AIDA. M. Joy et al. (Eds.). Zaragoza, 17-18 de mayo de 2011. pp. 452–454.

Alabart J.L., **Lahoz B.**, Folch J., Martí J.I., Sánchez P., Delahaut P., Colemonts Y., Beckers J.F., Melo de Sousa N. 2010. Diagnóstico precoz de gestación en ganado ovino mediante un kit de enzimoinmunoanálisis (EIA) de la glicoproteína asociada a la gestación (PAG) plasmática. XXXV Jornadas de la Sociedad Española de Ovinotecnia y Caprinotecnia. Valladolid, 16-19 de septiembre de 2010. pp. 199–202.

Lahoz B., Alabart J.L., Echegoyen E., Sánchez P., Calvo J.H., Martínez-Royo A., Jurado J.J., Fantova E., Folch J. 2009. Efecto del alelo *FecX*^{R} del gen *BMP15* sobre la tasa de ovulación y la prolificidad en ovejas Rasa Aragonesa. XIII Jornadas sobre Producción Animal AIDA. M. Joy et al. (Eds.). Zaragoza, 12-13 de mayo de 2009. pp. 678–680. Autor-presentador (oral).

Lahoz B., Alabart J.L., Martí J.I., Echegoyen E., Calvo J.H., Martínez-Royo A., Vijil E., Folch J. 2009. ¿Es necesario reducir la dosis de eCG en corderas prolíficas portadoras del alelo *FecX*^{R} (ROA)?: Efecto de dos dosis diferentes sobre la fertilidad y prolificidad. XXXIV Jornadas de la Sociedad Española de Ovinotecnia y Caprinotecnia. Barbastro (Huesca), 16-19 de septiembre de 2009. pp. 351–355. Autor-presentador (oral).

Alabart J.L., Martínez-Royo A., **Lahoz B.**, Calvo J.H., Martí J.I, Jurado J.J., Vijil E., Fantova E.., Folch J. 2009. Fertilidad y prolificidad de las ovejas portadoras o no portadoras del alelo *FecX*^{R} inseminadas en las ganaderías de UPRA-Grupo Pastores. XXXIV Jornadas de la Sociedad Española de Ovinotecnia y Caprinotecnia. Barbastro (Huesca), 16-19 de septiembre de 2009. pp. 387–392.

En cuclillas, ordeño
una cabrita y un sueño.

Glú, glú, glú,
hace la leche al caer
en el cubo. En el tisú
celeste va a amanecer.
Glú, glú, glú. Se infla la espuma,
que exhala
una finísima bruma.

(Me lame otra cabra, y bala).

En cuclillas, ordeño
una cabrita y un sueño.

(Miguel Hernández)