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Factores genéticos relacionados con la estacionalidad reproductiva en ovejas y la actividad sexual en moruecos de raza Rasa Aragonesa

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Tesis Doctoral

FACTORES GENÉTICOS RELACIONADOS CON LA ESTACIONALIDAD REPRODUCTIVA EN OVEJAS Y LA ACTIVIDAD SEXUAL EN MORUECOS DE RAZA RASA ARAGONESA

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Kenza Lakhssassi

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Memoria presentada por

Kenza LAKHSSASSI

Para optar al grado de doctor por la Universidad de Zaragoza

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À la mémoire de ma tante Souad & Oncle Houssine

En memoria de mi tía Souad y mi tío Houssine

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- Factores genéticos implicados en la estacionalidad reproductiva en ovino de carne con efecto macho en Rasa Aragonesa (RTA2015-00090-C03-01).
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« Certes, il y'a des travaux pénibles ; mais la joie de la réussite n'a - t - elle pas à compenser nos douleurs ? »

Jean de la Bruyère

Abreviaturas

A	
A	Activo / Active
AANAT	*Arilalquilamina-N-acetiltransferasa*
ABP	Proteína que liga andrógenos
ACTH	Hormona adenocorticotrópica
AIM	Anestro a la introducción del macho
AN	Anestrous
AR	Anestro reproductivo
ARC	Núcleo arcuato
AVP	*Arginina vasopresina* o *vasopresina*
B	
BCS	Body condition score
BDNF	Factor neurotrófico derivado del cerebro
C	
CC	Condición corporal
CD226	*Molécula CD226 / CD226 molecule*
cDNA	ADN complementario
CiC	Ciclicidad por celos
CIDR	Dispositivos intravaginales de liberación controlada
CiP4	Ciclicidad por progesterona
CITA	Centro de Investigación y Tecnología Agroalimentaria de Aragón
CNAG-CRG	Centro Nacional de Análisis Genómico - Centro de Regulación Genómica
CPM	Counts per million
Cq	Ciclo de amplificación
D	
DAVID	Base de datos de anotación, visualización y descubrimiento integrado / Database for Annotation, Visualization and Integrated Discovery
DE	Expresión diferencial
DEGs	Differentially Expressed Genes
Dio2	*Diodinasa tipo 2*
DL	Desequilibrio de ligamento
DMR	Regiones diferencialmente metiladas / Differentially methylated regions
DNAM-1	Proteína CD226 también conocida como DNAX accessory molecule-1
DTA	Número total de días de anestro
E	

eCG	Gonadotropina coriónica equina
EM	Energía metabolizable
ETL	Caracter de interes económico
F	
F	Folicular / Follicular
FAD	Fibra ácido detergente
FC	Fold-change
FDR	Tasa de falso descubrimiento / False discovery rate
FGA	Acetato de fluorogestona
FND	Fibra neutro detergente
FSH	Hormona folículo estimulante
G	
GABA	Ácido gamma-aminobutírico
GAS	Selección asistida por genes / Gene Assisted Selection
GCTA	Genome-wide Complex Trait Analysis
GDEs	Genes diferencialmente expresados
GH	Hormona del crecimiento
GnRH	Hormona liberadora de gonadotropina / Gonadotropin releasing hormone
GO	Ontología génica / Gene Ontology
GP	Glándula pineal
GR	Growth rate
GRM	Matriz de relaciones genómicas / Genomic Relationship Matrix
GS	Selección Genómica / Genomic Selection
GSEA	Análisis de enriquecimiento de conjuntos de genes / Gene set enrichment analysis
GWAS	Estudio de asociación de genoma completo / Genome-wide association study
H	
HK	Genes de referencia / Housekeeping genes
HPG	Eje hipotalámico-hipofisiario-gonadal
HT	Hipotálamo / Hypothalamus
I	
IA	Inseminación artificial
IBS	Distancia de identidad por estado
IGP	Indicación Geográfica Protegida
INIA-CSIC	Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria - Consejo Superior de Investigaciones Científicas
INRA Maroc	Institut National de la Recherche Agronomique du Maroc
K	
KASP	PCR competitiva específica de alelo / Kompetitive Allele Specific PCR

Kiss1	*Kisspeptina*
Kiss1R	*Receptor de kisspeptina*
L	
L	Luteal
LEPR	*Receptor de la leptina*
LH	Hormona luteinizante
LSMeans	Medias mínimo-cuadráticas
LW	Live weight
M	
MAF	Frecuencia del alelo menos frecuente
MAPA	Ministerio de Agricultura, Pesca y Alimentación
MDS	Análisis de escalamiento multidimensional
MNTR1A	*Receptor 1A de la melatonina*
MS	Materia seca
N	
NA	No activo / Non active
NK	Natural killer
NPY	*Neuropéptido Y / Neuropeptide Y*
NSQ	Núcleo supraquiasmático del hipotálamo
O	
OCM	Oestrous cycling months
P	
P4	Progesterona
P4CM	Progesterone cycling months
PB	Proteína bruta
PCA	Análisis de componentes principales / Principal Component Analysis
PG	Pineal gland
POA	Área preóptica
PRL	*Prolactina*
PT	*Pars tuberalis*
PV	Peso vivo
Q	
QTL	Quantitative trait loci
R	
RIA	Radioinmunoanálisis
RIN	Integridad del ARN / RNA Integrity Number
RT-qPCR	PCR cuantitativa en tiempo real / Reverse transcription quantitative real-time PCR
S	
SAM	Selección Asistida por Marcadores
SCT	Test de capacidad de cubrición / Serving Capacity Test

SMRT	Single-Molecule Real-Time
SNPs	Polimorfimos de un solo nucleótico / Single Nucleotide Polymorphism
T	
T3	Triyodotironina
T4	Tiroxina
TC	Tasa de crecimiento
TDA	Total days of anoestrus
trkB	Receptor tirosina quinasa B
TSH	Hormona estimulante de la tiroides
TSHβ	*Subunidad β de tirotropina*
TSS	Sitio de inicio de la transcripción / Transcription start site
U	
UE	Unión Europea
UPRA	Unión de Productores de Raza Rasa Aragonesa
UTH	Unidad trabajo hombre
V	
VE	Vitamina E
VEGF-A	Isoforma A del factor de crecimiento vascular endotelial
VEP	Variant Effect Predictor
VG	Valor genómico

Resumen

En la presente Tesis Doctoral hemos estudiado la base genética de la estacionalidad reproductiva en hembras y otros factores que influyen en la misma, así como de la capacidad de cubrición de los machos en época de anestro reproductivo (AR) en animales de raza Rasa Aragonesa, mediante herramientas genómicas de genotipado de SNPs y secuenciación masiva de nueva generación.

En primer lugar, mediante la aproximación de gen candidato se aisló parcialmente el gen *LEPR,* y se asociaron 2 SNPs a caracteres de estacionalidad reproductiva en ovejas. Se identificaron un total de 18 SNPs en ovejas con valores extremos para fenotipos de estacionalidad, que fueron el número total de días de anestro (DTA) y ciclicidad por progesterona (CiP4), ambos asociados a la función ovárica y basados en la medición de los niveles de progesterona (P4) en sangre; y el fenotipo ciclicidad por celos (CiC), basado en los signos de comportamiento estral. Seis de los SNPs fueron sustituciones no sinónimas, y dos de ellos se predijeron *in silico* como deletéreos: rs596133197 y rs403578195. El estudio de asociación de los polimorfismos encontrados en el gen fue llevado a cabo en 239 ovejas. Este estudio reveló que el SNP rs403578195, localizado en el exón 8 y que produce un cambio de alanina por glicina (Ala284Gly) en el dominio extracelular de la proteína, se asoció con un incremento del 12% del carácter CiC en las ovejas con el genotipo CC comparado con las heterocigotas GC. Los análisis de asociación haplotípica también sugirieron la implicación de otro SNP no sinónimo localizado en el exón 20 (rs405459906). Este SNP produce un cambio aminoacídico (Lys1069Glu) en el dominio intracelular de la proteína y segrega independientemente del rs403578195. En un segundo estudio, se llevó a cabo un análisis de asociación a genoma completo (GWAS) sobre los mismos caracteres de estacionalidad incluyendo 205 ovejas genotipadas con los chips Illumina de 50K y 600K. Solo un SNP (rs404991855) asociado con el fenotipo CiP4 superó el umbral de significación genómica. Nueve SNPs exhibieron asociaciones significativas a nivel cromosómico (FDR < 0,10), estando los SNPs rs404991855 y rs418191944, localizados en el gen de la *molécula CD226 (CD226)* relacionado con enfermedades reproductivas, asociados a los tres caracteres de estacionalidad reproductiva. Otros dos SNPs se

ubicaron cerca del gen *neuropéptido Y* (*NPY*), que está involucrado en los ritmos circadianos. Para validar los resultados del GWAS, se realizó la caracterización parcial de ambos genes por secuenciación SANGER en animales con valores extremos para estos caracteres, encontrando dos SNPs sinónimos y dos no sinónimos en los genes *NPY* y *CD226*, respectivamente. El análisis de asociación de estos SNPs mostró que sólo el SNP rs404360094 situado en el exón 3 del gen *CD226*, y que produce una sustitución aminoacídica de asparagina (polar sin carga) a ácido aspártico (ácido), se asocia con los tres caracteres de estacionalidad.

Por otra parte, dado que la condición corporal (CC) y el peso vivo (PV) de la oveja juegan un papel importante en el desempeño productivo y reproductivo, se llevó a cabo otro análisis GWAS para detectar variantes genéticas asociadas a los caracteres de crecimiento en 225 ovejas adultas genotipadas con los chips de media y alta densidad Illumina Ovine BeadChip. A estas ovejas se les controló y registró la CC, PV y tasa de crecimiento (TC) de enero a septiembre durante 2 años (2011 y 2012). Se estimaron los fenotipos corregidos para CC, PV y TC, que fueron utilizados posteriormente para el análisis GWAS. Sólo el SNP rs425509273 en el cromosoma 9 (OAR9), mostró una significación a nivel genómico para el fenotipo TC. Uno, tres y nueve SNPs se asociaron a nivel cromosómico con un FDR < 0,10 con los caracteres CC, PV y TC, respectivamente. El gen candidato *CYP7B1*, ubicado a 83 kb del SNP rs425509273 y asociado con la TC, se aisló parcialmente y se buscaron polimorfismos mediante secuenciacion SANGER en animales extremos para el fenotipo TC. Se detectaron 15 polimorfismos:12 SNPs, dos indels y un poliC, localizados todos en la región 5' del gen. El análisis de asociación de los polimorfismos ubicados cerca del sitio de inicio de la transcripción (TSS) mostró que una inserción de 22 pb ubicada a -58 nucleótidos del TSS (indel (-58)), un poliC (-25) y dos SNPs A/G (SNP3 (-114) y SNP5 (-63)) se asociaron con la TC, mientras que solo el indel (-58) se asoció con la CC. El análisis de asociación haplotípica confirmó estos resultados. La caracterización funcional de los polimorfismos del gen *CYP7B1* en hígado mediante la metodología de PCR cuantitativa en tiempo real tras transcripción inversa (RT-qPCR) confirmó que las mutaciones en la región promotora afectaban a la tasa de expresión del gen *CYP7B1*.

Con el objetivo de identificar nuevos genes y rutas metabólicas implicadas en la activación del ovario en AR se llevó a cabo un análisis del transcriptoma mediante RNA-

Seq del *pars tuberalis* (PT) e hipotálamo (HT) en 21 ovejas en diferentes fases ováricas, folicular (F) y luteal (L) del ciclo estral, y en AR. En HT, se encontraron 72 y 3 genes diferencialmente expresados (GDEs) al comparar F *vs.* AR y L *vs.* AR, respectivamente. En PT, se encontraron 6 y 4 GDEs en las comparaciones F *vs.* AR y L *vs.* AR, respectivamente. El análisis de enriquecimiento funcional con DAVID de GDEs entre las fases F y AR en el HT reveló grupos de genes significativamente asociados con la unión de actina y el citoesqueleto, relacionados con la plasticidad neuronal modulada por las hormonas esteroides gonadales, así como con la señalización de oxitocina. Los GDEs en PT mostraron mayores diferencias en los niveles de expresión que los encontrados en HT. En este sentido, el *ITLN* estaba altamente sobreexpresado en las fases F y L *vs.* AR, siendo *MRPL57* e *IRX4* altamente subexpresados en la comparación L *vs.* AR. El gen *DDC* en PT, relacionado con la regulación de LH, mostro una sobreexpresión en la fase F. El análisis de enriquecimiento funcional de conjuntos de genes (GSEA) reveló múltiples vías relacionadas con la neurotransmisión y la plasticidad neuronal.

Finalmente, para comprender los mecanismos del comportamiento sexual en moruecos, se utilizó también la tecnología de secuenciación masiva del transcriptoma para identificar GDEs en HT, PT, glándula pineal (GP) y sangre en 12 machos de Rasa Aragonesa con diferente actividad sexual (6 sexualmente activos (A) y 6 no activos (NA)). El análisis bioinformático de los 16.401 genes identificados por RNA-Seq reveló 103 y 12 GDEs en HT y GP, respectivamente, con un FDR < 0,05, y con un valor absoluto de log2FC ≥ 1. Sin embargo, no se encontraron GDEs en el PT. En sangre, de un total de 14.078 genes expresados solo cuatro fueron GDEs (FDR < 0,10) en la comparación de machos A frente a NA. La anotación funcional y el análisis de enriquecimiento de rutas mostraron que los GDEs de HT estaban enriquecidos principalmente en interacciones neuroactivas ligando-receptor y vías de señalización, incluyendo genes candidatos notables como *MTNR1A, CHRNA2, FSHB, LHB, GNRHR, AVP, PRL, PDYN, CGA, GABRD* y *TSHB*, que desempeñan un papel crucial en el comportamiento sexual. También se destacaron las vías de señalización GnRH y cAMP. Además, GSEA identificó vías potenciales, dominadas principalmente por la categoría de procesos biológicos, que podrían ser responsables de las diferencias en el comportamiento sexual observadas en los machos. El transporte intracelular de proteínas

y el proceso de especificación de patrones se enriquecieron en el PT, mientras que las vías de unión de factores de transcripción y ubiquitinación de proteínas en la GP. La validación de 5 GDEs mediante RT-qPCR confirmaron los resultados obtenidos por RNA-Seq. En sangre, sólo los genes, *inhibidor de acrosina 1* (*ENSOARG00020023278*) y *SORCS2* mostraron sobreexpresión (log2FC > 1) en machos activos, mientras que los genes *CRYL1* y la *isoforma X47 de la cadena ligera lambda-1 de inmunoglobulina* (*ENSOARG00020025518*) estaban subexpresados (log2FC < -1) en este mismo grupo. El GSEA realizado con todos los genes expresados en sangre identificó 428 vías de señalización, predominantemente relacionadas con procesos biológicos. La vía de los lisosomas (GO:0005764) fue la más enriquecida, que podría afectar a la fertilidad y al comportamiento sexual, dado el papel crucial que desempeñan los lisosomas en la esteroidogénesis, estando el gen *SORCS2* relacionado con esta vía de señalización. Además, la vía enriquecida de la regulación positiva de la cascada ERK1 y ERK2 (GO:0070374) se asocia con fenotipos reproductivos como la fertilidad a través de la modulación de la regulación hipotalámica y la producción de gonadotropinas hipofisarias mediada por GnRH. También se enriquecieron las vías del lado externo de la membrana plasmática (GO:0009897), el centro fibrilar (GO:0001650), la adhesión focal (GO:0005925) y el lamellipodio (GO:0030027), lo que sugiere que algunas moléculas de estas vías también podrían estar implicadas en el comportamiento sexual de los moruecos. Por lo tanto, estas vías en conjunto pueden jugar un papel importante en la regulación del comportamiento sexual en machos de raza Rasa Aragonesa a través del eje hipotálamo-pituitario-gonadal.

En conclusión, los estudios de asociación mediante GWAS y la aproximación de gen candidato permitieron asociar por primera vez polimorfismos en los genes *LEPR* y *CD226* con los caracteres de estacionalidad reproductiva. Por otra parte, los polimorfismos localizados en el promotor del gen *CYP7B1* se asociaron con los caracteres de crecimiento, que son factores que influyen de manera importante en la estacionalidad reproductiva de la raza Rasa Aragonesa. El estudio del transcriptoma en hembras reveló nuevos genes candidatos involucrados en las transiciones de las fases reproductivas en ovejas estacionales. Mientras que los resultados del estudio del transcriptoma en machos contribuyen a comprender la base genética de su comportamiento sexual demostrando que múltiples redes y vías orquestan el

comportamiento sexual en moruecos. Los genes descubiertos mediante estos estudios podrían constituirse en marcadores moleculares potenciales para ser utilizados en la selección asistida por marcadores para mejorar la estacionalidad reproductiva en hembras y el comportamiento sexual de machos en la especie ovina.

Abstract

In this Doctoral Thesis we studied the genetic basis of ewes' reproductive seasonality and other variables that influence it, as well as rams' sexual behaviour in Rasa Aragonesa breed using genomic tools as candidate gene approach, GWAS, and transcriptome analysis by RNA-Seq.

For the association analyses with reproductive traits, three phenotypes associated with either ovarian function based on blood progesterone levels (total days of anoestrus (TDA) and progesterone cycling months (P4CM)) or behavioural signs of oestrous (oestrous cycling months (OCM)) were studied. Firstly, the *LEPR* gene was partially isolated and a total of 18 SNPs were detected in ewes with extreme phenotypes values for TDA and OCM. Six SNPs were non-synonymous substitutions and two of them were predicted *in silico* as deleterious: rs596133197 and rs403578195. These polymorphisms were then genotyped in 239 ewes. SNP association study revealed that the SNP rs403578195, located in exon 8 and leading to a change of alanine to glycine (Ala284Gly) in the extracellular domain of the protein, was related to OCM trait, being the CC genotype associated with an increase of 12% of this trait compared with the GC genotype. Haplotype association analyses also suggested the involvement of other non-synonymous SNP located in exon 20 (rs405459906). This SNP also produces an amino acid change (Lys1069Glu) in the intracellular domain of the protein and segregates independently of rs403578195. Secondly, a genome-wide association study (GWAS) was also conducted with the three traits of reproductive seasonality including 205 ewes genotyped with the 50K and 600K Illumina Ovine Beadchips. Only one SNP associated with the P4CM overcame the genome-wide significance level (rs404991855). Nine SNPs exhibited significant associations at the chromosome level, being the SNPs rs404991855 and rs418191944, that are located in the *CD226 molecule* (*CD226*) gene related to reproductive diseases, associated with the three traits. Two other SNPs were located close to the *neuropeptide Y* (*NPY*) gene, which is involved in circadian rhythms. To validate the GWAS results, partial characterization of both genes by SANGER sequencing in ewes with extreme values for the traits studied were performed. Two synonymous and two non-synonymous SNPs in the *NPY* and *CD226* genes, respectively, were genotyped in the total population. SNP association analysis showed

that only SNP rs404360094 in the exon 3 of the *CD226* gene, which produces an amino acid substitution from asparagine (uncharged polar) to aspartic acid (acidic), was associated with the three seasonality traits, confirming the GWAS results.

As ewe body condition score (BCS) and live weight (LW) play a significant role in productive and reproductive performance, another GWAS was carried out to detect genetic variants associated with growth traits in 225 adult ewes by using the genotypes from the 50K and HD Illumina Ovine BeadChips. These ewes were measured for LW, BCS and growth rate (GR) from January to September for 2 years (2011 and 2012). Corrected phenotypes for BCS, LW and GR were estimated and used as input for the GWAS. Only one SNP rs425509273 in chromosome 9 (OAR9), associated with the GR, overcame the genome-wise significance level. One, three and nine SNPs were associated at the chromosome-wise level (FDR 10%) for BCS, LW and GR traits, respectively. The *CYP7B1* candidate gene, located 83 kb upstream from SNP rs425509273, was partial isolated and SANGER-sequenced. Fifteen polymorphisms comprising 12 SNPs, two indels and one polyC were detected in the 5' region. The SNP association analysis of the polymorphisms located close to the transcription start site (TSS) showed that a 22 bp insertion located at -58 nucleotides from the TSS (indel (-58)), a polyC (-25), and two A/G SNPs (SNP3 (-114) and SNP5 (-63)), were associated with the GR trait, whereas only the indel (-58) was associated with the BCS trait. The haplotype analysis confirmed these results. The functional characterisation of the polymorphisms at *CYP7B1* gene in liver by reverse transcription quantitative real-time PCR (RT-qPCR) analysis confirmed that the mutations in the promoter region affected *CYP7B1* gene expression.

In order to identify new genes and metabolic pathways involved in ewes' reproductive seasonality, the *pars tuberalis* (PT), and hypothalamus (HT) transcriptomes of 21 ewes in the follicular (F) and luteal (L) phases of estrous cycle, and anestrous (AN) phase, were studied. In HT, 72 and 3 Differentially Expressed Genes (DEGs) were found when comparing F *vs.* AN and L *vs.* AN, respectively. In PT, 6 and 4 DEGs were found in F *vs.* AN and L *vs.* AN comparisons, respectively. Enrichment analysis for DEGs between the F and AN phases in the HT revealed significant clusters, mainly associated with actin-binding, and cytoskeleton, that are related to neural plasticity modulated by gonadal steroid hormones, as well as with oxytocin signaling.

We found that DEGs in PT had higher differences in expression levels than those found in HT. In this sense, the *ITLN* was highly upregulated in the F and L *vs.* AN phases, being *MRPL57* and *IRX4* highly downregulated in L *vs.* AN comparison. The *DDC* gene in PT, related to LH regulation, was upregulated in the F phase. The gene set enrichment analysis (GSEA) revealed multiple pathways related to neurotransmission and neuronal plasticity.

On the other hand, to understand the mechanisms of rams' sexual behavior, transcriptomic sequencing technology was also used to identify DEGs in the HT, PT, pineal gland (PG) and blood in Rasa Aragonesa rams with different sexual behavior (6 sexually active (A) and 6 nonactive (NA)). Bioinformatics analysis of the 16,401 identified genes by RNA-Seq revealed 103 and 12 DEGs in the HT and the PG, respectively, at a false discovery rate (FDR) of 5% with an absolute value of expression ≥ 1 (log2FC). However, no DEGs were found in the PT. Whereas a total of 14,078 genes were expressed in blood but only four genes were DEGs (FDR < 0.10) in the A *vs.* NA rams comparison.

Functional annotation and pathway enrichment analysis showed that DEGs of HT were enriched mainly in neuroactive ligand-receptor interactions and signaling pathways, including notable candidate genes such as *MTNR1A, CHRNA2, FSHB, LHB, GNRHR, AVP, PRL, PDYN, CGA, GABRD*, and *TSHB*, which play a crucial role in sexual behavior. The GnRH and cAMP signaling pathways were also highlighted. In addition, GSEA identified potential pathways, dominated mainly by biological process category, that could be responsible for the differences in sexual behavior observed in rams. The intracellular protein transport and pattern specification process were enriched within the PT and the transcription factor binding and protein ubiquitination pathways for the PG. The validation of 5 DEGs using RT-qPCR showed expression patterns like the found with RNA-Seq. While in blood, the genes, *acrosin inhibitor 1 (ENSOARG00020023278)* and *SORCS2*, were upregulated (log2FC > 1) in active rams, whereas the *CRYL1* and *immunoglobulin lambda-1 light chain isoform X47 (ENSOARG00020025518)* genes were downregulated (log2FC < -1) in this same group. GSEA carried out with all the expressed genes identified 428 signaling pathways, predominantly related to biological processes. The lysosome pathway (GO:0005764) was the most enriched, and may affect fertility and sexual behavior, given the crucial

role played by lysosomes in steroidogenesis, being the *SORCS2* gene related to this signaling pathway. Furthermore, the enriched positive regulation of ERK1 and ERK2 cascade (GO:0070374) pathway is associated with reproductive phenotypes such as fertility via modulation of hypothalamic regulation and GnRH-mediated production of pituitary gonadotropins. Additionally, external side of plasma membrane (GO:0009897), fibrillar center (GO:0001650), focal adhesion (GO:0005925), and lamellipodium (GO:0030027) pathways were also enriched, suggesting that some molecules of these pathways might also be involved in rams' sexual behavior. Thus, these pathways together may play an important role in the regulation of the sexual behavior in Rasa Aragonesa rams through the hypothalamic-pituitary-gonadal axis.

In conclusion, we identified for the first-time polymorphisms in *LEPR* and *CD226* genes associated with reproductive seasonality traits using GWAS and candidate gene approaches. Furthermore, polymorphisms in the *CYP7B1* gene promoter region were found significantly associated with growth traits which are important factors influencing reproductive seasonality in Rasa Aragonesa sheep breed. Transcriptome study in ewes reveals new candidate genes involved in the reproductive stages' transitions in seasonal sheep. Whereas the results from the transcriptome study in rams contribute to understanding the genomic basis of their sexual behavior demonstrating that multiple networks and pathways orchestrate sexual behavior in sheep. Genes discovered through this study could be potential molecular markers to be used for genetic-assisted selection to improve female reproductive seasonality and male sexual behavior in the ovine species.

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I- Introducción

En España, la actividad ganadera ovina tiene una gran tradición, abarcando importantes aspectos económicos, sociales y ambientales (Bernués et al., 2018). El ganado ovino es el que presenta un mejor aprovechamiento de los pastos áridos o semiáridos (Zygoyiannis, 2006), generando bienes públicos derivados del pastoreo por su contribución a la conservación de los suelos, al incremento de materia orgánica en los mismos, al mantenimiento de la biodiversidad y a la prevención de incendios, reduciendo así los graves problemas de erosión del territorio y contribuyendo por tanto al equilibrio ecológico (Gabiña, 2006; Interovic, 2006; Pardos et al., 2022). Además, la capacidad de aprovechamiento de recursos pastables de zonas marginales, y la ocupación de una cuantiosa mano de obra de carácter familiar, alejada de los grandes centros urbanos, contribuye al asentamiento de la población rural evitando el despoblamiento de las zonas más desfavorecidas y ofreciendo una alternativa laboral en estas regiones. Sin embargo, en España, el censo ovino ha descendido dramáticamente en los últimos años, sufriendo una notable reducción del 2,3% respecto al 2020, y del 36% respecto al 1999, donde se contabilizaban un total de 23.965.000 cabezas (MAPA, 2021), encontrándose actualmente estabilizado en unos 15 millones de cabezas. Históricamente, la ganadería de ovino de carne ha sido la más representativa del campo, pero el envejecimiento de la población agraria y la expansión de sistemas de producción intensivos, como el porcino (más rentables en términos económicos), han provocado que en las últimas décadas el número de cabezas de ovino de aptitud cárnica haya descendido de forma significativa. De hecho, el censo del ovino de carne ha pasado de 7.950.757 en el 2020 a 7.772.186 cabezas en el 2021, lo que se traduce en una bajada del 2,2% (MAPA, 2021). Por otra parte, es destacable del descenso del consumo de carne de ovino y caprino en España, que se ha situado alrededor del 57% en los últimos 15 años (Figura 1).

En Aragón, el número de cabezas de ganado ovino se encuentran por encima del millón de ejemplares (1.546.372), que supone el 12% de ovejas de aptitud cárnica del total del ovino de carne en España (MAPA, 2021). La Indicación Geográfica Protegida (IGP) “Ternasco de Aragón” es la que más toneladas de carne vende al año en España, el 46,4% de toda la carne con denominación. Sin embargo, Aragón muestra una tendencia descendente del censo ovino como el resto de España, debido a motivos similares tales como el envejecimiento de los ganaderos, la escasez de relevo

generacional, la falta de innovación en las ganaderías, la disminución del consumo de carne de ovino, y la disminución de la rentabilidad económica de las explotaciones. Este último factor ha llevado a muchos ganaderos a abandonar la actividad debido al incremento de los costes de alimentación (precio de los cereales) y del combustible, así como a otros problemas como son encontrar mano de obra, o la menor disponibilidad de pastos por la intensificación de la agricultura.

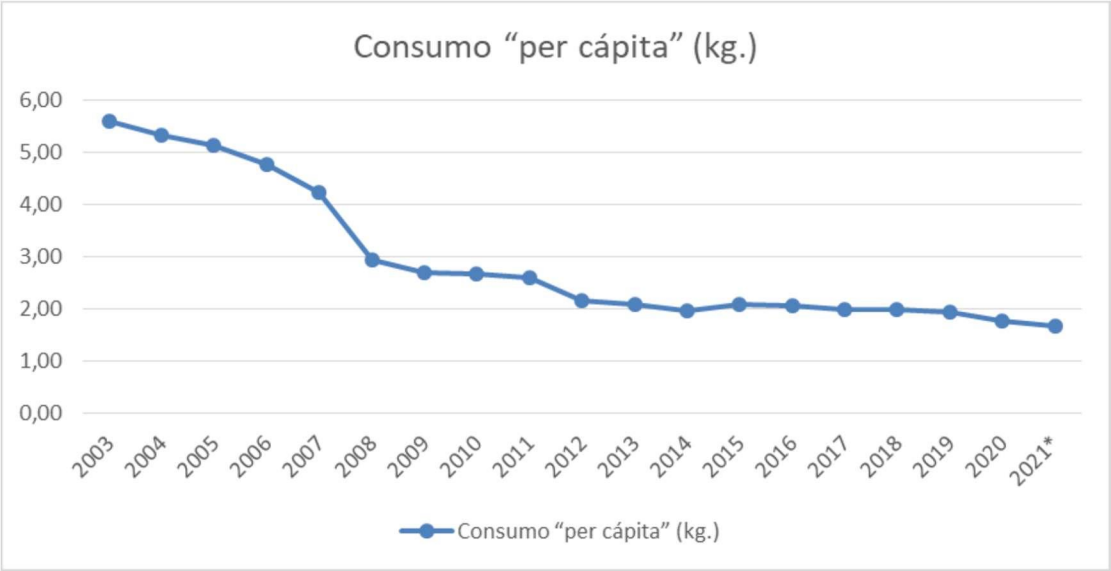


Figura 1. Evolución del consumo de carne fresca de ovino y caprino *per capita* en hogares españoles entre el 2007 y 2021. El año 2021 no tiene los datos completos (MAPA, 2021)

Una vía para aumentar la rentabilidad de las explotaciones, es mejorar la eficiencia productiva y reproductiva de los animales, sin incrementar, e incluso disminuyendo, los insumos. En Aragón, la Rasa Aragonesa es la principal raza ovina, y diversos estudios realizados con datos de gestión técnico-económicos en una muestra constante de explotaciones de esta raza, han demostrado que uno de los factores que influyen en la rentabilidad de las explotaciones depende del número de corderos vendidos por oveja y año (Pardos et al., 2012), y que un incremento del 1% en prolificidad supone un aumento de un 3,4% del margen bruto por oveja de la ganadería y por unidad trabajo hombre (UTH) (Fantova et al., 2016). Igualmente, en un trabajo reciente se muestra que las explotaciones con mayor número de corderos vendidos por oveja presentan mejores índices de rentabilidad (Pardos et al., 2022). En este mismo

sentido, se ha cuantificado que la disminución de los periodos improductivos de las ovejas en 0,5 meses (mediante la disminución de la edad al primer parto o la mejora de la fertilidad en primavera), conllevaría una reducción de los costes de 82,8 €/oveja y año (Pardos y Fantova, 2018). En resumen, podemos decir que las explotaciones que obtienen los mejores resultados económicos son aquellas que presentan unos mayores ingresos procedentes de la venta de corderos por oveja, mejorando sus índices técnicos, especialmente la prolificidad y la producción de corderos contra estación reproductiva.

La Rasa Aragonesa tiene unas características reproductivas y de movilización de grasa que permiten que sea explotada en ambientes más o menos desfavorables desde el punto de vista climático y de recursos alimenticios, por lo que puede ser sometida a distintos grados de extensificación con buenos resultados (Folch y Alabart, 2000). Esta raza presenta una prolificidad baja, de 1,37 corderos por oveja y parto (Jiménez y Serrano, 2022). Para mejorar la misma, se está llevando a cabo desde el año 1994 un Programa de Mejora Genética en las ganaderías controladas por UPRA-Grupo Pastores (Unión de Productores de Raza Rasa Aragonesa) cuyo objetivo es el incremento de la prolificidad. Gracias a este Programa de Mejora Genética, en el año 2007 se descubrió en esta raza ovina la existencia de un alelo de hiperprolificidad ($FecX^{R}$) en el gen *BMP15* localizado en el cromosoma X (Martinez-Royo et al., 2008). Recientemente, mediante un estudio de asociación de genoma completo (GWAS) se han detectado nuevas variantes génicas asociadas a la prolificidad: el alelo prolífico Grivette ($FecX^{Gr}$), y el nuevo alelo prolífico $FecX^{RA}$ localizado también en el gen *BMP15* (Calvo et al., 2020a). Estos alelos están siendo utilizados en la selección asistida por marcadores (SAM), mediante la preselección de reproductores en función de la presencia o ausencia de los mismos. Por otra parte, en cuanto a la fertilidad, hay que distinguir entre los fallos de fertilidad en cubriciones de primavera, debidos al anestro estacional, y los fallos durante la estación favorable, debidos a problemas reproductivos o esterilidad de las ovejas y/o de los machos.

La Rasa Aragonesa, como otras razas mediterráneas, presenta una marcada estacionalidad reproductiva que se prolonga de febrero a julio provocando una estacionalidad de la producción de corderos y variaciones de los precios de venta de los mismos a lo largo del año: precios muy bajos en primavera, cuando hay más oferta de corderos, y muy altos en otoño, cuando la oferta es mucho menor (Fantova y Casas,

2010; Hazard, 2010). Ello dificulta tanto la organización del mercado del cordero como la planificación de la producción en las ganaderías. Para superar este desequilibrio de oferta-demanda, se han desarrollado varias alternativas para inducir celos y ovulaciones en ovejas durante el anestro estacional, y para potenciar la actividad reproductiva de los machos fuera de la estación reproductiva. Actualmente, se emplean tratamientos hormonales (implantes de melatonina, uso de progestágenos, etc.) y/o de fotoperiodos de luz, estos últimos en menor medida. Estos tratamientos inducen el celo de manera eficiente pero agregan costes a la actividad ganadera (Posbergh et al., 2019). Por otro lado, la creciente exigencia de los consumidores y de la Unión Europea (UE) (Martin y Kadokawa, 2006) de productos de origen animal libres de hormonas, ha generado la necesidad de buscar métodos alternativos, como la introducción de machos en grupos de ovejas en anestro, previamente aisladas de los mismos durante un periodo de tiempo para asegurar la inducción de la ovulación y el celo (efecto macho), el flushing nutricional o el uso de marcadores genéticos para seleccionar como reproductores aquellos animales con alelos asociados a un aumento de la fertilidad en primavera. En el caso de Rasa Aragonesa, Folch y Alabart, (1999) demostraron que aproximadamente el 25% de las ovejas ovulan espontáneamente en primavera y pueden ser cubiertas de forma natural durante todo el año si las condiciones de manejo y nutrición son las adecuadas. Además, se ha descrito que esta actividad ovulatoria de primavera está bajo control genético con valores de heredabilidad y repetibilidad de 0,20 y 0,30, respectivamente (Hanocq et al., 1999). Por otra parte, entre los factores que afectan a la duración del anestro se han descrito algunos como la edad, la condición corporal (CC) y el peso vivo (PV) (Forcada et al., 1992; Macé et al., 2019). Por tanto, la selección de genotipos menos sensibles a la estacionalidad reproductiva en los Programas de Mejora, o que favorezcan caracteres de crecimiento como la CC, podrían constituirse en una alternativa para incrementar la rentabilidad y eficiencia del sector ovino.

Numerosos estudios han demostrado la eficacia de los implantes de melatonina y efecto macho en la mejora de parámetros reproductivos en épocas de anestro estacional (Abecia et al., 2006; Palacios et al., 2006). En concreto, en Rasa Aragonesa se ha detectado que, tras la realización de un efecto macho en anestro, entre un 91% y un 74% de hembras presentaron marcas de celo con machos de alta y baja capacidad de cubrición, respectivamente, durante los 33 días posteriores a la introducción del macho

(Mozo, 2015). Igualmente, se ha demostrado que los machos con mayor capacidad de cubrición muestran un efecto macho más eficiente durante el anestro estacional, que se traduce en un mayor porcentaje de ovejas cubiertas y una fertilidad más elevada (Mozo et al., 2013). Recientemente, en Rasa Aragonesa se ha descrito que el contacto permanente con machos estimulados con fotoperiodo artificial y melatonina es capaz de provocar un aumento de la actividad sexual de las ovejas, en lo que a la manifestación de celos se refiere (Abecia et al., 2015a). Además, la presencia continua de machos sexualmente activos evita la aparición del anestro estacional (Delgadillo et al., 2015). Los resultados obtenidos en estos estudios indican que la actividad sexual del macho juega un papel decisivo en el desarrollo de la actividad sexual anual de las hembras, y por lo tanto es muy interesante buscar biomarcadores asociados con una mayor actividad sexual de los machos.

Los avances en la investigación genómica, como la disponibilidad del genoma ovino, que ha proporcionado cientos de miles de marcadores genéticos, y las técnicas de genotipado de alto rendimiento, han mejorado la capacidad de los investigadores para detectar regiones genómicas asociadas a los caracteres de interés así como las mutaciones que subyacen a la variabilidad de los mismos (Porto-Neto et al., 2015; Van Binsbergen et al., 2015). En este contexto, los estudios de GWAS se han convertido en un método importante para identificar genes y mutaciones causales asociadas con fenotipos de interés en las especies ganaderas. Estos estudios se basan en la utilización de plataformas (chips) de genotipado masivo de polimorfismos de un solo nucleótido (SNPs por sus siglas en inglés “Single Nucleotide Polymorphism”). Por otra parte, la secuenciación masiva de nueva generación del transcriptoma realizada mediante la técnica conocida como RNA-Seq, permite analizar la gran complejidad del fenómeno de la expresión génica (Fernandez-Rodriguez et al., 2011; Mardis, 2008; Mortazavi et al., 2008). Esta metodología, presenta mayor sensibilidad y menor variación técnica que los microarrays de expresión, permitiendo caracterizar prácticamente todos los transcritos expresados en un tejido o tipo celular y la cuantificación de los mismos, y la detección de SNPs y otras variantes estructurales (Qian et al., 2014).

El presente trabajo tiene como fin la detección de marcadores genéticos del genoma ovino asociados a la estacionalidad reproductiva, y a otras variables que influyen en la misma, como es la CC, mediante estudios de gen candidato y análisis de

GWAS. Por otra parte, se profundizará en la base genética del restablecimiento de la actividad ovárica, así como de la capacidad de cubrición de los machos en época de anestro reproductivo (AR), mediante el análisis del transcriptoma. La determinación de los mecanismos moleculares implicados en el restablecimiento de la actividad ovárica en anestro y/o de la capacidad de cubrición de los machos permitirá localizar genes y/o rutas bioquímicas implicadas en la expresión de estos fenotipos, que pueden constituirse en el punto de partida para la búsqueda de polimorfismos genéticos asociados a la variabilidad de estos caracteres, o ser utilizados como biomarcadores. En definitiva, el uso de marcadores genéticos para seleccionar como reproductores aquellos animales con alelos asociados a un aumento de la fertilidad fuera de la estación reproductiva, o a un incremento de la capacidad de cubrición de los machos mediante SAM, se presenta como una herramienta muy prometedora para incrementar la rentabilidad y la eficiencia reproductiva del ovino de carne.

II- Objetivos

Mediante la realización de la presente Tesis Doctoral se pretende profundizar en la base genética de los fenotipos de estacionalidad reproductiva en hembras y de capacidad de cubrición de los machos en la especie ovina.

Para abordar este objetivo general, se propusieron los siguientes objetivos parciales:

1- Búsqueda de marcadores genéticos asociados al fenotipo estacionalidad reproductiva, y a otros caracteres que influyen en la misma:

- Estudios de asociación del gen *receptor de la Leptina* (*LEPR*) y el carácter estacionalidad reproductiva en hembras de raza Rasa Aragonesa.
- Búsqueda de nuevos polimorfismos asociados al carácter estacionalidad reproductiva mediante análisis GWAS.
- Búsqueda de polimorfismos asociados a los caracteres de crecimiento (PV, CC y crecimiento medio diario), que afectan a la duración del anestro estacional, mediante análisis GWAS.

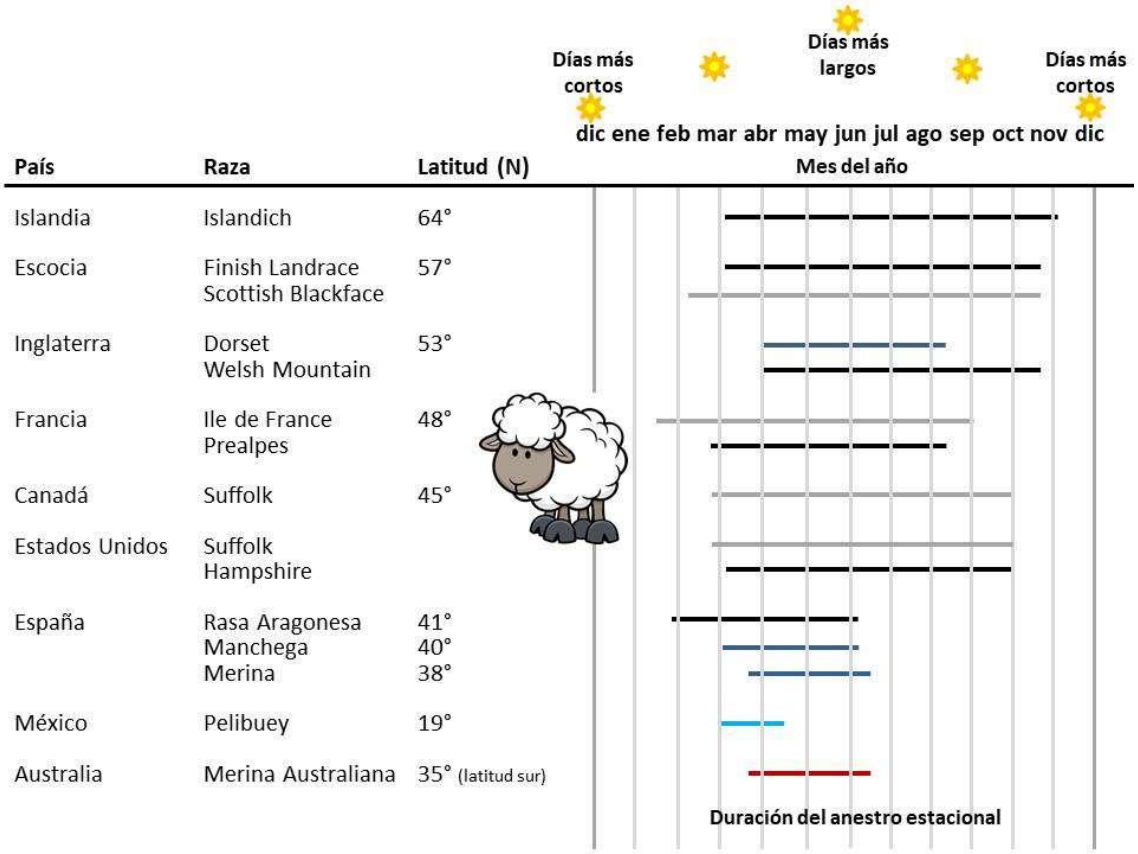
2- Estudio de los mecanismos moleculares que intervienen en el restablecimiento de la actividad ovárica en AR mediante el uso de secuenciación masiva del transcriptoma en muestras del *pars tuberalis* (PT) y del hipotálamo (HT), tejidos claves en el restablecimiento de la actividad ovárica.

3- Estudio de los mecanismos moleculares que modulan la capacidad de cubrición de los machos, mediante el uso de secuenciación masiva del transcriptoma en muestras del PT, HT, glándula pineal (GP) y sangre periférica.

III- Revisión bibliográfica

III-1 Estacionalidad reproductiva

Los cambios del fotoperiodo a lo largo del año (ritmos circanuales) y su variación en función de la latitud geográfica, son el factor más importante en la regulación de la estacionalidad reproductiva en la mayoría de las especies, incluyendo los pequeños rumiantes tanto domésticos como silvestres. El fotoperiodo es el factor con mayor influencia sobre las variaciones estacionales de la actividad ovulatoria en hembras y de la producción espermática en machos (Hafez, 1952; Lincoln y Short, 1980). En las razas originarias de climas templados, como son las de la cuenca Mediterránea, la disminución de las horas de luz induce la activación del eje neuro-endocrino-gonadal, determinando el establecimiento de los celos, ovulaciones y posibilidad de cubriciones fértiles en los días cortos del año (Ortavant et al., 1988, 1985; Sliwowska et al., 2004). De esta forma, el ciclo sexual suele comenzar en otoño, a medida que disminuye la duración del día (fotoperiodo decreciente), y la fase no reproductiva (anestro) se produce durante la primavera y el verano (Malpaux et al., 1989). La adaptación genética a las condiciones medioambientales donde se desarrolla una especie determinada, ha favorecido el desarrollo de estrategias reproductivas que garantizan que los partos coincidan con aquellas épocas del año óptimas por sus condiciones climáticas y disponibilidad de alimentos (Ortavant et al., 1985), lo que proporciona a las crías las condiciones más favorables para su crecimiento y supervivencia (Malpaux et al., 1996). En este sentido, y puesto que la duración de la gestación en ovino es de cinco meses aproximadamente, si se produce una gestación en otoño, los corderos nacerán en la primavera, que, en la zona climática de la cuenca Mediterránea con temperaturas más cálidas y alimentación abundante convierten a esta estación en una época favorable para el desarrollo de los corderos. Por otro lado, en las regiones tropicales y subtropicales, las ovejas son consideradas poliestrales continuas con una actividad reproductiva constante durante todo el año, si los factores ambientales, como la calidad y disponibilidad de alimento, lo permiten (Cambellas, 1993). No obstante, otros factores como la temperatura y la presencia del macho, influyen en la estacionalidad del ciclo estral de la oveja (Gündoğan et al., 2003; Malpaux et al., 1999). En general, como se puede ver en la Figura 2, cuanto mayor es la latitud, mayor es la foto-dependencia y más restringido es el período de actividad reproductiva (Ramírez Ramírez et al., 2021).



País	Raza	Latitud (N)
Islandia	Islandich	64°
Escocia	Finish Landrace Scottish Blackface	57°
Inglaterra	Dorset Welsh Mountain	53°
Francia	Ile de France Prealpes	48°
Canadá	Suffolk	45°
Estados Unidos	Suffolk Hampshire	
España	Rasa Aragonesa Manchega Merina	41° 40° 38°
México	Pelibuey	19°
Australia	Merina Australiana	35° (latitud sur)

Figura 2. Duración del anestro estacional en diferentes razas de ovino en función de la latitud (Tomado de Ramírez Ramírez et al., 2021)

III-1.1 Manifestación de la estacionalidad reproductiva en la oveja

La estacionalidad reproductiva en la oveja se caracteriza por cambios a nivel comportamental, endocrino y ovulatorio, dando lugar a una alternancia anual entre dos períodos distintos: época reproductiva (días cortos) caracterizada por una sucesión de ciclos sexuales de 16 a 18 días, que suele comenzar a finales de verano o principios del otoño, y termina a finales del invierno o principios de la primavera (días largos) (Forcada y Abecia, 2006). El ciclo consta de dos fases: la fase luteal (L) que comprende el metaestro y diestro (14 a 18 días), y la fase folicular (F) (4 a 6 días; proestro y estro). Tras la época reproductiva se produce la fase de anestro, periodo de inactividad sexual durante el cual cesan los ciclos estrales. En las ovejas existen tres tipos diferentes de anestro; el anestro estacional, comentado anteriormente, y modulado por el fotoperiodo principalmente; el anestro durante la lactancia, influenciado por el estímulo de succión de los corderos; y el anestro postparto (Arroyo, 2011; Rosa y Bryant, 2003). Ambos, tanto el de postparto como el de la lactación, suprimen la ciclicidad reproductiva en las

ovejas, es decir, conllevan una fase de anestro posterior. La fase anéstrica postparto, suele durar hasta el fin de la lactancia, es decir, se solapan, pudiendo ser su duración variable. Por otra parte, el anestro postparto puede coincidir con el anestro estacional cuando los partos se producen en primavera. La duración del periodo de anestro se ve influenciada, además de por el fotoperiodo, por la nutrición (Forcada y Abecia, 2006) y factores sociales (Lindsay, 1996), como el efecto macho (Abecia et al., 2015a).

III-1.2 Manifestación de la estacionalidad reproductiva en el macho

Los machos también muestran cambios estacionales en su comportamiento sexual, secreción de testosterona, peso testicular, y cantidad y calidad del esperma (Ortavant et al., 1985), aunque estos cambios son menos pronunciados que los experimentados por las ovejas. De hecho, mientras que la ovulación y el estro en la oveja se interrumpen al entrar en anestro, la espermatogénesis y la actividad sexual en los machos nunca se interrumpen. Generalmente, los valores de ambos son altos desde el final del verano hasta el otoño, disminuyendo mucho al final del invierno y permaneciendo bajos durante la primavera (Ortavant et al., 1985) (Figura 3). Esto se traduce en una menor fertilidad y libido durante el periodo de anestro.

Para mejorar la fertilidad y la libido en los machos, y por lo tanto la capacidad de cubrición, se han llevado a cabo diferentes aproximaciones como son el uso de implantes de melatonina con diferentes programas de fotoperíodo (Abecia et al., 2016, 2015a; Chemineau et al., 1996). Igualmente, los tratamientos lumínicos mediante la modificación del fotoperiodo sin el uso de hormonas, han demostrado ser una opción viable para mejorar los resultados de las cubriciones en primavera (Abecia et al., 2019). El llevar a cabo el efecto macho con animales activados con tratamientos lumínicos, y mantener estos machos activados sexualmente en el rebaño, puede suprimir el anestro en cabras y ovejas (Delgadillo et al., 2015). Estos estudios han demostrado que la intensidad del comportamiento sexual de los machos, puede influir en una mayor proporción de ovejas que ovulan en lotes expuestos a machos con un alto comportamiento sexual en relación con aquellos lotes de machos con un comportamiento sexual más pobre (Mozo et al., 2015, 2013; Perkins y Fitzgerald, 1994).

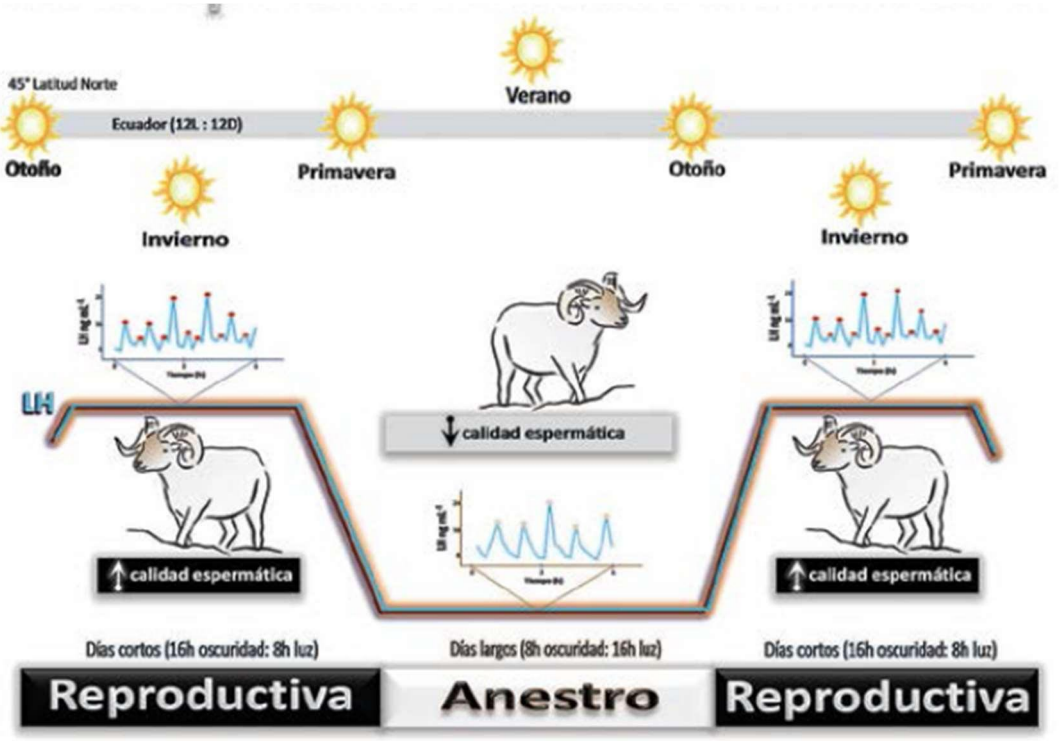


Figura 3. Ciclo reproductivo anual del macho (Tomado de Arellano et al., 2016)

III-2 Base neuroendocrina de la reproducción estacional

III-2.1 Hembra

La regulación de la estacionalidad reproductiva a través del fotoperiodo, está mediada por la melatonina. La melatonina, es producida en la GP con un patrón circadiano, que se caracteriza, en animales diurnos, por presentar niveles basales durante el día, y altos niveles de secreción durante la noche (Bittman et al., 1985; Karsch et al., 1984). Las variaciones en la secreción de esta hormona reflejan el fotoperiodo (Arendt, 1986; Tamarkin et al., 1985), haciendo de transductor de la información fotoperiódica a una respuesta hormonal, estimulando el eje hipotalámico-hipofisiario-gonadal (HPG) (Hoffmann, 1979). En los pequeños rumiantes, la información lumínica es percibida por la retina y trasmitida por vía nerviosa a la GP en varias etapas. En primer lugar, la información fotoperiódica es transmitida de la retina a los núcleos supraquiasmáticos del hipotálamo (NSQs) a través de la vía monosináptica retino-hipotalámica (Herbert et al., 1978). En el núcleo supraquiasmático reside el ritmo circadiano endógeno, que opera como reloj biológico interno. A partir de éstos, la señal es transportada al núcleo hipotalámico paraventricular, desde donde llega hasta los

ganglios cervicales superiores, y finalmente a la GP a través de las neuronas simpáticas postglanglionares. La GP a través de su secreción de melatonina, sirve como traductor: convierte la información neural en señal hormonal. La melatonina transmite la información del fotoperiodo en el área premamilar hipotalámica (Malpaux et al., 1998) regulando la secreción y liberación pulsátil de la hormona liberadora de gonadotropina (GnRH), que estimula la secreción de gonadotropinas hipofisarias, la hormona luteinizante (LH) y la hormona folículo estimulante (FSH) (Clarke et al., 2012). Su acción sobre la liberación de GnRH es realizada de forma indirecta, interviniendo diferentes neuromediadores, como son el sistema dopaminérgico y serotoninérgico (Zarazaga et al., 2009). En las hembras, estas hormonas actúan como reguladores ováricos, al mantener las funciones del cuerpo lúteo, estimulando la foliculogénesis y sintetizando progesterona (P4) y estrógenos (Plant, 2015). La LH produce un aumento en la secreción de estradiol en el ovario y a través de una cascada hormonal y enzimática se produce finalmente la ovulación (Foster y Jackson, 1994). En definitiva, la secreción de melatonina provoca una variación en la sensibilidad hipotalámica al efecto de retroalimentación negativa del estradiol. Como señal inductora, la melatonina estimula la secreción pulsátil de la GnRH, disminuyendo el poder inhibidor del estradiol (Malpaux et al., 2001).

En el AR, la reducción en la secreción de melatonina incrementa la retroalimentación negativa del estradiol y la secreción de la isoforma A del factor de crecimiento vascular endotelial (VEGF-A) sobre los gonadótropos. El VEGF-A está relacionado con la angiogénesis, y además estimula a los lactotropos para la síntesis y secreción de la prolactina (PRL). Esta última, junto con la dopamina, reducen el efecto de la GnRH sobre la secreción de FSH y LH, por parte de la adenohipófisis (Viguie et al., 1995). La retroalimentación negativa del estradiol sobre el HT actúa suprimiendo la liberación de ácido gamma-aminobutírico (GABA), que activa las neuronas dopaminérgicas e incrementa la síntesis y secreción de dopamina, la cual conduce a la disminución en la frecuencia de pulsos de secreción de la GnRH y por consiguiente de LH por parte de la hipófisis (Arroyo, 2011; Goodman et al., 2000; Karsch et al., 1980; Martin y Thiéry, 1987), reduciéndose el crecimiento folicular y la producción de estradiol, lo cual convierte a los folículos en atrésicos. Tampoco se presenta el pico preovulatorio de LH y por consiguiente la oveja no ovula.

La melatonina actúa a nivel hipotalámico estimulando la producción de GnRH, pero es en el PT de la hipófisis donde existe una mayor densidad de sus receptores, siendo en este sentido un tejido clave junto con el HT, para el estudio de la estacionalidad reproductiva (Bittman, 1993; de Reviers et al., 1989).

La hipófisis se divide en una porción nerviosa o neurohipófisis y en una porción glandular o adenohipófisis. La adenohipófisis produce la hormona del crecimiento (GH), la PRL, la hormona adenocorticotrópica (ACTH), la hormona estimulante de la tiroides (TSH), la FSH, y la LH entre otras; mientras que la neurohipófisis almacena arginina vasopresina (AVP o también conocida como vasopresina) y oxitocina, que son producidas en el HT. Sin embargo, diferentes estudios han demostrado que el PT no interviene en la acción de la melatonina en el eje reproductor neuroendocrino, y que su efecto estaría mediado por las hormonas tiroideas: triyodotironina y tiroxina (T3 y T4, respectivamente), ya que ovejas tiroidectomizadas permanecen en actividad ovulatoria (Dahl et al., 1994), y la aplicación de T4 restauró el estado de transición al anestro (Dahl et al., 1995). El modo de actuación propuesto de la melatonina en el PT estaría mediado por el factor de transcripción *Eya3*, cuya expresión es estimulada por la melatonina (Dardente et al., 2010; Dupré et al., 2010). El factor *Eya3* es un gen regulador circadiano, estimulándose la secreción de este fator con la menor concentración de melatonina, que se asocia con los días con mayor cantidad de horas luz, lo cual estimula la expresión de la *subunidad β de tirotropina*, *TSHβ* (Dardente et al., 2010), regulando de esta manera la actividad reproductiva en ovejas (Gorman, 2020; Qing et al., 2018).

La TSH se difunde cerca del tejido neural que contiene receptores para TSH para estimular la expresión de la *diodinasa tipo 2* (*Dio2*), que convierte la T4 en T3 (Hanon et al., 2008). Posteriormente, la T3 actúa induciendo los cambios neurales que impiden la secreción episódica de GnRH. La T3, además, participa en la plasticidad neuronal, incluso con efectos de neurogénesis (Thompson y Potter, 2000), pudiendo ser un posible mecanismo de retroalimentación negativa del estradiol sobre el HT, que se presenta durante la fase de anestro (Lehman et al., 2010a, 2010b).

De todo lo anterior, el anestro se establece por medio del efecto de la melatonina sobre el HT y el PT de la hipófisis. En el HT, se reduce la secreción pulsátil de GnRH y

en el PT se bloquea el efecto de GnRH sobre la secreción de las gonadotropinas (Hodson et al., 2012).

III-2.2 Macho

En el macho, al igual que en la hembra, durante los días crecientes, las concentraciones plasmáticas de FSH, LH y testosterona son más bajas que durante los días decrecientes, lo que conduce a una regresión del tamaño testicular, asociada a una disminución del diámetro de los túbulos seminíferos y, en definitiva, a marcados cambios en la producción espermática (Figura 4): volumen de eyaculado, concentración espermática, número total de espermatozoides, viabilidad de los espermatozoides, etc. (Delgadillo et al., 1991; Zarazaga et al., 2009). Esto se manifiesta en una menor fertilidad y menor actividad sexual durante el periodo de anestro.

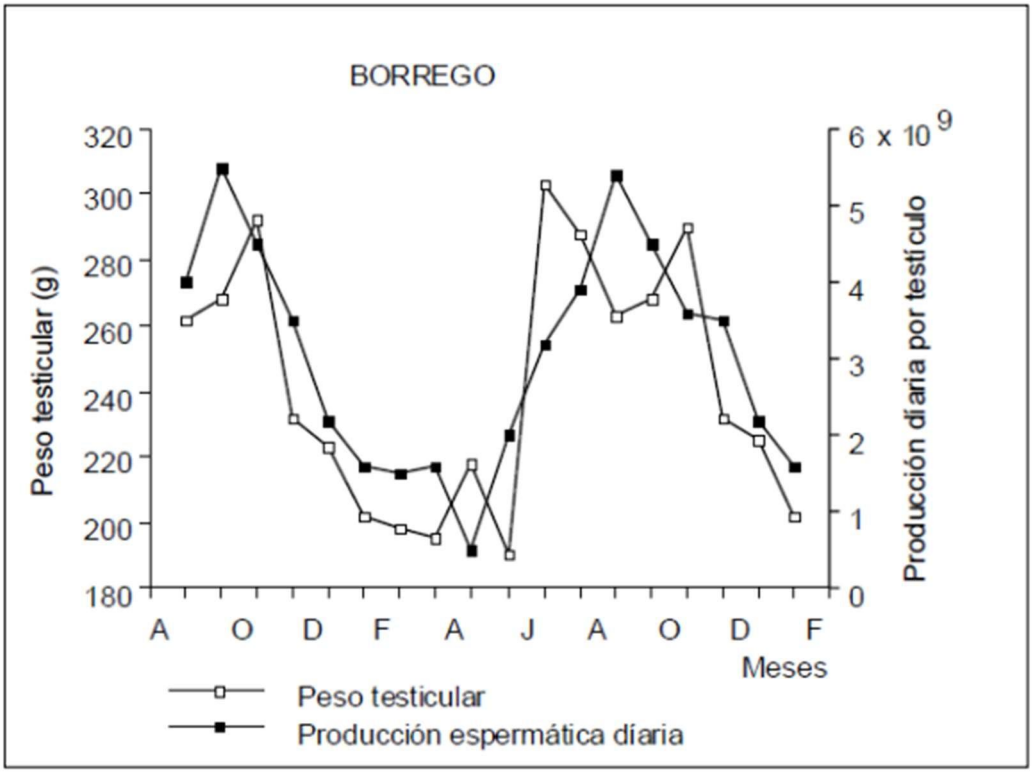


Figura 4. Variaciones estacionales de la producción espermática y del peso testicular en moruecos Île de France (Tomado de Chemineau et al., 2003)

La GnRH, estimula la producción de FSH y LH a nivel hipofisiario, y testicular, actuando la FSH sobre las células de Sertoli, mientras que la LH sobre las células de Leydig. Las células intersticiales de Leydig son las responsables de secretar testosterona,

que inhibe la secreción de LH, al actuar directamente sobre la secreción de GnRH en el HT. Las células de Sertoli, importantes por su papel trófico y metabólico sobre las células germinales, secretan activina e inhibina, que activan e inhiben la biosíntesis y secreción de FSH, respectivamente. Estas células también secretan muchas proteínas específicas entre las cuales se encuentra la proteína que liga andrógenos (ABP), importante porque concentra 100 veces la testosterona en el parénquima testicular (Bustos Obregón y Torres -Díaz, 2012). Estos andrógenos actúan en el área preóptica del HT que regula la actividad sexual de los machos, promovida por los niveles de estradiol en el cerebro que se producen después de la aromatización de la testosterona a nivel cerebral por el citocromo P-450, y no por los niveles de testosterona en sí mismos (Perkins y Roselli, 2007).

La especie ovina muestra variaciones significativas en la concentración de testosterona a lo largo del año, que puede alcanzar niveles mínimos en la época no reproductiva (días crecientes), que implican una falta de libido. La mayor capacidad de cubrición o actividad sexual se ha asociado a un incremento de la testosterona plasmática, considerando la testosterona un andrógeno predominante para expresar y mantener la libido en los machos (D'Occhio et al., 1985). Sin embargo, algunos estudios han demostrado que no existe una correlación significativa entre los niveles de testosterona y el comportamiento sexual en los machos (Howles et al., 1980; Schanbacher y Lunstra, 1976), no pudiendo considerarse a la testosterona como único indicador de la capacidad de cubrición de los mismos, y siendo interesante el estudio de otros biomarcadores asociados a la capacidad de cubrición.

Junto a la información hormonal, el área preóptica recibe información sensorial del órgano vomeronasal y genitales a través de la amígdala, induciendo reflejos genitales y patrones copulatorios (Carlson, 2007). Diversos estudios han mostrado diferencias estructurales y funcionales en distintas regiones del cerebro entre machos de mayor y de menor actividad sexual (machos con baja actividad sexual *per se* y machos que tienen preferencia sexual por otros animales de su mismo sexo). En este sentido, las diferencias en receptores de estrógenos en diferentes regiones del cerebro presentaron diferencias significativas entre moruecos de alta y baja actividad sexual cuando eran expuestos a ovejas en celo (Alexander et al., 1993). Del mismo modo, Alexander et al. (1999) observaron diferencias en el tamaño de las neuronas en las áreas involucradas en el

comportamiento sexual (área preóptica o el núcleo ventromedial del HT) de machos con mayor y menor capacidad de cubrición. Igualmente, Roselli et al. (2004) demostraron una asociación entre las preferencias de pareja sexual y la estructura cerebral, más concretamente en la región hipotalámica que incluye una área preóptica medial. Finalmente, Borja y Fabre-Nys (2012) observaron diferencias en la activación de las neuronas del área preóptica medial, la porción del lecho de la estría terminal, y en el núcleo paraventricular en moruecos de alta y baja actividad sexual expuestos a ovejas en celo. Estos estudios, muestran con especial énfasis la influencia de la actividad sexual en la inducción de la plasticidad cerebral.

III-3 Factores que afectan a la duración e intensidad de la estacionalidad reproductiva

Existen distintos factores tanto intrínsecos como extrínsecos que influyen de forma importante en la duración del anestro estacional de los individuos. Entre ellos encontramos factores genéticos, como la raza (ver Figura 2), la edad; factores medioambientales (humedad, temperatura, latitud y fotoperiodo, lluvias entre otros), la alimentación, la época de nacimiento; así como factores sociales.

III-3.1 Factores ambientales

Algunos de los factores medioambientales (fotoperiodo) o sociales (presencia de machos en los rebaños) han sido ya comentados en los apartados anteriores, y se revisarán con mayor profundidad en el apartado III.4 de esta memoria. De entre los enumerados anteriormente, podemos destacar la latitud y la alimentación.

Un factor medioambiental importante es la latitud, que modifica la regulación de la actividad reproductiva por el fotoperiodo. Es bien conocido que las razas que se explotan en latitudes elevadas tienen un anestro más largo y profundo. En las regiones geográficas en las que existen grandes diferencias de la duración del día entre el verano y el invierno, el fotoperiodo tiene una fuerte importancia en la regulación de la estacionalidad de los animales, mientras que los factores de manejo (nutrición) o las relaciones sociales (presencia de machos) apenas modifican la misma. Al contrario, sucede en las razas ubicadas en latitudes medias o bajas (Forcada et al., 2009). Sin embargo, se han observado interacciones entre la raza (genética) y la latitud (ambiente), de forma que razas con gran estacionalidad reproductiva que se mueven a latitudes

medias, siguen manteniendo una elevada estacionalidad reproductiva. Sin embargo, se ha observado una mayor variabilidad de resultados en relación a la duración del anestro, cuando se traslada una raza poco estacional, como las subtropicales, a latitudes superiores (Arroyo et al., 2007).

La alimentación es otro factor que influye sobre la estacionalidad reproductiva. En este sentido, se ha demostrado que una buena nutrición y un adecuado nivel de reservas pueden reducir la duración del anestro. El anestro estacional es más intenso en las ovejas que llegan a la primavera con una baja CC o que no se han recuperado plenamente de la lactación precedente (Gunn y Doney, 1975; Newton et al., 1980). En este sentido, estudios realizados en Rasa Aragonesa por Forcada et al. (1992) mostraron que las ovejas con una CC inferior o igual a 2,5 presentaron 113 días de anestro y manifestaron 1,7 celos en primavera, frente a los 64 días de anestro y 3,7 celos de ovejas con una CC igual o superior 2,75. Estos resultados se confirmaron al siguiente año (76 vs 92 días totales de anestro para ovejas con CC $\geq$ 2,75 vs $\leq$ 2,5, respectivamente). Rondon et al. (1996) encontraron que este efecto era debido a una entrada más tardía en AR, y a la menor duración del mismo. Las condiciones más desfavorables para la cubrición en primavera, se dan cuando coinciden el anestro de lactación y el anestro estacional. Si las necesidades nutritivas no se cubren correctamente, la oveja sufre una pérdida de peso más o menos importante que repercute en los resultados reproductivos de la cubrición siguiente (Forcada et al., 1992). Por otro lado, la presencia del cordero y el acto de tetar, alteran el funcionamiento del ovario dificultando la salida en celo y la fecundación en un porcentaje elevado de ovejas lactantes. Así, las ovejas que paren en primavera con una CC baja, aunque se les aporte un complemento alimenticio después del destete y antes de la introducción de los machos, generalmente no consiguen compensar las pérdidas de CC producidas en la lactación, lo que influye negativamente en el porcentaje de ovejas que salen cíclicas en primavera. Este problema es muy común en las zonas semiáridas en las que la disponibilidad de pastos en invierno es muy escasa.

III-3.2 Factores genéticos implicados en la estacionalidad reproductiva y en la capacidad de cubrición de los machos.

La mayoría de los caracteres de interés en Producción Animal presentan una base genética compleja, al estar determinados por un número elevado de genes con un efecto

individual pequeño y aditivo (poligenes o QTL), así como por el ambiente, e interacciones múltiples entre genes y ambiente.

III-3.2.1 Base genética de la estacionalidad reproductiva

La estacionalidad reproductiva en las ovejas es un carácter complejo con una gran influencia ambiental. Para el fenotipado de este carácter se utilizan medidas semanales de P4, la detección de los celos con machos vasectomizados (Martinez-Royo et al., 2017), y también medidas de la fertilidad de grupos de ovejas que han sido cubiertas en otoño o en primavera dentro de una misma población (Chu et al., 2003; Mura et al., 2010). La dificultad y el coste económico del fenotipado de este tipo de datos, implica que la selección genética de estos caracteres utilizando métodos tradicionales, sea poco eficiente (Notter y Cockett, 2005). Por otra parte, sólo se expresan en hembras (ovulaciones en primavera), tardíamente en la vida del animal y en sistemas de producción con parideras en otoño. Por todo ello, las estimas de heredabilidad de estos caracteres son bajas, alrededor de 0,10 (Al-Shorepy y Notter, 1996). Sin embargo, Hanocq et al. (1999) obtuvieron estimas de heredabilidad y repetibilidad más elevadas (0.20 y 0.30, respectivamente) para ovulaciones espontáneas en primavera en la raza Merinos d'Arles. Esta medición se basó en los niveles de P4 en sangre en dos muestras tomadas con 10 días de diferencia a principios de la primavera, antes de cualquier contacto con los machos. Igualmente, se encontró una heredabilidad similar para este carácter en la raza Chios en Grecia (Avdi et al., 2003) y en la raza Latxa en España (Beltrán De Heredia et al., 2002). Las estimas de los parámetros genéticos para ovulaciones espontáneas en época de anestro indican que este carácter puede ser sujeto a selección genética clásica (Hazard, 2010). La selección clásica de la estacionalidad reproductiva ha conseguido una reducción importante de la estacionalidad en razas ovinas americanas, pero siempre con un cuidadoso programa de cruzamientos y dejando de reposición las hijas de ovejas que quedaron gestantes en primavera (Notter, 2002). En general, la dificultad de la obtención del fenotipo, así como la baja heredabilidad, resulta en una selección ineficiente de estos caracteres en programas de selección tradicionales, con bajas fiabilidades y respuesta a la selección.

Es destacable, que las ovejas que exhiben actividad ovulatoria espontánea fuera del periodo reproductivo son de gran interés para identificar genes y mutaciones

involucradas en vías moleculares que controlan la estacionalidad reproductiva en ovejas. En Rasa Aragonesa, aproximadamente el 25% de las ovejas tienen ovulaciones espontáneas en primavera y pueden ser cubiertas durante todo el año, si las condiciones de manejo y alimentación son las adecuadas (Folch y Alabart, 1999).

Entre los estudios genéticos/genómicos relacionados con la estacionalidad reproductiva, destacan los trabajos llevados a cabo con **el gen del *receptor 1A de la melatonina* (*MNTR1A)*** que se ha encontrado asociado a la ovulación espontánea en AR en muchas razas distribuidas por todo el mundo (Abecia et al., 2020; Arjoune et al., 2023; Calvo et al., 2018; Carcangiu et al., 2011, 2009; Chu et al., 2003; Cosso et al., 2021; Faigl et al., 2009; He et al., 2019; Luridiana et al., 2020; Martínez-Royo et al., 2012; Mateescu et al., 2009; Mura et al., 2022, 2019, 2014, 2010; Notter et al., 2003; Pelletier et al., 2000; Pulinas et al., 2022; Starič et al., 2020; Teyssier et al., 2011; Yang et al., 2022). En general, la mayoría de estos estudios se basan en las posiciones 606 (*RsaI*) y 612 (*MnlI*) del exón 2 del gen, en los cuales estas dos mutaciones silentes y en desequilibrio de ligamento (DL), se han asociado con la reproducción en anestro estacional en diferentes razas ovinas (Carcangiu et al., 2009; Chu et al., 2003; Mateescu et al., 2009; Pelletier et al., 2000). En Rasa Aragonesa, Martínez-Royo et al. (2012) caracterizaron el gen *MTNR1A* incluyendo la región promotora, encontrando 11 SNPs en la región codificante y 17 SNPs en la región promotora. Por otra parte, Calvo et al. (2018) asociaron otra nueva mutación, el SNP rs403212791 del exón 2 del gen *MTNR1A*, que provoca un cambio de Arginina a Cisteína (p.R336C), con la estacionalidad reproductiva. Recientemente, Abecia et al. (2020) relacionaron los genotipos TT y GG de los polimorfismos *RsaI* y *MnlI*, respectivamente, con la edad a la primera cubrición de machos jóvenes de Rasa Aragonesa nacidos en septiembre, así como con la acctividad sexual de machos adultos.

La arilalquilamina-N-acetiltransferasa (AANAT) es una enzima crítica en la biosíntesis de melatonina en la GP (Klein y Berg, 1970). El aumento en la expresión y actividad de AANAT durante la noche conduce a una elevación significativa en los niveles de melatonina, mientras que la baja actividad durante el día limita severamente la cantidad de sustratos disponibles para la conversión a melatonina (Ganguly et al., 2002; Klein et al., 1996). De hecho, el gen *AANAT* se ha asociado con patrones reproductivos estacionales en la cabra Jining Grey de China (Chu et al., 2013), y en razas

de cabras indias (Sharma et al., 2015). En ovino, Ding-ping et al. (2012) encontraron una mutación en el exón 3 del gen *AANAT* responsable del cambio aminoacídico Arg por Gly. Las frecuencias genotipadas de este SNP fueron asociadas a la variabilidad en la estacionalidad reproductiva de distintas razas. Recientemente, Di et al. (2021) investigaron los perfiles de expresión y las funciones reguladoras de miARN pineales ovinos durante diferentes fases reproductivas. En este estudio, demostraron que la expresión del ARNm de *AANAT* variaba y podría ser regulada por miARN durante diferentes etapas de la reproducción. Del mismo modo, los niveles de expresión de *AANAT* en ovocitos fueron mayores en la fase F que en la fase L en ovarios durante el ciclo estral de rata (Sakaguchi et al., 2013).

Otro gen candidato ampliamente estudiado ha sido el de las **kisspeptinas (*Kiss1*) y su receptor (*Kiss1R*)**. Las kisspeptinas son unas proteínas implicadas en la reproducción, y en concreto en mecanismos de regulación de la estacionalidad reproductiva y de la pubertad (De Bond et al., 2013; Meza-Herrera et al., 2010). De hecho, la *kisspeptina* se expresa en el HT, principalmente durante el período de transición entre el anestro y la actividad sexual, y al comienzo de la pubertad (Smith y Clarke, 2010). Además, los polimorfismos encontrados en este gen se han asociado al tamaño de camada de ovejas y cabras (Cao et al., 2010; Chu et al., 2012). La vía de señalización de kisspeptina es esencial para la secreción de GnRH (Figura 5), estimulando la secreción de LH de manera dependiente de GnRH (Gottsch et al., 2004), y aumentando su secreción en la sangre portal hipofisaria (Smith et al., 2011). En las ovejas, las neuronas de kisspeptina (las que expresan el ARNm de *Kiss1*) se encuentran en el área preóptica dorsolateral (POA del inglés preoptic area) y en el núcleo arcuato (ARC) también conocido como A12. En el ARC, las neuronas de kisspeptina expresan receptores de estrógeno y progesterona (Franceschini et al., 2006; Smith et al., 2007), y están directamente reguladas por estos esteroides de manera consistente con la regulación por retroalimentación tanto positiva como negativa de la secreción pulsátil de GnRH (Clarke et al., 2009; Smith et al., 2009, 2008). Alternativamente, las neuronas de kisspeptina en el POA dorsolateral parecen estar involucradas en la señal de retroalimentación positiva para inducir el aumento preovulatorio de LH (Smith et al., 2009). Por otra parte, la expresión de *Kiss1* y la producción de péptidos está marcadamente sobreexpresado en el ARC al inicio de la temporada de reproducción

(Smith et al., 2008, 2007; Wagner et al., 2008). En otro estudio, se planteó la hipótesis de que las feromonas activan las neuronas de kisspeptina, cuyo producto es fundamental para la estimulación de las neuronas GnRH y la fertilidad, y se demostró que la introducción de macho a ovejas en anestro estacional activa las neuronas kisspeptina y otras células en el HT, lo que lleva a una mayor secreción de GnRH/LH (De Bond et al., 2013).

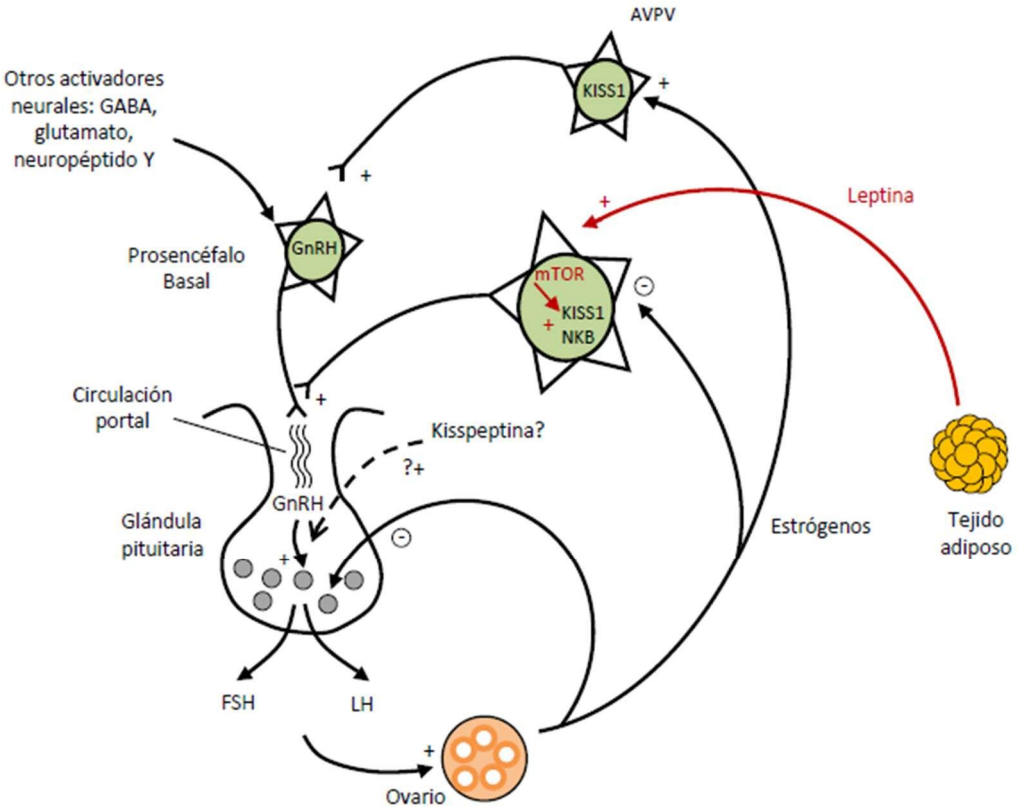


Figura 5. Diagrama de la señalización de kisspeptinas dentro del sistema nervioso central de la oveja (Tomado de Hameed et al., 2011)

El gen ***LEPR*** codifica para el receptor de la hormona leptina. La hormona leptina, secretada por el tejido adiposo y el receptor de leptina han sido ampliamente descritos por su importante papel en la regulación energética. Los polimorfismos del gen de la *leptina* han llamado mucho la atención de los investigadores por su posible relación con los caracteres productivos y reproductivos (van der Lende et al., 2005). La leptina es principalmente conocida por su papel en la regulación del equilibrio energético de todo el organismo al actuar sobre el sistema nervioso central e influir en la deposición

de grasa mediante el control del apetito y del gasto energético (Paczoska-Eliasiewicz et al., 2006). Por otra parte, se ha demostrado que algunos SNP en el gen *LEPR* están asociados con caracteres reproductivos (Israel y Chua, 2010; Liefers et al., 2004). Los efectos fisiológicos de *LEPR* en la reproducción, incluida la pubertad, el ciclo estral, la preñez, la lactancia e incluso las primeras etapas del desarrollo embrionario, han sido ampliamente demostrados (Moschos et al., 2002; Taheri y Parham, 2016). Numerosos estudios han confirmado que la leptina controla la madurez sexual a nivel del HT (Agarwal et al., 2009; Ehrhardt et al., 2002; Saleem, 2015). Además, la aparición y participación de la leptina en el HT con la liberación de la hormona gonadotrópica confirma su papel en la madurez sexual o la reproducción (Zieba et al., 2005). Por lo tanto, la leptina presenta un vínculo importante entre el estado metabólico y el eje neuroendocrino (Barb y Kraeling, 2004). También, se ha detectado la expresión de *LEPR* en el NSQ, el "reloj biológico" de los mamíferos, y en la GP de los rumiantes (Chelikani et al., 2003), lo que sugiere una interacción entre el fotoperíodo, la melatonina y la leptina (Malpaux et al., 1989). Los receptores de melatonina y leptina se han detectado en regiones hipotalámicas similares de ovejas (Chelikani et al., 2003; Dyer et al., 1997; Miller et al., 2002). Los polimorfismos en *LEPR*, y en particular, tres mutaciones (rs411478947 (R62H), rs428867159 y rs405459906), han sido asociadas con un retraso de la pubertad y con una disminución de las tasas de ovulación y fertilidad al parto en ovejas prolíficas Davisdale (Juengel et al., 2016a).

Por otra parte, el gen ***NPY*** juega un papel en la regulación de la secreción de la GnRH en ratas (Kalra, 1993), conejos (Khorram et al., 1987), monos (Francis Pau et al., 1995) y ovejas (Barker-Gibb et al., 1995; Porter et al., 1993). En ratas, se ha demostrado que las neuronas que expresan *NPY*, contienen receptores de estrógeno en el HT (Sar et al., 1990). De la misma manera, Skinner y Herbison (1997) indicaron que los cambios en el fotoperíodo pueden regular la expresión del receptor de estrógeno dentro del área preóptica. Estos autores sugirieron que las neuronas hipotalámicas NPY, y los niveles β-endorfinas están involucradas en la regulación estacional de la actividad reproductiva de las ovejas. Clarke et al. (2005) determinaron que el factor NPY estimula el consumo de alimento e inhibe la reproducción en ovejas. Sin embargo, Miner (1992) demostró que el factor NPY es un potente agente orexigénico en ovejas, como en otras especies,

y un cambio estacional en la expresión de este péptido puede estar relacionado con la estimulación del consumo de alimento, más que con la estacionalidad.

Otros genes interesantes son les **genes reloj o genes "Clock"**, que se expresan de forma oscilatoria a lo largo del día, principalmente en el NSQ y la PT en la hipófisis, siendo esta expresión funcionalmente importante para el mantenimiento de los ritmos circadianos (Hernández-Rosas y Santiago-García, 2010). Asimismo, se han descrito otros genes relacionados con los ritmos circadianos, como los genes *periodo* (*per1, per2 y per3*), *criptocromo* (*cry1 y cry2*), *clock*, *bmal1*, *CKIε* y *Rev-Erbα* (Reppert y Weaver, 2002). La expresión de estos genes es fuertemente rítmica y muestra respuestas dependientes de los estímulos lumínicos, lo que sugiere que el fotoperíodo puede modular la expresión de los genes *Per*, y el nivel de expresión de sus productos en el NSQ (Albrecht et al., 2001; Reppert y Weaver, 2001). Para el estudio de la expresión de los genes *clock* en mamíferos diurnos, los ovinos han sido el modelo más utilizado para las comparaciones con animales nocturnos (Correa y Riveros, 2017). En este sentido, Lincoln et al. (2002) llevaron a cabo un exhaustivo análisis de hibridación *in situ* de la expresión de genes reloj en el cerebro de las ovejas Soay, descubriendo que siete genes reloj (*Bmal1, Clock, Per1, Per2, Cry1, Cry2, y CKIε*) se expresaban en el NSQ y PT ovinos.

En relación a los estudios del transcriptoma, no existen muchos trabajos en ovino relacionados con el AR. Lomet et al. (2018) encontraron casi 3000 genes diferencialmente expresados (GDEs) en el HT entre la época reproductiva y no reproductiva. El análisis de enriquecimiento funcional mostró que estos genes estaban relacionados con rutas de señalización, epigenética y plasticidad neuronal. Sin embargo, el paso de días cortos a días largos, produjo 134 GDEs relacionados con la sincronización del fotoperiodo. Además, demostraron la importancia de las hormonas tiroideas en la regulación de la actividad reproductiva. Por otra parte, Xia et al. (2021) identificaron genes y lncRNA inducidos por el fotoperíodo en el PT de ovejas mediante comparaciones transcriptómicas entre animales sometidos a fotoperíodos cortos y largos. Entre los GDEs encontrados destacan los genes *EYA3*, *TSHB*, *SIX1*, *DCT*, *VMO1*, *AREG*, *SUV39H2*, y *EZH2.* Los análisis de enriquecimiento funcional mostraron el papel de estos genes en rutas relacionadas con el éxito reproductivo y los ritmos circadianos. Además, propusieron lncRNAs como reguladores epigenéticos de genes

claves como *TSHB* y *SIX1*. En este mismo sentido, Wang et al. (2022) identificaron un total de 41.088 lncRNAs inducidos por el fotoperiodo en la glándula tiroides en ovejas Sunite, mediante la comparación de los perfiles de expresión de ovejas sometidas a fotoperiodos cortos y largos. El análisis funcional de estos lncRNAs diferencialmente expresados reveló un enriquecimiento significativo de vías relacionadas con la respuesta a las hormonas reproductivas y al fotoperiodo, y también de vías de señalización de la PRL, de señalización del cAMP y del ritmo circadiano. Un análisis de interacción mRNA-lncRNA sugirió que el lncRNA *LOC1056153S88* trans regula los genes *ARG2* y *CCNB3*, y el lncRNA *LOC105607004* trans el *DMXL2*, y que ambos podrían estar implicados en la estacionalidad reproductiva en ovino. En otro estudio llevado a cabo por Du et al. (2022), se analizaron los mRNA y miRNA de las glándulas suprarrenales de la oveja Sunite bajo diferentes tratamientos de fotoperiodo artificial, e identificaron que los genes candidatos clave *GRHL2, CENPF, FGF16* y *SLC25A30* afectan a la reproducción. Estos autores propusieron diversos miARN (oar-miR-544-3p, oar-miR-411b-5p, oar-miR-376e-3p, oar-miR-376d, oar-miR-376b-3p, oar-miR-376a-3p) que se expresan específicamente en la glándula suprarrenal, y que podían afectar a caracteres reproductivos. En concreto, se identificó que el mRNA- *SLC25A30* estaba regulado por los miR554, miR555 y miR559, pudiendo afectar al celo estacional.

Finalmente, Zhong et al. (2021) en un estudio del transcriptoma del HT, pituitaria y ovario en ovejas en anestro y estación reproductiva, encontraron, en el análisis de enriquecimiento funcional, una sobrerrepresentación de rutas relacionadas con el fotoperiodo y la biosíntesis de leucina e isoleucina. Por otra parte, describieron los genes *ODC1*, *PRLH*, *CRYBB2*, *SMAD5*, *OPN1SW*, y *TPH1*, como claves en la regulación por estrógenos. En este sentido, los estrógenos son muy importantes en la plasticidad neuronal ya que contribuyen a la remodelación del citoesqueleto, induciendo modificaciones estructurales en las neuronas como la longitud de las dendritas o el número de sinapsis (Ferri et al., 2014; Hansberg-Pastor et al., 2015). Del mismo modo, Wei et al. (2022) analizaron la expresión mediante RNA-Seq de tejidos pineales, hipotalámicos, hipofisarios y ováricos en ovejas de raza Tan durante el estro y el anestro. Se identificaron 940, 1.638, 750 y 971 GDEs en GP, HT, hipófisis y ovario, respectivamente. El análisis de ontología génica (en inglés Gene Ontology cuya sigla es GO) de los GDEs mostró un enriquecimiento de procesos biológicos tales como el

transporte transmembrana, la biosíntesis de péptidos y amidas y la síntesis de ADN. El análisis de enriquecimiento funcional mostró un enriquecimiento significativo de las vías de secreción de ácidos biliares y de interacción ligando-receptor neuroactivo. Además, se identificaron cuatro posibles genes candidatos relacionados con la estacionalidad reproductiva (*VEGFA, CDC20, ASPM* y *PLCG2*) mediante perfiles de expresión génica y análisis de interacción proteína-proteína.

III-3.2.2 Factores genéticos implicados en la actividad sexual del macho

La capacidad de cubrición o el nivel de actividad sexual de los moruecos es un carácter de difícil medida (Blockey y Wilkins, 1984); además, del tiempo necesario para su valoración en condiciones de monta libre. Por este motivo, determinados autores han propuesto la utilización de métodos indirectos para su estimación, como es la utilización de biomarcadores. Uno de estos biomarcadores es el nivel de testosterona, no obstante, como se comentó en un apartado anterior, no se ha observado ninguna correlación entre los niveles de testosterona circulante u otros andrógenos y la actividad sexual de los moruecos, ni se han observado diferencias entre moruecos de alta o baja libido (Mozo, 2015). Perkins et al. (1992a) utilizaron la concentración plasmática de testosterona y LH en respuesta a la exposición a una oveja en celo durante varias horas, o a la administración de inhibidores opioides, como la naloxona (Fitzgerald y Perkins, 1994; Perkins et al., 2001). Aunque la administración de naloxona sólo es aplicable a animales adultos y durante la época reproductiva (Stellflug, 2003, 2002a; Stellflug et al., 2004). Roselli et al. (2002) utilizaron las diferencias en las concentraciones de testosterona y cortisol entre moruecos de alta actividad sexual y moruecos con actividad sexual nula o con preferencia por otros machos. Estos métodos resultan complejos por la aplicación de diferentes sustancias, y además pueden presentar problemas con relación al bienestar animal.

El método más habitual es el estudio del comportamiento de los moruecos en el aprisco, destacando los test de capacidad de cubrición (SCT por sus siglas en inglés; Serving Capacity Test). Los resultados de estos test se han correlacionado con el número de cubriciones que los machos realizan en condiciones de monta libre (Kilgour y Whale, 1980; Mattner et al., 1971; Perkins et al., 1992b; Stellflug, 2002) y también con la fertilidad del rebaño (Barwick et al., 1989; Kilgour, 1993). Los SCT consisten en la

observación del comportamiento de un morueco durante un determinado periodo de tiempo, cuando es expuesto a un grupo de ovejas en celo (Mattner et al., 1971). El número de cubriciones por unidad de tiempo que cada macho realiza durante estos SCT ha sido considerado como la medida predictiva más importante de su comportamiento sexual en monta libre. Cada macho es clasificado en función de su rendimiento sexual (Kilgour, 1984; Stellflug, 2002b). Uno de los problemas que se puede producir en la realización de los SCT es la falta de experiencia de los machos, ya que estos presentan pocas o ninguna cubrición cuando no han tenido contacto con hembras previamente. Igualmente, puede ocurrir una falta de adaptación a las condiciones del test (Kilgour y Whale, 1980), por lo que se aconseja que se hagan repeticiones. Además, en machos sin experiencia previa, la utilización de tiempos de evaluación muy reducidos puede producir resultados poco fiables (Folch, 1980; Kilgour, 1985).

La capacidad de cubrición es un fenotipo que presenta una heredabilidad moderada en ovinos ($h^2 = 0,33 \pm 0,62$, Kilgour, (1985); $h^2 = 0,22 \pm 0,04$, Snowder et al., (2002)), lo que sugiere la posibilidad de mejorar genéticamente este carácter mediante selección clásica, es decir a partir de las observaciones fenotípicas y las relaciones de parentesco entre los individuos. Sin embargo, este fenotipo presenta una elevada variabilidad, seguramente atribuible a las diferencias en las metodología utilizadas en los test realizados para su medición (Stellflug y Berardinelli, 2002).

Por otra parte, se ha estudiado la posibilidad de seleccionar la capacidad de cubrición del morueco a través de los niveles de hormonas implicadas en el carácter (Land, 1978). No obstante, estos estudios son escasos, como los de LH, debido a las dificultades que presenta su pulsatilidad (Land, 1978). Por ejemplo, Folch y Gabina (1980) observaron que las hijas de los machos de Rasa Aragonesa con mayor diámetro testicular y una mayor capacidad de cubrición presentaban un mayor número de picos de LH a las 7 y a las 24 semanas de edad. El caso de la testosterona, ha sido discutido con anterioridad, y en la mayoría de los casos no presenta una relación con la capacidad de cubrición.

Los estudios genético moleculares son escasos en caracteres reproductivos de machos de la especie ovina. Como se ha comentado anteriormente, los polimorfismos *RsaI* y *MnlI* del gen *MTNR1A* se han asociado con la actividad sexual de los machos en

Rasa Aragonesa (Abecia et al., 2022).. Estos mismos autores, han mostrado que determinados genotipos de este gen se asocian con una mayor capacidad de cubrición. Recientemente, Bhattacharya et al. (2022) llevaron a cabo un estudio con el objetivo de determinar factores epigenéticos asociados a la actividad sexual de los moruecos en relación a su preferencia por otros machos o por hembras, e identificaron 805 regiones diferencialmente metiladas (DMR, del inglés Differentially methylated regions), encontrando más regiones hipometiladas en machos con orientación sexual hacia otros machos. El análisis del transcriptoma identificó 15 GDEs entre los dos fenotipos, estando sólo el gen *BFSP1* relacionado con una DMR. La anotación funcional de estos genes identificó tres de ellos asociados con el término de actividad hormonal, la *PRL*, *CCK* y *NTS*. Estos tres genes han sido asociados previamente al comportamiento sexual en machos y en hembras (Giardino et al., 2018; Larsen y Grattan, 2012; McHenry et al., 2017; Salais-López et al., 2018).

Finalmente, Pal et al. (2014) llevaron a cabo un estudio con 493 toros cruzados (*Bos taurus* × *Bos indicus*) para estudiar los polimorfismos del gen de *la hormona del crecimiento* (*GH*) y su asociación con las características seminales y de comportamiento sexual. Detectaron doce cambios nucleotídicos, y una única sustitución no sinónima de leucina por valina, asociando este polimorfismo con el comportamiento sexual (libido, tiempo de reacción, respuesta de flehmen y otros) y las características seminales. Se ha hipotizado que el polimorfismo leucina/valina está relacionado a los niveles plasmáticos de GH, como sugieren Schlee et al. (1994).

La revisión llevada a cabo muestra que los trabajos relacionados con la capacidad de cubrición de los machos son escasos, y por lo tanto es interesante desarrollar estudios que permitan dilucidar los mecanismos moleculares que modulan la capacidad de cubrición de los moruecos.

III-4 Estrategias tradicionales utilizadas para inducir en anestro la actividad ovárica en las hembras y la activación sexual de los machos.

Reducir el anestro estacional, provocando que las hembras presenten actividad sexual durante todo este periodo de reposo reproductivo ha sido un objetivo prioritario en el control de la reproducción del ganado ovino. Dentro de las estrategias utilizadas para inducir la ovulación durante el anestro estacional se encuentran los tratamientos

hormonales, el efecto macho o la exposición a tratamientos de luz artificial (Martin et al., 1986; Thimonier, 1981). Igualmente, en los machos, se ha demostrado que aquellos que presentan una mayor actividad sexual presentan mayor fertilidad (Perkins et al., 1992b; Stellflug et al., 2008) y además producen un mayor estímulo en las hembras (Delgadillo et al., 2008). Estos tratamientos se pueden utilizar solos o combinados entre sí.

III-4.1 Tratamientos hormonales

El uso de los tratamientos hormonales para sincronización y/o inducción de celos y ovulaciones, especialmente esponjas intravaginales de FGA (Acetato de Fluorogestona) y eCG (Gonadotropina coriónica equina antiguamente llamada PMSG), es una práctica habitual y extendida en las ganaderías que ofrece buenos resultados, tanto en épocas de actividad sexual, como en épocas de anestro estacionario (Ungerfeld, 2016; Ungerfeld y Rubianes, 2002, 1999). Actualmente, también se están utilizando dispositivos intravaginales de liberación controlada "CIDR" impregnados en progestágenos. Estos tratamientos aumentan los niveles plasmáticos de P4, simulando la presencia de un cuerpo lúteo funcional (Córdova-Izquierdo et al., 2008; Latorre, 2018). La duración del tratamiento es variable (12-14 días), de manera que la retirada del progestágeno simula la rotura del cuerpo lúteo y desencadena el inicio de un nuevo ciclo sexual. La retirada del tratamiento va acompañada de una inyección de eCG (Menchaca et al., 2017). Estos tratamientos se pueden combinar con prostaglandinas (luteolíticos), utilizados para sincronizar ovejas que se encuentran cíclicas. Además, los tratamientos hormonales se pueden usar para adelantar la pubertad en las corderas (Abecia et al., 2011). Sin embargo, la utilización de estos tratamientos en forma masiva puede tener limitaciones en cuanto a su rentabilidad a largo plazo. De hecho, se han reportado casos de vaginitis en un número significativo de animales por el uso de estos dispositivos hormonales (Gatti et al., 2011; Suárez et al., 2006). Estas vaginitis se acompañan de un cambio en la composición y abundancia de la microbiota bacteriana presente en la vagina, lo que afecta directamente a la tasa de fertilidad obtenida tras los tratamientos (Manes et al., 2014).

Para mejorar la fertilidad de los animales en los meses de primavera, se suelen utilizar también los implantes de melatonina. Se ha demostrado el uso efectivo de este

tipo de implantes en estimular tanto la actividad de los machos como de hembras (Gatica et al., 2012; Ungerfeld, 2016). La melatonina, controla la secreción de las hormonas hipofisarias, tanto en ovino como caprino, y mimetiza los días cortos estimulantes de la actividad reproductiva. En los moruecos, el empleo de implantes de melatonina puede sacarlos del anestro estacional. Además, la melatonina mejora su libido e influye positivamente en los parámetros reproductivos (número total de espermatozoides en el eyaculado, mayor concentración espermática, mayor motilidad de los espermatozoides y mayor diámetro escrotal) (Casao et al., 2010; Palacín et al., 2008).

En relación a la eficacia de estos tratamientos hormonales, el análisis de resultados de desestacionalización durante 2021 en Oviaragón- Grupo Pastores, reveló que el número de corderos vendidos por oveja pasó de 0,82 en los casos en que no se realizó tratamiento, a 1,07 cuando se puso implante y a 1,15 si se combinó la melatonina con los dispositivos de sincronización (https://oviaragon.com/como-mejorar-la-cubricion-tratamientos-de-desestacionalizacion/). Sin embargo, por razones éticas, los ganaderos se ven obligados a reducir el uso de estos tratamientos, de acuerdo con las crecientes exigencias de los consumidores que demandan productos de alta calidad, saludables y libres de residuos y que estén producidos respetando el bienestar animal y el medio ambiente (Scaramuzzi y Martin, 2008).

A iniciativa de la Comisión Europea y para lograr una producción animal "limpia, verde y ética ("clean, green and ethical"), se está revisando el uso de hormonas, principalmente porque genera residuos hormonales en carne y leche (Martin y Kadokawa, 2006). De hecho, la UE ya prohíbe el uso de productos hormonales en las ganaderías ecológicas.

III-4.2 Efecto macho

La presencia permanente de machos en los rebaños, reduce notablemente la duración del anestro, incluso en razas muy estacionales. En este sentido, O'Callaghan et al. (1994) demostraron que la presencia de machos retrasaba casi un mes el inicio del anestro, y además adelantaba otro mes el inicio de la nueva estación reproductiva. El efecto macho es un fenómeno socio-sexual que consiste en la introducción súbita de machos en grupos de hembras en anestro que han sido previamente aisladas de los mismos, durante al menos 30-45 días, de todo contacto olfativo, físico, visual e incluso

auditivo. Esto estimula la ovulación y el estro en un porcentaje importante de hembras en anestro (Martin et al., 1986). De hecho, se sabe que la introducción de machos sexualmente activos en un rebaño de ovejas en anestro estimula la secreción de la hormona LH de la pituitaria anterior, lo que resulta en una rápido aumento en la frecuencia del pulso LH, y por lo tanto la activación de un pico preovulatorio de LH (Walkden-Brown et al., 1999), ocurriendo la ovulación tres días después. Sin embargo, la respuesta de las ovejas a la introducción de los machos depende de la CC de las ovejas, de la intensidad de la libido del macho (señales olfativas, vocalizaciones, etc.), y la proporción de machos y hembras (Arellano et al., 2010). Además, existe un porcentaje de hembras que no responden al efecto macho o responden con ovulaciones silenciosas, lo que indica una posible regulación genética de la respuesta dicho efecto. En concreto, en Rasa Aragonesa se ha detectado que, tras la realización de un efecto macho en anestro, entre un 91% y un 74% de hembras presentaron marcas de celo con machos de alta y baja capacidad de cubrición, durante los 33 días posteriores a la introducción del macho (Mozo, 2015). Igualmente, se ha demostrado que los machos con mayor capacidad de cubrición producen un mayor estímulo del efecto macho durante el anestro estacional, que se traduce en un mayor porcentaje de ovejas cubiertas y una fertilidad más elevada, mientras que la respuesta de la hembra al efecto del macho es escasa o incluso nula si la calidad de las señales de éstos disminuye significativamente (Delgadillo et al., 2008). Por este motivo, es interesante el detectar machos con una mayor capacidad de cubrición, que tendrán un mayor efecto sobre las hembras en anestro.

III-4.3 Tratamientos fotoperiódicos

Desde la determinación de que el fotoperiodo es el principal factor medioambiental que controla la actividad reproductiva en pequeños rumiantes, se ha intentado modificar la alternancia natural de este, controlando el número diario de horas de luz que reciben los animales, para desencadenar la actividad reproductiva (Chemineau et al., 1992, 1988; Delgadillo et al., 2002; Thimonier, 1981). Estos tratamientos fotoperiódicos inducen actividad reproductiva tanto en machos como en hembras, al igual que los tratamientos hormonales (Gatica et al., 2012).

La alternancia entre días largos y días cortos, es clave para lograr el control de la actividad sexual (Chemineau et al., 1992). Los días largos son necesarios para restaurar el efecto inductivo de los días cortos al final del invierno y para prevenir el efecto refractario a la estimulación fotoperiódica (Yellon y Foster, 1985). Este método, se utiliza esencialmente en centros de inseminación artificial (IA), donde se requiere una producción de semen constante a lo largo del año. Según Chemineau et al. (2003), los moruecos sometidos permanentemente a una alternancia de un mes de días cortos (8 h luz) con un mes de días largos (16 h) en edificios cerrados, mantienen a lo largo del año, una actividad sexual elevada con una producción seminal de buena calidad. En alojamientos abiertos, los animales tienen que ser sometidos de dos a tres meses de días largos seguidos por implantes de melatonina, siempre y cuando esos días largos se realicen en el momento adecuado del año (Delgadillo et al., 2001).

Ensayos en hembras primerizas han mostrado que el tratamiento fotoperiódico permite adelantar la temporada reproductiva en 60-80 días (BonDurant et al., 1981). Asimismo, este tratamiento, cuando es asociado al efecto macho, incrementa la fertilidad a un 76% (Chemineau et al., 2003, 1988). Fleisch et al. (2015) lograron inducir la actividad estral fértil durante la época no reproductiva en las ovejas Lacaune tras exponerlas a días largos artificiales durante el invierno, seguidos de la duración natural del día y la introducción del macho. Por otra parte, Abecia et al. (2015b) demostraron que la mayoría de las ovejas expuestas a los machos foto-estimulados y con un implante de melatonina presentaron signos de celo durante el anestro estacional. Estos mismos autores, demostraron que la presencia continua de machos activados sexualmente en primavera era capaz de adelantar 20 días la pubertad de las corderas expuestas a los mismos, que presentaban así sus primeros celos en primavera, momento de parada reproductiva (Abecia et al., 2016), así como de adelantar la activación del ovario tras el anestro postparto en primavera (Abecia et al., 2017). Igualmente, los tratamientos lumínicos mediante la modificación del fotoperiodo, sin el uso de hormonas, mejoran los resultados de las cubriciones en primavera (Abecia et al., 2019). En resumen, los machos que presentan una mayor capacidad de cubrición de forma natural o por activación sexual mediante tratamientos fotoperiódicos, mejoran los resultados reproductivos de las hembras en el periodo de anestro.

III-5 Estrategias genéticas utilizadas para mejorar la estacionalidad reproductiva en las hembras y la actividad sexual de los machos

Como antes se ha comentado en el epígrafe de la base genética de la estacionalidad reproductiva (apartado III-3.2.1), el avance de los programas de selección genética clásicos, basados exclusivamente en el control de rendimientos (fenotipos) y las relaciones genealógicas entre los animales, tendrán una eficiencia limitada en términos de respuesta a la selección ya que la mayoría de los caracteres reproductivos presentan heredabilidades bajas. Además, muchos de estos caracteres se miden en un solo sexo (actividad ovárica en las hembras y actividad sexual en los machos) por lo que se requerirán pruebas de descendencia para la predicción de los valores genéticos y éstos serán estimados con una baja fiabilidad. El uso de herramientas moleculares permite superar algunas de las limitaciones de los métodos tradicionales de predicción del mérito genético. Las plataformas de genotipado masivo de SNPs y secuenciación masiva permiten explorar el genoma e identificar genes y polimorfismos responsables de la variabilidad de caracteres de interés (por ejemplo, QTLs). La posibilidad de utilizar genes/marcadores responsables de una proporción significativa de la varianza fenotípica de caracteres de interés económico (ETLs) es una estrategia de gran interés para un desarrollo más eficiente de los Programas de Mejora Genética, y sobre todo para aquellos caracteres que presentan bajas heredabilidades, son difíciles de medir, y se recogen en un solo sexo. En este sentido, destacan la Selección Genómica (GS; por sus siglas en inglés "Genomic Selection"), y la SAM (Selección Asistida por Marcadores).

El objetivo de la Selección Genómica es predecir el valor genómico (VG) de los animales utilizando la información disponible de miles de marcadores (genotipos) distribuidos a lo largo del genoma, obtenidos mediante técnicas de genotipado de polimorfismos de un solo nucleótido (SNPs) en diversas plataformas comerciales (chips) (Meuwissen et al., 2001). En general, la Selección Genómica combina tres fuentes de información: control de producciones (fenotipos), relaciones genealógicas (pedigrí) y genoma (polimorfismos de ADN) de los animales. Una valoración genómica es, en el fondo, una valoración genética, pero con mucha más calidad, ya que, con esta tercera fuente de información, los genotipados, se aporta una mayor fiabilidad a las predicciones genéticas, algo que es muy importante para incrementar la respuesta a la selección, obteniendo el VG de todos los reproductores, tanto machos como hembras. En ovino de

carne no se está utilizando la Selección Genómica, aunque si en ovino de leche (Jurado et al., 2013). Para obtener los genotipos de los marcadores SNP se utilizan plataformas o chips de SNPs. En 2009, se desarrolló un chip de SNPs de densidad media (con alrededor de 50.000 SNPs) para el ganado ovino que fue denominado "Ovine SNP50 BeadChip" (desarrollado por el ISGC, International Sheep Genomics Consortium) que permitió realizar estudios de GWAS, así como la identificación de loci de carácter cuantitativo y estudios de genética comparativa (Kijas et al., 2012). En la actualidad, se ha desarrollado un chip de alta densidad que dispone de alrededor de 600.000 SNPs (Illumina AgResearch Sheep HD) distribuidos a lo largo de todo el genoma. En el caso de la Selección Genómica se utiliza el chip de 50K, y otros de menor tamaño (3K). La utilización de plataformas de genotipado de media y alta densidad para llevar a cabo la Selección Genómica, se basa en la existencia de DL entre los marcadores moleculares contenidos en los chips de genotipado (que no son causales) y los genes causales dispersos por el genoma ovino. El DL entre los SNPs y los genes causales de los caracteres de interés, consiste en que los alelos de ambos no segregan de forma independiente, ya que su frecuencia de recombinación es menor del 50%. Su utilización en Programas de Mejora Genética ovina permite la reducción del intervalo generacional y el aumento de la ganancia genética (Duchemin et al., 2012). La posibilidad de genotipar animales a edades tempranas permite acortar el intervalo generacional (Rohrer, 2004). Además, dado que la información genómica incrementa la fiabilidad de las predicciones genéticas de los animales, las pruebas de testaje de machos y hembras son más cortas, ya que se requiere menos información fenotípica de cada animal para alcanzar una fiabilidad aceptable.

La SAM, se basa en el uso de marcadores moleculares QTL o ETLs, responsables de un efecto significativo sobre la varianza de una o varias características fenotípicas de los animales, de forma que el genotipo de dicho locus puede utilizarse directamente en la selección de reproductores. En este caso, la mutación genotipada no suele ser la mutación responsable, sino que está en DL con la misma. Finalmente, si localizamos la mutación responsable del efecto fenotípico observado, y por lo tanto hacemos una selección basada en el marcador directamente responsable del fenotipo, la selección se denomina selección asistida por genes (GAS; del inglés "Gene Assisted Selection"); cuya aplicación es relativamente sencilla desde el momento en que se conoce

exactamente la mutación causal, su función y el efecto que tiene sobre los caracteres. Un ejemplo de este proceso de GAS en genética animal es la utilización del genotipo del gen *PRNP* de resistencia al scrapie en los programas de selección del ganado ovino. En este sentido, la estrategia es incrementar la frecuencia del alelo favorable por selección o introgresión génica, mediante la preselección de reproductores con alelos favorables de resistencia a la enfermedad. Esta metodología se ha utilizado con caracteres de prolificidad en ovino (Calvo et al., 2020a). Un problema que puede presentar este tipo de selección es la disminución de la variabilidad de la población si seleccionamos sólo en función de unos pocos marcadores.

Para llevar a cabo la detección de variantes génicas asociadas a los caracteres de interés en especies ganaderas, podemos distinguir dos tipos de estrategias, en función de si el estudio implica todo el genoma (sin tener en cuenta condiciones previas, es decir con un enfoque libre de hipótesis), o solo incluyendo un número de genes limitado (gen candidato), previamente seleccionados por estar potencialmente (funcional o posicionalmente) relacionados con el fenotipo de interés (enfoque con una hipótesis previa) como prolificidad, fertilidad, sensibilidad o resistencia a padecer una determinada enfermedad, etc. Los estudios de hipótesis previa tienen la desventaja de requerir un conocimiento biológico previo que permita la selección de los genes candidatos objeto de análisis, pero también la fortaleza de un gran conocimiento biológico-fisiológico de las diferencias genéticas y fenotípicas observadas. La mayor ventaja es que permite la aplicación directa de los resultados en nuestra raza en estudio, lo cual es muy útil ya que, en el caso de las aproximaciones a genoma completo, generalmente no se detecta directamente el gen causal, sino que, haciendo uso del DL, se marcan regiones del genoma asociadas a la variabilidad del fenotipo estudiado (por ejemplo, en el caso de GWAS).

En cuanto a los estudios de expresión génica con hipótesis previa destaca la PCR cuantitativa a tiempo real (RT-qPCR), que es una técnica que amplifica y cuantifica simultáneamente. Esta técnica es ideal, para el estudio de genes o rutas bioquímicas específicas, ya que es muy sensible y ofrece la posibilidad de llevar a cabo reacciones múltiples (Bustin, 2002). Además, es la técnica de elección para validar los resultados obtenidos mediante las técnicas de RNA-Seq.

En cuanto a los estudios libres de hipótesis, la aparición y adopción de técnicas de secuenciación de alto rendimiento, que permiten la secuenciación de varias gigabases de genoma en poco tiempo, ha abierto nuevas perspectivas en muchos campos de la genómica. Los avances en las técnicas genómicas, la disponibilidad del genoma ovino que ha proporcionado cientos de miles de marcadores genéticos (actualmente la versión Rambouillet 2.0), y las técnicas de genotipado de alto rendimiento han mejorado la capacidad de los investigadores para detectar regiones genómicas asociadas a la variabilidad de los caracteres de interés así como de las mutaciones subyacentes a la misma (Porto-Neto et al., 2015; Van Binsbergen et al., 2015). En este contexto, los GWAS se han convertido en un método de referencia para identificar genes y mutaciones causales estadísticamente asociadas a fenotipos de interés en ganadería (Hirschhorn y Daly, 2005). Estos estudios se basan en la utilización de chips de genotipado masivo de SNPs. Una vez que se han identificado esas variantes genómicas, que por lo general no son las variantes causales de la variabilidad del fenotipo, sino que están en DL con el polimorfismo causal, se buscan en regiones adyacentes al SNP significativo, las posibles variantes causales implicadas directamente en los cambios observados en el fenotipo. Para esto, se pueden utilizar metodologías de imputación genómica o incrementar la densidad de marcadores en la región detectada (Schaid et al., 2018). Si la región genómica a estudiar es muy grande, se pueden combinar los resultados del GWAS con los de secuenciación masiva de nueva generación, como el RNA-Seq (Suárez-Vega et al., 2015) o secuenciación masiva de genoma completo (Ben Braiek et al., 2021) para identificar la mutación responsable. Por otra parte, si la región genómica no es muy grande, y hay genes candidatos que por función o por posición en otras especies o poblaciones son interesantes, se pueden utilizar técnicas de bajo rendimiento como la secuenciación SANGER.

La secuenciación de segunda generación utiliza superficies de fijación de moléculas de ADN, que permiten la secuenciación en paralelo de millones de secuencias de ADN (Slatko et al., 2018). Esta secuenciación ha permitido disminuir, por un lado, el tiempo necesario para secuenciar genomas enteros y, por otro, los costes de esta (Pettersson et al., 2009). Este tipo de secuenciación lleva a cabo una PCR para aumentar el número de copias del material genético a analizar. Dentro de las diferentes tecnologías de secuenciación masiva en paralelo, la tecnología Illumina es la más utilizada, gracias

a la alta capacidad de los instrumentos de Illumina que los hace ideales para una gran variedad de aplicaciones de secuenciación, entre las que se incluyen el mapeo frente a un genoma de referencia (resecuenciación), la secuenciación del transcriptoma, la detección de SNPs o la secuenciación utilizada para estudios metagenómicos (Figura 6). En general, tras la fragmentación de los ácidos nucleicos, se preparan librerías de fragmentos de ADN de un tamaño que puede variar entre 35 y 500 pb (Martin y Wang, 2011), que al ser secuenciados producirán millones de lecturas.

La secuenciación masiva de nueva generación del transcriptoma realizada mediante la técnica conocida como RNA-Seq junto con las herramientas bioinformáticas apropiadas, permiten analizar la gran complejidad del transcriptoma (Fernandez-Rodriguez et al., 2011; Mardis, 2008; Mortazavi et al., 2008) (Figura 7). En este sentido, presentan mayor sensibilidad y menor variación técnica que los microarrays de expresión, y por otra parte, mediante RNA-Seq es posible detectar prácticamente todos los transcritos presente en una muestra, lo que permite caracterizar nuevos genes o transcritos. Con esta técnica es posible cuantificar cada transcrito y de ARNs no codificantes, así como detectar SNPs y otras variables estructurales.

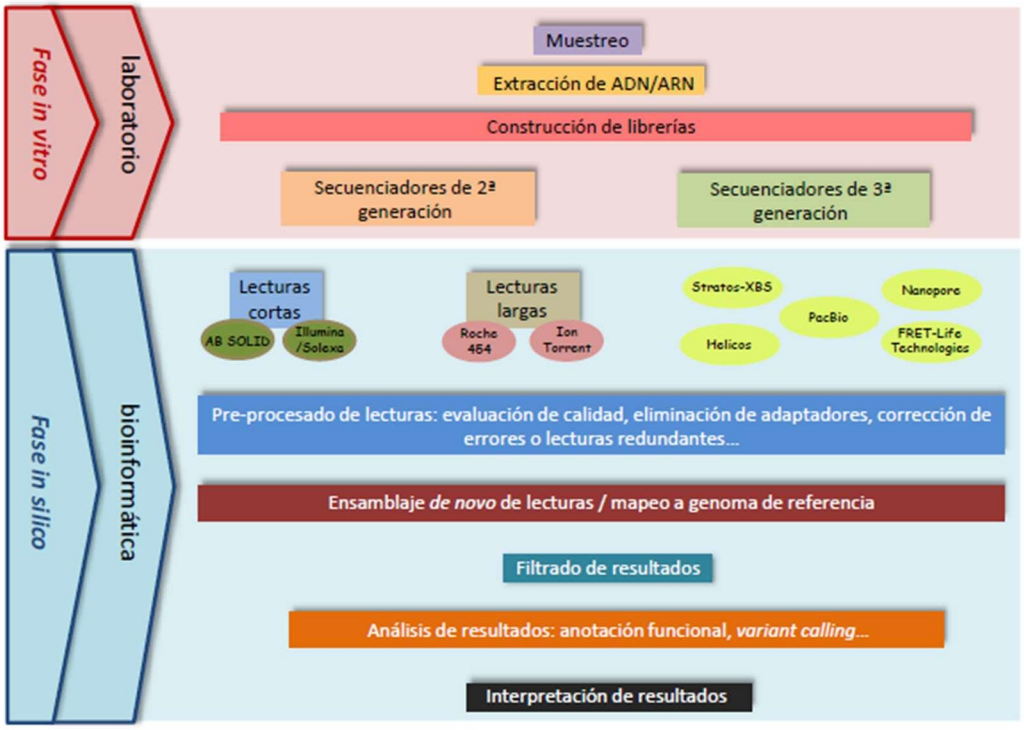


Figura 6. Esquema general de los procesos a llevar a cabo para realizar un experimento de secuenciación masiva (Tomado de López de Heredia, 2016)

Los secuenciadores de tercera generación se fundamentan en la idea de eliminar la etapa de PCR, y tratar de realizar la secuenciación a partir de una única molécula de ADN. Al mismo tiempo que se trata de abaratar los costes, se obtienen lecturas de secuencia más largas (10-30 Kb), que facilitan el posterior ensamblado de secuencias, lo que amplía sus aplicaciones, como es el caso de la detección de grandes variaciones en la estructura de los cromosomas. Esta tecnología requiere una cantidad relativamente grande de ADN (entre 250 y 5.000 ng, en función de la tecnología). En el mercado existen dos tecnologías destacadas de este tipo, basadas en la técnica SMRT (Single-Molecule Real-Time) (Eid et al., 2009), y en la secuenciación con nanoporos de Oxford Nanopore Technologies (ONT) (Branton et al., 2008) (Figura 6).

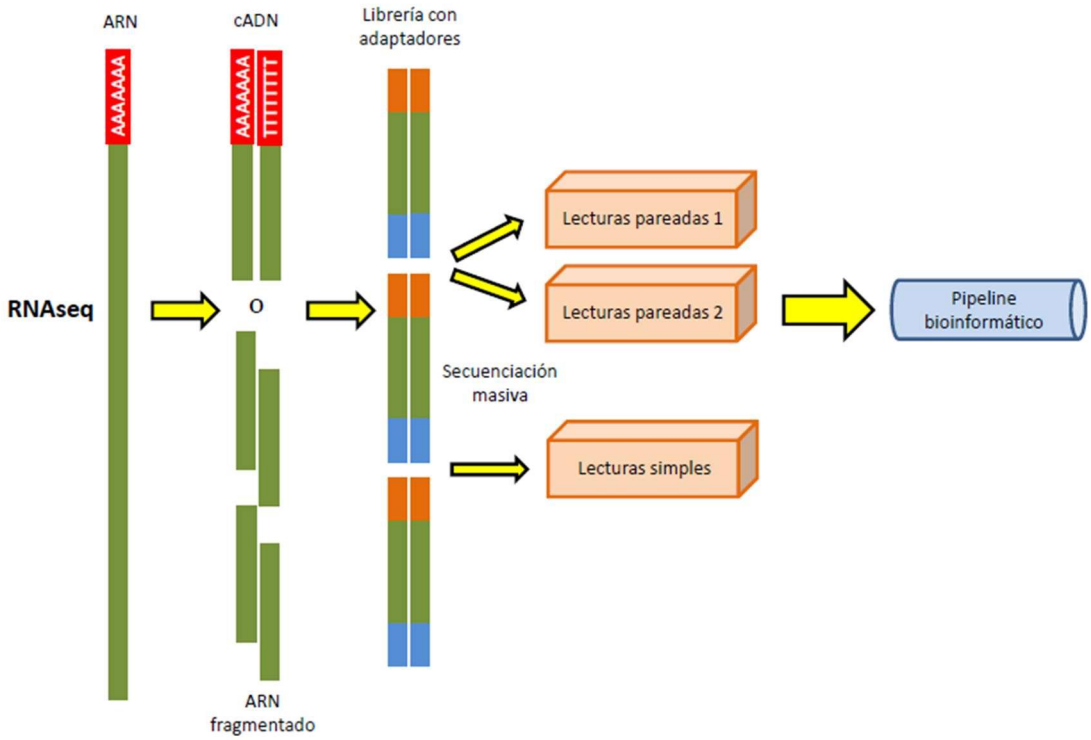


Figura 7. Esquema de la metodología para el análisis de RNA-Seq (Tomado de López de Heredia, 2016)

En este trabajo se ha utilizado la secuenciación masiva del transcriptoma (RNA-Seq) para el estudio de la base genética del restablecimiento de la actividad ovárica, así como de la capacidad de cubrición de los machos en época de AR. Como se ha descrito en esta revisión, la literatura a este respecto es escasa, y la determinación de los

mecanismos moleculares implicados en el restablecimiento de la actividad ovárica en anestro y/o de la capacidad de cubrición de los machos permitirá localizar genes y/o rutas bioquímicas implicadas en la expresión de estos fenotipos. Por otra parte, se llevarán a cabo análisis GWAS con los chips Illumina de 50K y 600K con el fin de detectar nuevos marcadores genéticos asociados a la estacionalidad reproductiva, y a otras variables que influyen en la misma, como es la CC.

IV- Material y métodos

El material animal y la metodología adoptada para el desarrollo de esta Tesis Doctoral están descritos en detalle en cada uno de los manuscritos. Con el objetivo de completar la información proporcionada en el material y métodos de los diferentes manuscritos y facilitar así la interpretación de los resultados, vamos a describir brevemente los grupos experimentales y métodos utilizados en este trabajo.

IV-1 Grupos experimentales

Se han utilizado cuatro grupos experimentales de raza Rasa Aragonesa. En el grupo experimental 1, la población experimental proviene de "La Pardina de Ayés", una granja experimental propiedad de Oviaragón-Grupo Pastores, situada en el pre-Pirineo (Ayés, Huesca; 42º 29' 48,55" N 0º 23' 37,54", a una altitud de 790 m sobre el nivel del mar). En los grupos experimentales 2, 3 y 4, los animales provienen del rebaño experimental del CITA, Gobierno de Aragón en Montañana (Zaragoza; 41° 3´N, 0° 47 ´W; a una altitud de 225 m).

El material y diseño animal utilizado en este estudio cumple los principios éticos y de protección de los animales utilizados en experimentación y otros fines científicos, según resolución de la Comisión Ética Asesora para la Experimentación Animal del CITA, con expedientes 2011/08, 2015/03, 2017/02 y 2009/01_MJT para los modelos animales 1, 2, 3 y 4, respectivamente.

IV-1.1 Grupo experimental 1

Este material animal proviene de un estudio detallado en Martinez-Royo et al. (2017), en el cual se realizó un estudio GWAS con caracteres de estacionalidad reproductiva usando el chip Illumina de 50K. Para los estudios de asociación del gen *LEPR* con los caracteres de estacionalidad reproductiva (manuscrito 1), el material animal estuvo constituido por 239 ovejas de Rasa Aragonesa repartidas en dos grupos de edad: 155 ovejas adultas con un promedio de 5,5 ± 0,5 años y 84 ovejas jóvenes todas de la misma edad al inicio del estudio (1,9 ± 0,0 años). En ambos grupos, las medias del PV y CC fueron 52,5 ± 7,7 kg y 2,9 ± 0,3 respectivamente, durante todo el periodo experimental, que duró desde enero hasta agosto del 2012.

Para la búsqueda de nuevos polimorfismos asociados a la estacionalidad reproductiva mediante GWAS (manuscrito 2), se consideraron los dos grupos de ovejas

adultas y jóvenes descritas anteriormente (155 ± 84), junto con un tercer grupo de 26 corderas de la misma edad (0,94 ± 0,0 años) constituyendo un total de 265 ovejas. Sin embargo, para el análisis GWAS se utilizaron 205 ovejas distribuidas de la siguiente manera: 122 adultas (5,4 ± 0,45 años), 66 jóvenes (1,9 ± 0,0 años) y 17 corderas (0,94 ± 0,0 años). La selección de las 205 ovejas se basó en la disponibilidad de ADN en calidad y cantidad suficiente para ser genotipado, y en la existencia de datos reproductivos durante todo el periodo de duración del experimento. De las 205 ovejas, 108 fueron genotipadas en el trabajo de Martinez-Royo et al. (2017) con el chip de 50K (Illumina OvineSNP50 BeadChip), y 97 con el chip de 600K (Illumina AgResearch Sheep HD de 600K). Para los estudios de validación y asociación génica, se utilizaron las 265 ovejas.

Para la búsqueda de polimorfismos asociados a los caracteres de crecimiento mediante GWAS (manuscrito 3), el período experimental duró de enero a agosto durante los años 2011 y 2012. El estudio experimental estuvo compuesto por 225 ovejas repartidas en tres grupos de edad al inicio del experimento: 123 adultas (5,4 ± 0,5 años), 67 jóvenes (1,9 ± 0,0 años) y 35 corderas (0,94 ± 0,0 años). La diferencia de animales genotipados para el GWAS entre este estudio y el anterior estuvo relacionada con la existencia de datos completos de CC y PV durante todo el periodo experimental.

Las ovejas de raza Rasa Aragonesa fueron criadas según el sistema tradicional local. Brevemente, las ovejas se mantuvieron estabuladas y se alimentaron con un concentrado comercial de octubre a marzo. Desde marzo hasta el final del período experimental (agosto), las ovejas pastorearon en pastos de montaña y recibieron el mismo concentrado comercial *ad libitum*. Todas las ovejas fueron manejadas en un solo lote y fueron sometidas a las mismas condiciones de manejo, nutrición y medio ambiente. A las ovejas no se les suministró ningún tratamiento hormonal.

IV-1.2 Grupo experimental 2

Estos animales pertenecen al rebaño del CITA de Aragón que consta de aproximadamente 400 ovejas con una tasa de renovación anual del 30% y criadas en condiciones de manejo homogéneas (alimentación, tratamientos veterinarios, etc.).

Para el estudio del transcriptoma de muestras del PT e HT en hembras con diferentes fases ováricas (F, L o AR) (manuscrito 4), se seleccionaron del rebaño del

CITA 50 ovejas con una CC (2,98 ± 0,31), PV (53,50 ± 6,13 kg) y edad (7,97 ± 0,56 años) similares. Estas ovejas habían parido en otoño (septiembre-diciembre), y fueron sometidas a las mismas condiciones de manejo y nutrición para limitar los efectos ambientales sobre el patrón de expresión génica.

IV-1.3 Grupo experimental 3

En cuanto al estudio del transcriptoma de muestras del PT, HT, GP (manuscrito 5) y sangre periférica (manuscrito 6) en machos con diferente actividad sexual, se utilizaron 59 machos de raza Rasa Aragonesa con edades comprendidas entre 1,8 y 8,3 años (3,7 ± 2,05 años), una CC media de 3,3 ± 0,3, PV medio de 81,5 ± 14,44 kg y un diámetro testicular máximo anteroposterior medio de 6,00 ± 0,58 cm. Estos machos eran los machos utilizados en las diferentes cubriciones a fecha de 1 de abril de 2019. Todos los machos tenían experiencia sexual previa, es decir, habían sido expuestos previamente a las ovejas durante la temporada de reproducción.

IV-1.4 Grupo experimental 4

El grupo experimental 4 se utilizó para el estudio de expresión génica diferencial en muestras de hígado del gen *CYP7B1* con diferentes alelos en la región promotora (manuscrito 3). Para ello se utilizó un material animal que proviene de estudios previos, que tenían como objetivo el estudio del efecto del tiempo de suplementación con vitamina E o pastoreo con alfalfa sobre la vida útil de la carne, perfil de ácidos grasos y expresión de genes en músculo y grasa subcutánea en corderos de la raza Rasa Aragonesa (González-Calvo et al., 2017; Ripoll et al., 2013). Brevemente, el material animal utilizado estuvo constituido por 48 corderos machos de raza Rasa Aragonesa. Todos ellos procedían de una cubrición controlada de un rebaño de 120 ovejas. La edad promedio de los corderos al inicio del estudio fue de 48,7 ± 0,21 días, momento en el que se destetaron y se inició la alimentación a base de pienso concentrado comercial (Control; 175 g proteína bruta (PB); 180 g fibra neutro detergente (FND); 45 g fibra ácido detergente (FAD); 13,2 MJ energía metabolizable (EM) por kg de materia seca (MS) y 27 mg de acetato de dl-α-tocoferol por kg de concentrado). Posteriormente, este pienso concentrado fue reemplazado por otro pienso de las mismas características, pero enriquecido con vitamina E (VE concentrado; 179 g PB; 190 g FND; 52 g FAD; 13,2 MJ EM por kg de MS y 480 mg de acetato de dl-α-tocoferol por kg de concentrado),

que les fue administrado durante diferente número de días, que variaban de 4 a 28 días antes del sacrificio (n = 36). Igualmente, hubo 12 animales que sólo recibieron el pienso control durante todo el periodo de cebo hasta el sacrificio. Los animales se pesaron semanalmente a primera hora de la mañana, además se tomó la CC. Cuando los corderos alcanzaron los 22-24 kg de PV, se sacrificaron en el matadero experimental que el CITA posee en Zaragoza siguiendo la normativa de la IGP del Ternasco de Aragón (Regulación (EC) Nº. 1107/96). Esta, exige que los animales deben alcanzar los 22-24 kg de PV y tener menos de 90 días de edad. La edad y PV promedio al sacrificio fue de 75,20 ± 1.84 días y 23 ± 0,24 kg, respectivamente. Los sacrificios fueron organizados semanalmente por grupos de animales según el peso final establecido. Tras el sacrificio se aislaron muestras de hígado, que fueron inmediatamente envueltas en papel de aluminio debidamente identificado e introducidas en nitrógeno líquido para, posteriormente, almacenarlas a -80º C hasta la extracción de ARN.

IV-2 Fenotipos estudiados y medidas controladas

IV-2.1 Estacionalidad reproductiva

Para los estudios de asociación y GWAS (grupo experimental 1), los caracteres controlados fueron principalmente relacionados con la estacionalidad reproductiva en hembras de Rasa Aragonesa (manuscritos 1 y 2). Desde enero hasta septiembre se tomaron datos de PV y CC cada 3 semanas, así como una muestra de plasma sanguíneo para medir la concentración de P4 semanalmente usando el kit ELISA de RIDGEWAY (Ridgeway Science, St. Briavels, Gloucestershire, UK). La CC de las ovejas se estimó siguiendo las recomendaciones de Russel et al. (1969). Igualmente, durante todo el periodo se llevó a cabo la detección de celos en las hembras mediante machos vasectomizados provistos de arneses con pastillas marcadoras. El control de celos se llevó a cabo diariamente de forma que, tras la monta natural, se identificaban visualmente las ovejas que habían manifestado el celo con una marca de color en la grupa. Para no confundir las marcas de celo en el tiempo, se procedió al cambio de color del marcador de los arneses cada dos semanas, ya que los ciclos ovulatorios suceden aproximadamente cada 17 días. Los caracteres analizados fueron los fenotipos descritos por Martinez-Royo et al. (2017): número total de días de anestro (DTA); ciclicidad por progesterona (CiP4) y ciclicidad por celos (CiC). Brevemente, DTA serían los días

totales de anestro reproductivo para cada oveja, considerando como tales aquellos periodos con 3 o más medidas de P4 por debajo del umbral de actividad ovárica, situado en 0,5 ng/ml (Figura 8). CiP4 es el porcentaje de meses cíclicos por P4, considerando un mes cíclico para cada oveja aquel en el que al menos hay una medida de concentración de P4 superior al umbral de actividad ovárica. Por último, CiC corresponde al porcentaje de meses cíclicos por celos, considerando un mes cíclico para cada oveja aquel en el que ha mostrado al menos un celo (Figura 9).

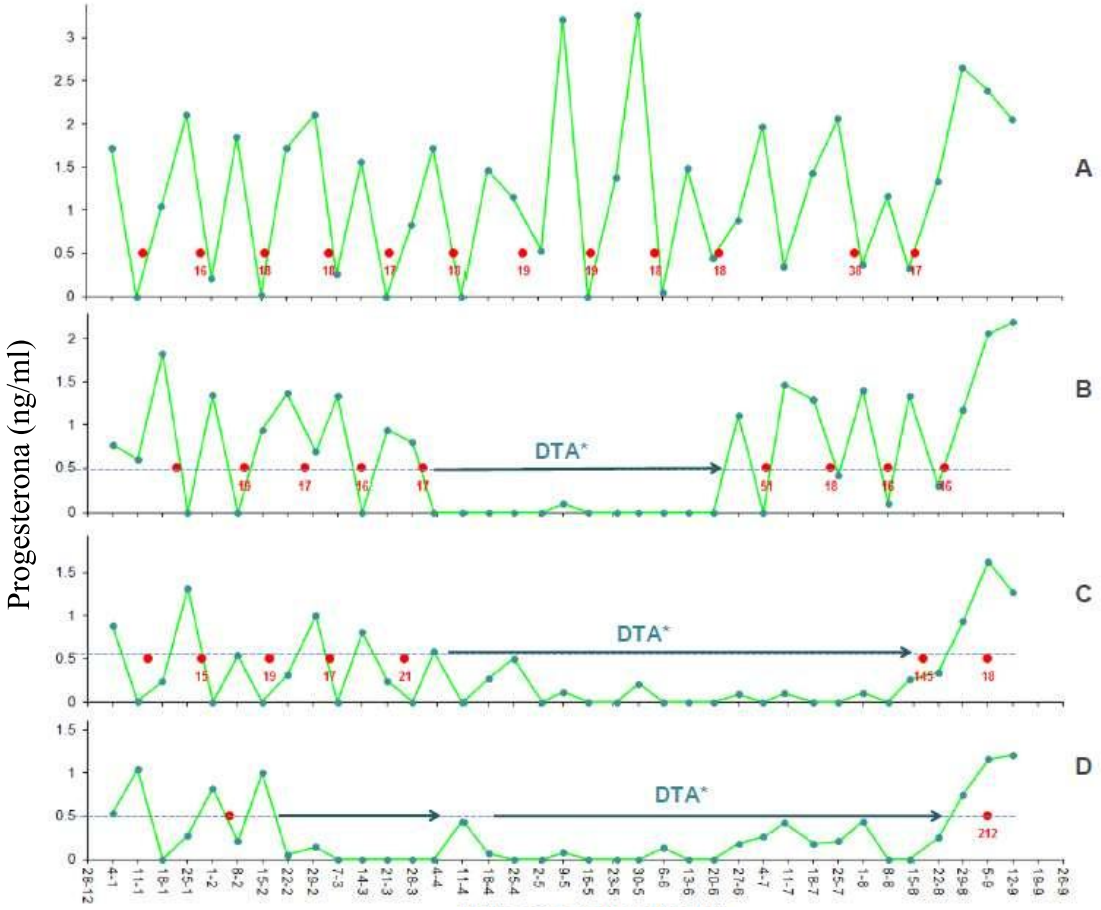


Figura 8. Perfiles de progesterona obtenidos en 4 ovejas. Los puntos rojos indican la detección de celo. La oveja A fue cíclica durante todo el estudio (días totales de anestro: DTA = 0). Las ovejas B (DTA = 77), C (DTA = 126) y D (DTA = 168) presentaron diferentes periodos de anestro durante el estudio.

IV-2.2 Caracteres de crecimiento

Como se ha indicado en el apartado III-3.1 de revisión bibliográfica, los caracteres de crecimiento están muy relacionados con la estacionalidad reproductiva. En este sentido se utilizó el grupo experimental 1, del que se disponían medidas cada 3 semanas de CC y PV de enero a agosto durante dos años (2011 y 2012), para llevar a cabo un estudio GWAS sobre estos caracteres, así como con la TC (manuscrito 3).

Los fenotipos corregidos usados para el GWAS se obtuvieron a partir de los datos de CC y PV recogidos a lo largo de los 2 años de los experimentos (ver apartado IV-4.1.1). Para el estudio de validación mediante expresión génica del gen *CYP7B1* en muestras de hígado, se utilizó en grupo experimental 4 (apartado IV-1.4).

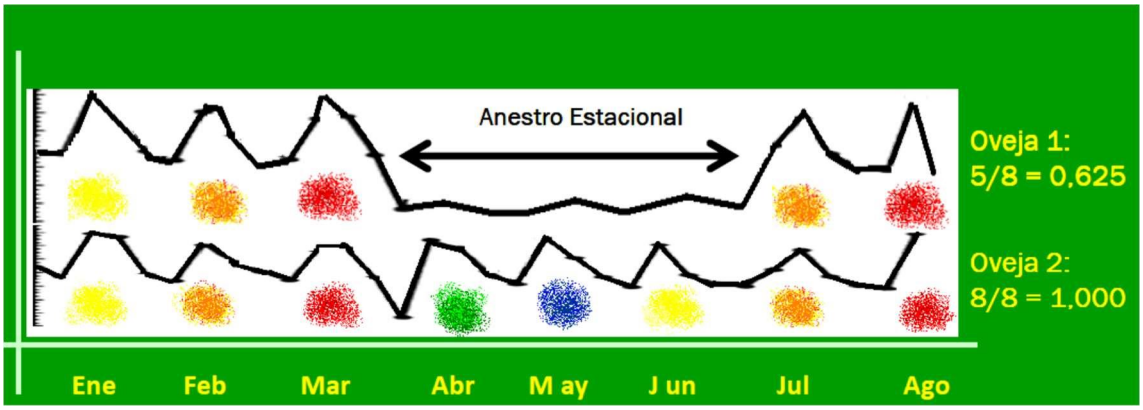


Figura 9. Porcentaje de meses cíclicos mediante detección de celos (CiC). Las marcas de color simulan la detección de celo realizada mediante machos vasectomizados provistos de arneses con pastillas marcadoras. La oveja 1 presenta anestro estacional con un porcentaje de meses cíclicos de 0,625, mientras que la oveja 2 es cíclica durante todo el periodo de estudio.

IV-2.3 Fases de actividad ovárica en anestro reproductivo

Para el estudio del transcriptoma en hembras adultas de Rasa Aragonesa (grupo experimental 2; manuscrito 4), se controló la actividad ovárica en 50 ovejas del rebaño del CITA. Dicho control se llevó a cabo midiendo los niveles de P4 en sangre periférica mediante el test ELISA descrito anteriormente (Ridgeway Science, St. Briavels, Gloucestershire, UK), para seleccionar las ovejas a sacrificar en función de su fase ovárica. Siguiendo el protocolo de Thimonier (2000), se tomaron un total de cinco

muestras de sangre dos veces por semana (con 3-4 días de intervalo). La última toma se realizó 2 días antes del sacrificio, durante la segunda quincena de abril. En función del perfil de P4 y siguiendo los criterios de Teyssier et al. (2011) se seleccionaron 30 ovejas que estaban en diferentes fases de actividad ovárica. Para ello se consideró que una oveja estaba en AR cuando los niveles de P4 durante las 5 tomas se situaban por debajo del umbral de actividad ovárica (0,5 ng/ml). Se consideró que una oveja estaba en la fase F en el momento del sacrificio cuando la última muestra estaba por debajo del umbral de actividad ovárica y era precedida por muestras por encima del umbral, o cuando la última muestra estaba por encima del umbral y la penúltima muestra tenía una concentración de P4 de al menos 1,3 veces más alta. Se considera que una oveja estaba en fase L cuando las dos últimas muestras estaban por encima del umbral de actividad ovárica, siendo la última superior a la anterior, y ambas precedidas por lo menos por una muestra con concentración de P4 por debajo del umbral.

Las ovejas se sacrificaron con sobredosis de pentotal sódico. Tras el sacrificio, se confirmó la fase del ciclo ovárico en la que se encontraba el animal mediante observación ovárica. Tres y 4 ovejas consideradas en AR y fase F, respectivamente, según los niveles de P4, mostraron ovulaciones recientes tras la disección del ovario. Igualmente, en una oveja en AR se sospechó de hidrosálpinx. Finalmente, en una oveja considerada en fase L, el cuerpo lúteo maduro estaba acompañado de un quiste ovárico. Todas estas ovejas no fueron consideradas para el estudio RNA-Seq. Las ovejas consideradas en AR incluidas en este estudio no tenían ni cuerpos luteos ni tampoco estos mismos en regresión (*corpora albicantia*) en la superficie de los ovarios (n = 7). En las ovejas consideradas en fase F incluidas en este estudio encontramos *corpora albicantia* y ningún cuerpo lúteo en la superficie ovárica, junto con folículos pequeños (3-5 mm) en todas las ovejas (n = 7), y folículos grandes (> 5 mm) en todas las ovejas menos en una. Las ovejas consideradas en fase L para este estudio tenían al menos un ovario con al menos un cuerpo lúteo maduro (> 7 d; n = 7).

En total, se seleccionaron 21 ovejas, 7 por cada estado reproductivo (AR, F y L). Las muestras de los tejidos HT (n = 21), y PT de la pituitaria (n = 21) se diseccionaron, fueron inmediatamente envueltas en papel de aluminio debidamente identificadas e introducidas en nitrógeno líquido para, posteriormente, almacenarlas a -80º C hasta la extracción de ARN.

IV-2.4 Actividad sexual en machos

El comportamiento sexual de los moruecos se determinó mediante un test de aprisco en todos los machos adultos del CITA (n = 59) (grupo experimental 3; manuscritos 5 y 6). Este primer test de aprisco se llevó a cabo en mayo, durante el anestro estacional. Antes del comienzo de los test de capacidad de cubrición, los machos se sometieron a una fase previa de aclimatación para que se adaptaran a las condiciones de la experiencia (Kilgour, 1985), basada en exponer a cada macho durante 10 minutos a dos ovejas que no presentaban signos de celo en el lugar donde posteriormente se iban a realizar los test. La prueba consistió en introducir a cada macho durante 20 minutos en un apartado de 3x3 m con 2 ovejas adultas en celo sincronizadas (esponjas vaginales de 30 mg de acetato de fluorogestona 14 días + 480 UI de eCG en el momento de su retirada) y con libertad de movimiento. Los test se iniciaron 42 horas después de la retirada de las esponjas. Se registró el número de montas (incorporaciones sobre la grupa de la oveja) y de cubriciones (montas con eyaculación, observadas mediante la ejecución de un "golpe de riñón") realizadas durante el test de aprisco (Mozo, 2015; Mozo et al., 2013). El fenotipo utilizado para evaluar la actividad sexual de los moruecos fue la suma de montas y cubriciones. Además, se categorizo el fenotipo de estos animales en activos (A; al menos 1 monta + cubrición) o no activos (NA; 0 montas + cubriciones). El día anterior, se tomaron tres tomas seriadas de sangre separadas por una hora en tubos de heparina litio para medir testosterona mediante radioinmunoanálisis (RIA) (Hochereau-De Reviers et al., 1990). Dicho análisis, se llevó a cabo en el Laboratoire Phénotypage-Endocrinologie, INRAE. Se utilizó este protocolo de muestreo considerando que el valor medio de las tres muestras es una estimación fiable de la concentración de testosterona de cada animal, ya que se sabe que se producen grandes variaciones en los niveles de testosterona en sangre periférica en periodos cortos debido a la naturaleza pulsátil de la secreción de testosterona en los moruecos (Santiago-Moreno et al., 2005). También, se midieron al inicio del experimento la CC (3,3 ± 0,3) y PV (81,5 ± 14,44 kg), así como el diámetro testicular medio (6,00 ± 0,58 cm). Este último se calculó como la media entre los diámetros antero-posteriores máximos de ambos testículos menos el grosor correspondiente al pliegue cutáneo de esa zona, medidos con un calibre. Posteriormente, se realizó un segundo test de capacidad de cubrición, quince días después de la primera prueba, en un grupo de machos con edad y CC similares (n = 21), seleccionados

mediante un modelo de regresión basado en árboles de decisión (ver apartado IV-4.2.1). Como en el primer test de aprisco, se midieron la CC (3,4 ± 0,3) y el PV (84,1 ± 10,5 kg), así como el diámetro testicular medio (6,61 ± 0,54 cm). También se tomaron tres muestras de sangre seriadas con una hora de intervalo para medir la testosterona. Finalmente, los animales de este grupo también se clasificaron como A (9,0 ± 7,5, media de montas más cubriciones ± desviación estándar; n = 11) o NA (sin montas ni cubriciones; n = 10). Este segundo test de aprisco se realizó para confirmar que los machos seguían A o NA, llevándose a cabo el día anterior al sacrificio. Finalmente, se seleccionaron 6 animales de cada grupo que fueron sacrificados con sobredosis de pentotal sódico, e inmediatamente, tras el sacrificio se procedió a la extracción del cerebro y disección del PT, HT y GP, tejidos clave en la activación sexual de los machos. Las muestras fueron inmediatamente envueltas en papel de aluminio debidamente identificadas e introducidas en nitrógeno líquido para, posteriormente, almacenarlas a -80ºC hasta la extracción de ARN.

Igualmente, se tomó una muestra de sangre en tubos Tempus (Applied Biosystems, Fisher, Madrid, España), que se utilizan para la estabilización y extracción del ARN total de sangre. Cuando la sangre (3 ml) se extrae en el tubo y se mezcla con el reactivo de los tubos Tempus (6 ml), la lisis se produce casi inmediatamente. El reactivo de estabilización desactiva las ribonucleasas (ARNasas) y precipita de forma selectiva el ARN y el ADN genómico mientras que las proteínas permanecen en la solución. Tras la extracción de sangre, se agitaron los tubos con fuerza para producir la lisis y se mantuvieron durante 2 horas a temperatura ambiente, para posteriormente congelar los tubos a -80ºC, hasta proceder a la extracción del ARN. Las muestras se tomaron los días del primer test de aprisco (n = 59) a todos los moruecos, y el día del sacrificio tras el segundo test de aprisco a los 12 moruecos en los que se estudió el transcriptoma (n = 12).

IV-3 Análisis de laboratorio

IV-3.1 Estudios de asociación

IV-3.1.1 Extracción de ADN

Para la extracción de ADN en muestras sanguíneas, se utilizó el Kit SpeedTools DNA Extraction (Biotools, Madrid, España), siguiendo los protocolos del fabricante (manuscritos 1, 2 y 3). La concentración y presencia de contaminantes del ADN se determinó con el nanoespectrofotómetro (Implen, Fisher, Madrid, España) mediante la cuantificación de la densidad óptica a 260 y 280 nanómetros. La presencia de proteínas y otros inhibidores se estimó a partir de los ratios de absorbancia 260/280 y 260/230, considerando valores aceptables de [1,8 ± 0,2]. Igualmente, la concentración de ADN se estimó mediante el sistema fluorométrico Qubit 4 (Fisher, Madrid, España).

IV-3.1.2 Análisis de asociación de genoma completo mediante chips de SNPs

Se utilizaron los chips de media (OvineSNP50 Infinium Beadchip de 50K) y alta densidad (AgResearch Sheep HD beadchip de 600K) de Illumina. El chip OvineSNP50 Infinium Beadchip de 50K tiene 54.241 SNPs distribuidos por todo el genoma con una distancia media entre SNPs de 46 kb, mientras que el AgResearch Sheep HD beadchip de 600K tiene 606.006 SNPs. Los servicios de genotipado de SNPs con el chip de media y alta densidad fueron proporcionados por el Centro Nacional de Genotipado (CEGEN-ISCIII) (https://www.usc.es/cegen/) y la empresa Xenetica Fontao (https://www.xeneticafontao.com/), respectivamente (manuscritos 2 y 3). Para ello, se les envío 1 μg de ADN genómico.

IV-3.1.3 Búsqueda de polimorfismos y genotipado en los genes candidatos para los estudios de asociación con los fenotipos analizados

IV-3.1.3.1 Búsqueda de polimorfismos

Para la búsqueda de polimorfismos en el gen ***LEPR*** asociados con la estacionalidad reproductiva (manuscrito 1), se seleccionaron 33 animales extremos del total de 239 ovejas para cada uno los caracteres DTA (bajos valores de DTA = 0 días, n = 15; altos valores de DTA: 149,3 ± 22,3 días, n = 18) y CiC (bajos valores de CiC: 0,24 ± 0,12, n = 18; altos valores de CiC: 0,88 ± 0,09, n = 15) del grupo experimental 1. El

gen *LEPR* está localizado en el cromosoma OAR1, con un tamaño de aproximadamente 99 kb con 20 exones (GenBank acc. nº NC_019458). Se secuenciaron mediante SANGER los exones 4, 6, 7, 8, 9, 10, 11, 12, 15, 16, 17 y 20 del gen *LEPR* (manuscrito 1). Estos exones se seleccionaron por tener polimorfismos no sinónimos en la base de datos Ensembl Variation (https://www.ensembl.org/info/genome/variation/index.html) en la versión Oar 3.1 del genoma ovino.

Por otra parte, se eligieron los genes candidatos ***CD226*** y ***NPY*** (manuscrito 2) para validar los resultados de GWAS sobre los caracteres de la estacionalidad reproductiva. Como en el caso del gen *LEPR*, para la búsqueda de polimorfismos responsables de parte de la varianza aditiva de los caracteres reproductivos, se seleccionaron 18 ovejas con valores extremos e intermedios de DTA (DTA baja: 0 días, n = 6; DTA intermedio: 56 ± 19,8 días, n = 6; DTA alta: 142,3 ± 15,7 días, n = 6). Los genes *CD226* y *NPY* presentan 6 y 4 exones y un tamaño de 100,7 y 6,8 kb, respectivamente. Del gen *NPY* se amplificaron y secuenciaron mediante SANGER todos los exones, mientras que del gen *CD226* se eligieron 4 exones (del 1 al 4). La selección de estos exones se realizó de la misma forma que en el caso del gen *LEPR*.

Finalmente, para validar los resultados de los estudios GWAS y caracteres de crecimiento, se aisló parcialmente el gen ***CYP7B1*** (manuscrito 3) con el objetivo de aislar polimorfismos responsables de la variabilidad de los caracteres. En este caso se seleccionaron 13 ovejas con diferentes genotipos para el SNP rs425509273 (5 CC, 5 CT y 3 TT), y con diferentes fenotipos para el carácter TC. Este SNP resultó estar asociado a la TC con significación genómica en los estudios GWAS. El gen *CYP7B1* se localiza en el cromosoma OAR9, con una longitud de aproximadamente 173,7 kb con 6 exones (GenBank acc. Number NC_056062). Los cebadores diseñados a partir de las secuencias NC_056062 y XM_004011703 se utilizaron para amplificar la región 5'UTR y la región codificante (exones 1-6), además de aislar parcialmente las regiones promotora y 3'UTR.

Los cebadores se diseñaron mediante el programa informático Primer3 (https://primer3.ut.ee/). Las reacciones de PCR se llevaron a cabo siguiendo un protocolo estándar en un volumen de 25 ml. Los cebadores, las condiciones de amplificación y el tamaño de amplificación para cada gen están descritos en cada uno de los manuscritos.

La visualización de los productos generados por PCR se realizó mediante electroforesis en geles de agarosa al porcentaje adecuado para el tamaño de ADN amplificado en tampón TBE (Tris-Bórico 0,045mM, EDTA 1mM) teñidos con SYBR Safe (Invitrogen, Carlsbad, CA, EE.UU.). Para comprobar el tamaño de los productos amplificados se utilizó marcador de talla de 1 kb y de 100 pb (Biotools, Madrid, España). La visualización de los fragmentos se realizó en un analizador de imágenes BioRAD modelo Gel Doc XR+ equipado con una pantalla de conversión Xcita Blue y con el software ImageLab vs. 4.0 (Sumalsa, Zaragoza, España). La purificación de los fragmentos de PCR se realizó mediante el kit comercial Favorprep GEL/PCR purification Minikit (FAVORGEN, IBIAN, Zaragoza, España), conforme a las instrucciones del fabricante. Posteriormente a la purificación de los fragmentos de interés, las muestras fueron preparadas y enviadas según las especificaciones del servicio de secuenciación automática SANGER de STABVIDA (Caparica, Portugal), y secuenciadas en ambos sentidos utilizando un secuenciador ABI 3730XL (Applied Biosystems, Foster City, CA, EE.UU.). Una vez recibidos los cromatogramas, se analizaron las secuencias nucleotídicas con los programas bioinformáticos Bioedit (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) y CLUSTAL Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Los estudios de homología se llevaron a cabo mediante el programa BLAST del NCBI (http://www.ncbi.nlm.nih.gov/BLAST/), mientras que la predicción del impacto de la sustitución aminoacídica fue llevada a cabo mediante los softwares PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) y Variant Effect Predictor (VEP: http://www.ensembl.org/Ovisaries/Tools/VEP?db=core). Para el análisis de la modificación de posibles sitios de unión para factores de transcripción, se utilizó el programa Alibaba 2.1 (http://gene-regulation.com/pub/programs/alibaba2/). La localización de los SNPs se determinó a partir de la versión del genoma de *Ovis aries* Oar_v3.1 (GCA_000298735.1) para los genes *LEPR, CD226* y *NPY*. Mientras que para el gen *CYP7B1*, la localización de los SNPs se determinó a partir del genoma Rambouillet v1.0 (GCA_002742125.1).

IV-3.1.3.2 Genotipado de los polimorfismos en los genes candidatos

Los polimorfismos de los genes ***LEPR***, y ***CD226*** se genotiparon mediante KASP (del inglés "Kompetitive Allele Specific PCR"). KASP es una PCR competitiva específica de alelo que se lleva a cabo en un termociclador a tiempo real, y permite la

identificación de SNP y de inserciones y deleciones (indels) en loci específicos. En resumen, la técnica se basa en la extensión de cebador específico de alelo y la liberación de una fluorescencia al hibridar los cebadores, que será posteriormente detectada por el sistema óptico del termociclador a tiempo real. En esta Tesis Doctoral, la discriminación alélica mediante la tecnología KASP se llevó a cabo en el termociclador a tiempo real Bio-Rad CFX96 (Bio-Rad, Madrid, España). Los cebadores para KASP fueron diseñados por LGC Genomics, proveedor de los ensayos KASP, mediante el envío de las secuencias flanqueantes al SNP (LCG Genomics, Beverly, MA, USA). La amplificación se realizó en placas de 96 micropocillos en un volumen final de 10 µl, conteniendo: 1 µl de ADN (50-100 ng), 5 µl de la mezcla de reacción KASP V4.0 2x con ROX estándar (LCG Genomics, Beverly, MA, USA), 0,14 µl de la mezcla de cebadores optimizada para la discriminación alélica (KASP-by-Design assay mix; LGC Genomics, Beverly, MA, USA), y 3,86 µL de agua libre de nucleasas. Las condiciones de amplificación fueron: una fase de activación a 94ºC durante 15 minutos; 10 ciclos de desnaturalización a 94ºC durante 20 segundos de hibridación y elongación a 57 y 61ºC según el SNP (con una bajada de 0,6 ºC por ciclo); 20 ciclos de desnaturalización durante 20 segundos a 94ºC e hibridación y elongación a 55ºC durante 1 minuto; y, finalmente, un ciclo a 30ºC durante 1 minuto. Finalmente, se hace una lectura de la fluorescencia a tiempo final tras este ciclo para obtener los genotipos Las temperaturas de hibridación específicas para cada uno de los SNPs se pueden consultar en los manuscritos 1 y 2. En todos los ensayos KASP se incluyeron controles negativos (sin ADN), y controles positivos de animales de genotipos conocidos previamente mediante secuenciación SANGER. En total se han analizado 8 SNPs mediante KASP: 6 y 2 SNPs no sinónimos en los genes *LEPR* y *CD226,* respectivamente (Tabla 1). Se diseñaron sondas para genotipar los 2 SNPs (OAR4: g.71593018G > T, y rs594346709) del gen *NPY*, pero fallaron. Por lo tanto, los dos SNPs silentes localizados en el exón 2 del gen *NPY* fueron genotipados por secuenciación SANGER, con los mismos cebadores y condiciones utilizadas para la búsqueda de polimorfismos (manuscrito 2) (Tabla 1).

Los polimorfismos detectados en la región 5' del gen *CYP7B1* fueron genotipados también mediante secuenciación SANGER en los 225 y 48 animales de los estudios GWAS (grupo experimental 1) y de validación funcional (grupo experimental 4), respectivamente. Para ello, se diseñaron dos cebadores (5′-

GCTCATGTCTTCCGCTGTC -3′; 5′- AAATCTCAGCCCCTTCCCC -3′), que amplificaban un fragmento de 360 pb. Mediante la secuenciación de este fragmento se genotiparon 5 polimorfismos (Tabla 1). Las condiciones de PCR están descritas en el manuscrito 3. La purificación y secuenciación están descritas en el apartado anterior.

IV-3.2 Estudios de expresión génica

IV-3.2.1 Extracción de ARN y secuenciación masiva del transcriptoma

El ARN total se extrajo de muestras de tejido (HT, PT, GP e hígado) utilizando el mini kit RNeasy Lipid Tissue (QIAGEN, Madrid, España), siguiendo las instrucciones del fabricante (manuscritos 3, 4 y 5). Para la extracción del ARN total de sangre periférica, se utilizó el kit TempusTM Spin RNA Isolation Reagent (Applied Biosystems, Fisher, Madrid, España) siguiendo las instrucciones del fabricante (manuscrito 6). Previamente, el tejido (HT, PT, GP e hígado) se disgregó y homogenizó utilizando el homogenizador OMNITIP (Grupo Taper, Madrid, España). El ARN extraído se congeló a -80ºC hasta la síntesis de cDNA. Como en el caso del ADN, la presencia de proteínas y otros inhibidores se estimó a partir de los ratios de absorbancia 260/280 y 260/230 considerando valores aceptables de [1,8 ± 0,2]. Igualmente, la concentración del ARN se estimó mediante el sistema fluorométrico Qubit 4, mientras que la integridad del ARN (RIN por sus siglas en inglés RNA Integrity Number) se comprobó mediante un bioanalizador Agilent 2100 (Agilent Technologies, Palo Alto, CA, EE.UU.) utilizando el chip RNA 6000 Nano LabChip. Para la construcción de las librerías, sólo se consideraron las muestras con un RIN > 7. La secuenciación se llevó a cabo en el Centro de Regulación Genómica que forma parte del Centro Nacional de Análisis Genómico (CNAG-CRG; https://www.cnag.crg.eu/).

IV-3.2.2 Síntesis de ADN complementario (cDNA)

En primer lugar, tratamos el ARN total (1 μg) de cada muestra con DNAsa (Invitrogen, Fisher, Madrid, España) para evitar la posible amplificación de ADN genómico. La síntesis de cDNA se llevó a cabo utilizando el kit SuperScript III Reverse Transriptase (Invitrogen, Fisher, Madrid, España), siguiendo las recomendaciones del fabricante.

IV-3.2.3 PCR en tiempo real

La caracterización funcional de los polimorfismos localizados en la región promotora del gen *CYP7B1* en muestras de hígado se hizo mediante qPCR (manuscrito 3). Para normalizar los resultados, se utilizaron tres genes candidatos de referencia (HKs: housekeeping; *GUSB*, *RPL37*, y *RPL19*).

Por otra parte, con el fin de confirmar y validar la expresión diferencial de los genes detectados mediante el análisis masivo del transcriptoma (RNA-Seq), se utilizaron GDEs entre tratamientos en cada uno de los modelos animales utilizados:

- En hembras de Rasa Aragonesa en diferente fase ovárica (F, L o AR): *ASPH, CAVIN3, CTGF, FBN3, HTR2B, ITPR3, LTA4H, MYLK, PTGIS* y *WFDC1* en HT*;* y *DDC, ITLN, MRPL57, PCDH15 y PLCXD2* en PT (manuscrito 4).
- En machos de raza Rasa Aragonesa con diferente capacidad de cubrición: *AVP, CGA, PRL_HT y GABRD* en HT*; CRABP1, GNG8, NPY* y *PRL_PG* en GP (manuscrito 5); e *inhibidor de la acrosina 1, CRYL1* y *SORCS2* en sangre periférica (manuscrito 6).

Además, en las hembras, seleccionamos un conjunto de cinco genes HKs *(RPL19, RPL27, RPL32, B2M* y *GAPDH)* utilizados habitualmente como genes de referencia en los estudios de expresión cerebral (manuscrito 4). En el caso de los machos, los resultados de los GDEs en tejidos cerebrales se normalizaron con los genes de referencia más estables en los estudios con estos tejidos en hembras: *RPL19* y *RPL32* para HT y GP. En el caso de la sangre, se utilizaron los HKs *B2M, GAPDH* y *RPL32*.

Los cebadores se diseñaron utilizando el programa Primer3 vs. 4.0 (http://primer3.ut.ee/). Cuando era posible, los cebadores se diseñaban en la unión exón-exón, o en exones diferentes con el fin de poder evitar la amplificación de ADN genómico. Una vez identificada la secuencia ovina de los genes a analizar mediante qPCR. Los cebadores de los genes de interés (*CYP7B1* y GDE en los estudios transcriptómicos) y de expresión constitutiva (HKs), el tamaño de amplicón y las condiciones de la PCR a tiempo real están descritos en los manuscritos 3, 4, 5 y 6.

Tabla 1. Información sobre los SNPs genotipados para los estudios de asociación en los genes *LEPR*, *CD226, NPY* y *CYP7B1.* Se indica la localización, identificador según la base de datos dbSNP (SNP) o la utilizada en los manuscritos, y el efecto de sustitución aminoacídica de los SNPs según los softwares "Variant Effect Predictor" y "PolyPhen-2" (los valores de predicción se indican entre paréntesis). Los SNPs se ordenan según sus posiciones en la versión del genoma Oar_rambouillet_v1.0

Gen	Identificador SNP	Localización	Posición en versión Oar_rambouillet_v1.0	Cambio nucleótido	Cambio aminoacídico	VEP (valor de SIFT)	PolyPhen-2 (valor)	Método de genotipado
LEPR	rs411478947	Exón 4	OAR1: g.43133198	C>T	Arg62Cys	Tolerado (0,05)	Posiblemente dañino (0,74)	KASP
	rs596133197	Exón 7	OAR1: g.43159974	C>T	Thr248Ile	Deletéreo (0)	Posiblemente dañino (0,98)	KASP
	rs403578195	Exón 8	OAR1: g.43164920	C>G	Ala284Gly	Deletéreo (0)	Posiblemente dañino (0,77)	KASP
	rs412929474	Exón 20	OAR1: g.43203879	G>A	Val923Ile	Tolerado (0,5)	Benigno (0,04)	KASP
	rs428867159	Exón 20	OAR1: g.43204167	C>T	Pro1019Ser	Tolerado (0,77)	Benigno (0,06)	KASP
	rs405459906	Exón 20	OAR1: g.43204317	A>G	Lys1069Glu	Tolerado (1)	Benigno (0)	KASP
CD226	rs588529642	Exón 2	OAR23: g. 8412570	A>G	Met60Val	Tolerado (0,1)	Benigno (0,03)	KASP
	rs404360094	Exón 3	OAR23: g.8482720	A>G	Asn243Asp	Tolerado (1)	Benigno (0,008)	KASP
NPY	-	Exón 2	OAR4: g. 78547891	G>T	Leu21 = [1]	-	-	SANGER
	rs594346709	Exón 2	OAR4: g. 78547941	G>A	Ser18 =	-	-	SANGER
CYP7B1	SNP3 (-114)	Promotor	OAR9: g.45801590	G>A				SANGER
	SNP4 (-97)	Promotor	OAR9: g.45801573	C>A				SANGER
	SNP5 (-63)	Promotor	OAR9: g.45801539	A>G				SANGER
	Indel (-58)	Promotor	OAR9: g.45801534	insACCCACACGCACCGCCCCGCTC				SANGER
	PolyC (-25)	Promotor	OAR9: g.45801510	PolyC				SANGER

[1] Sin cambio de aminoacídico

Previo a la cuantificación mediante PCR a tiempo real, los productos de amplificación se secuenciaron mediante SANGER, con el fin de verificar la correcta amplificación de los genes a estudiar mediante PCR convencional y condiciones estándar. Las muestras fueron purificadas mediante el kit comercial Favorprep GEL/PCR purification Minikit, siguiendo las especificaciones de preparación y envío indicadas por el Servicio de Secuenciación Automática de STABVIDA.

Para cuantificar la expresión de los genes de interés se utilizó el termociclador Applied BioSystems 7500 (Applied Biosystems, Fisher, Madrid, España) y placas de 96 micropocillos para qPCR selladas con film adhesivo para qPCR. La cuantificación se realizó utilizando cebadores específicos para cada gen y el fluoróforo SYBR green. Previamente a la cuantificación, se estudió la eficiencia de la amplificación mediante la realización de una curva estándar, construida a partir de seis diluciones seriadas 1, 1/10, 1/100, 1/1000 y 1/10000, 1/100000, a partir de un stock de cDNA, formado por 3 µl de cada uno de los cDNAs extraídos de cada tejido. La eficiencia de amplificación de todos los genes se ha calculado a partir de la fórmula $E = [10(^{-1/\ slope})]$, siendo "slope" la pendiente obtenida. La optimización de la concentración de los cebadores se ha realizado mediante la matriz de cebadores.

Cada muestra ha sido analizada por triplicado para cada gen de interés y HKs, cada una en un volumen final de 10 µl, conteniendo 1 µl de cDNA, 5 µl de mezcla de SYBR Green Master Mix: SYBR Premix Ex Taq II (Tli RNase H Plus, Takara, Demlab, Zaragoza, España), la concentración correspondiente de cada pareja de cebadores y un volumen de agua DEPC (dietilpirocarbonato, libre de ribonucleasas) hasta 10 µl. Las condiciones de amplificación están descritas en los diferentes manuscritos. En cada experimento se incluyó un control negativo indicador de posible contaminación. También se han incluido dos muestras comunes para cada gen y placa, que nos han servido para conectar placas para cada gen y evaluar el efecto placa en el modelo experimental. Las muestras y controles negativos se han colocado en diferentes posiciones en la placa para evitar posibles errores sistemáticos. La especificidad de la reacción de la PCR se ha verificado mediante la curva de disociación, la temperatura de disociación y el tamaño de producto amplificado para cada gen. La estabilidad de la expresión génica se determinó mediante NormFinder (Andersen et al., 2004) y los

resultados de la PCR a tiempo real se han normalizado con los genes de referencia HKs más estables.

Finalmente, se han estimado las variaciones de expresión relativa normalizada entre los diferentes grupos en ciclos de amplificación o ("Cq"), y se expresarán en n-veces de cambio de expresión (en inglés fold- change: "FC") entre dichos grupos.

IV-4 Análisis bioinformáticos y estadísticos

IV-4.1 Estudios de asociación

IV-4.1.1 Estimación del fenotipo corregido para los caracteres de crecimiento

Para los estudios de asociación se utilizaron como fenotipos los datos brutos sin corregir para los caracteres reproductivos, y la estimación de un valor corregido en función de los efectos conocidos para los fenotipos de crecimiento. Los fenotipos corregidos usados para el GWAS con los caracteres de crecimiento se obtuvieron a partir de los datos de CC y PV recogidos a lo largo de los 2 años de los experimentos. En total, se registraron 4.172 datos de CC y PV pertenecientes a las 225 ovejas. Los fenotipos corregidos se estimaron mediante un análisis de regresión lineal mixta con BLUPF90 (Misztal et al., 2018). El tercer fenotipo, la TC, se estimó mediante un análisis de regresión lineal con el programa informático estadístico R v3.5.1 (R Core Team, 2020), considerando el PV de las ovejas como variable dependiente y su edad como variable independiente. Las estimaciones individuales de la pendiente obtenidas fueron el fenotipo estimado por la TC (kg/día),

IV-4.1.2 GWAS

Para la detección de regiones genómicas y/o genes asociados a la estacionalidad reproductiva (manuscrito 2) y a los caracteres de crecimiento (manuscrito 3), se realizaron estudios GWAS incluyendo animales genotipados con el chip de 50K (Illumina OvineSNP50 BeadChip) y otros con el chip de 600K (Illumina AgResearch Sheep HD Beadchip de 600 K) diseñado por el Consorcio Internacional del Genoma Ovino. En la presente Tesis Doctoral, todo el flujo de análisis de los datos de genotipado para la edición y filtrado de calidad de los datos se realizó con el software PLINK 1.9 (Chang et al., 2015), eliminando aquellos SNPs que no cumplieran las siguientes condiciones: SNPs con una frecuencia del alelo menos frecuente (MAF) < 0,01; SNPs

que no estuviesen en equilibrio de Hardy – Weinberg (P < 0,001); SNPs con una frecuencia de individuos genotipados < 0,97; SNPs sin localización cromosómica en el genoma ovino; SNPs duplicados. Se eliminaron todos los animales con un porcentaje de SNPs genotipados inferior al 90%. A continuación, los dos conjuntos de datos, provenientes de los dos chips, se fusionaron con PLINK 1.9. El proceso de imputación de los genotipos del chip de alta densidad ausentes en los animales de la población genotipada con el chip de 50K se realizó con el programa Beagle 4.0 (Browning y Browning, 2007). Se llevó a cabo un análisis de escalamiento multidimensional (MDS) para conocer la posible estructuración de la población con el software PLINK 1.9. Se calcularon las distancias de identidad por estado (IBS) entre pares de individuos utilizando los SNPs que presentaron un DL entre SNPs inferior a 0,2 (r^2<0,2), con un tamaño de ventana de 50 SNPs, y avanzando 10 SNPs en cada interacción. Finalmente, se representó gráficamente el componente 1 frente al componente 2 en función de estos coeficientes. Igualmente, se llevó a cabo un análisis cluster aplicando una prueba de concordancia poblacional aplicando la opción -ppc.

Para la detección de regiones genómicas asociadas a los caracteres estudiados, se realizó un análisis GWAS con el programa GCTA (Genome-wide Complex Trait Analysis) (Yang et al., 2011) usando un modelo lineal mixto (MLMA) y con el MLMA-LOCO (leaving-one-chromosome-out; en el cuál dejan un cromosoma fuera del análisis al azar), incluyendo la edad y los clusters al que pertenecen los animales como covariable discreta, y la CC y PV como covariables cuantitativas en el caso del GWAS con fenotipos de estacionalidad reproductiva (manuscrito 2). En el caso del GWAS para los caracteres de crecimiento, se utilizaron fenotipos corregidos por las variables conocidas para el GWAS (ver apartado IV-4.1.1) (manuscrito 3). La matriz de relaciones genómicas (GRM, del inglés Genomic Relationship Matrix) estimada se incluyó en el análisis del modelo mixto para corregir el efecto de la posible subestructura de la población. Finalmente, los SNPs significativos fueron elegidos en base al valor P obtenido, utilizando la corrección de Bonferroni y de Benjamini-Hochberg (Benjamini y Hochberg, 1995) para comparaciones múltiples a nivel genómico. La significación de la asociación a nivel cromosómico también se evaluó utilizando un umbral del 10% (FDR = 0,1) (Hochberg, 2015). Para controlar el número de falsos positivos, se calcularon los factores de inflación genómica en el software R v3.5.1 (R Core Team,

2020) para cada carácter y GWAS. El factor de inflación genómica se estimó como la mediana $\chi 2$ observada dividida por la mediana $\chi 2$ esperada. La visualización de los datos de asociación en gráficos Manhattan y gráficos cuantil-cuantil se realizó utilizando el software SNPEVG (Wang et al., 2012). La anotación de genes en las regiones significativas (SNPs significativos para el GWAS), considerando un intervalo de 0,50 Mb y situando el SNP significativo en la posición media del intervalo, se realizó utilizando el ensamblaje del genoma ovino Oar_v3.1 basado en la versión 81 de Ensembl para los caracteres de estacionalidad reproductiva, y Oar_rambouillet 1.0 basado en la versión 101 de Ensembl para los caracteres de crecimiento. Como se ha descrito en el apartado IV-3.1.3, se seleccionaron los genes *CD226*, *NPY* y *CYP7B1* para validar los resultados de GWAS, por su localización en este intervalo y su función.

IV-4.1.3 Estudios de asociación de SNP con genes candidatos

Como se ha indicado en el apartado IV-3.1.3.2, para los estudios de asociación del gen *LEPR*, y la validación de los resultados de GWAS en los genes *CD226*, *NPY* y *CYP7B1,* se llevó a cabo el genotipado con KASP y secuenciación SANGER de los polimorfismos indicados en la Tabla 1. Las heterocigosidades observadas y esperadas, la frecuencia alélica menor (MAF) de cada SNP, así como el análisis del DL, se llevaron a cabo mediante el programa Haploview v4.2 (Barrett et al., 2005). Los estudios de asociación estadística entre los SNPs y los fenotipos en cuestión, se realizaron ajustando un modelo lineal mediante el paquete Rcmdr del paquete informático R (Fox et al., 2020) incluyendo diferentes efectos fijos y covariables:

- *LEPR*: genotipo, edad, la interacción de la edad y el genotipo como efectos fijos, y el PV y CC como covariables (n = 239).
- *CD226* y *NPY*: genotipo, edad y el genotipo como efectos fijos, y el PV y CC como covariables (n = 265).
- *CYP7B1*: el genotipo y el cluster al que pertenece el animal como efectos fijos (n = 205).

Finalmente, se estimaron las medias mínimo-cuadráticas (LSMeans) y error estándar de cada genotipo y SNP para cada fenotipo estudiado. Se utilizó el test de Bonferroni de comparaciones múltiples para comparar entre las LSMeans. Todos los polimorfismos de cada gen se analizaron independientemente con el mismo modelo

estadístico. Igualmente, y con el objetivo de estudiar la posible interacción entre los SNPs rs403212791 (*MTNR1A*; SNP asociado previamente a la estacionalidad reproductiva en Rasa Aragonesa; Calvo et al., 2018), rs403578195 (*LEPR*) y rs404360094 (*CD226*), se ha realizado un estudio de asociación para los fenotipos DTA y CiC, que incluyen como efectos fijos los genotipos de los tres SNPs conjuntamente, y como covariables la edad, PV y CC. Se llevaron a cabo varios modelos en el que se incluyeron adicionalmente como efectos fijos la interacción entre los genotipos de los 3 SNPs, así como interacciones por pares de SNPs. El análisis se llevó a cabo con el paquete Rcmdr ajustando un modelo lineal en 263 ovejas con genotipos para los 3 SNPs.

IV-4.1.4 Estudios de asociación haplotípica

Con el objetivo de confirmar los análisis de asociación y detectar otras posibles asociaciones que los análisis de SNPs individualmente no pudiesen capturar, se llevó a cabo un análisis de asociación haplotípica. Para predecir los bloques haplotípicos, se utilizó el programa Haploview utilizando el algoritmo basado en los 4 gametos (Barrett et al., 2005). Se utilizó el algoritmo de maximización de expectativas (E-M) para asignar los diferentes alelos de los SNPs a haplotipos individuales con PLINK1.9 (Purcell et al., 2007). Se descartaron aquellos diplotipos con una probabilidad posterior de predicción inferior a 0,7. Los estudios de asociación haplotípicas se realizaron utilizando el paquete Rcmdr ajustando un modelo lineal similar al utilizado para los estudios de asociación de SNP, pero incluyendo el efecto haplotipo (H; diplotipo) en lugar del efecto SNP (genotipo). El número de copias de cada haplotipo para cada animal se codificó como 0, 1 o 2 copias. Aquellos haplotipos con una frecuencia <1% no se incluyeron en el análisis. Como en el caso anterior, se estimaron las LSMeans y error estándar para cada haplotipo y fenotipo estudiado, utilizando la corrección de Bonferroni.

IV-4.2 Estudios del transcriptoma

IV-4.2.1 Caracterización de los diferentes grupos de comparación para el análisis transcriptómico

La caracterización de los diferentes **fenotipos de fase ovárica** en las hembras, y el establecimiento de los grupos de comparación se describen en el apartado IV-2.3 y manuscrito 4.

En el caso de los **fenotipos de actividad sexual en los machos**, el fenotipo de concentración plasmática de testosterona se analizó ajustando un modelo mixto mediante el paquete estadístico SAS v. 9.3 (SAS Institute, Cary NC, EE.UU.) incluyendo la edad, la CC, el PV, el diámetro testicular y la actividad sexual (suma de montas y cubriciones) como covariables, y el macho como efecto aleatorio. Se realizó un segundo modelo que incluía la actividad sexual como efecto fijo. En este caso, se definieron dos niveles para este factor: NA, sin ningún monta ni cubrición; y A, con al menos una monta o cubrición. El fenotipo de actividad sexual también se analizó ajustando un modelo de análisis de varianza utilizando la función "proc glm" de SAS e incluyendo como covariables la edad, la CC, el PV, el diámetro testicular y el contenido de testosterona. La concentración plasmática de testosterona y los fenotipos de actividad sexual también se analizaron transformando las variables continuas en categóricas, utilizando los cuantiles de las variables continuas.

Por último, se ejecutó un modelo de regresión basado en árboles de decisión para el fenotipo actividad sexual utilizando el "paquete Party" en R (Hothorn et al., 2006). Las variables analizadas fueron: PV, CC, edad, testosterona media, y peso testicular medio. Los animales se clasificaron en 3 nodos finales en función de la edad y CC, que fueron las variables significativas. Debido a la posible influencia de la edad y la CC en el fenotipo capacidad de cubrición, se eligieron animales pertenecientes a un único nodo (edad inferior o igual a 2.52, y una CC mayor de 3). Sobre estos animales (n = 21) se realizó un segundo test de aprisco (15 días después del primer test) en las mismas condiciones y con los mismos controles que el anterior. También se calculó una matriz de correlación de Pearson por pares entre variables, y un análisis de componentes principales (PCA; del inglés Principal Component Analysis) utilizando el paquete FactoMineR en R (Lê et al., 2008) con los datos del primer test de aprisco.

IV-4.2.2 Análisis de datos RNA-Seq

Existen diversas herramientas bioinformáticas que nos permiten hacer el análisis de los datos de un experimento de RNA-Seq y determinar así qué genes se expresan en diferentes condiciones y comparar sus niveles de expresión. La elección correcta del conjunto de herramientas informáticas es fundamental para realizar las comprobaciones de calidad necesarias, abordar los sesgos y, en última instancia, responder a las preguntas

planteadas por el estudio. Una vez obtenidos los datos de secuenciación del transcriptoma, el flujo general de trabajo comienza con el control de calidad de muestras secuenciadas (FastQC) y el alineamiento de las lecturas generadas frente al genoma de referencia. Del mismo modo, existen multitud de programas comerciales que permiten realizar el flujo de análisis completo de datos de RNA-Seq. Uno de los más populares es el OmicsBox (https://www.biobam.com/omicsbox), el cuál ha sido utilizado en la presente Tesis Doctoral. A continuación, vamos a describir los dos flujos de trabajo que hemos adoptado en esta Tesis Doctoral para analizar los datos de RNA-Seq.

IV-4.2.2.1 Flujo de trabajo RNA-Seq en hembras

El objetivo de este análisis fue conocer los diferentes factores genéticos y rutas bioquímicas que pueden estar implicadas en las diferentes fases ováricas y que, por lo tanto, podrían influir en la activación o inactivación del ovario, y de este modo estar relacionados con la estacionalidad reproductiva. La secuenciación masiva del transcriptoma en 21 muestras y 2 tejidos se hizo en un laboratorio externo generando unas lecturas pareadas de 72 pb. La empresa encargada de realizar los análisis de RNA-Seq nos envió tras la secuenciación masiva archivos FASTQ (formato basado en texto para almacenar tanto una secuencia de nucleótido y sus marcadores de calidad correspondientes codificadas con un solo carácter ASCII para mayor brevedad). El flujo de trabajo llevado a cabo aparece en la Figura 10. Se usó el programa TRIMMOMATIC v 0.38 (Bolger et al., 2014) para eliminar los adaptadores que se hayan secuenciado, y otras secuencias de mala calidad sobrerrepresentadas o contaminantes. Las lecturas pareadas fueron examinadas con FastQC versión 0.11.5 (Anders, 2010). Una vez comprobada la calidad de los ficheros FASTQ, se realizó el alineamiento contra el genoma de referencia Texel Oar_v3.1 (GCA_000298735.1) utilizando el software HISAT2 versión 2.1.0 (Kim et al., 2015). Posteriormente, se utilizó el software StringTie versión 1.3.5 (Pertea et al., 2015) para ensamblar los transcritos y cuantificar los mismos. A continuación, los niveles de expresión génica diferencial de los recuentos de lecturas obtenidos de StringTie se midieron en el entorno estadístico R versión 3.5.1, utilizando el paquete DESeq2 (Love et al., 2014). Para ambos tejidos (HT y PT), el diseño empleado estudió las siguientes comparaciones entre las fases ováricas: F vs. AR y L vs. AR. Antes de examinar la expresión, se realizó un paso de filtrado previo. Para mantener el gen en el análisis de expresión diferencial debe haber al menos cinco

muestras con la suma de recuentos de transcritos mayor o igual a 5. La identificación de los GDEs se realizó comparando los recuentos entre grupos de muestras para el mismo gen, y se consideraron GDEs cuando el valor p ajustado fue inferior a 0,05, tras la corrección de Benjamini y Hochberg para controlar el FDR (Benjamini y Hochberg, 1995), y |log2FoldChange| superior a 1. Para la interpretación biológica de los genes que han resultado GDEs en nuestro experimento se realizó un análisis de enriquecimiento funcional mediante la base de datos de anotación, visualización y descubrimiento integrado DAVID (del inglés Database for Annotation, Visualization and Integrated Discovery) (Huang et al., 2009).

Finalmente, se llevó a cabo un análisis de enriquecimiento funcional con GSEA (del inglés Gene Set Enrichment Analysis), que usa todo el conjunto de genes expresados en cada tejido y no sólo los GDEs. GSEA se utilizó para caracterizar cambios modestos, robustos, y coordinados, biológicamente relevantes en las vías de señalización molecular, y para explorar e identificar términos GO más significativos y sobrerrepresentados. GSEA clasifica las funciones biológicas del conjunto de genes basándose en tres factores: funciones moleculares, proceso biológico y componente celular. Para ello calcula la expresión diferencial (DE) por gen, y los genes se ordenan en una lista según su DE entre los grupos/tratamientos (Figura 11).

Para identificar los genes sobrerrepresentados en los extremos (superior o inferior) de toda la lista clasificada, se calculó un estadístico normalizado, denominado NES, para cada conjunto de genes. El nivel de significación se ajustó para tener en cuenta las pruebas de hipótesis múltiples utilizando FDR 5%. Este análisis se llevó a cabo dentro la plataforma OmicsBox de BioBam.

IV-4.2.2.2 Flujo de trabajo RNA-Seq en machos

La secuenciación masiva del transcriptoma en 12 muestras, 3 tejidos (HT, PT y GP) y sangre se hizo en un laboratorio externo, generando lecturas pareadas de 151 pb. El flujo de análisis bioinformático llevado a cabo aparece en la Figura 12. En primer lugar, se llevó a cabo un control de calidad de las secuencias con el programa FASTQC, que permite visualizar estadísticas de calidad de la secuenciación. Como en el caso de las hembras, se usó el programa TRIMMOMATIC y una vez comprobada la calidad de

los ficheros FASTQ, se realizó el alineamiento contra el genoma de referencia Oar_rambouillet 1.0 con el programa STAR.

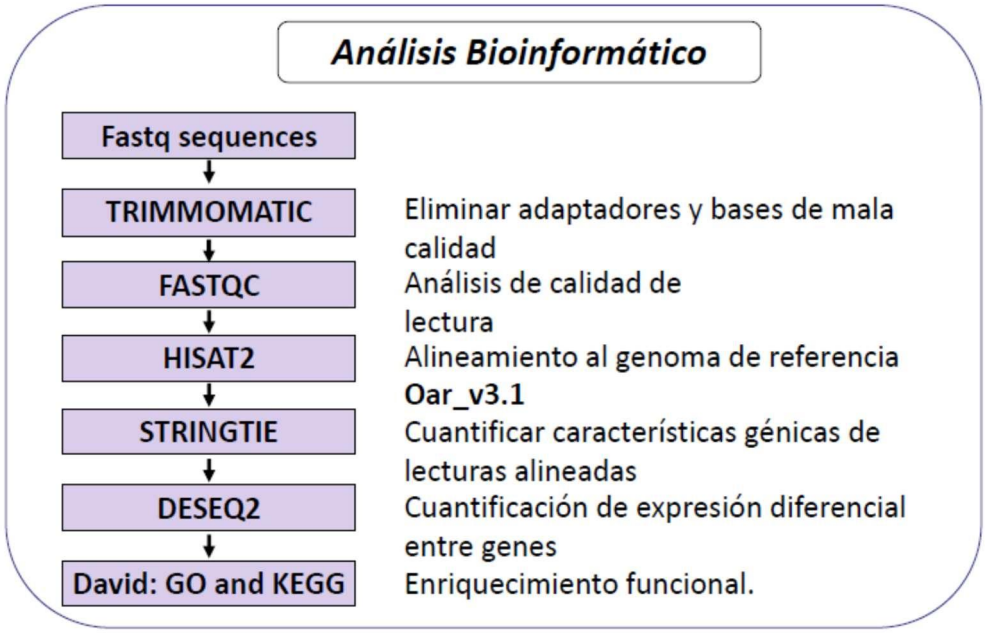


Figura 10. Flujo de trabajo llevado a cabo para el análisis del transcriptoma mediante secuenciación de nueva generación en hembras en diferentes fases del ovario.

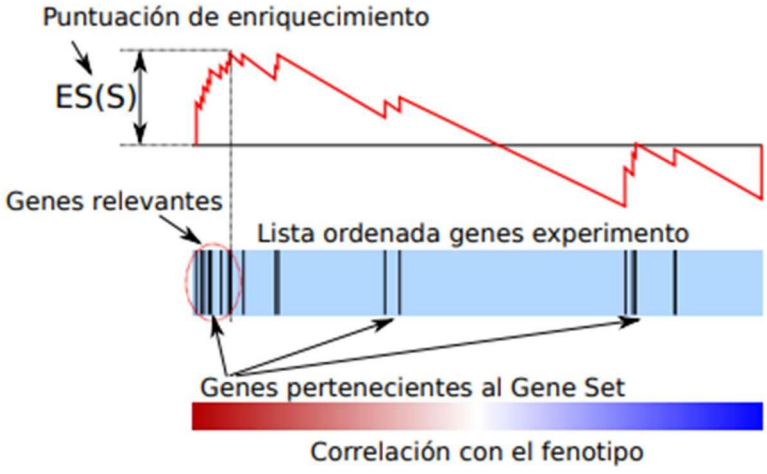


Figura 11. Esquema y resultados del método de análisis de enriquecimiento de conjuntos de genes (GSEA) (Tomado de Tabas Madrid, 2018)

Las salidas de este programa son ficheros BAM que se usaron para medir y cuantificar la expresión génica mediante el software HTSeq. Para juzgar si una lectura pertenece a un determinado gen, la función "HTSeq-count" proporciona tres modos: unión, intersección estricta e intersección no vacía (Figura 13). En la presente Tesis Doctoral, se ha utilizado el modelo de unión, recomendado por la mayoría los autores según la bibliografía. La detección de GDEs se llevó a cabo con el programa EdgeR. El diseño empleado estudió el contraste entre machos A *vs* NA. Sin embargo, para no alterar la potencia del análisis, se hizo un filtrado de los genes con recuentos bajos donde mantuvimos genes con valores de CPM (del inglés counts per million) superiores a 0,5 en al menos 5 casos para el análisis de HT, PT y GP. En la sangre, un umbral de CPM mayor o igual a 0,5 en al menos dos muestras y la media recortada de valores M (TMM) con el método de normalización de emparejamiento cero se aplicaron para filtrar los genes de recuento bajo. El análisis de enriquecimiento funcional se hizo con GSEA como se ha descrito en el apartado anterior. Todos los análisis se llevaron a cabo dentro la plataforma OmicsBox de BioBam.

IV-4.2.3 Análisis estadístico de la expresión génica por RT-qPCR

Se utilizó para la caracterización funcional de los polimorfismos localizados en la región promotora del gen *CYP7B1* en muestras de hígado (manuscrito 3) y para validar los resultados de RNA-Seq (manuscritos 4, 5 y 6). Se han estimado las variaciones de expresión relativa normalizada entre los diferentes grupos a partir de los Cq umbral. El método utilizado para la cuantificación ha sido el de cuantificación relativa mediante la utilización de modelos mixtos propuestos por Steibel et al., (2009). Los modelos utilizados aparecen detallados en los diferentes manuscritos. Además, se han calculado las correlaciones de Pearson entre los resultados de expresión mediante RNA-Seq y qPCR. Los resultados obtenidos permiten validar, o no, los resultados obtenidos mediante la técnica de RNA-Seq.

Fastq sequences	Lecturas en paired end de 151 bp
TRIMMOMATIC	Quitar adaptadores y bases de mala calidad
FASTQC	Analizar la calidad de lectura
STAR	Alinear al genoma de referencia **Oar_rambouillet 1.0**
HTseq	Cuantificar la expresión génica a partir de lecturas alineadas
EdgeR	Identificar genes expresados diferencialmente (DEGs)
GSEA	Análisis de enriquecimiento del conjunto de genes

Figura 12. Flujo de trabajo de RNA-Seq en OmicsBox

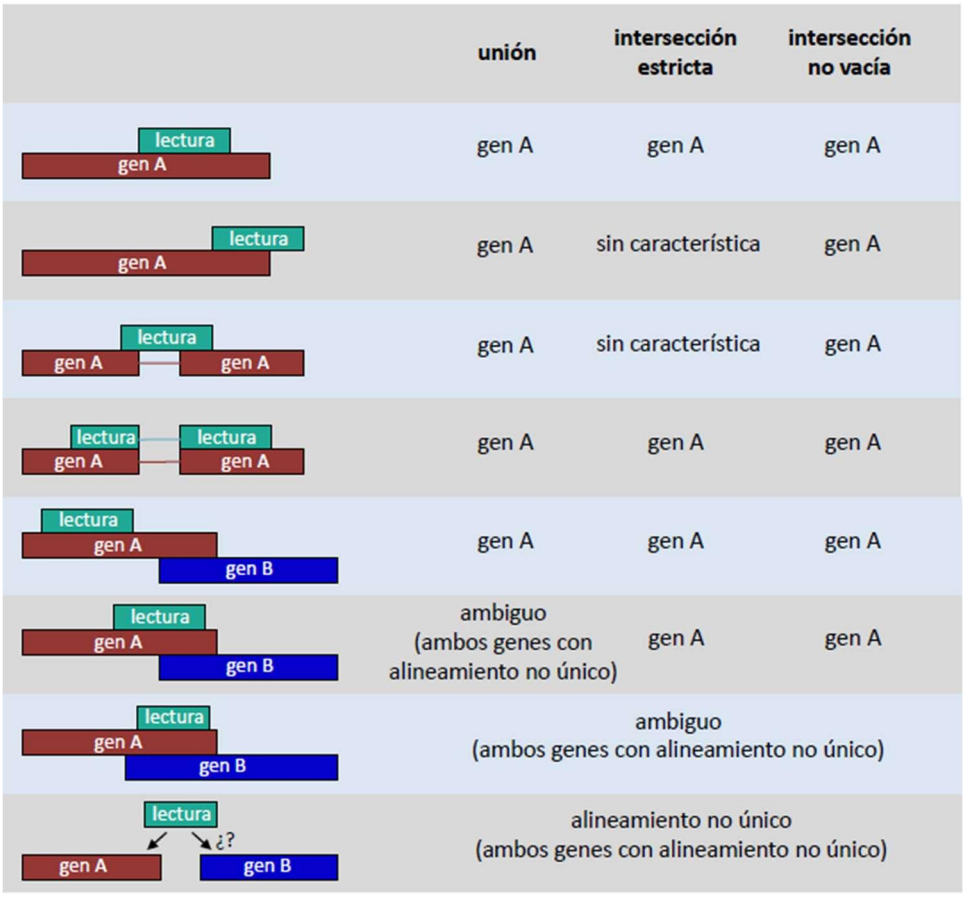


Figura 13. Los tres modos del programa HTSeq Count (Tomado de la página web del programa HTSeq: https://htseq.readthedocs.io/en/release_0.8.0/count.html**)**

IV-5 Disponibilidad de datos

Los conjuntos de datos analizados durante el estudio actual están disponibles en el repositorio Gene Expression Omnibus de NCBI y se puede acceder a ellos a través de los siguientes números de acceso:

- GEO Series GSE191213: (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE191213) Para RNA-Seq hembras en tejidos HT y PT.
- GEO Series GSE204861: (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE204861) para RNA-Seq machos en tejidos HT, PT y GP.
- GEO Series GSE218667: (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE218667) para RNA-Seq machos en sangre periférica.

En cuanto a los datos del GWAS, no fueron depositados en un repositorio oficial. Sin embargo, los datos que respaldan los hallazgos de estos estudios están disponibles bajo demanda.

V- Resultados y discusión

Los resultados y discusión de la presente Tesis Doctoral han sido presentados en el formato de 6 manuscritos científicos que se numeran a continuación:

- **Manuscrito 1:** Lakhssassi K, Serrano M, Lahoz B, Sarto MP, Iguácel LP, Folch J, Alabart JL, Calvo JH. 2020. "The *LEPR* gene is associated with reproductive seasonality traits in Rasa Aragonesa sheep". Publicado en la revista "Animals" 10(12): 2448. https://doi.org/10.3390/ani10122448.
- **Manuscrito 2:** Lakhssassi K, Lahoz B, Sarto P, Iguácel LP, Folch J, Alabart JL, Serrano M, Calvo JH. 2021. "Genome-Wide Association Study demonstrates the role played by the *CD226* gene in Rasa Aragonesa sheep reproductive seasonality". Publicado en la revista "Animals" 11(4):1171. https://doi.org/10.3390/ani11041171.
- **Manuscrito 3:** Lakhssassi, K., C. Meneses., Serrano, M. Calvo, J.H. "Genome-wide analysis reveals that the Cytochrome P450 Family 7 Subfamily B Member 1 (*CYP7B1*) gene is implicated in growth traits in Rasa Aragonesa ewes". En revisión en la revista "Animal".
- **Manuscrito 4:** Lakhssassi, K., Ureña, I., Marín, B., Sarto, M.P., Lahoz, B., Alabart, J.L., Calvo, J.H., Serrano, M. 2022. "Characterization of the *pars tuberalis* and hypothalamus transcriptome in female sheep under different reproductive stages". Publicado en la revista "Animal Biotechnology" (Online ahead of print). https://doi.org/10.1080/10495398.2022.2155174.
- **Manuscrito 5:** Lakhssassi, K., Sarto, M.P., Marín, B., Lahoz, B., Folch, J., Alabart, J.L., Serrano, M., Calvo, J.H. 2023. "Exploring differentially expressed genes in hypothalamic, *pars tuberalis* and pineal gland transcriptomes in different sexual behavior phenotypes in rams using RNA-Seq". Publicado en la revista "Journal of Animal Science" 101: skac365. https://doi.org/10.1093/jas/skac365.
- **Manuscrito 6:** Lakhssassi, K., Sarto, M.P., Lahoz, B., Alabart, J.L., Folch, J., Serrano, M., Calvo, J.H. 2023. "Blood transcriptome of Rasa Aragonesa rams with different sexual behavior phenotype reveals *CRYL1* and *SORCS2* as genes associated with this trait". Publicado en la revista "Journal of Animal Science" 101: skad098. doi: 10.1093/jas/skad098.

Manuscrito 1: The *LEPR* gene is associated with reproductive seasonality traits in Rasa Aragonesa sheep

animals

MDPI

Article

The *LEPR* Gene Is Associated with Reproductive Seasonality Traits in Rasa Aragonesa Sheep

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Simple Summary: Seasonality of reproduction is one of the limiting factor of sheep production, with the *leptin receptor* (*LEPR*) gene associated with some reproductive traits in different species. Thereby, we searched for polymorphisms in the ovine *LEPR* gene and associated them with three reproductive seasonality traits: the total days of anoestrous (TDA) and the progesterone cycling months (P4CM), both based on blood progesterone level and related to seasonal ovarian function; and the oestrous cycling months (OCM) as an indicator of oestrous behaviour. Two non-synonymous and non-linked single nucleotide polymorphisms (SNPs) in the *LEPR* gene were involved in the OCM trait (rs403578195 and rs405459906). These findings show for the first time the involvement of *LEPR* gene in seasonality reproduction in sheep and will help to improve genetic selection programs by implementing the genotyping of reproducers, which might increase the productivity of meat sheep.

Abstract: The aim of this study was to characterize and identify causative polymorphisms in the *leptin receptor* (*LEPR*) gene responsible for the seasonal variation of reproductive traits in sheep. Three reproductive seasonality traits were studied: the total days of anoestrous (TDA), the progesterone cycling months (P4CM) and the oestrous cycling months (OCM). In total, 18 SNPs were detected in 33 ewes with extreme values for TDA and OCM. Six SNPs were non-synonymous substitutions and two of them were predicted in silico as deleterious: rs596133197 and rs403578195. These polymorphisms were then validated in 239 ewes. The SNP rs403578195, located in exon 8 and leading to a change of alanine to glycine (Ala284Gly) in the extracellular domain of the protein, was associated with the OCM trait, being the G allele associated with a decrease of 12 percent of the OCM trait. Haplotype analyses also suggested the involvement of other non-synonymous SNP located in exon 20 (rs405459906). This SNP also produces an amino acid change (Lys1069Glu) in the intracellular domain of the protein and segregates independently of rs403578195. These results confirm for the first time the role of the *LEPR* gene in sheep reproductive seasonality.

Keywords: leptin receptor; reproductive seasonality; Rasa Aragonesa; SNP; haplotype

1. Introduction

The seasonality of reproduction in sheep is a general phenomenon in breeds originating from temperate climates, such as those raised in the Mediterranean basin. Changes in the photoperiod at temperate latitudes provide the main external cue that controls the timing of out-of-season fertility [1].

In Rasa Aragonesa, an autochthonous Mediterranean sheep breed from northeastern Spain reared for meat purposes, the maximal reproductive activity is associated with short days, with the highest percentage of ewes exhibiting ovulatory activities from August to March. This reproductive seasonality induces major variation in lamb production and therefore in the market price, which suffers a decline in the price of lambs from late spring to early fall when the lamb supply is the highest. To improve the reproductive efficiency of sheep, some producers use hormonal treatments and lighting manipulation, alone or in combination, to induce ewes to lamb out of season. Both treatments efficiently induce oestrous but add expenses to producers [2]. However, the increasing demand for hormone-free products has led to the search for alternative methods, such as the introduction of rams to previously isolated anoestrous ewes to ensure the induction of ovulation and oestrous (ram effect), nutritional flushing or the use of genetic markers to select as reproducers those animals with alleles associated with an increase in out-of-season fertility. In the case of Rasa Aragonesa, Folch and Alabart [3] showed that approximately 25% of ewes have spontaneous ovulations in spring and can be naturally mated throughout the year if management conditions and nutrition are suitable. It was proven that this spring ovulatory activity is under genetic control with heritability and repeatability values of 0.20 and 0.30, respectively [4]. Seasonality is a complex trait with a strong environmental influence, expressed only in ewes, and manifested relatively late throughout life, and only in some management systems [5]. In this context, genomic approaches have been used to detect genes or genome regions influencing the ability of ewes to lamb out of season [5–19]. Including the selection of genotypes less sensitive to reproductive seasonality in breeding programs would be an alternative to increase the profitability and efficiency of the ovine sector.

Leptin gene polymorphisms have drawn much attention from animal scientists for their possible roles in economically important productive and reproductive traits [20]. In fact, leptin is primarily known for its role in the regulation of whole-body energy balance by acting on the central nervous system and influencing fat deposition in animals through the control of appetite and energy expenditure [21]. Recent experimental evidence has shown that some SNPs in the *leptin receptor* (*LEPR*) gene are associated with reproductive traits [22,23]. The physiological effects of *LEPR* on reproduction, including puberty, the oestrous cycle, pregnancy, lactation, and even the early stages of embryonic development, have been proven [24,25]. Numerous research studies have shown that leptin controls sexual maturity at the hypothalamus level [26–28]. Moreover, the occurrence and involvement of leptin in the hypothalamus with the release of gonadotropic hormone confirms its role in sexual maturity or reproduction [29]. Thus, leptin seems to be an important link between metabolic status and the neuroendocrine axis [30]. However, melatonin influences reproductive function via activation of receptor sites within the hypothalamic-pituitary-gonadal axis [31]. The *melatonin receptor subtype 1A* (*MTNR1A*) is considered a key gene that mediates photoperiodic reproductive seasonality in sheep [5–8,12–19]. Furthermore, expression of *LEPR* was detected in the suprachiasmatic nucleus (SCN), the mammalian "biological clock", and the pineal gland of ruminant species [32], suggesting an interaction between photoperiod, melatonin, and leptin [1]. Although receptors for leptin and melatonin have not yet been colocalized, their presence has been demonstrated in similar hypothalamic regions in sheep [32–34].

Leptin and its receptor have been suggested as markers for enhancing productivity in livestock and are also potential candidates for marker-assisted selection [35]. In sheep, polymorphisms in *LEPR* have been associated with delayed onset of puberty and with decreased ovulation and lambing rates in prolific Davisdale sheep [36], but no studies have been performed concerning the *LEPR* gene and its involvement in seasonality reproduction in sheep. Therefore, this study aimed to identify polymorphisms in several regions of the *LEPR* gene in Rasa Aragonesa sheep and to test their association with reproductive seasonality traits.

2. Materials and Methods

2.1. Ethics Statement

All experimental procedures were performed in accordance with the guidelines of the European Union (2003/65/CE) and Spanish regulations (RD 1201/2005, BOE 252/34367e91) for the use and care of animals in research. No hormonal treatments were applied to the ewes during the study.

2.2. Animal Samples

As described by Martinez-Royo et al. [11], phenotypic seasonality data were obtained from a Rasa Aragonesa sheep flock managed in an experimental farm ("Pardina de Ayés") owned by Oviaragón S.C.L. The experimental period lasted from January to August 2012. The experimental flock was composed of 239 ewes in two age groups: 155 mature (5.2–7.2 y, 5.5 ± 0.5; mean ± SD) and 84 young (all 1.9 y, 1.9 ± 0.0) at the beginning of the experiment. Every three weeks, individual live weight (LW) and body condition score (BCS) on a 1 to 5 scale [37] were assessed. The mean LW and BCS were similar in both age groups. The pooled overall means and standard deviations for the whole experimental period were 52.5 ± 7.7 kg and 2.9 ± 0.3 for LW and BCS, respectively. Management of the ewes was described by Martinez-Royo et al. [11]; all ewes were handled in a single lot and subjected to the same management, nutrition and environmental conditions.

2.3. Measurement of Reproductive Seasonality Traits

Three reproductive seasonality traits were considered and described by Martinez-Royo et al. [11]. Briefly, the first one was the total days of anoestrous (TDA), based on weekly individual plasma progesterone levels. TDA was the sum of days in anoestrous, with anoestrous defined as those periods with three or more consecutive progesterone concentrations lower than 0.5 ng/mL. Likewise, ewes were not considered for this study if they were not cycling in the preceding breeding season (based on three samples taken one week apart in October), with progesterone levels under the threshold in all samples taken in January and with more than 4 consecutive samples higher than or equal to the threshold (possible pathological ewes). The second reproductive seasonality trait was the progesterone cycling months (P4CM), defined for each ewe as the rate of cycling months based on progesterone determinations. When the progesterone level was higher than or equal to 0.5 ng/mL in at least one blood sample in that month, the ewe was considered cyclic in a particular month. Finally, the third trait considered was the oestrous cycling months (OCM), defined as the rate of months cycling based on daily oestrous records for each ewe. Eight vasectomised rams fitted with harnesses and marking crayons were mixed with the ewes, and daily oestrous detection was performed [38]. Thus, after natural mating, oestrous was recorded as a colour mark on the rump of the ewes.

2.4. LEPR Gene Characterization

Genomic DNA was extracted from blood samples using standard protocols. The ovine *LEPR* gene is located on the chromosome OAR1, covering approximately 99 kb with 20 exons (GenBank acc. number NC_019458). Twelve exons were chosen to characterize the *LEPR* gene. These exons were selected based on having non-synonymous polymorphisms in the Ensembl Variation database (https://www.ensembl.org/info/genome/variation/index.html) on the Oar 3.1 version of the sheep genome. Primers were designed using Primer3 software version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/), and were designed in the intron regions around the targeted exon. The details of the oligonucleotide sequences, the annealing temperature and expected product size are summarized in Table 1. Polymerase chain reactions (PCRs) were performed in a 25 μL reaction including 25 ng of genomic DNA, 5 pmol of each primer, 200 nM dNTPs, 2.4 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100 and 1 U Taq polymerase (Biotools, Madrid, Spain). The cycling conditions were as follows: an initial denaturation step of 94 °C for 3 min, 35 cycles of 94 °C for 30 s, annealing temperature for 30 s, and 72 °C for 30 s except for fragments 5, 6 and 11, which were 45 s, and a final extension step

of 72 °C for 5 min. Direct Sanger sequencing of the PCR products from the 12 exons of 33 ewes with extreme values for TDA (low TDA: 0 days, n = 15; high TDA: 149.3 ± 22.3 days, n = 18) and OCM (low OCM: 0.24 ± 0.12, n = 18; high OCM: 0.88 ± 0.09, n = 15) were used to search for polymorphisms in the experimental population. The PCR products from genomic DNA were purified using the FlavorPrep Gel/PCR purification mini kit (Flavorgen, Ibian, Zaragoza, Spain) according to the manufacturer's instructions. The PCR products were sequenced in both directions by STAB Vida (Caparica, Portugal) using an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA, USA). The homology searches were performed using BLAST (National Centre for Biotechnology Information: https://blast.ncbi.nlm.nih.gov/Blast.cgi). For alignment of the sequences, CLUSTAL Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and BioEdit [39] software were used. For prediction of the possible impact of an amino acid substitution on the structure and function of a protein, Variant Effect Predictor software (VEP: http://www.ensembl.org/Ovisaries/Tools/VEP?db=core) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) [40] were used. Locations of SNPs were identified based on the genome version of *Ovis aries* Oar_v3.1. The secondary structure of the protein was determined from the amino acid sequence using CFSSP software (http://www.biogem.org/tool/chou-fasman/) [41].

Table 1. Primer sequences, location, annealing temperatures and amplification fragment sizes.

PCR	Primer Sequence (5′–3′) [1]	Site	[2] AT (°C)	Size (bp)
1	F: TTTTTCTGTGTCTTTTGAATGTCC R: AAGTAACAACTAATGCTTGGAACA	Exon 4	57	397
2	F: GCTCTTTAAGCTGGGTGTGC R: TTCAGCCTGTTTGAATGACTG	Exon 6	55	386
3	F: TGCTAAAAATTCATTTTGACTTCG R: GGAGGGCATCTCACCTTTTC	Exon 7	55	293
4	F: CTGTCGCCAGCTAACTCCTC R: CCTCCTTTTGAGTTACCACCA	Exon 8	55	378
5	F: TGCCTGGTGAATCCTTTTTA R: TCTCACCATATCCACAGAAAAAT	Exons 9–10	53	700
6	F: AGAGCTGGGAATTCAGAAATG R: TCTTTTCAATCCCACTGCAA	Exon 11	53	496
7	F: CTGCTTGGCAGGTGGATT R: CAGGAGGATGTATTTTATGCCAGT	Exon 12	55	392
8	F: TGCCTACCAATGGGAAATGT R: ATGGGAGGGGTTTGAAAGAT	Exon 15	55	383
9	F: CCTGCTTTCTCTTCCTTCTTCC R: TTTTTGAAGTTTTCATTAACTGTGTT	Exon 16	55	389
10	F: CCAGTTTCAATCCATAAATCATCA R: TGGCAGCATTGTTGCTAACT	Exon 17	55	299
11	F: TGAAGCAAAACAAAACAAAACA R: ACTCTCCTAACCAATGGTGAAA	Exon 20	52	974

[1] F: forward; R: reverse; [2] AT: annealing temperature.

2.5. LEPR Polymorphism Genotyping

Genomic DNA was extracted from blood samples of 239 ewes from the total ewes of the flock using standard protocols. Six non-synonymous SNPs were selected for genotyping the whole population: one in exon 4 (rs411478947), exon 7 (rs596133197), and exon 8 (rs403578195) and three in exon 20 (rs412929474, rs428867159, and rs405459906) (Table 2). Only non-synonymous SNPs were selected because they produce changes in the translated amino acid residue sequence and are more likely to affect the structure and function of the encoded protein and so may influence the phenotype of interest. These SNPs were genotyped by Kompetitive Allele-Specific PCR (KASP) following the manufacturer's instructions. Sequences flanking SNPs for the SNPs were submitted for assay design to the genotyping platform provider (LGC Genomics, Biotools, Spain). For all samples, the KASP assay was carried out in a 10 µL volume containing 20 ng of genomic DNA, 5 µL of KASP V4.0 2x Master mix standard

ROX (LCG Genomics, Beverly, MA, USA) and 0.14 µL of KASP-by-Design assay mix (LGC Genomics). Reactions were carried out in a CFX96 Bio-Rad thermocycler (Bio-Rad, Madrid, Spain) under the following conditions: 15 min at 94 °C followed by 9 cycles of 94 °C for 20 s and 57 °C for 1 min (dropping −0.6 °C per cycle to achieve a 55 °C annealing temperature), followed by 25 cycles of 94 °C for 20 s and 55 °C for 1 min. Following PCR, fluorescence was detected using a single quantification cycle for 1 s after cooling at 30 °C for 2 min.

2.6. Statistical Analysis

2.6.1. SNP Association Studies

The Hardy–Weinberg equilibrium exact test was applied and the observed and expected heterozygosities and the minor allele frequency (MAF) for each SNP were calculated using Haploview software v4.2 [42]. Statistical analyses were carried out as a regression of the phenotype values of the three reproductive seasonality traits on the SNP genotypes by fitting a linear model using the Rcmdr package of R software (http://socserv.socsci.mcmaster.ca/jfox/Misc/Rcmdr/) [43]. The model included the genotype of the SNPs (S), the age (mature and young) (A), and the interaction of age by genotype of the SNPs (A × S) as fixed effects and the live weight (LW) and body condition score (BCS) as covariates. To test differences between genotypes, we estimated the least square means (LSMs) for each pairwise comparison for the SNP and SNP x age effects. A Bonferroni correction was fitted to take into account multiple tests. All SNPs were independently analysed with the same statistical model.

2.6.2. Haplotype Association Studies

Blocks of linkage disequilibrium (LD) were evaluated with Haploview software v4.2 using the 4-gamete rule [42]. D′ and r^2 within the *LEPR* were calculated and visualized in Figure 1. SNPs were phased with PLINK1.9 [44] using the expectation–maximization (E–M) algorithm to assign individual haplotypes. We considered diplotypes with a posterior probability higher than 0.7. Associations between the haplotypes and reproductive seasonality traits were performed by fitting a linear model using the Rcmdr package of R software. The model fit was similar to that used for the SNP association studies but included the haplotype (H) effect and the interaction of age by haplotype (A × H). Haplotypes for each individual were codified as 0, 1 or 2, indicating the number of copies of each haplotype. Only haplotypes with a frequency greater than or equal to 1% were considered. To test differences between haplotypes, we estimated the LSMs for each pairwise comparison. The Bonferroni correction was applied to take into account multiple tests.

Table 2. Information about the location and amino acid substitution effect of the identified SNPs according to the Variant Effect Predictor and PolyPhen-2 software in the *LEPR* gene. Scores for these programs are indicated in brackets. The SNPs are ordered according to their positions in the Oar3.1 genome version (Oar3.1: GenBank acc. number NC_019458). The amino acid positions are ordered according to their positions in GenBank acc. number ENSOARP00000011154 sequence.

SNP	Alias [1]	Location	Position in OAR Version 3.1	Nucleotide Change	Amino Acid Change	VEP (SIFT Score)	PolyPhen-2 (Score)
rs411478947	snp_ex4	Exon 4	Oar1: g.40787726	C > T	Arg62Cys	Tolerated (0.05)	Possibly damaging (0.74)
rs159694506			Oar1: g.40787782	T > C	Asn80 = [2]	-	-
rs159694508			Oar1: g.40787821	T > C	Ser93 =	-	-
rs596133197	snp_ex7	Exon 7	Oar1: g.40813963	C > T	Thr248Ile	Deleterious (0)	Probably damaging (0.98)
rs403578195	snp_ex8	Exon 8	Oar1: g.40818703	C > G	Ala284Gly	Deleterious (0)	Possibly damaging (0.77)
rs416296450		Intron 9	Oar1: g.40825576	G > A	-	-	-
rs404892216		Intron 10	Oar1: g.40828606	A > G	-	-	-
rs407234698		Exon 12	Oar1: g.40833201	A > G	Pro561 =	-	-
rs421946862		Exon 16	Oar1: g.40840634	C > T	Ser791 =	-	-
rs401262081		Intron 16	Oar1: g.40840703	C > T	-	-	-
rs403654953		Exon 20	Oar1: g.40857538	C > T	Gly908 =	-	-
rs412929474	snp_ex20_1		Oar1: g.40857581	G > A	Val923Ile	Tolerated (0.5)	Benign (0.04)
rs426037269			Oar1: g.40857583	C > T	Val923 =	-	-
rs415715948			Oar1: g.40857634	C > T	Ala940 =	-	-
rs428867159	snp_ex20_2		Oar1: g.40857869	C > T	Pro1019Ser	Tolerated (0.77)	Benign (0.06)
rs405459906	snp_ex20_3		Oar1: g.40858019	A > G	Lys1069Glu	Tolerated (1)	Benign (0)
rs414501727			Oar1: g.40858045	C > T	Val1077 =	-	-
rs427778198			Oar1: g.40858219	G > A	Gln1135 =	-	-

[1] Nomenclature used for each SNP in this work. [2] No amino acid change.

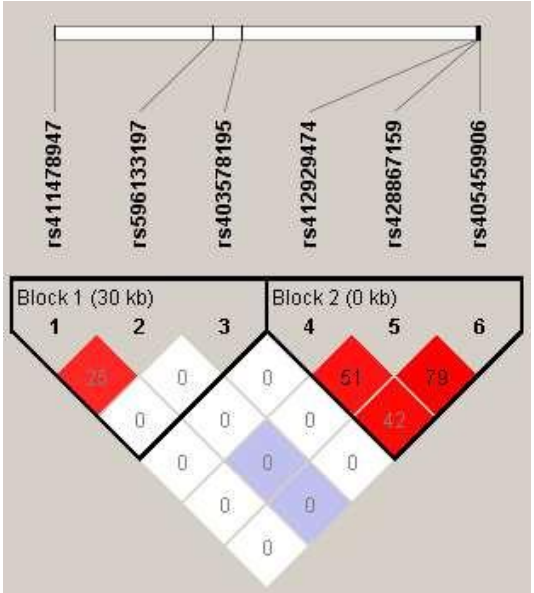

Figure 1. Linkage disequilibrium plot among the six non-conservative SNPs in *LEPR* using Haploview. The linkage disequilibrium colour scheme and values correspond with the D′ and r^2 parameters, respectively. Strong LD (D′ = 1, LOD ≥ 2) is indicated in red. Red indicates varying degrees of LD with lighter shades displaying less than darker shades (D′ < 1, LOD ≥ 2), and white indicates low LD (D′ < 1, LOD < 2).

3. Results

3.1. Isolation of the Partial Ovine LEPR Gene and Polymorphism Genotyping and Linkage Disequilibrium (LD)

To identify polymorphisms in the *LEPR* gene, we sequenced twelve exons that have non-synonymous polymorphisms in the Ensembl Variation database (Table 1) (https://www.ensembl.org/info/genome/variation/index.html). These exons were located at the beginning, middle and end regions of the gene. In total, 18 SNPs were detected: 3 and 8 SNPs in exons 4 (rs411478947, rs159694506 and rs159694508) and 20 (rs412929474, rs428867159, rs405459906, rs403654953, rs426037269, rs415715948, rs414501727 and rs427778198), respectively, and 1 SNP in exons 7 (rs596133197), 8 (rs403578195), 12 (rs407234698) and 16 (rs421946862) and in introns 9 (rs416296450), 10 (rs404892216) and 16 (rs401262081) (Table 2). Six of these SNPs were non-synonymous substitutions and were genotyped in the whole population, with 2 (snp_ex7 and snp_ex8) and 3 (snp_ex4, snp_ex7 and snp_ex8) of them predicted as deleterious or possibly/probably damaging by VEP or PolyPhen-2 software, respectively (Table 2). These SNPs showed low MAFs, ranging between 0.023 (snp_ex7) and 0.065 (snp_ex4) (Supplementary Table S1).

To determine the extent of LD among these markers, we estimated the parameters D′ and r^2 between all pairwise combinations of the six non-synonymous SNPs. The results of the LD analysis are shown in Figure 1, in which two LD blocks were predicted. Block 1 is composed of SNPs located in the extracellular domain of the protein (snp_ex4, snp_ex7 and snp_ex8), and Block 2 is composed of three missense mutations located in the cytoplasmic domain of the receptor in exon 20.

3.2. SNP Association Studies

For the association analyses, we used 239 ewes from which thirty-five ewes (29 adults and 6 young ewes) did not present anoestrous during the experiment based on TDA trait (TDA = 0). Similarly,

seventy-seven (60 adult and 17 young) and nine (7 adult and 2 young) ewes were cycling during all the experiments based on P4CM and OCM traits, respectively. All SNPs were in Hardy–Weinberg equilibrium. Only snp_ex8 showed a significant association with OCM (CC vs. GC genotypes, $p = 0.0027$). Ewes with the CC genotype showed more oestrous records (+0.12) than heterozygous ewes (Table 3). Details of type III test and LSMs for the SNP and the SNP x A effects for each SNP are provided in Supplementary Table S2. For the interaction effect between SNP and age, only the TDA phenotype differed among genotypes ($p < 0.05$) in young ewes for snp_ex20_1 after the Bonferroni correction (see Supplementary Table S2 for further details). Significant difference was found between the GG genotype and AG genotype ($p = 0.04$). Indeed, young ewes with the GG genotype had higher TDA values than heterozygotes. No significant differences were detected between the AA ($n = 2$) and AG ($n = 14$) genotypes in young ewes. It is worth noting that in our population, this SNP showed a low frequency for the A allele (0.10).

Table 3. Type III test for the significant SNPs and SNP by age (A) effects on the *LEPR* gene using the seasonal traits from Rasa Aragonesa ewes. The least square means (LSMs) and standard errors for the SNP and the SNP x A effects on the *LEPR* gene are also shown. Only significant SNPs after Bonferroni correction are shown. Different letters indicate significant differences: ^{a,b}: $p < 0.05$.

SNP	Trait	*p* Value SNP	A	SNP LSMs		
				CC	GC	GG
snp_ex8	OCM	0.003	All	0.54 ± 0.01 ^{a}	0.42 ± 0.03 ^{b}	-
SNP	**Trait**	***p* Value SNP × A**	**A**	**SNP × A LSMs**		
				AA	**AG**	**GG**
snp_ex20_1	TDA	0.0004	Young	80.6 ± 31.09 ^{a,b}	45.6 ± 11.98 ^{a}	83.3 ± 6.41 ^{b}

3.3. Haplotype Association Studies

Haplotype association studies was performed taking into account the two LD blocks predicted with Haploview (Figure 1) and a block containing all SNPs (Block 0). In total, 17, 5 and 6 haplotypes were found for blocks 0, 1 and 2, respectively (Supplementary Table S3). We only considered diplotypes with a posterior probability higher than 0.7 and haplotype frequency > 0.01. Thus, haplotype analysis was conducted considering 237/8, 237/4 and 235/4 ewes/haplotypes for blocks 0 (Supplementary Table S4), 1 (Supplementary Table S5) and 2 (Supplementary Table S6), respectively.

The significant association previously found between snp_ex20_1 by age and TDA phenotype was confirmed by haplotype association studies. In this sense, young ewes with no copies of the h1 (**A**TG) haplotype (in bold, snp_ex20_1) in block 2 had higher TDA values than those with 1 copy (Table 4). Moreover, in block 0, a significant effect was found for the h1 (GCC**A**TG) haplotype containing allele A of the snp_ex20_1, showing that young ewes with one copy of h1 had more oestrous events than those without copies (Table 4).

Haplotypes h2 and h8 of block 0 were also associated with OCM considering the whole population. Ewes with 0 copies of the h2 (GC**C**GT**G**) or h8 (GC**G**AT**G**) haplotypes (in bold, snp_ex8 and snp_ex20_3) showed more oestrous records than those with 1 copy. Notably, the h8 haplotype has the G allele (deleterious allele) of snp_ex8. Similarly, the analysis of block 1 showed that haplotype h1 (GC**G**), which also contains the G allele of snp_ex8, is associated with OCM. However, ewes with haplotype h2 for block 0 (GCCGT**G**) and block 2 (GT**G**) carry the G allele of snp_ex20_3, which was associated with less oestrous records (OCM), although this SNP did not show a significant *p*-value after Bonferroni correction in SNP association studies.

Table 4. Type III test for the haplotype and haplotype by age (A) effects for blocks 0, 1 and 2 on the *LEPR* gene using the seasonal phenotypic data from Rasa Aragonesa ewes. The least square means (LSMs) and standard errors for the haplotype effect on the *LEPR* gene are also shown. Only significant haplotypes after Bonferroni correction are shown. Different letters indicate significant differences: [a,b]: $p < 0.05$.

Haplotype Block [1]	Trait	Haplotype	Frequency	*p* Value Haplotype	A	Haplotype LSMs [2]		
						0 Copies	1 Copy	2 Copies
Block 0	OCM	h2 (GCCGTG)	0.07	0.002	All	0.53 ± 0.01 [a]	0.44 ± 0.03 [b]	-
	OCM	h8 (GCGATG)	0.01	0.004	All	0.52 ± 0.01 [a]	0.26 ± 0.10 [b]	-
Block 1	OCM	h1(GCG)	0.05	0.003	All	0.55 ± 0.01 [a]	0.42 ± 0.03 [b]	-
Block 2	OCM	h2 (GTG)	0.07	0.002	All	0.54 ± 0.01 [a]	0.44 ± 0.03 [b]	-
Haplotype Block [1]	**Trait**	**Haplotype**	**Frequency**	***p* Value Haplotype × A**	**A**	**Haplotype ×A LSMs [2]**		
						0 Copies	**1 Copy**	**2 Copies**
Block 0	OCM	h1(GCCATG)	0.09	0.004	Young	0.46 ± 0.02 [a]	0.66 ± 0.05 [b]	0.55 ± 0.14 [a,b]
Block 2	TDA	h1 (ATG)	0.10	0.0003	Young	83.9 ± 6.38 [a]	46.3 ± 11.97 [b]	81.4 ± 31.09 [a,b]

[1] Block 0: snp_ex4–snp_ex7–snp_ex8–snp_ex20_1–snp_ex20_2–snp_ex20_3; Block 1: snp_ex4–snp_ex7–snp_ex8; and Block2: snp_ex20_1–snp_ex20_2–snp_ex20_3. [2] 0 copy: LSMs and SE for 0 copies of the haplotype; 1 copy: LSMs and SE for 1 copy of the haplotype; and 2 copies: LSMs and SE for 2 copies of the haplotype.

4. Discussion

We detected 18 SNPs in the *LEPR* gene using 33 ewes with extreme values of TDA and OCM; six were non-synonymous substitutions, that were validated in 239 ewes. SIFT values varying from 0 to 1 were predicted for these SNPs by VEP software. SIFT scores lower than 0.05 suggest a potential intolerable amino acid substitution and a potential influence on protein function. In exon 4, a non-synonymous polymorphism (snp_ex4) promoting a change of arginine to cysteine at position 62 (according to their positions in GenBank acc. number ENSOARP00000011154) was detected, with this substitution predicted as tolerated but with a low SIFT value (0.05) and possibly damaging by VEP and PolyPhen-2 software, respectively (Table 2). Furthermore, arginine is a positively charged amino acid, whereas cysteine is polar in nature. This SNP was previously described by Haldar et al. [45] in Davisdale ewes. Of particular interest were two non-synonymous SNPs located in exons 7 and 8 and predicted as deleterious (SIFT = 0) by VEP analysis. The first SNP in exon 7 (snp_ex7) produces an amino acid change from threonine (polar) to isoleucine (non-polar) at position 248, whereas the second SNP in exon 8 (snp_ex8) produces a change from alanine (non-polar) to glycine (non-polar) at position 284. These three SNPs found in exons 4, 7 and 8 are located in the extracellular domain of the protein, where different amino acid substitutions have been associated with obesity in humans [46]. The three SNPs found in exon 20 were located in the cytoplasmic domain of the receptor. Two of them (snp_ex20_2 and snp_ex20_3) were previously described by Haldar et al. [45]. None of the three non-synonymous substitutions found in exon 20 were predicted as deleterious, being considered tolerated or benign, with SIFT values ranging from 0.5 (snp_ex20_1) to 1 (snp_ex20_3).

We also studied whether these mutations alter the secondary structure of the protein. In fact, four of these mutations alter the predicted secondary structure of the mature protein. Snp_ex4 promotes a putative loss of two turns, which increases the length of the random coil structure in two amino acids. Snp_ex7 and snp_ex8 putatively change one alpha helix motif by a β-pleated sheet and a turn motif, respectively. Finally, snp_ex20_3 should promote a change of a random coil by an alpha helix motif.

SNP association analysis showed that the non-conservative SNPs found in exons 4 and 7 were not associated with reproductive seasonality traits. These SNPs were predicted as tolerated (but with a low SIFT value) and deleterious, respectively, but they showed low MAF values (0.06 and 0.02 for snp_ex4 and snp_ex7, respectively) (Supplementary Table S1). Only one homozygous and no animals were found for the predicted tolerated and deleterious alleles (T alleles for both SNPs) of snp_ex4 and snp_ex7, respectively. However, Haldar et al. [45] found a strong association between snp_ex4 and puberty phenotypes ($p < 0.001$) but found a higher frequency for the T allele (0.47). These researchers reported that ewe lambs homozygous for the T allele in the *LEPR* gene were less likely to attain puberty at 1 year of age than those that did not carry the mutation in Davisdale sheep. Therefore, statistically significant effects were found concerning OCM and the deleterious SNP mutation in exon 8, showing a low MAF (0.06) and no homozygous animals for the putative deleterious allele. Heterozygous animals for this SNP showed fewer oestrous records (OCM trait) than homozygous animals. As OCM indicates behavioural signs of oestrous, it could be inferred that natural selection against homozygous animals for the deleterious allele has led to a low frequency of this allele. Haplotype association analysis confirmed these results. In fact, ewes whose haplotype contains the G allele (deleterious mutation) for snp_ex8 showed less oestrous events.

The interaction between snp_ex20_1 and age affected TDA in young ewes. However, the opposite effect was found in adult ewes (Supplementary Table S2). This finding could indicate that this mutation is not responsible for the observed effect but could be in LD with some causative mutation. In this sense, snp_ex20_1 was in LD with snp_ex20_2 ($r^2 = 0.51$) and snp_ex20_3 ($r^2 = 0.42$) in the predicted haplotype block 2 (Figure 1). Moreover, ewes with haplotype h2 for block 0 (GCCGT**G**) and block 2 (GT**G**), carrying the G allele of snp_ex20_3, had significantly lower OCM values after Bonferroni correction, indicating the putative involvement of this SNP of the *LEPR* gene on the seasonal phenotypes. The SNPs at exon 20 segregate independently from those located in the extracellular domain, and different effects in two different regions of the LEPR protein were found in this study. One of them, snp_ex8, is located in the CRHI/immunoglobulin-like domain of the extracellular domain of the protein, where different amino acid substitutions have been associated with obesity and disrupted pubertal development in humans [46]. The second mutation, snp_ex20_3, was not associated with puberty traits in the work of Haldar et al. [45]. This mutation was not predicted as deleterious and was located in the cytoplasmic domain close to a conserved region (called box 3) around position 1079 in the amino acid sequence. In humans, multiple splice variants of *LEPR* mRNA have been identified encoding an identical ligand binding domain but differing in the length of the cytosolic domain [47,48]. The LEPR isoforms A, B, C, and D have the same JAK binding motif encoded by exon 17. However, only the LEPR-B isoform contains the Box 3 motif encoded by exon 20 for STAT activation [47,49,50]. This isoform is expressed ubiquitously and constitutes up to 35% of the *LEPR* transcripts in the hypothalamus [51]. Only the full-length LEPR isoform (LEPR-B isoform) is able to fully transduce an activating JAK/STAT signal into the cell. Remarkably, the intracellular domain of the B isoform contains three tyrosine residues (Y986, Y1079 and Y1141) that activate the intracellular STAT signal transduction pathway. Y1079 plays a dominant role in activating STAT5, and Y1141 activates STAT3 [46]. Then, snp_ex20_3 (Lys1069Glu) could modify the STAT5 binding motif and disrupt the JAK/STAT signalling pathway. However, in Y1138S LEPR-B mutant females in mice, this mutation induced impaired STAT3 signalling with residual STAT5 function, but it did not cause infertility [52]. In livestock species, Almeida et al. [53] investigated the SNP (T945M) polymorphism in exon 20 of the *LEPR* gene in Angus, Brangus and Charolais cattle and found no associations with reproductive characteristics. The authors reported that blood leptin levels were influenced by this *LEPR* SNP in late pregnancy but not during lactation. It is important to note that associations between a mutation and the observed phenotypes are not direct evidence that the mutations caused the observed changes in phenotype. The observed relationship could indicate that the SNP is in linkage disequilibrium with the true causative mutation [36]. In our study, we used a small sample size of ewes ($n = 33$) with extreme values for reproductive seasonality traits to look for polymorphisms that could be segregating in this population. This design could

increase the power to detect polymorphic SNPs associated with the trait but minimized the probability of detecting other polymorphic SNPs. Furthermore, we did not sequence the complete coding region or regulatory regions, such as the promoter or 5′ and 3′ UTRs.

Although the relationships between individuals in the sample were unknown and then population stratification cannot be checked, the results with the SNP located in exon 8 are very consistent. This consistency is justified by the significant association with the OMC trait (in SNP and haplotype association analysis), the in silico prediction of the functional and structural consequences of this non-synonymous SNP (predicted as deleterious and affecting the secondary structure of the protein) and the location in the extracellular domain of the protein, where different amino acid substitutions have been associated with phenotype effects.

In summary, these results confirmed for the first time the involvement of the *LEPR* gene in reproductive seasonality. In this sense, several studies suggest that *LEPR* influences GnRH neuron activity and GnRH secretion by crosstalk with kisspeptin [54]. Kisspeptin cells are determinants of GnRH/LH secretion in the different seasons and are responsible for activation of reproductive function. Clarke et al. [55] reported that kisspeptin expression in the arcuate nucleus is markedly reduced during the nonbreeding period and increased in ewes exposed to a short photoperiod and in the follicular phase of the cycle in the breeding season, suggesting the involvement of kisspeptin neurons in this activation. Kisspeptin neurons regulate GnRH neurons and transmit sex-steroid feedback to the reproductive axis (the trigger of increased LH secretion and gonadal activation), whereas a negative feedback of oestrogen on GnRH secretion is characteristic of the nonbreeding season [55]. This finding is comparable to that reported by other authors [56,57] about pubertal development events. In knockout mice and individuals with impaired LEPR function, disruption of pubertal development was found, as in the case of Davisdale ewes [45].

5. Conclusions

In conclusion, one SNP predicted as deleterious located in the extracellular domain of the *LEPR* gene (snp_ex8) was strongly associated with the oestrous cycling months in Rasa Aragonesa sheep, confirming for the first time the role of the *LEPR* gene in reproductive seasonality in ruminants. Furthermore, another non-linked SNP in exon 20 was associated with this trait, as shown in the haplotype association analysis. This SNP could be in linkage disequilibrium with other SNPs not detected in this study. The G alleles of snp_ex8 and snp_ex20_3 are associated with higher OCM values, which indicate behavioural signs of oestrous in the Rasa Aragonesa breed. Genetic selection programs can be enhanced by implementing the genotyping of reproducers for these alleles related to reproductive seasonality, which might increase the productivity of meat sheep.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-2615/10/12/2448/s1, Table S1: Genotypic and allelic frequencies of the identified SNPs, Table S2: Type III test for the body condition (BC), live weight (LW), age (A), SNP, and SNP × age effects for the LEPR polymorphisms using the seasonality phenotype data from Rasa Aragonesa ewes. The least square means (LSMs) and standard errors of the *LEPR* polymorphisms in the seasonality phenotype data in Rasa Aragonesa ewes are also shown, Table S3: Haplotypes combination and frequency for block 0 (rs411478947-rs596133197-rs403578195-rs412929474-rs428867159-rs405459906), block 1 (rs411478947-rs596133197-rs403578195) and 2 (rs412929474-rs428867159-rs405459906). Only haplotypes with a frequency higher than 1% are shown, Table S4: Type III test for the body condition (BC), live weight (LW), age (A), haplotype (H), and haplotype × age (H × A) effects for the Block 0 haplotype using the seasonality phenotype data from Rasa Aragonesa ewes. The least square means (LSMs) and standard errors of the *LEPR* polymorphisms in the seasonality phenotype data in Rasa Aragonesa ewes are also shown, Table S5: Type III test for the body condition (BC), live weight (LW), age (A), haplotype (H), and haplotype × age (H × A) effects for the Block 1 haplotype using the seasonality phenotype data from Rasa Aragonesa ewes. The least square means and standard errors of the LEPR polymorphisms in the seasonality phenotype data in Rasa Aragonesa ewes are also shown, Table S6: Type III test for the body condition (BC), live weight (LW), age (A), haplotype (H), and haplotype × age (H × A) effects for the Block 2 haplotype using the seasonality phenotype data from Rasa Aragonesa ewes. The least square means and standard errors of the *LEPR* polymorphisms in the seasonality phenotype data in Rasa Aragonesa ewes are also shown.

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

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Supplementary Materials*

Table S1. Genotypic and allelic frequencies of the identified SNPs.

Table S2. Type III test for the body condition (BC), live weight (LW), age (A), SNP, and SNP× age effects for the *LEPR* polymorphisms using the seasonality phenotype data from Rasa Aragonesa ewes. The least square means (LSMs) and standard errors of the *LEPR* polymorphisms in the seasonality phenotype data in Rasa Aragonesa ewes are also shown.

Table S3. Haplotypes combination and frequency for block 0 (rs411478947-rs596133197-rs403578195-rs412929474-rs428867159-rs405459906), block 1 (rs411478947-rs596133197-rs403578195) and 2 (rs412929474-rs428867159-rs405459906). Only haplotypes with a frequency higher than 1% are shown.

Table S4. Type III test for the body condition (BC), live weight (LW), age (A), haplotype (H), and haplotype × age (H × A) effects for the Block 0 haplotype using the seasonality phenotype data from Rasa Aragonesa ewes. The least square means (LSMs) and standard errors of the *LEPR* polymorphisms in the seasonality phenotype data in Rasa Aragonesa ewes are also shown.

Table S5. Type III test for the body condition (BC), live weight (LW), age (A), haplotype (H), and haplotype × age (H × A) effects for the Block 1 haplotype using the seasonality phenotype data from Rasa Aragonesa ewes. The least square means and standard errors of the *LEPR* polymorphisms in the seasonality phenotype data in Rasa Aragonesa ewes are also shown.

Table S6. Type III test for the body condition (BC), live weight (LW), age (A), haplotype (H), and haplotype × age (H × A) effects for the Block 2 haplotype using the seasonality phenotype data from Rasa Aragonesa ewes. The least square means and standard errors of the *LEPR* polymorphisms in the seasonality phenotype data in Rasa Aragonesa ewes are also shown

*Para visualizar el material suplementario, se puede acceder a través del siguiente link: http://www.mdpi.com/2076-2615/10/12/2448/s1

Manuscrito 2: Genome-Wide Association Study demonstrates the role played by the *CD226* gene in Rasa Aragonesa sheep reproductive seasonality

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Genome-Wide Association Study Demonstrates the Role Played by the *CD226* Gene in Rasa Aragonesa Sheep Reproductive Seasonality

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Simple Summary: To elucidate the genetic basis of reproductive seasonality in Rasa Aragonesa sheep breed, we performed a genome-wide association study (GWAS) in order to detect single nucleotide polymorphisms (SNPs) or regions associated with traits related to ovarian function and behavioural signs of estrous. The GWAS included 205 ewes with genotypes for 583882 SNPs. Only one SNP overcame the genome-wide significance level. Nine potential SNPs overcame the chromosome-wise significance level (FDR 10%). Gene annotation demonstrated that *CD226 molecule* (*CD226*) and *neuropeptide Y* (*NPY*) genes that could be involved in reproductive seasonality were close to the significant SNPs. To validate the results, we sequenced the entire coding region of the *NPY* gene and four exons of the *CD226* gene to search for polymorphisms that could be involved in the phenotypes studied. Two synonymous and two nonsynonymous SNPs in the *NPY* and *CD226* genes, respectively, were genotyped in the whole population. We demonstrated that the AA genotype of the SNP rs404360094 located in exon 3 of the *CD226* gene was associated with higher and lower total days of anoestrus and oestrous cycling months, respectively. Therefore, this SNP could be utilized as a genetic marker for assisted selection marker to reduce seasonality.

Abstract: A genome-wide association study (GWAS) was used to identify genomic regions influencing seasonality reproduction traits in Rasa Aragonesa sheep. Three traits associated with either ovarian function based on blood progesterone levels (total days of anoestrus and progesterone cycling months) or behavioral signs of oestrous (oestrous cycling months) were studied. The GWAS included 205 ewes genotyped using the 50k and 680k Illumina Ovine Beadchips. Only one SNP associated with the progesterone cycling months overcame the genome-wide significance level (rs404991855). Nine SNPs exhibited significant associations at the chromosome level, being the SNPs rs404991855 and rs418191944, that are located in the *CD226 molecule* (*CD226*) gene, associated with the three traits. This gene is related to reproductive diseases. Two other SNPs were located close to the *neuropeptide Y* (*NPY*) gene, which is involved in circadian rhythms. To validate the GWAS, partial characterization of both genes by Sanger sequencing, and genotyping of two synonymous and two nonsynonymous SNPs in the *NPY* and *CD226* genes, respectively, were performed. SNP association analysis showed that only SNP rs404360094 in the exon 3 of the *CD226* gene, which produces an amino acid substitution from asparagine (uncharged polar) to aspartic acid (acidic), was associated with the three seasonality traits. Our results suggest that the *CD226* gene may be involved in the reproductive seasonality in Rasa Aragonesa.

Keywords: GWAS; reproduction; seasonality; oestrous; *CD226*; *NPY*

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1. Introduction

Sheep reproduction at temperate latitudes is widely known to exhibit marked seasonality [1]. The photoperiod represents a temporal signal to initiate changes in sheep reproductive status [2,3]. Lambing normally occurs at the end of winter-early spring, which causes seasonal variation in lamb production throughout the year. Accordingly, there is an imbalance between the availability of animal products and consumer demand. To overcome this disequilibrium, several alternatives have been developed, such as hormonal and/or photoperiod treatments. Nevertheless, at the initiative of the European Commission and to achieve "clean, green, and ethical" animal production [4], the use of hormones is being reviewed, primarily because it generates hormonal residues in animal carcasses. Therefore, breeders need other alternatives that minimize or completely avoid the use of hormonal treatments.

Differences in the duration of the breeding season between breeds and between individuals within a breed raised in the same region have been reported [5,6]. Ewes exhibiting spontaneous out-of-season ovulatory activity (SOA) are of considerable interest for identifying genes and mutations involved in molecular pathways controlling reproductive seasonality in sheep. In Rasa Aragonesa, Folch and Alabart [7] reported that approximately 25% of ewes have spontaneous ovulations in spring and can be naturally mated throughout the year with good management and feeding conditions. Therefore, developing a genetic approach for improving the out-of-season breeding ability of animals may represent a useful way to address this challenge. Hanocq et al. [8] reported relatively high heritability and repeatability estimates (0.20 and 0.30, respectively) for SOA in the Merinos d'Arles breed. Similar heritability of SOAs was found in the Chios breed in Greece [9] and in the Latxa breed in Spain [10]. Hence, the estimation of genetic parameters for SOA indicated that this trait could be used in selection [11].

Advances in genomic research and high-throughput genotyping techniques have enhanced the ability of researchers to search for mutations that underlie variations in complex traits [12,13]. Genome-wide association studies (GWAS) have become an important method for identifying genes and genomic regions associated with economically important traits in livestock. These studies are used to screen the whole genome for target genes that correlate with phenotypic traits using single nucleotide polymorphisms (SNPs) as genetic markers [14]. In sheep, progress has been made in the genetic basis of reproductive seasonality. In fact, three genes have been reported to be associated with out-of-season breeding: *melatonin receptor subtype 1A (MTNR1A)* [15–25], *arylalkylamine N-acetyltransferase (AANAT)* [26], and *leptin receptor (LEPR)* [27]. The first gene acts through high-affinity G-protein coupled receptors, one of which is melatonin receptor 1A encoded by the *MTNR1A* gene. Calvo et al. [28] and Lakhssassi et al. [27] showed that the SNPs rs403212791 in exon 2 of the *MTNR1A* gene and rs403578195 in exon 8 of the *LEPR* gene were associated with reproductive seasonality traits in the Rasa Aragonesa sheep breed. *AANAT* is involved in the biosynthesis of melatonin and controls daily changes in melatonin production. Additional candidate genes involved in seasonal breeding in sheep, such as the *aryl hydrocarbon receptor nuclear translocator-like protein (ARNTL)*, *casein kinase 1 epsilon (CSNK1E)*, *clock circadian regulator (CLOCK)*, *cryptochrome circadian regulator 1* (*CRY1)*, *period circadian regulator 1* (*PER1)*, *period circadian regulator 2* (*PER2)*, and *neuronal pas domain protein 4* (*NPAS4)*, have been reported in functional genomic studies of genes associated with circadian and circannual rhythms [29–31], although association studies of these genes with seasonality in ovine species have not been published to date.

As an initial attempt to identify genomic regions associated with reproductive seasonality in Rasa Aragonesa, our research group carried out a GWAS using 110 ewes genotyped by the Illumina OvineSNP50 Beadchip [32]. Several genes were identified near the significant SNPs, namely, *neuropeptide s receptor 1* (*NPSR1)*, *heparan sulfate-glucosamine 3-sulfotransferase 5* (*HS3ST5)*, *regulatory associated protein of mtor complex* 1 *(RPTOR)*, and *neuronal pentraxin 1* (*NPTX1)*, which are related to circadian and circannual rhythms. The aims of this study were the following: first, to perform a GWAS with a larger animal

sample (205 ewes) using medium (50k) and high-density (680k) arrays from an Illumina Ovine Beadchip to identify new SNPs underlying sheep reproductive seasonality traits. Second, we confirmed the results obtained in the GWAS analysis through partial characterization of the genes located close to significant SNPs with putative functions related to seasonal reproduction and subsequent genotyping of some SNPs identified in these genes for association studies.

2. Materials and Methods

2.1. Animal Samples

Phenotypic seasonality data were collected from an experimental flock of Rasa Aragonesa sheep, described by Martinez-Royo et al. [32]. Flock management was the same for all ewes. Briefly, 3 different flock age groups were considered for a total number of 265 ewes: 155 mature ewes (5.2–7.2 y; 5.5 ± 0.5; mean ± SD), 84 young ewes (1.9 ± 0.0 y), and 26 lambs (0.94 ± 0.0 y). From these 265 ewes, 205 were used for GWAS distributed as follows: 122 mature (5.4 ± 0.45 y), 66 young (1.9 ± 0.0 y) and 17 ewe lambs (0.94 ± 0.0 y). Every three weeks, individual live weight (LW) and body condition score (BCS) on a 1 to 5 scale [33] were assessed. The mean LW and BCS were similar in the mature and young ewe age groups. The pooled overall means and standard deviations for mature and young ewes for the entire experimental period were 52.5 ± 7.7 kg and 2.9 ± 0.3 for LW and BCS, respectively. Ewe lambs had LW and BCS values of 40.6 ± 3.8 kg and 2.8 ± 0.1, respectively. The experimental period lasted from January to August 2012.

2.2. Measurement of Reproductive Seasonality Traits

In Martinez-Royo et al. [32] are reported the three reproductive seasonality phenotypes analysed in this study in detail. The first two phenotypes were based on weekly individual plasma progesterone level measurements. The total days of anoestrous (TDA) were the sum of days in anoestrous. This period of ovarian inactivity was characterised by three or more consecutive progesterone concentrations lower than a threshold of 0.5 ng/mL. The second reproductive seasonality phenotype was the progesterone cycling months (P4CM), defined for each ewe as the rate of cycling months according to the progesterone level measurements. Progesterone was determined using a commercial ELISA kit designed for ovine plasma (Ridgeway Science, St. Briavels, Gloucestershire, UK). An ewe was considered cyclic in a month when the progesterone concentration was higher than or equal to the threshold of 0.5 ng/mL in at least one progesterone determination in that month. It is outstanding that ewes with progesterone levels below the 0.5 ng/mL threshold in all samples taken in January and with more than 4 consecutive samples higher than the threshold (potential pathological ewes) were discarded for the GWAS. In the same way, the ewes had to be cycling in the preceding breeding season, according to three progesterone determinations performed one week apart in October. Finally, the oestrous cycling months (OCM) was the third phenotype analysed. This trait was explained for each ewe as the rate of months cycling based on daily oestrous records, using eight vasectomised rams fitted with breeding harnesses with a replaceable marking crayon [34]. Consequently, oestrous was recorded daily as crayon marks on the rumps of the ewes.

2.3. Sampling and Genotyping Analysis

The GWAS included 110 ewes genotyped with the OvineSNP50 Infinium Beadchip (Illumina Inc., San Diego, CA, USA) designed by the International Sheep Genome Consortium [35], as employed in the study performed by Martinez-Royo et al. [32], and 97 ewes genotyped with the 680k (IlluminaAgResearchSheep HD). Genomic DNA was extracted from blood samples using the SpeedTools DNA Extraction kit (Biotools, Madrid, Spain). SNP genotyping services were provided by the Spanish centre "Centro Nacional de Genotipado (CEGEN-ISCIII)" (https://www.usc.es/cegen/; accessed on 19 April 2021) and "Xenetica Fontao" company (https://www.xeneticafontao.com; accessed on 19 April 2021).

2.4. Data Quality Control and Genome-Wide Association Analysis

The software Plink1.9 [36] was used for quality control (QC) of each genotyped data. Individuals with a low call rate (< 0.90) were excluded from additional analysis. The SNPs that met the following criteria were selected: call rate > 0.97 and minor allele frequency (MAF) > 0.01. SNPs that failed Hardy–Weinberg equilibrium (HWE) (p-Value < 0.001) were excluded. Next, the two datasets were merged with PLINK 1.9, and the Beagle4.0 program [37] was used to impute 50k to 680k genotypes. We performed clustering and multidimensional scaling (MDS) to check for population stratification using PLINK 1.9. SNPs that passed QC were pruned using the linkage disequilibrium (LD) pruning parameters of $r^2 < 0.2$ over a window size of 50 SNPs, and a step of 10 SNPs. Genome-wide identity-by-state (IBS) pairwise distances were calculated using all SNPs that remained after pruning. A pairwise population concordance test constraint was applied to the clustering procedure (-ppc option). GWAS was performed using the GCTA (Genome-wide Complex Trait Analysis) program [38] running a mixed linear model association (MLMA) and excluding the chromosome on which the candidate SNP is located (leaving-one-chromosome-out, or LOCO). The model also considered age and clusters obtained by MDS as a fixed effect, and BCS and LW effects as quantitative covariates. The estimated genetic relationships matrix (GRM) was included in the mixed model analysis to correct the effect of population substructure. The significance of association was assessed using Bonferroni correction and the false discovery rate (FDR) multitest correction tests. Chromosome-wise significance association was also assessed using a false discovery rate (FDR = 0.1) multitest correction threshold [39]. The choice of a threshold of 10% can be explained by the fact that in this study, the objective is mainly exploratory in order to identify new SNPs underlying sheep reproductive seasonality traits, and these are low heritable female traits. Visualization of the association data in Manhattan plots and quantile-quantile plots was performed using SNPEVG software [40]. To control the number of false positives, genomic inflation factors were calculated in R v3.5.1 software for each reproductive seasonality trait. The genomic inflation factor was estimated as the observed median χ^2 divided by the expected median χ^2.

2.5. Gene Identification

Genes located within a 500 kb-long interval centered on the significant SNPs associated with the three seasonality traits were obtained according to the sheep genome assembly (Oar_v3.1) and based on Ensembl release 81.

2.6. Validation of GWAS Results

2.6.1. CD226 and NPY Gene Characterization

Gene annotation based on the results of GWAS demonstrated that *CD226* and *NPY* genes could be involved in reproductive seasonality. The ovine *CD226* gene is located on chromosome 23, covering approximately 100.7 kb with 6 exons (GenBank acc. Number NC_040274), whereas the *NPY* gene is located on chromosome OAR4 and covers approximately 6.8 kb with 4 exons (GenBank acc. Number NC_040255). All exons of the *NPY* gene were characterized, while four exons were chosen to characterize the *CD226* gene. The significant SNPs were located in intron 2 of *CD226*, approximately 52 and 4 kb from exons 2 and 3, respectively. These exons were selected because they have nonsynonymous polymorphisms in the Ensembl Variation database (https://www.ensembl.org/info/genome/variation/index.html; accessed on 19 April 2021) of the Oar 3.1 version of the sheep genome. Primer3 software was used to design the primers (https://bioinfo.ut.ee/primer3--0.4.0/; accessed on 19 April 2021). Target-specific primers were designed in intron-flanking regions around the targeted exon. Table 1 shows the oligonucleotide sequences, the amplified exon, the annealing temperature, and expected product sizes. Genomic DNA was extracted from blood samples using standard protocols. The genomic DNA (25 ng) was amplified in a final polymerase chain reaction (PCR) volume of 25 µL containing 5 pmol of each primer, 200 nM dNTPs, 2.4 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, and 1 U

Taq polymerase (Biotools, Madrid, Spain). The following cycling conditions were used for all amplification fragments: an initial denaturation step of 94 °C for 3 min followed by 35 cycles of PCR, with cycling conditions of 30 s at 94 °C, 30 s at annealing temperature, and 30 s at 72 °C, and a final extension step of 72 °C for 5 min.

Table 1. Primer sequences, amplified exon (site), annealing temperatures (AT), and amplification fragment sizes.

Target Gene	Site	Primer Sequence (5′–3′) [1]	AT (°C) [2]	Size (bp)
CD226	Exon 1	F: GCATGATGGCAAGGATTTTT	52	486
	-	R: GCGTCATAAATTCTGAACGTG	-	-
	Exon 2	F: TTTCTGATATTTCTCTGGTGTTTCA	52	493
	-	R: GACCCCAAAATGGGATAAGG	-	-
	Exon 3	F: CCTCATATCCAAGAACTTGAGGA	52	498
	-	R:TGTATAAGAAAGTCATGAGAAAGACAA	-	-
	Exon 4	F: TCCCAACTTCCTCTCTATTCTAGC	55	212
	-	R: GCATCAGAATTACTCAGGAGGAG	-	-
NPY	Exon 1	F: CACAGGGGTTAGGGATCG	55	236
	-	R: AGCCATAAAAACCCTGTTGC	-	-
	Exon 2	F: AAGATGCCCATGATCTCCAG	55	300
	-	R: GAATTCCCTAAGCCCCCTTC	-	-
	Exon 3	F: CTTTCCCTGACCACCTTGAG	55	188
	-	R: AAGAACTTTTACTCCCCCAACC	-	-
	Exon 4	F: TGACGACAAAGGGAAACTGC	55	220
	-	R: TCTTACAAGCCTCCCAGGAA	-	-

[1] F: forward; R: reverse; [2] AT: annealing temperature.

Direct Sanger sequencing of the PCR products from the 8 exons in 18 ewes with extreme and intermediate values for TDA (low TDA: 0 days, n = 6; intermediate TDA: 56 ± 19.8 days, n = 6; high TDA: 142.3 ± 15.7 days, n = 6) was utilized to look for polymorphisms in the experimental population. The PCR products were purified using the FavorPrep Gel/PCR purification mini kit (Favorgen, Ibian, Zaragoza, Spain), and sequenced in both directions by STAB Vida company (Caparica, Portugal) using an ABI 3730XL sequencer (Applied Biosystems, Foster City, CA, USA). Homology searches were performed using BLAST (National Centre for Biotechnology Information: https://blast.ncbi.nlm.nih.gov/Blast.cgi; accessed on 19 April 2021). To align the sequences, BioEdit [41] software and CLUSTAL Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/; accessed on 19 April 2021) were used. The impact of amino acid substitution on the structure and function of the protein was predicted using the PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/; accessed on 19 April 2021) [42] and Variant Effect Predictor (VEP: http://www.ensembl.org/Ovisaries/Tools/VEP?db=core; accessed on 19 April 2021) softwares. The locations of SNPs were determined based on the genome version of *Ovis aries* Oar_v3.1.

2.6.2. CD226 and NPY Polymorphism Genotyping

Genomic DNA was extracted from blood samples of 265 ewes of the flock using standard protocols. Two nonsynonymous SNPs in the *CD226* gene were selected for genotyping the whole population by Kompetitive allele-specific PCR (KASP) following the manufacturer's instructions: one in exon 2 (rs588529642) and the second in exon 3 (rs404360094). The sequence surrounding the target polymorphism was submitted for assay design to the KASP genotyping provider (LGC Genomics, Biotools, Spain). Assays were performed in 96-well formats in a 10 µL volume containing 1 µL of DNA (20 ng final concentration of DNA), 5 µL of 2X KASP V4.0 master mix standard ROX (LGC Genomics, UK), 0.14 µL of assay mix (KASP by Design assay mix, LGC Genomics, UK) and 3.86 µL nuclease-free water. Reactions were performed in a CFX96 Bio-Rad thermocycler (Bio-Rad, Madrid, Spain) with conditions as follows: 94 °C for 15 min followed by 9 touchdown cycles of 94 °C for 20 s and 57 °C for 60 s (decreasing −0.6 °C per cycle) followed by 25 additional cycles of 20 s at 94 °C and 60 s at 55 °C. Following PCR, the plate was cooled to 30 °C, and fluorescence was read using a single quantification cycle for 1 s.

The assay designed to genotype the *NPY* gene failed. Therefore, the two synonymous SNPs located in exon 2 of the *NPY* gene (OAR4: g.71593018G > T, and rs594346709) were genotyped by Sanger sequencing (as described above in Section 2.6.1).

2.6.3. SNP Association Studies

Haploview software v4.2 [43] was used to calculate the Hardy–Weinberg equilibrium exact test, the observed and expected heterozygosities and the minor allele frequency (MAF) for each SNP. Statistical association studies between the SNPs and reproductive seasonality traits (TDA, P4CM, and OCM) were performed by fitting a linear model using the Rcmdr package of R software (http://socserv.socsci.mcmaster.ca/jfox/Misc/Rcmdr/; accessed on 19 April 2021) [44], including the genotype of the SNPs (S) and age (mature, young and ewe lambs) (A) as fixed effects. The LW and (CS were fitted as covariates. The least square means (LSMs) for each pairwise comparison were calculated to test differences between genotypes. The Bonferroni correction was used to consider multiple tests. The *CD226* and *NPY* SNPs were independently analysed with the same statistical model.

2.6.4. Haplotype Association Studies

Haploview software v4.2 was used to define blocks of LD based on the 4-gamete rule [43]. D′ and r^2 within the *CD226* and *NPY* were estimated. SNPs were phased using the expectation-maximization (E–M) algorithm to assign individual haplotypes with PLINK1.9 [45]. Diplotypes with a posterior probability lower than 0.7 were discarded. Haplotype association studies were performed using the Rcmdr package by fitting a similar lineal model to that used for the SNP association studies but included the haplotype (H) effect instead of the S effect. The number of copies of each haplotype were codified as 0, 1, or 2 copies. Haplotypes with <1% frequency were not included in the analysis. The LSMs for each pairwise comparison were calculated to test differences between haplotypes. The Bonferroni correction was used to consider multiple tests.

3. Results

3.1. GWAS Results

After the QC was performed on the imputed genotypes, 205 ewes with genotypes for 583882 SNPs distributed on the 27 ovine chromosomes were retained for subsequent analyses. In the MDS analysis, 188633 autosomal SNPs were used to calculate the pairwise IBS distance after SNP pruning. MDS analysis revealed a substructure within the total dataset and identified 4 principal clusters in the analysed population (Figure S1). These clusters were taken into account for subsequent association analysis. The GWAS results obtained through MLMA and LOCO were very similar. The genomic inflation factors for each trait were less than 1 (TDA: 0.96; P4CM: 0.97; OCM: 0.95), and then no more adjustment was needed.

Only one significant SNP (rs404991855) for P4CM was found at the genome-wise significance level after Bonferroni correction (Table S1). In the same way, a trend was observed for the same SNP and TDA trait ($p = 0.07$). Figure S2 presents the Manhattan and Q-Q plots for the three traits. Furthermore, 2, 7, and 4 SNPs were significantly associated at the chromosome-wise level (FDR $p < 0.10$) with TDA, P4CM, and OCM traits, respectively (Figure 1 and Table 2). Two SNPs (rs404991855 and rs418191944) located on chromosome 23 were found to be significantly associated with the three traits at the chromosome level (Figure 1 and Table 2). The seven SNPs associated with P4CM variability were located on chromosomes 4 (rs424340754, rs410373132), 6 (rs409834034), 7 (rs428238419 and rs405959180), and 23 (rs404991855 and rs418191944) (Figure 1c). Finally, four SNPs on chromosome 23 (rs405024177, rs404991855, rs418191944 and rs410842314) were associated with OCM (Figure 1b). The genes located 250 kb upstream and downstream of the most significant SNPs for each seasonality trait are shown in Table 2. Two genes were of interest because they may be related to reproductive seasonality trait variation. The *NPY* gene, and *CD226* gene that is also known as *DNAM-1* (*DNAX Accessory Molecule-1*). The significant

SNPs for P4CM in chromosome 4 were located 35 (rs424340754) and 47 kb (rs410373132) from *NPY* and were completely linked. The SNPs rs404991855 and rs418191944 were located in intron 2 of *CD226*.

3.2. Validation Studies

We sequenced the entire coding region of the *NPY* gene and four exons of the *CD226* gene to search for polymorphisms that could be involved in the phenotypes studied. No deleterious mutations were detected. For the *NPY* gene, we found two synonymous SNPs in exon 2. One of these mutations was not previously described (OAR4:g.71593018 G > T) (Table 3). These SNPs showed low MAFs, ranging between 0.025 (g.71593018 G > T) and 0.082 (rs594346709) (Table S2). Although these mutations were synonymous, they were genotyped in the whole population, since they might be in linkage disequilibrium with putative causative mutations.

For the *CD226* gene, three SNPs were detected in exon 2 (rs427511555, rs403900117 and rs588529642), and one SNP was detected in exon 3 (rs404360094). Two of these SNPs (rs588529642 and rs404360094) were nonsynonymous substitutions and were selected to be genotyped in the whole population. These SNPs were predicted in silico to be tolerated by VEP and benign by PolyPhen-2, showing that the SNP located in exon 2 (rs588529642) had a lower SIFT value (0.1) (Table 3). This SNP produces a change of methionine to valine (Met60Val), both of which belong to hydrophobic groups (nonpolar). Conversely, the SNP located in exon 3 (rs404360094) produces an amino acid substitution from asparagine (uncharged polar) to aspartic acid (acidic) at position 243.

To determine the extent of LD among these markers, we estimated the parameters D′ and r^2 between the pairwise combination of the SNPs in the same chromosome. Very low LD was found. LD indices for SNPs located in the *CD226* gene were 0.06 and 0 for D′ and r^2, respectively. For SNPs located in the *NPY* gene, 1 and 0.002 were observed for D′ and r^2, respectively. One haplotype block was predicted for SNPs located in exon 2 of the *NPY* gene.

3.3. SNP Association Studies

Association analyses included 265 ewes. Table 4 shows results after Bonferroni correction. Only the SNP located in exon 3 of the *CD226* gene was significantly associated with the three reproductive seasonality traits (see Table S3 for further details). In fact, significant differences were observed among alternative genotypes. Ewes with the AA genotypes had higher TDA values than ewes with AG (50.80 $\pm$ 12.76; $p < 0.01$) or GG (42.52 $\pm$ 12.81; $p < 0.05$) genotypes. Similarly, ewes with the AA genotype showed fewer oestrous records than those with AG (-0.17 ± 0.05; $p < 0.01$) or GG (-0.13 ± 0.05). Similar results were observed for the P4CM trait.

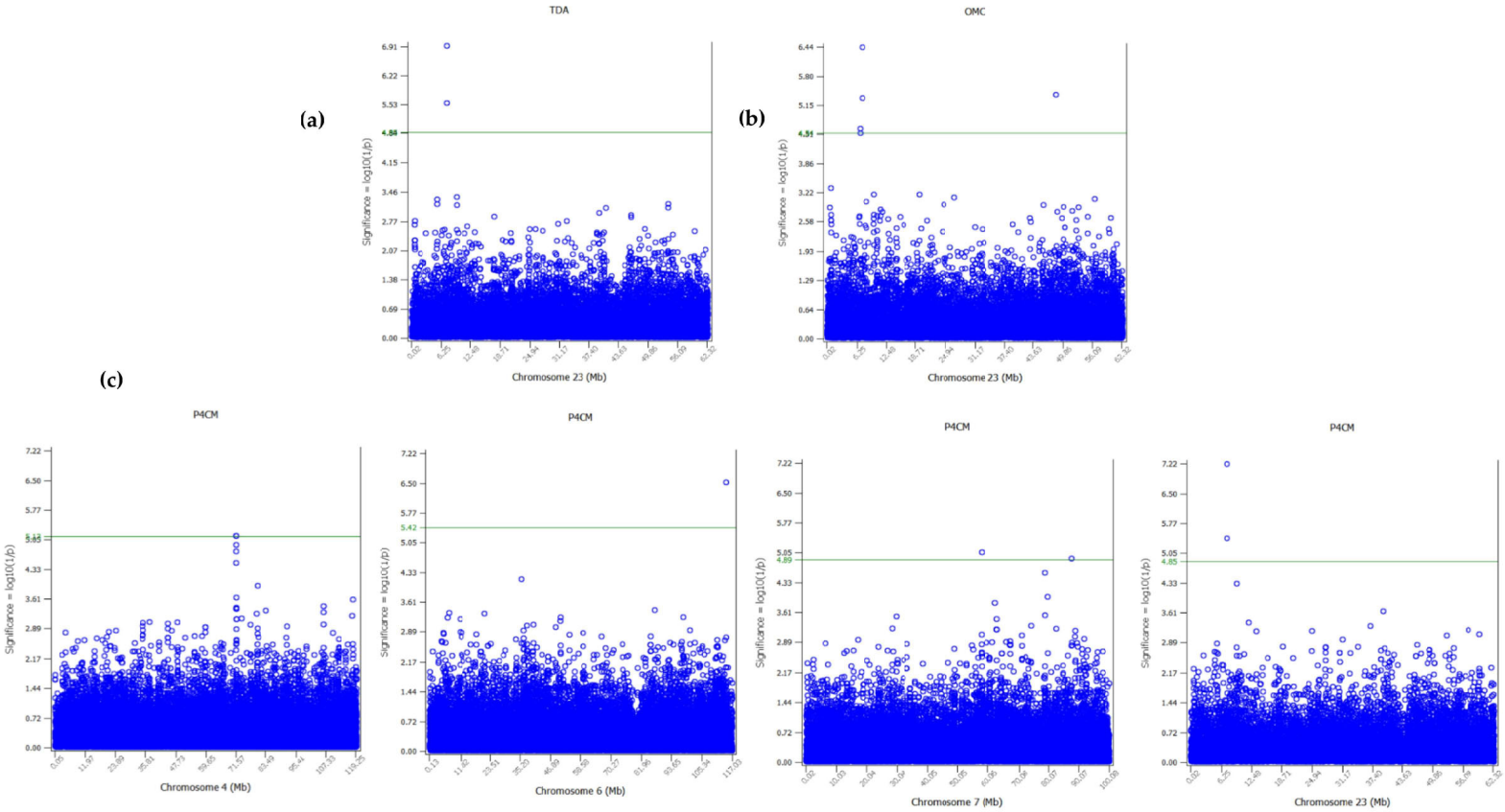

Figure 1. Manhattan plots of chromosome-wise association studies of (**a**) total days of anoestrous (TDA), (**b**) oestrous cycling month (OCM) and (**c**) progesterone cycling month (P4CM) traits. Only chromosomes with significant SNPs are shown. The horizontal green line in Manhattan plots corresponds to the average threshold value for an FDR of 10% evaluated at the chromosomal level.

Table 2. Significant SNPs at the chromosome-wise level associated with each seasonality trait. The SNPs are ordered according to their positions in the Oar 3.1 genome version (Ensembl release 81). Minor allele frequency (MAF) is also indicated. Putative causal genes located in the 250 kb region on both sides of the significant SNPs are indicated.

Trait	SNP	dbSNP	Chr	Position	MAF	*p*-Value	Genes within 250 kb on either Side
TDA	oar3_OAR23_7427625	rs404991855	23	7427625	0.40	1.22×10^{-7}	*RTTN -CD226*-DOK6
	oar3_OAR23_7428353	rs418191944	23	7428353	0.39	2.77×10^{-6}	*RTTN -CD226-DOK6*
P4CM	oar3_OAR4_71540823	rs424340754	4	71540823	0.22	7.12×10^{6}	*NPY*
	oar3_OAR4_71552651	rs410373132	4	71552651	0.22	7.12×10^{-6}	*NPY*
	oar3_OAR6_114690755	rs409834034	6	114690755	0.21	3.01×10^{-7}	*ENSOARG00000011847-ENSOARG00000013314-LRPAP1-ENSOARG00000013472-ENSOARG00000013494 -ENSOARG00000013502- RGS12*
	oar3_OAR7_57807908	rs428238419	7	57807908	0.04	8.59×10^{-6}	*DTWD1- ENSOARG00000020999- FGF7-GALK2*
	oar3_OAR7_87670575	rs405959180	7	87670575	0.12	1.22×10^{-5}	*ENSOARG00000002769*
	oar3_OAR23_7427625	rs404991855	23	7427625	0.40	6.07×10^{-8}	*RTTN -CD226-DOK6*
	oar3_OAR23_7428353	rs418191944	23	7428353	0.39	3.83×10^{-6}	*RTTN -CD226-DOK6*
OCM	oar3_OAR23_6962033	rs405024177	23	6962033	0.08	2.30×10^{-5}	*SOCS6- RTTN*
	oar3_OAR23_7427625	rs404991855	23	7427625	0.40	3.63×10^{7}	*RTTN -CD226-DOK6*
	oar3_OAR23_7428353	rs418191944	23	7428353	0.39	4.86×10^{-6}	*RTTN -CD226-DOK6*
	oar3_OAR23_48239663	rs410842314	23	48239663	0.21	4.09×10^{-6}	*ZBTB7C-CTIF*

Table 3. Information regarding the location and amino acid substitution effect of the identified SNPs according to the Variant Effect Predictor and PolyPhen-2 software in the *NPY* and *CD226* genes. Scores are indicated in brackets. The SNPs are ordered according to their positions in the Oar3.1 genome version (Oar3.1: GenBank acc. Numbers NC_019461 and NC_019480 for *NPY* and *CD226*, respectively).

Gene	dbSNP	Location	Position in OAR Version 3.1	Nucleotide Change	Amino Acid Change	VEP (SIFT Score)	Polyphen-2 (Score)
NPY	-	Exon 2	OAR4:g.71593018	G > T	Leu21 = [1]	-	-
-	rs594346709	Exon 2	OAR4:g.71593068	G > A	Ser18 =	-	-
CD226	rs427511555	Exon 2	OAR23:g.7375331	G > A	Thr25 =	-	-
-	rs403900117	Exon 2	OAR23:g.7375377	T > C	Leu41 =	-	-
-	rs588529642	Exon 2	OAR23:g.7375434	A > G	Met60Val	Tolerated (0.1)	Benign (0.03)
-	rs404360094	Exon 3	OAR23:g.7432390	A > G	Asn243Asp	Tolerated (1)	Benign (0.008)

[1] No amino acid change.

Table 4. Type III test for the SNP located at exon 3 of the *CD226* gene on seasonal traits (total days of anoestrous (TDA), oestrous cycling month (OCM) and progesterone cycling month (P4CM)) of Rasa Aragonesa ewes. The least square means (LSMs) and standard errors of alternative genotypes are also shown. Only significant SNPs after Bonferroni correction are shown. Different letters indicate significant differences: **a**, **b**: $p < 0.05$.

Trait	*p*-Value SNP	SNP LSMs		
		AA	AG	GG
TDA	0.0003	120.7 ± 12.21 **a**	69.9 ± 4.88 **b**	78.1 ± 4.94 **b**
P4CM	0.0006	0.64 ± 0.04 **a**	0.83 ± 0.01 **b**	0.79 ± 0.01 **b**
OCM	0.001	0.29 ± 0.05 **a**	0.50 ± 0.02 **b**	0.46 ± 0.02 **b**

3.4. Haplotype Association Studies

Haplotype association studies were performed, considering block 1, which was predicted with Haploview (the two SNPs in exon 2 of the *NPY* gene), and a second block containing the two nonsynonymous SNPs of the *CD226* gene (block 2). Three haplotypes were identified for each block (Table S4). Only diplotypes with a posterior probability higher than 0.7 and haplotype frequency > 1% were considered. The results of haplotype

analysis are presented in detail in Tables S5 and S6 for blocks 1 and 2, respectively. After Bonferroni correction, only haplotypes H1 and H2 of block 2 were associated with the three reproductive seasonality traits, thereby confirming the previous association found in SNP analysis (Table 5). In this sense, ewes with one or 2 copies of haplotype H1 (A**G**) had lower TDA and more oestrous records than those without copies (in bold the SNP allele associated with the trait). This haplotype contains the G allele of the SNP located in exon 2 (rs418191944), which is associated with lower TDA and higher P4MC and OCM values. Conversely, ewes with 2 copies of haplotype H2 (A**A**) had more TDA and fewer oestrous records.

Table 5. Type III test for the haplotype effects for block 2 of the *CD226* gene using seasonal phenotypic data from Rasa Aragonesa ewes (total days of anoestrous (TDA), oestrous cycling month (OCM) and progesterone cycling month (P4CM)). The least square means (LSMs) and standard errors for the haplotype effect are also shown. Only significant haplotypes after Bonferroni correction are shown. Different letters indicate significant differences: **a**, **b**: $p < 0.05$.

Trait	Haplotype [1]	Frequency	*p*-Value Haplotype	Haplotype LSMs [2]		
				0 copies	1 copy	2 copies
TDA	H1(AG)	0.70	0.002	110.3 ± 11.17 **a**	70.7 ± 4.83 **b**	79 ± 5.08 **b**
P4CM	-	-	0.002	0.67 ± 0.04 **a**	0.82 ± 0.01 **b**	0.79 ± 0.01 **b**
OCM	-	-	0.004	0.32 ± 0.04 **a**	0.49 ± 0.02 **b**	0.46 ± 0.02 **b**
TDA	H2(AA)	0.25	0.0007	77.5 ± 4.88 **a**	71.1 ± 4.94 **a**	122.1 ± 12.69 **b**
P4CM	-	-	0.0005	0.79 ± 0.01 **a**	0.83 ± 0.01 **a**	0.63 ± 0.04 **b**
OCM	-	-	0.003	0.46 ± 0.02 **a**	0.49 ± 0.02 **a**	0.29 ± 0.05 **b**

[1] Block 2 rs588529642–rs404360094. [2] 0 copy: LSMs and SE for 0 copies of the haplotype; 1 copy: LSMs and SE for 1 copy of the haplotype; and 2 copies: LSMs and SE for 2 copies of the haplotype.

4. Discussion

Nine potential SNPs reaching the chromosome-wise level of significance were identified to be associated with the TDA, P4CM, and OCM reproductive seasonality traits. Only one SNP (rs404991855) associated with P4CM overcame the genome-wide significance level. The SNPs rs404991855 and rs418191944 on chromosome 23 appeared to be associated with the three traits and were located in intron 2 of the *CD226* gene. This gene has been related to such reproductive diseases as cystic ovarian teratoma and mature teratoma of the ovary in humans [46,47]. On the other hand, SNPs (rs424340754 and rs410373132) in chromosome 4 were significantly associated with P4CM. These SNPs are located in an intergenic region approximately 34 kb from the *NPY* gene, which influences many physiological processes, including circadian rhythms, anorexia, and weight loss [48]. Three other SNPs (rs409834034, rs428238419, and rs405959180) in chromosomes 6 and 7 were also significantly associated with this trait. However, none of the genes annotated in the 250 kb region around the significant SNPs was related to reproductive traits. Finally, four SNPs located in chromosome 23 were associated with the OCM trait: two in intron 2 of the *CD226* gene (rs404991855 and rs418191944), one in an intergenic region approximately 412 kb from the *CD226* gene (rs405024177), and one in a different intergenic region in chromosome 23 (rs410842314).

The *NPY* and *CD226* genes have been reported in other studies to be potentially associated with reproductive processes. *NPY* plays a role in the regulation of the secretion of gonadotropin releasing hormone (GnRH) in rats [49], rabbits [50], monkeys [51], and sheep [52,53]. It has been shown that NPY-containing neurons in the hypothalamus hold oestrogen receptors in rats [54]. In the same way, Skinner and Herbison [55] indicated that changes in photoperiod may regulate oestrogen receptor expression within the preoptic area. These authors suggested that hypothalamic NPY and β-endorphin neurons are involved in the seasonal regulation of ewe reproductive activity. Barker-Gibb and Clarke [56] reported a reduction in the number of NPY cells detectable by immunohistochemistry in the nonbreeding season compared to the breeding season in ovariectomized ewes either treated or not treated with oestrogen. Williams et al. [57] noted that NPY and other unidentified neurons in the hypothalamus interact with leptin, which is an important mediator of

energy homeostasis and reproductive status in sheep. In pigs, NPY modulates hypothalamic neuronal activity (LH secretion) and serves as a putative link between the metabolic state and the reproductive axis [58]. Clarke et al. [48] determined that *NPY* stimulates feed consumption and inhibits reproduction in sheep. However, Miner [59] showed that NPY is a potent orexigenic agent in sheep, as in other species, and a seasonal change in the expression of this peptide may be related to stimulation of feed intake, rather than seasonality. On the other hand, the *CD226* gene encodes the glycoprotein CD226, also known as DNAM-1 (DNAX Accessory Molecule-1), which is expressed on the surface of natural killer (NK) cells and is involved in T cell-mediated cytotoxicity against certain tumours [60]. NK cells express a repertoire of activating and inhibitory receptors that control NK cell cytotoxicity and interferon-γ (IFNG) production to ensure self-tolerance while allowing efficacy against such insults as viral infection and tumour development [61,62]. NK cells are also known to play an important role in human reproduction. These cells can be categorized into two main populations based on the relative expression of the surface markers CD^{16} and CD^{56}: $CD^{56bright}/CD^{16-}$ functioning to primarily produce cytokines in the circulating blood, and CD^{56dim}/CD^{16+} performing cytotoxicity in the tissues [63,64]. Lukassen et al. [65] have shown that the proportion of CD^{56dim}/CD^{16+} NK cells in follicular fluid (FF) in women suffering from idiopathic infertility is significantly higher than that in FF from patients undergoing in vitro fertilization (IVF) for tubal or male factor infertility. Furthermore, Křížan et al. [66] reported that the FF of patients with successful IVF outcomes was enriched with $CD^{56bright}/CD^{16-}$ NK cells. Additionally, Fainaru et al. [67] indicated that $CD^{56bright}$ CD^{16-} NK cells are abundant in maturing ovarian follicles and that their presence correlates with the ovarian response to gonadotropins. These cells have been shown to be proangiogenic through the secretion of such cytokines as vascular endothelial growth factor and placental growth factor [68]. In patients with a good response to ovarian stimulation, $CD^{56bright}$ NK cells migrate into the ovarian follicle, supporting follicular angiogenesis and oocyte development [67]. Recent research conducted by Stannard et al. [46] described a new CD^{56dim} NK cell subset characterized by a lack of expression of DNAM-1. These researchers reported that $CD^{56dim}DNAM\text{-}1^{neg}$ NK cells displayed reduced motility, poor proliferation, lower production of interferon-γ, and limited killing capacities compared with $CD^{56bright}$ and $CD^{56dim}DNAM\text{-}1^{pos}$ NK cell subsets.

According to the aforementioned findings, it can be inferred that mutations in *CD226* could lead to glycoprotein alteration and could therefore affect follicle development. Additionally, mutations in the *NPY* gene would explain one of the neuroendocrine and physiological mechanisms that link nutrition and reproduction. Accordingly, the *NPY* and *CD226* genes were selected for validation studies for their possible involvement in seasonal reproduction in sheep. Using 18 ewes with extreme values of TDA, we detected 2 and 4 SNPs in *NPY* and *CD226*, respectively (Table 3). This design could increase the power to detect polymorphic SNPs associated with the trait but minimize the probability of detecting other polymorphic SNPs.

In this study, no association was found with the *NPY* gene. These SNPs show a low MAF (0.025 and 0.08 for OAR4: g.71593018 G > T and rs594346709, respectively). Notably, we did not sequence regulatory regions, such as the promoter or 5′ and 3′ UTR regions. Therefore, we could have missed the responsible mutation, as it was not in LD with the detected mutations. Similarly, no association was found with the SNP located in exon 2 of the *CD226* gene. Importantly, no homozygous animals were found for the G allele, and a low MAF was found for this SNP (0.042). However, the analysis demonstrated that the SNP located in exon 3 of *CD226* (rs404360094) was associated with the three reproductive seasonality traits. Notably, this SNP is close and in LD with the significant SNPs found in intron 2 in the GWAS analysis (4 and 4.7 kb from SNPs rs418191944 and rs404991855, respectively). In this sense, the GWAS analysis could have detected the effect found in exon 3 of *CD226*. Heterozygous and homozygous ewes for the G allele had less TDA and showed more oestrous events than those with the AA genotype (Table 4), which were observed at low frequency (4.7%) in our sample (Table S2). These results

were confirmed by haplotype analysis such that animals carrying 1 or 2 copies of the H1 haplotype (containing the G allele) showed less TDA and higher P4CM and OCM values. Similarly, animals with two copies of the H2 haplotype (containing the A allele) showed higher TDA and lower P4CM and OCM values. This SNP produces an amino acid substitution from asparagine (uncharged polar) to aspartic acid (acidic) at position 243 at the end of the second immunoglobulin V-like domain in the extracellular region of the protein and close to the transmembrane motif [69]. However, the real effect of this variant is not clear because it has been predicted in silico to be a tolerant nonsynonymous substitution. It should be noted that we did not sequence the complete coding or regulatory regions of the *CD226* gene; thus, the observed relationship could indicate that the SNP should be in LD with the true causative mutation.

In summary, GWAS results were confirmed using a candidate gene approach. First, two SNPs located in intron 2 of the *CD226* gene reached the chromosome-wise significance threshold on chromosome 23 using the GWAS approach. As noted above, because this gene could be related to follicular angiogenesis and oocyte development in humans, we decided to confirm the GWAS results using a candidate gene approach, determining that the SNP rs404360094 located in exon 3 of the *CD226* gene was associated with reproductive seasonality traits in Rasa Aragonesa ewes. Therefore, this SNP could be utilized as a genetic marker for assisted selection to reduce seasonality. These results should be confirmed in more animals and in other breeds.

5. Conclusions

This study employed a GWAS approach to identify genomic regions associated with traits involved in reproductive seasonality in sheep. We demonstrated that the G allele of the SNP rs404360094 located in exon 3 of the *CD226* gene is associated with lower TDA and higher P4CM, which are both traits related to ovarian function based on blood progesterone levels. This allele was also associated with higher OCM scores, which is an indicator of oestrous behaviour. These findings enabled us to validate the GWAS results and demonstrated the involvement of the genomic region where the *CD226* gene is located. This SNP could be utilized as a genetic marker along with other SNPs already characterized in Rasa Aragonesa as being associated with traits related to reproductive efficiency, such as prolificacy or reproductive seasonality.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ani11041171/s1, Figure S1: Multidimensional scaling (MDS) analysis performed with 188,633 SNPs after pruning in 205 ewes. The analysis showed the 4 clusters of animals included in analysis., Figure S2: Manhattan and Q-Q plots from GWAS analysis for a) TDA, b) P4CM and c) OCM traits in Rasa Aragonesa sheep breed. Chromosomes 1–26 and X (27) are shown. Horizontal green line in the Manhattan plot corresponds to the average threshold value for a FDR of 10% evaluated at the chromosomal level, Table S1: Genome-wide Complex Trait Analysis (GCTA) results for significant Single Nucleotide Polymorphisms (SNPs) at the genome (Bonferroni correction), and chromosome (FDR 0.10) levels for the reproductive seasonality traits studied. Threshold for chromosome level (FDR 0.10) was indicated (pval_FDR10), Table S2: Genotypic and allelic frequencies of the genotyped SNPs for the validation studies, Table S3: Type III test for the body condition score (BCS), live weight (LW), the age (A), and SNP effects for the *CD226* and *NPY* polymorphisms using the seasonality phenotype data from Rasa Aragonesa ewes. The least square means (LSMs) and standard errors are also shown. Different letters indicate significant differences: a, b: $p < 0.05$ after Bonferroni correction, Table S4: Haplotypes combination and frequency block 1 (OAR4:g.71593018- rs594346709) and for block 2 (rs588529642–rs404360094). Only haplotypes with a frequency higher than 1% are shown, Table S5: Block 1 Type III test for the body condition (BC), live weight (LW), the age (A), and Haplotype (H) for the *NPY* polymorphisms using the seasonality phenotype data from Rasa Aragonesa ewes. The least square means (LSMs) and standard are also shown. Different letters indicate significant differences: a, b: $p < 0.05$ after Bonferroni correction, Table S6: Block 2 Type III test for the body condition (BC), live weight (LW), the age (A), and Haplotype (H) for the *CD226* polymorphisms using the seasonality phenotype data from Rasa Aragonesa ewes. The least square

means (LSMs) and standard are also shown. Different letters indicate significant differences: a, b: $p < 0.05$ after Bonferroni correction.

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Institutional Review Board Statement: All experimental procedures were performed in accordance with the guidelines of the European Union (2003/65/CE) and Spanish regulations (RD 1201/2005, BOE 252/34367e91) for the care and use of animals in research and were approved by the Animal Welfare Committee of the Centro de Investigación y Tecnología Agroalimentaria (CITA) (protocol number code 2011-08). No hormonal treatments were applied to the ewes during the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Supplementary Materials*

Figure S1. Multidimensional scaling (MDS) analysis performed with 188,633 SNPs after pruning in 205 ewes. The analysis showed the 4 clusters of animals included in analysis.

Figure S2. Manhattan and Q-Q plots from GWAS analysis for a) TDA, b) P4CM and c) OCM traits in Rasa Aragonesa sheep breed. Chromosomes 1–26 and X (27) are shown. Horizontal green line in the Manhattan plot corresponds to the average threshold value for a FDR of 10% evaluated at the chromosomal level.

Table S1. Genome-wide Complex Trait Analysis (GCTA) results for significant Single Nucleotide Polymorphisms (SNPs) at the genome (Bonferroni correction), and chromosome (FDR 0.10) levels for the reproductive seasonality traits studied. Threshold for chromosome level (FDR 0.10) was indicated (pval_FDR10).

Table S2. Genotypic and allelic frequencies of the genotyped SNPs for the validation studies, **Table S3**. Type III test for the body condition score (BCS), live weight (LW), the age (A), and SNP effects for the *CD226* and *NPY* polymorphisms using the seasonality phenotype data from Rasa Aragonesa ewes. The least square means (LSMs) and standard errors are also shown. Different letters indicate significant differences: a, b: $p < 0.05$ after Bonferroni correction.

Table S4. Haplotypes combination and frequency block 1 (OAR4:g.71593018-rs594346709) and for block 2 (rs588529642–rs404360094). Only haplotypes with a frequency higher than 1% are shown.

Table S5. Block 1 Type III test for the body condition (BC), live weight (LW), the age (A), and Haplotype (H) for the *NPY* polymorphisms using the seasonality phenotype data from Rasa Aragonesa ewes. The least square means (LSMs) and standard are also shown. Different letters indicate significant differences: a, b: $p < 0.05$ after Bonferroni correction

Table S6. Block 2 Type III test for the body condition (BC), live weight (LW), the age (A), and Haplotype (H) for the *CD226* polymorphisms using the seasonality phenotype data from Rasa Aragonesa ewes. The least square

*Para visualizar el material suplementario, se puede acceder a través del siguiente link: https://www.mdpi.com/article/10 .3390/ani11041171/s1

Manuscrito 3: Genome-wide analysis reveals that the *Cytochrome P450 Family 7 Subfamily B Member 1* (*CYP7B1*) gene is implicated in growth traits in Rasa Aragonesa ewes

Genome-wide analysis reveals that the *Cytochrome P450 Family 7 Subfamily B Member 1* (*CYP7B1*) gene is implicated in growth traits in Rasa Aragonesa ewes

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Abstract

Sheep are very well adapted to changing environments and are able to produce and reproduce with low inputs in feed and water better than other domestic ruminants. Indeed, the ewe body condition score (BCS) and live weight (LW) play a significant role in productive and reproductive performance. This work conducts a genome-wide association study (GWAS) to detect genetic variants associated with growth traits in 225 adult ewes of the Rasa Aragonesa breed by using the genotypes from 50k and HD Illumina Ovine BeadChip. These ewes were measured for LW, BCS and growth rate (GR) for 2 years, from January to September. Corrected phenotypes for BCS, LW and GR were estimated and used as input for the GWAS. Only one SNP rs425509273 in chromosome 9 (OAR9), associated with the GR, overcame the genome-wise significance level. One, three and nine SNPs were associated at the chromosome-wise level (FDR 10%) for traits BCS, LW and GR, respectively. The *CYP7B1* candidate gene, located 83 kb upstream from SNP rs425509273 in OAR9, was partial isolated and Sanger-sequenced. Fifteen polymorphisms comprising 12 SNPs, two indels and one polyC were detected. The SNP association analysis of the polymorphisms located close to the transcription start site (TSS) showed that a 22 bp insertion located at -58 nucleotides from the TSS (indel (-58), a polyC (-25), and two A/G SNPs (SNP3 (-114) and SNP5 (-63)), were associated with the GR trait, whereas only the indel (-58) was associated with the BCS trait. The haplotype analysis confirmed these results. The functional characterisation of the polymorphisms at *CYP7B1* gene in liver by real-time quantitative PCR analysis confirmed that the mutations in the promoter region affected *CYP7B1* gene expression. Our results demonstrated the involvement of the *CYP7B1* gene promoter on GR and BCS traits in Rasa Aragonesa. These findings suggest that variations in ovine *CYP7B1* may serve as potential genetic markers to be used in breeding programmes to improve growth characteristics that could influence reproductive traits.

Keywords: GWAS, Sheep, *CYP7B1* gene, growth traits, polymorphism.

Implications

Climate change had a devasting effects on sheep productivity and reproductive efficiency through the exposure of animals to heat stress, water scarcity and lower grazing lands. Finding genetic markers associated with resilience and environmental stress resistance may help sheep breeding programs to achieve animals with better adaptive ability to manage their body reserves to cope with environmental stressors.

Introduction

The effect of climate change is expected to directly and indirectly affect livestock physiology and health by increasing exposure to environmental stressors like heat (Rojas-Downing et al., 2017) and water stress (Chedid et al., 2014). Therefore, sheep farming systems should look at working with more robust animals that combine productivity and adaptive ability, and a more efficient use of natural resources. Body reserves in adult animals can be considered an important trait, and both, life weight (LW) and body condition score (BCS) are related traits to this. In this context, Macé et al., (2019) showed different profiles in BCS and LW dynamics in a flock suggesting the existence of proven intra-flock variability in ewes' adaptive capacity to manage their body reserves. Furthermore, ewes' BCS plays an important role in both fertility and growth rate (GR) of its progeny. Many studies have investigated the sheep BCS in relation to productive and reproductive performances (Macé et al., 2019; Newton et al., 1980; Russel et al., 1969) to define the suitable BCS profile throughout a ewe's breeding cycle. In Masham ewe breed the number of lambs weaned per ewe exposed to the ram was positively correlated with the BCS upon breeding and/or at mid-pregnancy (Newton et al., 1980). Likewise, ewes' LW upon mating has been shown to influence subsequent litter size and ewes' productivity (Paganoni et al., 2022). In the Romane sheep breed reared under extensive conditions, a study confirmed that LW and BCS levels are influenced by the age at first lambing, parity or litter size (Macé et al., 2019), and breeding season is also influenced by the BCS and LW. The literature suggests an association between a poorer BCS and a shorter breeding season, which implies that the BCS can influence ewes' response to seasonal signals (Kenyon et al., 2014). Gunn and Doney, (1975), reported that the delay or suppression of oestrus in the Scottish Blackface

ewes breed is associated with poor body condition, although the Masham ewe breed with a higher BCS seems to display oestrus late in the breeding season (Newton et al., 1980).

Rasa Aragonesa is a meat local Mediterranean sheep breed from northeast Spain, reared in extensive or semi-extensive farming systems. This breed shows a marked reproductive seasonality where maximal breeding activity is associated with short days from August to March. Forcada et al., (1992) have reported that Rasa Aragonesa ewes with a higher BCS have a longer breeding season, which is predominately due to later seasonal anoestrous onset and a shorter whole anoestrous period (Rondon et al, 1996). Therefore, the objective of this study was to detect genetic variants associated with growth traits in ewes by using the genotypes from 50k and HD Illumina Ovine BeadChip and investigated the association between polymorphisms in the *CYP7B1* gene and growth traits.

Material and methods

Animals and phenotypes

GWAS animals

Two hundred and twenty-five Rasa Aragonesa ewes were kept indoors and fed a commercial concentrate from October to March. From March to the end of the experimental period (August), ewes were grazed on mountain pastures and received the same commercial concentrate *ad libitum*. All the ewes were handled in a single lot. The experimental period lasted from January to August in 2011 and 2012. Every 3 weeks, the individual LW and BCS on a scale from 1 to 5 (Russel et al., 1969) were measured. The pooled overall means and standard deviations for LW and the BCS were 45±9.5 kg and 2.9±0.3, respectively in 2011, and 52.4±8.4 kg and 3±0.2 in 2012, with an age of 3.98±1.85 years at the beginning of the experiment.

Functional validation studies in animals

Liver samples were obtained from a previous study (Ripoll et al., 2013) in which 48 Rasa Aragonesa breed lambs were fed *ad libitum* a commercial concentrate from weaning to slaughter, supplemented with 500 mg of dl-α–tocopheryl acetate kg^{-1} (n = 36; ranging from 4 to 28 days) and without this supplementation (n = 12). Ripoll *et al.*

(2013) study was performed to evaluate the effects of finishing period length with vitamin E (dl-α–tocopheryl acetate) in the meat colour and lipid oxidation of light lambs. Briefly, the experiment began upon weaning (48.7 ± 0.21 days old) with an average LW of 18.5 ± 0.16 kg and finished at a slaughter age and weight of 75.20 ± 1.84 days old and 23 ± 0.24 kg, respectively, according to the Ternasco de Aragón Protected Geographical Indication specifications (Regulation (EC) No. 1107/96). Lambs were slaughtered in a commercial abattoir in accordance with EU laws. Immediately after slaughter, a piece of liver was cut, frozen in liquid nitrogen and stored at -80ºC until RNA isolation.

GWAS

Genomic DNA from blood samples was obtained using the SpeedTools DNA Extraction kit (Biotools, Madrid, Spain). The 225 ewes were genotyped using the OvineSNP50 Infinium Beadchip (n =110; Illumina Inc., San Diego, CA, USA), and the 680k IlluminaAgResearchSheep HD chip (n =115). Genotyping services were conducted at the "Centro Nacional de Genotipado (CEGEN-ISCIII)" (http://www.usc.es/cegen/) and the "Xenetica Fontao" company (http://www.xeneticafontao.com/).

Validation of the GWAS results

Structural characterisation of the CYP7B1 gene

The gene annotation based on the GWAS results showed that the *CYP7B1* gene could be involved in growth traits. The *CYP7B1* gene is located in OAR9, covering approximately 173.7 kb with 6 exons (GenBank acc. Number NC_056062). The primers designed from sheep sequences NC_056062 and XM_004011703 were used to amplify total 5'UTR and coding region (exons 1-6), and also partial promoter and 3'UTR regions (Supplementary Table S1). The primers for PCR were designed with the Primer3 software (https://primer3.ut.ee/).

Genomic DNA (50 ng) from 13 ewes with different genotypes for SNP rs425509273 (5 CC, 5 CT, and 3 TT) was amplified in a final PCR volume of 25 µl, which contained 5 pmol of each primer, 200 nM dNTPs, 2.25mM $MgCl_2$, 50mM KCl, 10mM Tris-HCl, 0.1% Triton X-100 and 1 U Taq polymerase (Biotools, Madrid, Spain) for fragments 3 to 7. DNA AmpliTools Master Mix (Biotools, Madrid, Spain) was used for fragments 1

and 2. The PCR cycling conditions were set according to the protocol provided in the manufacturer's recommended procedures: an initial denaturation cycle at 94 °C for 3 min, 35 cycles of 94°C for 30 s, 55°C for 30 s (57ºC for fragments 1 and 2), and 72°C for 30s, with a final elongation step at 72°C for 5 min. PCR products were sequenced in both directions using Sanger technology in an ABI 3730XL sequencer (STAB VIDA. Caparica, Portugal). Previously, PCR clean-up was performed using the FavorPrep Gel/PCR purification mini kit (Favorgen, Ibian, Zaragoza, Spain). BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) tools were used for searching regions of similarity between sequences and multiple sequence alignment, respectively. The SNPs position in the genome were determined based on the ovine Rambouillet v1.0 genome (GCA_002742125.1). The software Alibaba 2.1 (http://gene-regulation.com/pub/programs/alibaba2/) was used to predict transcription factor binding sites within the surrounding sequence where the SNP was located (plus and minus 30 nucleotides).

Genotyping of the polymorphism in the CYP7B1 gene

The polymorphisms detected in the 5' region of the gene were genotyped by Sanger sequencing in the 225 and 48 animals of the GWAS and the functional validation studies, respectively. For this purpose, a pair of primers (forward: 5′-GCTCATGTCTTCCGCTGTC -3′; reverse: 5′- AAATCTCAGCCCCTTCCCC -3′), was designed by amplifying a 360 bp fragment. The PCR conditions were the same as those for fragments 3 to 7 (Supplementary Table S1), but at the annealing temperature of 57ºC. PCR was purified and Sanger-sequenced as described above.

Functional characterisation of the polymorphism in the CYP7B1 gene by a real-time quantitative PCR analysis (RT-qPCR)

Total RNA was extracted from approximately 500 mg of liver using the RNeasy Tissue mini kits (QIAGEN, Madrid, Spain) according to the recommended manufacturer's protocol. The RNA concentration was determined by a Qubit® fluorometer (Fisher, Madrid, Spain). Integrity of the RNA was assessed by visualization of the 28S and 18S ribosomal RNA bands after agarose gel electrophoresis, being both bands clearly visible without observed degradation. To completely remove genomic DNA, 1 μg RNA was

treated with deoxyribonuclease (Invitrogen, Carlsbad, CA, USA), and first-stranded cDNA was performed using the SuperScript III Reverse Transcriptase kit (Invitrogen) following the manufacturer's instructions. Gene expression levels were determined by RT-qPCR. To normalise the results of the *CYP7B1* gene, three candidate housekeeping (HK) genes (*GUSB*, *RPL37, RPL19*) were used. Gene expression stability was determined using NormFinder (Andersen et al., 2004). Specific exon-spanning primers for genes for RT-qPCR were designed with the Primer3 software (https://primer3.ut.ee/). RT-qPCR was performed in a 10 µl PCR total reaction mixture containing the SYBR Green Master Mix: SYBR Premix Ex Taq II (Tli RNase H Plus, Takara, Demlab, Zaragoza, Spain) on an ABI Prism 7500 platform (Applied Biosystem, Madrid, Spain). All qPCR reactions were run in triplicate following the manufacturer's instructions. Post-amplification melting-curve analysis were carried out for each gene to ensure primer specificity and to confirm the presence of a unique PCR product. The PCR amplification efficiency (E) were assessed by generating standard curves for each target gene using a 10-fold serial dilution of pooled cDNA that were included on each plate. The correlation coefficient (R^2) and slope were calculated. The formula $E = 10^{(-1/slope)}$ was used. The annealing temperature, primer concentration, primer sequences, efficiencies and correlation coefficient (R^2) for these genes are described in Supplementary Table S2.

Statistical analysis

Corrected phenotype values for growth traits

The phenotypes used in the GWAS were obtained from the BCS and LW data collected over the 2-year experiment. In all, 4,172 BCS and LW data belonging to the 225 ewes were recorded. The corrected phenotypes were estimated by running a mixed linear regression analysis. To estimate the BCS-corrected phenotype, LW and ewes age were taken as covariates. For the LW-corrected phenotype, the BCS and age were considered to be covariates. The fitted model included the collection date of growth traits as a fixed effect (21 levels) and ewes' permanent environmental effect as random (225 levels). The individual BCS and LW estimates, adjusted by the factors included in the model, were obtained and used as inputs in the GWAS. BLUPF90 (Misztal et al., 2018) was used to run the mixed linear regression analyses.

The third phenotype was estimated by a linear regression analysis with the lm command in the R v3.5.1 software by taking ewes' LW as the dependent variable and their age as the independent variable. The obtained individual slope estimates were the GR-estimated phenotype (kg/day) utilised for the GWAS.

GWAS

The quality control (QC) criteria applied to the raw genotypes using PLINK 1.9 software (Chang et al., 2015) were based on excluding the individuals with a low genotype call rate (< 0.90), the SNPs with a genotype call rate < 0.97, SNPs with a minor allele frequency (MAF) < 0.01, and the SNPs with a failure of Hardy-Weinberg equilibrium (HWE) (p-value < 0.001). PLINK 1.9 and Beagle 4.0 program (Browning and Browning, 2007) were used to merge the datasets from the two genotyping platforms and for imputation, respectively. The Genome-wide Complex Trait Analysis software (GCTA) (Yang et al., 2011) was run for the GWAS analysis using the mixed linear model association (MLMA) approach. The genomic relationship matrix (GRM) was constructed and included in the mixed model analysis to account for population structure (PS). Bonferroni ($p < 0.05$) and the false discovery rate (FDR < 0.1) multitest correction tests were applied to adjust the threshold of the genome and chromosome-wide significant values. The genomic inflation factor was calculated in the R v3.5.1 software for each trait as the observed median χ^2 over the expected median χ^2. The Rambouillet v1.0 genome assembly based on Ensembl release 101 was employed to identify any candidate genes within a window of 500 kb centred on the significant SNP.

CYP7B1 gene association analysis

Statistical analyses were carried out as a regression of the corrected phenotypes for the three growth traits on the polymorphism genotypes by fitting a linear model with the Rcmdr package of the R software (Fox et al., 2020). The model included the genotype of SNPs (S) and ewes' clusters to which ewes belonged as a fixed effect. These clusters were obtained with PLINK 1.9 (Chang et al., 2015) using genotypes utilised for the GWAS analysis. Briefly, the SNPs that passed QC were subsequently pruned for linkage disequilibrium in PLINK, using a 50 SNP window size, a 10 SNP window shift, and r^2 < 0.2. The SNPs that remained after pruning were used to calculate the genome-wide identity-by state (IBS) pairwise distances. Clustering analysis was performed applying

the pairwise population concordance test constraint (-ppc option). Multidimensional scaling (MDS) analyses were performed on IBS pairwise distances to examine for PS. Differences between genotypes were estimated by the least square means (LSMs) for each genotype pairwise comparison. Bonferroni correction was applied, and all the polymorphisms were independently analysed with the same statistical model.

A haplotype association analysis was performed between haplotypes and the corrected phenotypes for the growth traits using the Rcmdr package by fitting a similar linear model to that employed for the polymorphism association studies but including the number of copies of each haplotype (H) instead of the genotype of SNP (this was recoded as 0, 1 or 2 copies). The same statistical model was applied for analysing independently each haplotype. The LD blocks were evaluated with the Haploview software v4.2 using the 4-gamete rule (Barrett et al., 2005). Polymorphisms were phased with PLINK1.9 (Chang et al., 2015) using the expectation-maximisation (E-M) algorithm to assign individual haplotypes, considering only those haplotypes with a frequency ≥ 1%. The diplotypes with a posterior probability bellow 0.7 were discarded. LSMs were applied to estimate the differences between the number of copies of each haplotype. To adjust for multiple testing, Bonferroni correction was applied.

Analysis of expression results

Differences in the ovine *CYP7B1* gene expression rates of the alternative genotypes/haplotypes located at the gene promoter were estimated by the approach described by Steibel et al., (2009). The Haploview software v4.2 was used to construct haplotype blocks, and the LD analysis was done as previously mentioned. The quantification cycle (Cq) values were rescaled with the equation proposed by Steibel et al., (2009) because amplification efficiencies of all the genes differs from 2. A mixed model using the SAS statistical package v. 9.3 (SAS Institute, Cary NC, USA) was employed:

$$y_{gomr} = \mu + MG_{og} + b_1(SA)_m + b_2(SW)_m + b_3(VE)_m + A_m + e_{gomr}$$

where y_{gomr} is the Cq for the g^{th} gene (*CYP7B1* and the three HK genes) from the r^{th} well, corresponding to the m^{th} animal; MG_{og} is the fixed interaction between the o^{th} genotype (AA, AB and BB) or the haplotype (0, 1 and 2 copies) and the g^{th} gene (M is the effect of the o^{th} genotype or haplotype of the *CYP7B1* gene, and G is the effect of the g^{th} gene). The SA (slaughter age), SW (slaughter weight) and VE (number of days of concentrate

enriched with dl-α-tocopheryl acetate intake) effects were included as a covariate; A_m ($A_m \sim N(0, \sigma^2_a)$ and e_{gomr} were the random effects of the animal and residual, respectively. Gene-specific residual variance (heterogeneous residual) was also included in the model ($e_{gomr} \sim N(0, \sigma^2_{eg})$).

The *CYP7B1* expression was normalise using the three HK genes as suggested by Steibel et al., (2009). The expression rate differences ($diff_{CYP7B1}$) between genotypes/haplotypes and the fold change (FC) values were estimated from MG fixed interaction differences. The t statistic was determined to calculate the significance of the $diff_{CYP7B1}$ estimates. The *P* values were adjusted for multiple testing according to Bonferroni correction. The standard error (SE) for each FC value of $diff_{CYP7B1}$ were used to calculate asymmetric 95% confidence intervals (upper and lower).

Results

GWAS results

The 225 ewes genotyped for 582,880 SNPs passed QC and were included in the GWAS analysis. For the MDS analysis, 184,076 autosomal SNPs were used to calculate the pairwise IBS distance after SNP pruning. The MDS analysis revealed a substructure in the total dataset and identified four principal clusters in the population (Supplementary Figure S1). The GRM was included in the mixed model analysis to correct the putative effect of the population substructure. This was checked by calculating the genomic inflation factors for each trait, which were ≤ 1 (LW: 0.95; BCS: 0.96; GR: 1). The GWAS results obtained by the MLMA analysis for the significant SNPs at the chromosome and genome-wide levels for the studied growth traits are reported in Supplementary Table S3. Only one SNP (rs425509273), located in OAR9 and associated with GR, overcame the genome-wise significance level after Bonferroni correction. At the chromosome-wise level of significance (FDR 10%), 1, 3 and 9 SNPs were identified for the BCS, LW and the GR respectively. The nine SNPs associated with GR variability were located in eight chromosomes. In most cases, the less frequent allele (A1) of the associated SNP showed a negative effect on the trait with allele substitution values (b) ranging between −0.008 and −0.04, and frequencies from 0.01 to 0.13. In general, the A1 alleles showed a negative effect on the LW trait, with b values

from -2.01 to -2.85 and frequencies ranging between 0.13 and 0.38. For the BCS, the associated SNP (rs424629620) was located in chromosome 6 and had an effect of 0.09 and an MAF of 0.38. Several annotated genes were found near the significant SNPs for GR and LW, whereas only one gene was found for BCS (Supplementary Table S3).

Validation studies

Structural characterisation of the CYP7B1 gene

To validate the GWAS results, the SNP that overcame the genome-wise significance level was chosen. This SNP was 83 kb downstream from the *CYP7B1* gene. To search for the polymorphisms that could be involved in the studied phenotypes, the total 5'UTR and coding region (exons 1-6), partial promotor and 3'UTR regions of the *CYP7B1* gene were sequenced. Fifteen polymorphisms were found: 12 SNPs, two indels and one polyC (Table 1). None of these polymorphisms produced amino acid changes. Eight of the total polymorphisms, not previously described, were located in the promoter region. It is noteworthy that one of the polymorphisms was an insertion of 22 bp at position -58 in relation to the transcription start site (TSS) (Table 1).

This insertion was a repeat of the 22 nucleotides located between OAR9: g.45801535 and OAR9: g.45801556 according to the ovine genomic map Oar_rambouillet_v1.0. *In-silico* analysis of the transcription factor-binding motifs identified several overlapping motifs containing polymorphisms indel (-621), SNP3 (-114), SNP4 (-97), SNP5 (-63), indel (-58) and polyC (-25). For SNP3 G>A (-114), the CACCC-bi and RAP1 binding consensus sites were predicted with the A allele, while these motifs were replaced with an AP-2alpha motif for the G allele. For SNP5 A>G (-63), the substitution of the G by the A allele resulted in the loss of an SP1 and the Adf-1 binding consensus site. However, the strongest effect was found for the 22 bp insertion (indel (-58)), which led to several TF-binding motifs appearing: SP1, REB1 and AP-2alph. PolyC and the other polymorphisms were not predicted to produce any major changes in the binding consensus sites.

Table 1

The *CYP7B1* gene polymorphisms. The position in the ovine genomic map Oar_rambouillet_v1.0., its identifier in the dbSNPs variant database, and the polymorphism are indicated. The distance in bp from the transcription start site (TSS) to the polymorphism in the SNPs located in the promoter is indicated in parentheses.

Name	dbSNP	Region	Position	Polymorphism
Indel (-621)	-	promoter	g.45802097	insC
SNP1 (-558)	-	promoter	g.45802034	A/G
SNP2 (-199)	-	promoter	g.45801675	A/G
SNP3 (-114)	-	promoter	g.45801590	G/A
SNP4 (-97)	-	promoter	g.45801573	C/A
SNP5 (-63)	-	promoter	g.45801539	A/G
indel (-58)	-	promoter	g.45801534	insACCCACACGCAC CGCCCCGCTC
PolyC (-25)	-	promoter	g.45801510	polyC
SNP6	-	exon 1	g.45801489	C/A
SNP7	rs417515909	intron 1	g.45660532	C/T
SNP8	rs404047939	intron 2	g.45660360	A/T
SNP9	rs421906566	intron 3	g.45652963	G/T
SNP10	rs408370224	intron 3	g.45652933	T/C
SNP11	-	exon 3	g.45652588	T/C
SNP12	rs160653444	exon 5	g.45642126	T/C

CYP7B1 SNP association studies

Polymorphisms SNP3 G>A (-114), SNP4 C>A (-97), SNP5A>G (-63), indel (-58), and polyC (-25) located at the promoter region were genotyped in the GWAS population. Table 2 provides the association results for each polymorphism after Bonferroni correction. SNP3 (-114), SNP5 (-63), the indel (-58), and polyC (-25) were associated with the GR trait. Lower GR was observed for the indel (-58) in the homozygous animals for the 22 bp insertion (0.010 ± 0.006) in respect to the homozygous animals for the alternative allele (0.017 ± 0.015). Likewise, the GR of the ewes carrying the GG

genotype for SNP5 (-63) (0.018 ± 0.001) and SNP3 (-114) (0.016 ± 0.001) was higher than in the AA animals for the two SNPs (0.009 ± 0.002 and 0.004 ± 0.004 for SNP5 (-63) and SNP3 (-114), respectively). For SNP5 (-63), the heterozygous ewes (0.016 ± 0.001) obtained a higher GR than the AA ewes. Finally, a lower GR was observed in the homozygous animals with seven repetitions (0.008 ± 0.019) compared to the homozygous ones with eight repetitions (0.018 ± 0.001) or to the heterozygous (0.016 ± 0.001) ewes for these two alleles for polyC (-25). The indel (-58) was also associated with the BCS trait insofar as the homozygous animals for the 22 bp insertion had a estimated lower BCS value (-0.036 ± 0.026) than the homozygous animals for the alternative allele (0.043 ± 0.019).

CYP7B1 haplotype association studies

Two haplotypic blocks in the promoter region of the *CYP7B1* gene were detected with the Haploview software (Supplementary Figure S2). The first one comprised polyC (-25), the indel (-58) and SNP5 (-63), while the second one included SNP3 (-114) and SNP4 (-97). Considering that one block included all the SNPs, 22 haplotypes were defined (Supplementary Table S4).

The haplotype association analysis confirmed the SNP association results. Only haplotypes H2 (G-C-A-Ins22-C7 for SNP3 (-114) - SNP4 (-97) - SNP5 (-63) - indel (-58) - polyC (-25) polymorphisms) and H12 (G-C-G-N-C8) presented a frequency above 5%. Ins22 indicated the allele with the insertion of 22 bp, and otherwise N as a normal allele. C7 and C8 indicated alleles with seven and eight cytosine repetitions, respectively. These haplotypes were significantly associated with the GR trait in such a way that having two copies of the H2 haplotype led to a lower GR than for the animals with one or no copies ($P<0.05$) (Table 3). Similarly, this haplotype was also significant for the BCS, with significant differences between having two and no copies. The H2 haplotype had the A, Ins22, and C7 alleles for the SNP5 (-63), indel (-58) and polyC (-25) polymorphisms, which were significantly associated with a lower GR in the SNP association studies. The H12 haplotype, which was associated with a higher GR, had alleles G, G, N and C8 for the SNP3 (-114), SNP5 (-63), indel (-58) and polyC (-25) polymorphisms, which were also significantly associated with an increased GR. In this case, having the H12/H12 diplotype implied a higher GR than the diplotypes with one

or no copies. These results confirmed the involvement of the *CYP7B1* promoter in both the GR and BCS in Rasa Aragonesa.

Functional characterisation of the polymorphism in CYP7B1

The genotype frequencies for the SNPs located in the 5' region of gene *CYP7B1* in the population used for the expression studies are shown in Supplementary Table S5. Housekeeping genes *GUSB, RPL37* and *RPL19* were employed to normalise gene expression because they were all stabler than the *CYP7B1* gene (Supplementary Figure S3). The *CYP7B1* expression was affected by the number of days of concentrate enriched with dl-α-tocopheryl acetate intake ($P < 0.05$), SNP5 (-63) ($P < 0.0001$) and polyC (-25) ($P < 0.0001$). The insertion of 22 nucleotides (indel (-58)) was not significant. SNP5 (-63) and polyC (-25) were completely linked ($r^2 = 1$). The *CYP7B1* expression in the AA lambs for SNP5 (-63) (n = 13) (homozygous animals for the C7 allele at the polyC (-25) polymorphism) was 2.9-fold higher than that in the AG lambs (n = 17; $p < 0.0001$) (heterozygous for seven and eight cytosine repetitions at the polyC (-25) polymorphism). Furthermore, *CYP7B1* expression in the GG lambs for SNP5 (n = 17) (homozygous animals for eight cytosines at the polyC (-25) polymorphism) was 3.21-fold higher than that in the AG lambs (n = 17; $p < 0.0001$) (heterozygous for seven and eight cytosine repetitions at the polyC (-25) polymorphism) (Table 4). Whether the *CYP7B1* expression was affected by the haplotypes defined with PLINK 1.9 was also tested (Chang et al., 2015). Haplotypes were built using the five polymorphisms genotyped in the 5' region of the gene. In Supplementary Table S6, both haplotypes and their frequency in the population for the functional studies are shown. The *CYP7B1* expression was also affected by the number of days of concentrate enriched with dl-α-tocopheryl acetate intake ($P < 0.05$) and haplotype H3 (G-C-**A**-Ins22-C7 for the SNP3 (-114) - SNP4 (-97) - SNP5 (-63) - indel (-58) - polyC (-25) polymorphisms) ($P<0.0001$). The animals with two copies (n = 3) had a gene expression that was 6.05- and 3.44-fold higher than the animals with one (n = 16) or no (n = 28) copies, respectively (Table 5). Finally, the lambs with one copy had a gene expression that was 1.76-fold lower than the animals with no copies.

Table 2

CYP7B1 gene polymorphisms effect on growth traits in the Rasa Aragonesa breed. Different letters indicate significant differences after Bonferroni correction: a,b: <0.10; c,d: <0.05; e,f:< 0.01; g,h:<0.001

Polymorphism	Trait[1]	P value polymorphism	Lsmeans Genotype					
			A/A	A/G	G/G			
SNP3 (-114)	GR	2.00E-03	0.004 ± 0.004e	0.012 ± 0.002	0.016 ± 0.001f			
	BCS	1.60E-01	0.044 ± 0.048	0.050 ± 0.027	-0.004 ± 0.014			
	LW	9.16E-01	0.115 ± 1.27	-0.412 ± 0.71	-0.137 ± 0.36			
			A/A	A/C	C/C			
SNP4 (-97)	GR	3.54E-01	0.008 ± 0.005	0.014 ± 0.004	0.015 ± 0.001			
	BCS	9.16E-01	0.031 ± 0.07	-0.005 ± 0.05	0.010 ± 0.01			
	LW	6.12E-01	-0.692 ± 1.80	1.017 ± 1.28	-0.236 ± 0.32			
			A/A	A/G	G/G			
SNP5 (-63)	GR	1.89E-04	0.009 ± 0.002e,g	0.016 ± 0.001f	0.018 ± 0.001h			
	BCS	2.70E-01	-0.017 ± 0.023	0.009 ± 0.017	0.036 ± 0.022			
	LW	9.74E-01	-0.053 ± 0.61	-0.228 ± 0.45	-0.193 ± 0.59			
			N/N	Ins22/N	Ins22/Ins22			
Indel (-58)[2]	GR	5.17E-03	0.017 ± 0.015e	0.015 ± 0.012a	0.010 ± 0.006f,b			
	BCS	4.25E-02	0.043 ± 0.019c	-0.001 ± 0.019	-0.036 ± 0.026d			
	LW	5.76E-01	0.120 ± 0.67	-0.594 ± 0.51	0.057 ± 0.49			
			C7/C7	C7/C8	C7/C9	C8/C8	C8/C9	C9/C9
PolyC (-25)[3]	GR	2.24E-04	0.008 ± 0.019e,g	0.016 ± 0.001f	-0.008 ± 0.012	0.018 ±0.001h	0.011 ± 0.006	0.008 ± 0.007
	BCS	1.26E-01	-0.032 ± 0.026	0.002 ± 0.020	-0.271 ± 0.017	0.041 ± 0.019	-0.001 ± 0.084	0.029 ± 0.10
	LW	5.11E-01	0.451 ± 0.67	-0.897 ± 0.52	-0.328 ± 4.37	0.087 ± 0.49	1.737 ± 2.19	-1.865 ± 2.52

[1]GR: growth rate; BCS: Body condition score; LW: Live weight

[2]Ins22 indicates the allele with the insertion of 22 bp, otherwise N as normal allele.

[3]C7, C8 and C9 indicate alleles with 7, 8, and 9 cytosine repetitions, respectively.

Table 3

Haplotype effects for the promoter region of the *CYP7B1* gene (SNP3 (-114)-SNP4 (-97)- SNP5 (-63)- ins22 (-58)- polyC (-25)) on growth traits in the Rasa Aragonesa breed. The Lsmeans indicate 0 copies of the haplotype (0), 1 copy of the haplotype (1), or 2 copies of the haplotype (2). Different letters indicate significant differences after Bonferroni correction: a,b: <0.10; c,d: <0.05; e,f:< 0.01; g,h:<0.001

			Lsmeans		
Haplotype[1]	Trait[2]	P value	0	1	2
H2	GR	1.26E-02	0.016 ± 0.002c	0.016 ± 0.002c	0.008 ± 0.001d
	BCS	1.39E-02	0.035 ± 0.02c	-0.004 ± 0.02	-0.068 ± 0.03d
	LW	3.95E-01	-0.115 ± 0.413	-0.663 ± 0.556	0.694 ± 0.85
			0	1	2
H12	GR	8.84E-05	0.009 ± 0.002g	0.016 ± 0.001h	0.019 ± 0.002h
	BCS	1.22E-01	-0.014 ± 0.02	0.006 ± 0.02	0.053 ± 0.03
	LW	8.12E-01	0.0498 ± 0.551	-0.175 ± 0.451	-0.509 ± 0.668

[1]H2: G-C-A-Ins22-C7; H12: G-C-G-N-C8.

[2]GR: growth rate; BCS: Body condition score; LW: Live weight

Table 4

Differences in the *CYP7B1* expression rate between the different genotypes for the SNP5 (-63). Estimates, standard error (SE), p values, fold change (FC) and the 95% FC confidence interval (FCup-FClow) between genotype contrasts are included.

Genotype contrast	Estimate	SE	P value	FC	FCup	FCdown
AA vs AG	-1.5351	0.3815	<0.0001	2.90	4.87	1.73
GG vs AA	-0.1458	0.397	0.7136	1.11	1.90	0.65
GG vs AG	-1.6808	0.3391	<0.0001	3.21	5.08	2.02

Table 5

Differences in the *CYP7B1* expression rate between the different number of haplotype 3 copies (H3). Haplotype 3: G-C-A-Ins22-C7 alleles for SNP3 (-114) - SNP4 (-97) - SNP5 (-63) - indel (-58) - polyC (-25) polymorphisms. Estimates, standard error (SE), p values, fold change (FC) and the 95% FC confidence interval (FCup-FClow) between 0 copies of the haplotype (0), 1 copy of the haplotype (1), or 2 copies of the haplotype (2) contrasts are included.

Haplotype contrast	Estimate	SE	P value	FC	FCup	FCdown
0 vs 1	-0.8129	0.3157	0.0103	1.76	2.70	1.14
2 vs 0	-1.7835	0.5979	0.003	3.44	7.76	1.53
2 vs 1	-2.5964	0.6152	<0.0001	6.05	13.95	2.62

Discussion

In this study, a GWAS performed for BCS, LW and GR traits in the Rasa Aragonesa sheep breed, showed a single SNP rs425509273 associated with GR that overcame the genome-wise significance level. This SNP is located approximately 83 kb downstream from the *CYP7B1* gene in OAR9. This gene has been reported to be involved in the metabolism of endogenous oxysterols, which are key mediators of cholesterol and lipid homeostasis (Guillemot-Legris et al., 2016). Several studies have reported that *CYP7B1* inhibition leads to fatty liver (Dai et al., 2014) because its role in the conversing cholesterol into bile acids pathways. Some studies have reported that *CYP7B1* expression is related to different physiological functions depending on the species and tissue where it is expressed (liver, reproductive tract and brain) (Stiles et al., 2009). In fact, loss of function mutations in the *CYP7B1* human gene causes spastic paraplegia 5A (SPG5A), a progressive neuropathy that is due to defects in cholesterol and neurosteroid metabolism (Stiles et al., 2009). In the reproductive tract, the CYP7B1 enzyme metabolises the androgens that antagonise oestrogen action so that mice without *CYP7B1* have abnormal prostates and ovaries (Stiles et al., 2009). We also identified at a chromosome-wise level of significance (FDR 10%) one, three and nine SNPs for BCS,

LW and GR, respectively. For GR trait, SNP rs405607259 located in chromosome 7 was 21.6 kb from the *NPC2* gene, which is involved in the intracellular trafficking of cholesterol and other lipids (Kim et al., 2010). The *VRTN* and *SYNDIG1L* genes were near this SNP. *SYNDIG1L* has been reported in Landrace pigs to be a factor that affects final body weight and back-fat thickness (Lee et al., 2018), whereas *VRTN* variants have been associated with the number of thoracic vertebrae in sheep (Cunyuan et al., 2019). The *ARHGAP24* gene was the only candidate gene found for the BCS at 210 kb from the significant SNP, which has been reported to be associated with pigs' growth performance (Meng et al., 2017). SNP rs422199592 in chromosome 13, related to LW, was 191.8 kb from *ZEB1* gene, which is associated with obesity in human and adipogenesis in mice (Gubelmann et al., 2014). It is noteworthy that most of the significant SNPs were found at very low frequencies, which could indicate a spurious association.

Because of the putative functional effect of the *CYP7B1* gene on body reserves, we decided to isolate the gene to look for the polymorphisms that could be involved in growth traits.

Therefore, all the exons of the *CYP7B1* gene were isolated, as well as 996 bp of the promoter region. None of the 15 polymorphisms detected produced amino acid changes. Six polymorphisms were located in the promoter region, including a 22 bp insertion (indel (-58)) in the promoter at -58 bp from the TSS, as well as modifying transcription factor-binding motifs. No TATA box-like sequences were found in the human *CYP7B1* gene promoter, whose transcription is activated through SP1-type elements (Wu et al., 1999). According to the literature, there is a CpG island of 1200 bp that includes exon 1 and 600 bp of the promoter region, and the region between bases -291 and +189 from the TSS is critical for its transcription. In this way, transcription factors SP1 and Sp3 would be responsible for the binding to the promoter and gene activation (Wu and Chiang, 2001). Accordingly, SNP5 (-63) was predicted to modify an SP1 consensus site and also the indel (-58). It has been hypothesised that oxysterols regulate cholesterol synthesis negatively by inhibiting transcription factor SREBP, and by positively regulating bile acid synthesis by binding to Liver X receptor (LXR) (Peet et al., 1998). In addition, LXRs regulate SREBP-1c expression and lipogenesis (Repa et al., 2000), and oxysterols regulate endogenous LXR ligands (Janowski et al., 1999). So, if *CYP7B1*

reduces the activity of oxysterols and their cytotoxicity, *CYP7B1* would be a regulator of LXRs and SREBPs activation and, consequently, of lipogenesis. Increased *CYP7B1* activity would lead to a reduction in oxysterols and in lipogenesis. In this study, five *CYP7B1* polymorphisms were selected for validation due to their location near the TSS of the gene that could alter the transcription factor-binding sites and consequently the gene expression rate. The SNP association studies demonstrated that SNP3 (-114), SNP5 (-63), the indel (-58) and polyC (-25) were associated with GR, and the indel (-58) was also associated with BCS. These results were confirmed by the haplotype analysis and showed that animals carrying two copies of H2 (G-C-A-Ins22-C7 for SNP3 (-114)-SNP4 (-97)- SNP5 (-63)- indel (-58)- polyC (-25)) had lower GR compared to animals with one or no copies ($P<0.05$). One outstanding finding was that the Ins22 and A alleles for the indel (-58) and SNP5 (-63) polymorphisms were, respectively, the alleles associated with a lower GR, and were predicted to modify the SP1 consensus sites. On the contrary, the H12/H12 diplotype implied a higher GR than diplotypes with one or no copies. H12 (G-C-G-N-C8) had the alleles of polymorphisms SNP3 (-114), SNP5 (-63), indel (-58) and polyC (-25), associated with an increased GR.

Because of the putative modification of the TF consensus sites, we further explored the *CYP7B1* expression by RT-qPCR. The statistical analysis considered the number of days of concentrate enriched with dl-α-tocopheryl acetate intake as a fixed effect because our previous work had demonstrated that vitamin E supplementation causes different responses in gene expression (González-Calvo et al., 2017). The present research work demonstrated that *CYP7B1* expression was affected by SNP5 (-63). The A allele caused a modification of SP1 and Adf-1 binding consensus sites, and could, thus, affect the gene expression rate. No effect was found for the indel (-58), but genotypes were unbalanced in the population studied with 28, 15 and 4 ewes being homozygous without the insertion, and heterozygous and homozygous with the insertion, respectively. The haplotypes analyses confirmed these results and showed that the *CYP7B1* expression appeared to vastly differ between animals with two copies of the H3 haplotype (n = 3; G-C-A-Ins22-C7) and the animals with one (n = 16) or no copies (n = 28). However, there were only three homozygous ewes for this haplotype. Furthermore, the *CYP7B1* expression was 1.76-fold higher in the ewes with no copies than for the animals with one copy. In this research, the animal model for validation differed from that of the

association analysis, mainly because of animals' age and sex. The gene expression studies used male lambs slaughtered at 75.20±1.84 days, while the association studies included adult ewes managed according to the local traditional system. Despite these possible limitations, the results still demonstrated the involvement of the *CYP7B1* gene promoter in the variability of GR and BCS traits in the Rasa Aragonesa ewes. This is the first study demonstrating the association between some polymorphisms in the *CYP7B1* gene promoter and growth traits in sheep.

Ethics approval

All the experimental procedures, including the care of animals and euthanasia, were performed in accordance with the guidelines of the European Union and with Spanish regulations for the use and care of animals in research, and were approved by the Animal Welfare Committee of the Centro de Investigación y Tecnología Agroalimentaria (CITA). Protocol number codes 2011-08 and 2009-01_MJT are for the animals used in the GWAS and the functional studies, respectively.

Data and model availability statement

The data were not deposited in an official repository. The data supporting the findings of this study are available from the corresponding author upon request.

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Declaration of interest

None

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Supplementary material:

Supplementary Table S1. Primer sequences, amplified exon (site), annealing temperatures (AT), and amplification fragment sizes for isolation of *CYP7B1*.

Fragment	Site	Primer Sequence (5' -> 3') [1]	AT (ºC)[2]	Size (bp)
1	Promoter	F:CCTGGCGCTTCTGGAACG R:TACCTGCTCAGTCATGTGGG	57	923
2	Promoter-exon 1	F:AGGGGAAGCTTTGTGTCAAC R:CCCTTCTCTTGGGCCTTTAG	57	669
3	Exon 2	F:AACAACATTAAAGAAAAGGTGAGAAA R:GGATAAATGACCCACAGGTGTT	55	276
4	Exon 3	F:CAAGGTCGCCATTTTGTCTT R:GGAAACATGTAGTGGAATTTTGAA	55	847
5	Exon 4	F:TGTGTCCTCCACCTCTTGGT R:GGCTGGGTCTGTGTCCTAAC	55	399
6	Exon 5	F:GGAAGGAATGTGCTTGCAG R:AAATGAGAGTACTTTTCAAGAGCAG	55	299
7	Exon 6	F:GGCATCTTTAAAACAAACAAAGC R:TTAAGATGGGCAAGGAGAGC	55	492

[1] F: forward; R: reverse.
[2] AT: annealing temperature.

Supplementary Table S2. Genes and forward (F) and reverse (R) primers used for relative quantification by real-time qPCR.

Gene[2]	Forward and reverse primers	Amplicon (bp)	RT-qPCR conditions[1]			
			AT (ºC)	[nM]	E	R^2
CYP7B1	F:5'-TGCTGGTCTTGTGCCTGAG-3' R:5'-CCAAAGTAAGGAAGCCAGCC-3'	82	60	600/600	1.98	0.99
GUSB	F: 5′-GCTTCGAGCAGCAGTGGTA-3′ R: 5′-CACGTCGTTGAAGCTGGAC-3′	86	60	600/600	1.99	0.99
RPL19	F: 5′-CAACTCCCGCCAGCAGAT-3′ R: 5′-CCGGGAATGGACATGCACA-3	76	60	200/200	1.97	0.99
RPL37	F: 5′-GAAGTCGACCTGTGGCAAGT-3′ R: 5′-CTCATTCGACCAGTCCCAGT-3′	105	60	900/900	1.97	0.99

[1]RT-qPCR conditions: AT = annealing temperature; nM = primer concentrations; E = amplification efficiency; R^2 = correlation coefficient.
[2]*CYP7B1 = Cytochrome P450 Family 7 Subfamily B Member 1; RPL37 = ribosomal protein L37; RPL1 9= ribosomal protein L19; GUSB = β-glucuronidase*

Supplementary Table S3. Significant SNPs at the chromosome-wise level associated with each growth traits in Rasa Aragonesa breed. Minor allele frequency (MAF) is also indicated. Putative causal genes located in the 250 kb region on both sides of the significant SNPs are indicated. Position of markers are based on Oar_rambouillet_v1.0 genome assembly in bp.

Traits[1]	Chr[2]	SNP	dbSNP	Position	MAF	b	p-value	p-Bonf[3]	Fdr_thr[4]	Genes within 250 kb on either Side
GR	7	oar3_OAR7_82708460	rs405607259	89550547	0.01	-0.040 ± 0.008	5.90E-07	0.34	4.40E-06	*LIN52-VSX2-ABCD4-VRTN-SYNDIG1L-ISCA2-NPC2-LTBP2-AREL1-FCF1-YLPM1*
	9	**oar3_OAR9_42035112**	rs425509273	45546608	0.06	-0.014 ± 0.003	4.63E-08	**0.03**	9.50E-06	*BHLHE22-**CYP7B1***
	9	oar3_OAR9_88434565	rs427506082	97921953	0.03	-0.017 ± 0.004	1.60E-06	0.93	9.50E-06	*CNBD1-CNGB3*
	15	oar3_OAR15_47073330	rs430194623	51439713	0.01	-0.037 ± 0.008	7.43E-06	1	7.50E-06	*ZNF667-TRIM5 like (LOC105614607)-TRIM5 Like (LOC114118421)-OR51I2-TRIM34-TRIM6-LOC101102669-OR52B6-OR52B4 like-OR52H1(LOC101119670)-OR52H1 (LOC105602420)-UBQLNL-UBQLN3-LOC101120692-OR52D1-LOC101120949-OR51I2 like-LOC101121967-LOC101103421-LOC101122215-LOC101122462*
	20	oar3_OAR20_22070146	rs417043327	23839961	0.06	-0.012 ± 0.003	2.07E-06	1	8.40E-06	*MEP1A like-MMUT-CENPQ-GLYATL3-C20H6orf141-RHAG-CRISP2-CRISP3-PGK2*
	23	oar3_OAR23_51434165	rs427416766	56897122	0.06	-0.014 ± 0.003	2.23E-06	1	7.20E-06	-
	25	oar3_OAR25_6873226	rs425329469	6487542	0.05	-0.013 ± 0.003	1.13E-05	1	1.20E-05	*SLC35F3*
	26	oar3_OAR26_3943857	rs398535453	4490102	0.13	-0.008 ± 0.002	8.35E-06	1	1.00E-05	*CSMD1*
	27	oar3_OARX_6627610	rs422252289	7456631	0.08	-0.011 ± 0.002	1.64E-06	0.96	3.80E-06	*MAGEB2 like-MAGEB1 like*
BCS	6	oar3_OAR6_99760786	rs424629620	110458659	0.38	0.092 ±0.02	2.29E-06	1	3.90E-06	*ARHGAP24*
LW	12	oar3_OAR12_22059564	rs430155342	25998332	0.13	-2.850 ± 0.608	2.77E-06	1	5.50E-06	*RAB3GAP2-MARK1-C12H1orf115*
	13	oar3_OAR13_33247804	rs422199592	35228082	0.14	-2.671 ± 0.592	6.35E-06	1	6.40E-06	*ZEB1-ZNF438*
	24	oar3_OAR24_28384805	rs408865776	29194526	0.38	-2.013 ± 0.448	6.87E-06	1	1.00E-05	*RABGEF1-TMEM248-LOC101111335- SBDS-CALN1*

[1]GR: growth rate; BCS: Body condition score; LW: Live weight. [2] Chr: chromosome. [3] p_Bonf: p value after Bonferroni correction. [4] Fdr_thr: False Discovery rate threshold =10%

Supplementary Table S4. Haplotypes frequency for haplotype SNP3 (-114)-SNP4 (-97)-SNP5 (-63)-indel (-58)-polyC (-25)

Id haplotype	Alleles haplotype[1]	Frequency
H1	A-A-A-Ins22-C7	0.025
H2	G-C-A-Ins22-C7	0.285
H3	G-C-G-Ins22-C7	0.012
H4	A-C-A-N-C7	0.017
H5	A-C-G-Ins22-C7	0.002
H6	G-C-A-N-C7	0.030
H7	A-C-A-Ins22-C7	0.005
H8	G-C-G-N-C7	0.005
H9	A-A-A-Ins22-C8	0.015
H10	A-C-G-Ins22-C8	0.005
H11	A-C-A-Ins22-C8	0.012
H12	G-C-G-N-C8	0.448
H13	G-C-A-Ins22-C9	0.002
H14	G-C-G-Ins22-C8	0.015
H15	A-C-A-N-C8	0.050
H16	G-A-G-N-C8	0.012
H17	G-C-A-N-C8	0.025
H18	A-C-G-N-C8	0.007
H19	G-C-A-Ins22-C8	0.002
H20	A-C-A-N-C9	0.007
H21	G-C-G-Ins22-C9	0.007
H22	A-A-A-Ins22-C9	0.010

[1]Ins22 indicates the allele with the insertion of 22 bp, otherwise N as normal allele. C7 and C8 indicate alleles with 7 and 8 cytosine repetitions, respectively.

Supplementary Table S5. Frequency of the genotypes for the polymorphisms located in the 5' region of the gene *CYP7B1* in the population for the functional studies (n = 48)

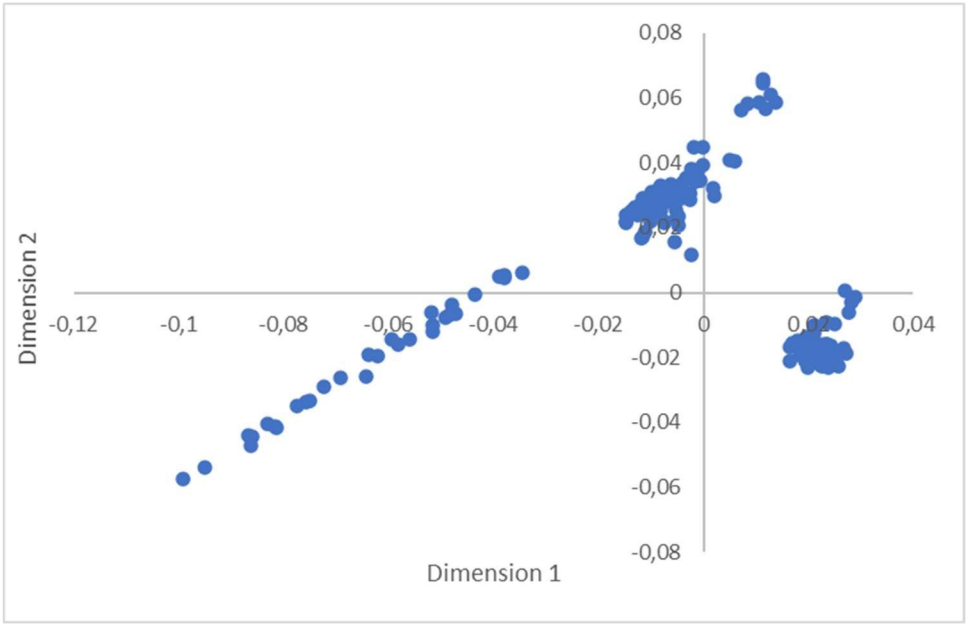
Polymorphism	Genotype		
SNP3 (-114)	G/G	A/G	A/A
	0.83	0.17	0
SNP4 (-97)	C/C	C/A	A/A
	1	0	0
SNP5 (-63)	A/A	A/G	G/G
	0.28	0.36	0.36
Indel (-58)[1]	N/N	N/Ins22	Ins22/Ins22
	0.60	0.32	0.08
PolyC (-25)[2]	C7/C7	C7/C8	C8/C8
	0.28	0.36	0.36

[1]Ins22 indicates the allele with the insertion of 22 bp, otherwise N as normal allele. [2]C7, and C8 indicate alleles with 7, and 8 cytosine repetitions, respectively.

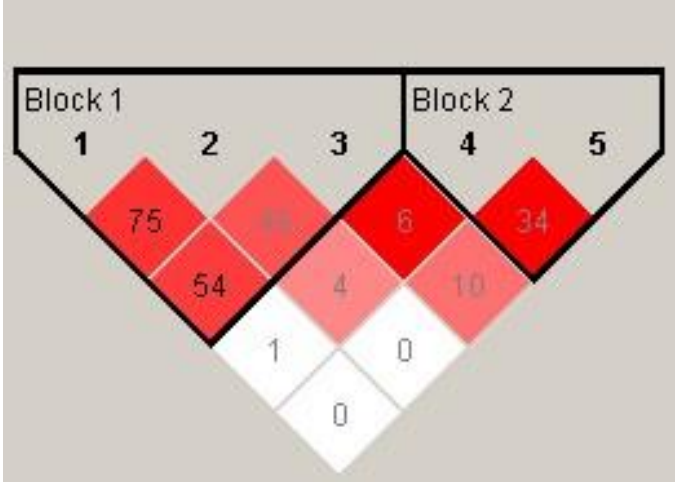
Supplementary Table S6. Haplotypes frequency for the polymorphisms SNP3 (-114)-SNP4 (-97)- SNP5 (-63)-indel (-58)-polyC (-25) of the *CYP7B1* gene in the population for the functional studies

Haplotype[1]	**Frequency**
H1 (G-C-A-N-C7)	0.03
H2 (G-C-G-N-C8)	0.53
H3 (G-C-A-Ins22-C7)	0.22
H4 (G-C-A-N-C7)	0.17
H5 (A-C-A-Ins22-C7)	0.01
H6 (A-C-G-N-C8)	0.03
H7 (G-C-G-Ins22-C8)	0.01

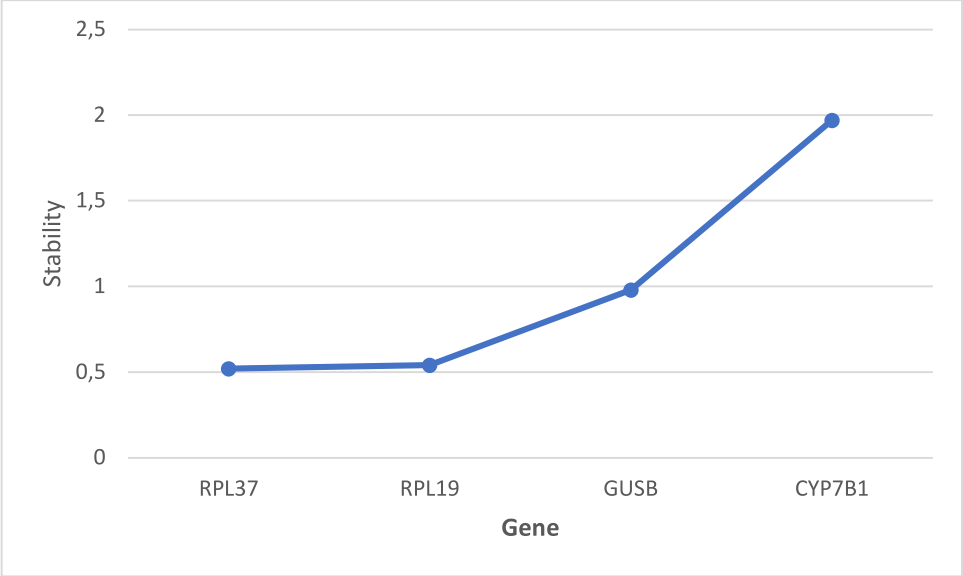
[1]Ins22 indicates the allele with the insertion of 22 bp, otherwise N as normal allele. C7 and C8 indicate alleles with 7 and 8 cytosine repetitions, respectively.



Supplementary Figure S1. Multidimensional scaling (MDS) analysis performed with 184,076 SNPs after pruning in 225 ewes.



Supplementary Figure S2. Linkage disequilibrium plot among the five polymorphisms: 1 (polyC, -25); 2 (indel, -58); 3 (SNP5, -63); 4 (SNP4, -97); 5 (SNP3, -114). The linkage disequilibrium (LD) colour scheme and values correspond with the D' and r2 parameters, respectively. Strong LD (D' = 1, LOD ≥ 2) is indicated in red. Red indicates varying degrees of LD with lighter shades displaying less than darker shades (D' < 1, LOD ≥ 2), and white indicates low LD (D'< 1, LOD < 2).



Supplementary Figure S3. Gene expression stability of genes *CYP7B1, GUSB, RPL37* and *RPL19* using NormFinder in liver tissue.

Manuscrito 4: Characterization of the *pars tuberalis* and hypothalamus transcriptome in female sheep under different reproductive stages

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Characterization of the *pars tuberalis* and hypothalamus transcriptome in female sheep under different reproductive stages

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ABSTRACT

For understanding the molecular events underlying the follicular (F) and luteal (L) phases of estrous cycle, and anestrous (A) phase, the *pars tuberalis* (PT), and hypothalamus (HT) transcriptomes of 21 ewes were studied. In HT, 72 and 3 differential expression genes (DEGs) were found when comparing F *vs.* A and L *vs.* A, respectively. In PT, 6 and 4 DEGs were found in F *vs.* A and L *vs.* A comparisons, respectively. Enrichment analysis for DEGs between the F and A phases in the HT revealed significant clusters, mainly associated with actin-binding, and cytoskeleton, that are related to neural plasticity modulated by gonadal steroid hormones, as well as with oxytocin signaling. We found that DEGs in PT had higher differences in expression levels than those found in HT. In this sense, the *ITLN* was highly upregulated in the F and L *vs.* A phases, being *MRPL57* and *IRX4* highly downregulated in L *vs.* A comparison. The *DDC* gene in PT, related to LH regulation, was upregulated in the F phase. The gene set enrichment analysis (GSEA) revealed multiple pathways related to neurotransmission and neuronal plasticity. Our study reveals new candidate genes involved in the reproductive stages' transitions in seasonal sheep.

KEYWORDS

RNA sequencing; *pars tuberalis*; hypothalamus; enrichment; anestrous

Introduction

Reproductive seasonality is one of the limiting factors significantly affecting the development of sheep industry as it induces disequilibrium between the availability of animal products and consumer demand.[1] The breeding season occurs during the short days of autumn and winter whereas the long days (spring and summer) in the Northern Hemisphere inhibit the reproductive activity (anestrous).[2–4] However, literature shows that the duration of the breeding season varies between geographic locations and breeds. Therefore, in breeds raised in the Mediterranean basin, the maximal estrous activity is associated with short days from September to February. The principal mechanism that activates the onset of the breeding season is associated with a complex combination of endogenous circannual rhythm driven and synchronized by light and melatonin.[5] Indeed, the reproductive process is primarily controlled by the hypothalamic–pituitary–gonadal (HPG) axis in vertebrates. Neurons in the Hypothalamus (HT) generate a gonadotropin-releasing hormone (GnRH) signal which acts downstream on *the pars tuberalis* (PT), to modulate the secretion of the gonadotropins (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) in the anterior pituitary to stimulate gonadal activity.[6,7] These hormones act as ovary regulators by sustaining the functions of the corpus luteum, driving folliculogenesis, and synthesizing progesterone and estrogen, but the release of GnRH is also regulated by gonadal hormone feedback.[8] Temporal changes in GnRH and gonadotropin are driven by seasonal changes in day length. This light signal is converted into a daily cycle of melatonin secretion in the pineal gland that produces photoperiod-induced signals modifications in pituitary PT. Then, the PT and HT tissues play a key role in controlling gonadal functions in mammals, such as follicular (F) development, and ovulation, as well as tissues mechanistic changes responsible for seasonal reproduction in ewes regulated by photoperiod.[9]

In Rasa Aragonesa breed, a north-eastern Spain meat breed, it has been shown that 25% of ewes have spontaneous ovulations in spring and can be naturally

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mated all over the year under appropriate management and feeding conditions.[10] For improving breeding rate during anestrous, studies providing detailed analysis on the molecular mechanisms regulating anestrous and breeding season are needed. With the advances in next-generation sequencing and bioinformatics tools, high throughput RNA deep sequencing (RNA-seq) offers a platform to measure large-scale gene expression patterns.[11–13] Thus, using high-throughput sequencing platforms, a single experiment can capture the expression levels of thousands of genes at once with high accuracy.[14,15] However, reports on the sheep transcriptome information related to fertility that have applied RNA-seq technology are very limited. In this sense, Lomet et al.[16] found almost 3000 differentially expressed genes (DEGs) in HT between the breeding and non-breeding seasons. Moreover, Xia et al.[17] identified photoperiod-induced lncRNAs and genes in pituitary PT of ewes by transcriptome comparisons between short and long photoperiod. Transcriptome studies comparing ewes in different ovarian phases during the non-breeding season are also scarce. Zhong et al.[18] believed that *ODC1*, *PRLH*, *CRYBB2*, *SMAD5*, *OPN1SW*, and *TPH1* are key genes involved in the estrogen process. According to these authors, these genes may regulate downstream genes derived by annual rhythm, and finally exhibit reproductive cycles. The identification of new DEGs between, anestrous and F or luteal (L) phases can help in selecting these genes to look for polymorphisms (SNP) within them that could be involved in the variability of this phenotype. This kind of research may provide a better understanding of the molecular regulation of reproductive stages' transitions to manage better the breeding period and reproductive efficiency (E) in sheep production systems. In this study, we used high-throughput sequencing technology to explore transcriptional changes in the PT and the HT tissues during the F and L phases of estrous cycle, and anestrous (A) phase of 21 Rasa Aragonesa ewes. Finally, several candidate genes were chosen for validation by reverse transcription real-time quantitative PCR (RT-qPCR) analysis and confirmation of the RNA-seq results consistency.

Materials and methods

Ethics statement

All experimental procedures including care of animals and euthanasia were performed in accordance with the guidelines of the European Union regulations for the use and care of animals in research (Directive 2010/63/EU) and approved by the Animal Ethics Committee of the Research Center (protocol number 2015/03). No hormonal treatments were applied to ewes during the study.

Animals and tissues samples

The experiment was conducted in a Rasa Aragonesa sheep flock managed at the experimental farm of the Agrifood Research and Technology Center of Aragón (CITA). From this flock, which was composed of 450 Rasa Aragonesa ewes, 50 ewes with similar body condition score (2.98 ± 0.31), live weight (53.50 ± 6.13 kg), and age (7.97 ± 0.56 y) were selected to control the ovarian activity. These ewes had lambed in the autumn (September–December). Ewes were subjected to the same management and nutrition conditions to limit the effect of environmental influences on gene expression pattern. Plasmatic progesterone profiles were monitored during the second fortnight of April (within the non-breeding season) to determine the reproductive status of ewes to be slaughtered: anestrous, F, or L phases of the estrous cycle. A total of five blood samples were taken twice a week (3–4 d apart), the last one 2 d before slaughter. This protocol was based on the article of Thimonier.[19] Progesterone was determined using a commercial ELISA kit designed for ovine plasma (Ridgeway Science, St. Briavels, Gloucestershire, UK). A ewe was considered in anestrus (A) when the progesterone concentration was lower than the threshold (0.5 ng/mL) in the five samples. In previous works[20,21] we used the criterion of Teyssier et al.,[22] who considered that a ewe was in anestrus when progesterone was below the threshold in three samples taken one week apart. The current sampling protocol is more exhaustive, since it includes the three samples from the previous one, plus an additional sample between every two samples. A ewe was considered to be in the F phase at slaughter when either, the last sample was below the threshold and was preceded by samples above the threshold, or when the last sample was above the threshold and the second last was at least 1.3 times greater. A ewe was considered to be in the L phase when the last two samples were above the threshold, the last being higher than the previous one, and both were preceded by at least one sample below the threshold. We selected 30 ewes (ten from each group) for slaughtering. The reproductive status was confirmed or disconfirmed after animal's sacrifice through reproductive tract examination by an expert. There were observed

recent ovulations in the ovaries of 3 ewes considered in anestrus as well as in 4 ewes considered in F phase. Hydrosalpinx was suspected in one more ewe considered in anestrus. In one ewe considered in L phase, the mature corpus luteum was accompanied by an ovarian cyst. All these ewes were not considered for the RNA-seq study. The ewes considered to be in anestrus included for this study had neither corpora lutea nor regressed corpora lutea (corpora albicantia) in the ovaries surface ($n = 7$). In the ewes considered to be in F phase included in this study we found corpora albicantia and no corpora lutea in the ovarian surface, together with small (1–2.5 mm) and medium (3–5 mm) follicles in all ewes ($n = 7$), and large follicles (> 5 mm) in all but one ewe. The ewes considered to be in L phase for this study had at least one ovary with at least one healthy, mature (>7 d) corpus luteum ($n = 7$). In total, 21 ewes (7 for each reproductive status) were selected for RNA extraction from HT ($n = 21$) and pituitary PT ($n = 21$) tissues samples. Samples were dissected, rapidly frozen in liquid nitrogen and stored at −80 °C.

RNA isolation and sequencing

Total RNA was extracted from approximately 0.5 g of tissue using RNeasy Lipid Tissue mini kit (QIAGEN, Madrid, Spain), following the manufacturer's instructions. The RNA samples were resuspended in DEPC-water and were quantified with Nanodrop spectrophotometer (Implen, Madrid, Spain). DNase I (Invitrogen, Carlsbad, CA) was used to degrade the remaining DNA according to the manufacturer's instructions. The RNA concentration and RNA integrity number (RIN) of the samples was assessed using an Agilent 2100 Bioanalyzer machine (Agilent Technologies, Palo Alto, CA). Only RNA samples with a RIN > 7 were used for library construction. Sequencing was carried out at the CNAG (Centro Nacional de Análisis Genómico, Spain; https://www.cnag.crg.eu/). The RNA-Seq libraries were prepared from total RNA using KAPA Stranded mRNA-Seq Kit Illumina Platforms (Roche, Basel, Switzerland) according to the manufacturer's protocol with Illumina Truseq indexed adapters (Illumina, San Diego, CA). The ligation product was enriched with 15 PCR cycles and the final library was validated on an Agilent 2100 Bioanalyzer with the DNA 7500 assay. The libraries were sequenced in paired-end mode (2 × 76 bp) on HiSeq 4000 (Illumina) in a fraction of a HiSeq 4000 PE Cluster kit sequencing flow cell lane, following the manufacturer's protocol. Image analysis, base calling and quality scoring of the run were processed using the manufacturer's software Real Time Analysis (RTA) version 2.7.7 (Illumina) and followed by generation of FASTQ sequence files. The data sets supporting the results and discussion in this publication have been deposited in NCBI's Gene Expression Omnibus repository[23] and are accessible through GEO Series accession number GSE191213 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE191213).

Validation of RNA-seq data by reverse transcription quantitative real-time PCR analysis (RT-qPCR)

In order to validate the RNA-Seq data, 15 DEGs were selected for qRT-PCR analysis: *ASPH*, *CAVIN3*, *CTGF*, *DDC*, *FBN3*, *HTR2B*, *ITLN*, *ITPR3*, *LTA4H*, *MRPL57*, *MYLK*, *PCDH15*, *PLCXD2*, *PTGIS*, and *WFDC1*. Moreover, we selected a set of five housekeeping genes (*RPL19*, *RPL27*, *RPL32*, *B2M*, and *GAPDH*) commonly used as reference genes in brain expression studies. One microgram of RNA from each sample ($n = 21$) was treated with DNAse (Invitrogen, Carlsbad, CA), and was used to synthesize single-stranded cDNA using the Super-Script®III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA), following the manufacturer's recommendations. Prime version 3 software was used to design the primers in specific exon-spanning regions (https://bioinfo.ut.ee/primer3–0.4.0/; last accessed on May 14 2022). The specificity of the primers was confirmed using BLAST (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/BLAST/; last accessed on May 14 2022). Conventional PCR was performed using standard conditions for all the genes to test the primers and verify the amplified products. The PCR products were Sanger sequenced by the company Stabvida (https://www.stabvida.com/es) to confirm gene identity. Homology searches were performed with BLAST (National Center for Biotechnology Information: https://blast.ncbi.nlm.nih.gov/Blast.cgi). The real-time PCR was carried out in a 10 µl PCR total reaction mixture containing SYBR Green Master Mix: SYBR Premix Ex Taq II (Tli RNase H Plus, Takara, Demlab, Zaragoza, Spain), and an ABI Prism 7500 platform (Applied Biosystem, Madrid, Spain), running the samples in triplicate. The correlation coefficient (R^2) and slope for each gene were estimated using standard curves through 10-fold serial dilution of pooled cDNA. The slope was used to assess the efficiency (E) of PCR amplification using the formula $E = 10^{(-1/\text{slope})}$. Two 'connector samples' were

replicated in all plates to remove technical variation from this source of variability. The annealing temperatures, primer concentrations, and primer sequences are described in Supplementary Table S1. The gene stability was evaluated across the two analyzed tissues. Determinations of the gene expression stability of HT and PT genes (DEGs and housekeeping genes) included in this study were calculated using NormFinder to select the best housekeeping genes.[24]

Data analysis

RNA-Seq data processing

Quality control of the raw and trimmed paired-end reads were examined with FastQC version 0.11.5.[25] The reads were trimmed with Trimmomatic version 0.38[26] to remove adaptor sequences and low-quality bases using the options '-leading:20', '-slidingwindow:40:20', and '-minlen:35'. Clean reads were aligned to the ovine reference genome Texel Oar_v3.1 (GCA_000298735.1) using HISAT2 version 2.1.0 software[27] with default parameters except the options '–max seed 30' and '–k 2', and were converted to sorted bam files using Samtools version 1.7.[28] Subsequently, StringTie version 1.3.5[29] software was used to assemble transcripts and quantify RNA-seq, using the '-G' option for annotation (Ensembl Release 104). Thereafter, differential gene expression levels of read counts obtained from StringTie were measured in the R version 3.5.1 statistical environment using the DESeq2 package.[30] Prior to examining expression, a pre-filtering step was performed. There must be at least five samples with the sum of gene counts greater or equal to 5 to keep the gene in the differential expression analysis. For both tissues (HT and PT), the design employed studied the following contrasts between the ovarian phases: F *vs.* A and L *vs.* A. The identification of DEGs was performed by comparing the counts between sample groups for the same gene, and were considered DEG when the adjusted *p* value was lower than 0.05, after Benjamini and Hochberg correction[31] for controlling the false discovery rate (FDR), and |log2FoldChange| was higher than 1. Annotation of DEGs was retrieved with BiomaRt (https://www.ensembl.org/biomart). The volcano Plots were generated using the R version 3.5.1 programming language[32] (https://www.R-project.org/; last accessed on May 14 2022).

GO and pathways enrichment analysis

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were used to perform enrichment analysis of DEGs using the Database for Annotation, Visualization and Integrated Discovery (DAVID)[33] through the Functional Annotation Cluster (FAC) tool. Medium stringency EASE score parameters were selected, and an enrichment score (ES) of 1.3 was used as a threshold for cluster significance.[33] As enrichment analysis of DEGs in certain comparisons were not possible due to the low number of DEGs, we decided to run the gene set enrichment analysis (GSEA)[34] implemented in OmicsBox (https://www.biobam.com/omicsbox) including all the expressed genes. GSEA was used to characterize both modest and robust, coordinated, biologically relevant changes in molecular signaling pathways, and to explore and identify the most significant and over-represented GO terms considering as input all expressed genes. The entire unfiltered data set is used in GSEA, without fold cutoff filtering or significance test as it seeks to point out modest, coordinated transcriptomic changes. In this study, this tool was used to determine whether an a priori-defined set of genes is statistically significant between the ovarian phases. It classifies the biological functions of the set genes based on three factors: molecular functions, biological process and cellular component. Differential expression scores (DE) were calculated and the genes ordered in a ranked list, according to their DE between the classes. To identify genes that are over-represented at the extremes (top or bottom) of the entire ranked list, an ES and normalized ES (NES) for each gene set were calculated. The significance level was adjusted to account for multiple hypothesis testing using FDR 5%.

Statistical analysis of gene expression validated by RT-qPCR

The method proposed by Steibel et al.[35] was used to analyze differences in the expression rate using their quantification cycle (Cq). Because amplification efficiencies of all genes were different from 2, Cq values were rescaled using the equation proposed by Steibel et al.[35] The mixed model fitted was as follows:

$$y_{rigkm} = OG_{gi} + P_k + BC_m + A_m + LW_m + E_m + e_{rigkm},$$

where, y_{rigkm} is the Cq value (transformed data taking into account gene amplification efficiencies) of the gth gene (DEGs and housekeeping genes) from the rth well (reactions were run in triplicate) in the kth plate corresponding to the mth animal and to the ith

ovarian phase (A, F and L); OG_{gi} is the fixed interaction among the ith ovarian phase and the gth gene; P_k is the fixed effect of the kth plate; BC_m, A_m, and LW_m are the effects of body condition score, age, and the live weight of the mth animal, respectively, included as covariates; Em is the random effect of the mth animal from which samples were collected (Em $\sim (0,\sigma^2_E)$); and e_{rigkm} is the random residual. Gene-specific residual variance (heterogeneous residual) was fitted to the gene by ovarian phase ($e_{rigkm} \sim N(0, \sigma^2_{egi})$). To test differences ($diff_{DEGs}$) in the expression rates for each selected gene between ovarian phases in terms of fold changes (FCs), the approach suggested by Steibel et al.[35] using two housekeeping genes was carried out. The *t* statistic after Benjamini and Hochberg correction was used to determine the significance of the $diff_{DEGs}$ estimates ($p < 0.05$).

The Pearson correlation coefficient was calculated between Log2FC values estimated in RNA-seq analysis and qPCR expression measures to validate the RNA-seq results.[36]

Results

Mapping statistics summary

Approximately, 834 and 874 million (M) paired-end reads were generated from RNA sequencing of HT and PT, respectively, from 42 libraries (21 from each tissue), ranging from 30.5 to 57.6 M per sample (Supplementary Table S2). After trimming, around 746 M and 741 M were retained from HT and PT samples, respectively, that were mapped against the ovine reference genome (Oar_v3.1; GCA_000298735.1) using HISAT2 version 2.1.0. The statistics of the sequencing read alignments are shown in Supplementary Table S2. On average, the overall alignment rate to reference genome was 90%. Moreover, 69% of all the clean reads were uniquely mapped to the ovine reference genome whereas 15.90% were multiple aligned. These results might be explained by sequencing errors, imperfections in the reference genome (Oar_v3.1, assembly GCA_000298735.1), and the mapping criteria used (stringent).

Identification and analysis of differentially expressed genes (DEGs)

The DESeq2 package based on read counts was used to evaluate the gene expression levels. As a result, the expression of 18,895 and 18,960 genes was detected in PT and HT, respectively. The DEGs identified by DESeq2 in the HT and PT are shown in Tables 1 and 2, respectively. The greatest number ($n = 72$) of DEGs was detected in the pairwise comparison between the F and A phases in HT, in which 14 and 58 genes were up and down-regulated, respectively. In Table 1, the top 20 DEGs for F *vs.* A in HT are shown. The detailed list is provided in Supplementary Table S3. In the comparison of L *vs.* A phases, only three significant DEGs were found (*p*-adjusted < 0.05) in the same tissue, being all of them down-regulated.

In PT, 6 and 4 DEGs were found significant in the comparisons of F *vs.* A and L *vs.* A phases,

Table 1. Differentially expressed gene in the hypothalamus through the three-stage comparison.

Phase[a]	Genes	Description	Log2FC[b]	P-adj[c]
L *vs.* A	*SMC6*	*Structural maintenance of chromosomes 6*	−0.62	0.0010
	HDGFL3	*HDGF like 3*	−0.41	0.0289
	ASPH	*Aspartate beta-hydroxylase*	−0.47	0.0329
F *vs.* A*	*MYLK*	*Myosin light chain kinase*	−2.17	0.0004
	SYNPO2	*Synaptopodin 2*	−2.47	0.0011
	CTGF	*Connective tissue growth factor*	−2.12	0.0011
	CNN1	*Calponin 1*	−3.82	0.0047
	DES	*Desmin*	−3.71	0.0066
	MUSTN1	*Musculoskeletal, embryonic nuclear protein 1*	−3.13	0.0091
	SYNC	*Syncoilin, intermediate filament protein*	−0.75	0.0125
	PLEKHA4	*Pleckstrin homology domain-containing A4*	−3.71	0.0130
	ENSOARG00000001181	*HLA class I histocompatibility antigen, Cw-4 alpha chain-like*	−2.52	0.0130
	LGALS1	*Galectin 1*	−1.33	0.0130
	ASB2	*Ankyrin repeat and SOCS box containing 2*	−3.25	0.0140
	DCLK3	*Doublecortin like kinase 3*	−2.52	0.0140
	CEP85L	*Centrosomal protein 85 like*	−1.10	0.0140
	SMC6	*Structural maintenance of chromosomes 6*	−0.49	0.0194
	ACTA2	*Actin, alpha 2, smooth muscle, aorta*	−3.53	0.0199
	NEXN	*Nexilin F-actin binding protein*	−1.95	0.0199
	ANGPTL5	*Angiopoietin like 5*	−1.90	0.0207
	MYH11	*Myosin heavy chain 11*	−3.59	0.0207
	TPM1	*Tropomyosin 1*	−1.65	0.0207
	TSKS	*Testis-specific serine kinase substrate*	−1.85	0.0207

[a]L: luteal; A: anestrous; F: Follicular; [b]Log2Fold change; [c]P-adjusted.
*Only the top 20 DEGs are shown for this comparison. The complete list is provided in Supplementary Table S3.

Table 2. Differentially expressed gene in the *pars tuberalis* through the three stages comparison.

Phase[a]	Genes	Description	Log2FC [b]	P-adj[c]
F *vs.* A	*DDC*	*Dopa decarboxylase*	1.92	0.008
	ITLN	*Intelectin*	17.72	0.008
	POU6F1	*POU class 6 homeobox 1*	−0.57	0.008
	CRYBG1	*Crystallin beta-gamma domain containing 1*	−0.73	0.018
	PCDH15	*Protocadherin related 15*	−3.96	0.020
	COA1	*Cytochrome c oxidase assembly factor 1 homolog*	0.54	0.036
L *vs.* A	*MRPL57*	*Mitochondrial ribosomal protein L57*	−22.43	0.00001
	ITLN	*Intelectin*	20.80	0.00006
	IRX4	*Iroquois homeobox 4*	−20.85	0.00006
	PLCXD2	*Phosphatidylinositol-specific phospholipase C X domain containing 2*	−5.28	0.004

[a]L: luteal; A: anestrous; F: follicular; [b]Log2Fold change; [c]P-adjusted.

respectively, being four genes up-regulated and six genes down-regulated (Table 2). The magnitude of gene expression differences between F and L phases was greater in PT than in HT. *Intelectin* (*ITLN*) was upregulated in the F (Log2FC = 17.72) and L (Log2FC = 20.80) phases compared to A phase. By contrast, *Mitochondrial Ribosomal Protein L57* (*MRPL57*, Log2FC= −22.43) and *Iroquois Homeobox 4* (*IRX4*), Log2FC= −20.85) were highly down-regulated in the L compared to the A phases (Table 2). The volcano plots of DEGs between the different conditions of estrous cycle and anestrous for the two tissues are shown in Fig. 1.

Enrichment analysis

Using DAVID with the DEGs, only ESs above the threshold of 1.3 was found in HT between the F and A phases. The analysis showed nine significant clusters enrichments, mainly related to actin-binding, and cytoskeleton, but also to oxytocin signaling, smooth muscle contraction, cGMP-PKG signaling pathway, or acetylation (Supplementary material; Supplementary Table 4). Furthermore, we conducted GSEA to determine whether an a priori-defined set of genes is statistically significant and to identify modest, coordinated transcriptomic changes. In the HT, GSEA identified 195 significantly enriched gene sets at FDR of 5% for the F *vs.* A comparison, whereas in the L *vs.* A 350 pathways were identified. The most enriched terms were centrosome (GO:0005813), endoplasmic reticulum (ER) membrane (GO:0005789), and the structural constituent of ribosome (GO:0003735). Another interesting GO was the Heparin-binding pathway (GO:0008201). Within this pathway, we found the *Cellular communication network factor 2* (*CCN2*) gene which related gene is *CTGF* that was differentially expressed in the HT. Regarding to the PT, 63 pathways were identified for F *vs.* A and 129 for L *vs.* A comparisons. The most enriched pathways were the intracellular protein transport (GO:0006886) and centrosome (GO:0005813). The GO term related to the response to ER stress pathway (GO:0034976) was also enriched. The top 30 GOs among ovarian phases comparisons in the HT and PT are shown in Figs. 2 and 3, respectively. The gene set size and the ESs details for these GOs are reported in Supplementary Tables S5–S8.

Validation of differentially expressed genes

In this study, 15 genes were selected to confirm their expression profiles obtained by RNA-seq analysis using RT-qPCR. The genes *RPL19*, *RPL27*, *RPL32*, *B2M*, and *GAPDH* were used as housekeeping genes to normalize gene expression. NormFinder program was used to choose the two most stable housekeeping genes for each tissue taking into account all genes (DEGs and housekeeping genes). As it is shown in Supplementary Fig. S1, the *RPL19* and *RPL32,* and the *B2M* and *RPL27* pair genes were the most stable genes for the HT and PT, respectively. The expression patterns were in general consistent with the RNA-seq results, although the magnitude of Log2FC obtained by RNA-seq and RT-qPCR was slightly different in some instances. These results demonstrated the reliability of RNA-seq technique. The Pearson correlation between the expression results by RNA-seq and RT-qPCR were 0.69 and 0.97 for the HT and PT, respectively, considering all genes. Furthermore, 10 genes of the 15 analyzed were also differential expressed between ovarian phases within tissues by RT- qPCR (Table 3).

Discussion

In sheep farming, improving the fertility in the out-of-breeding season has always been an important economic objective. In the context of research for an alternative approach that meets modern criteria for 'clean, green and ethical' production methods without the use of exogenous hormones, a better

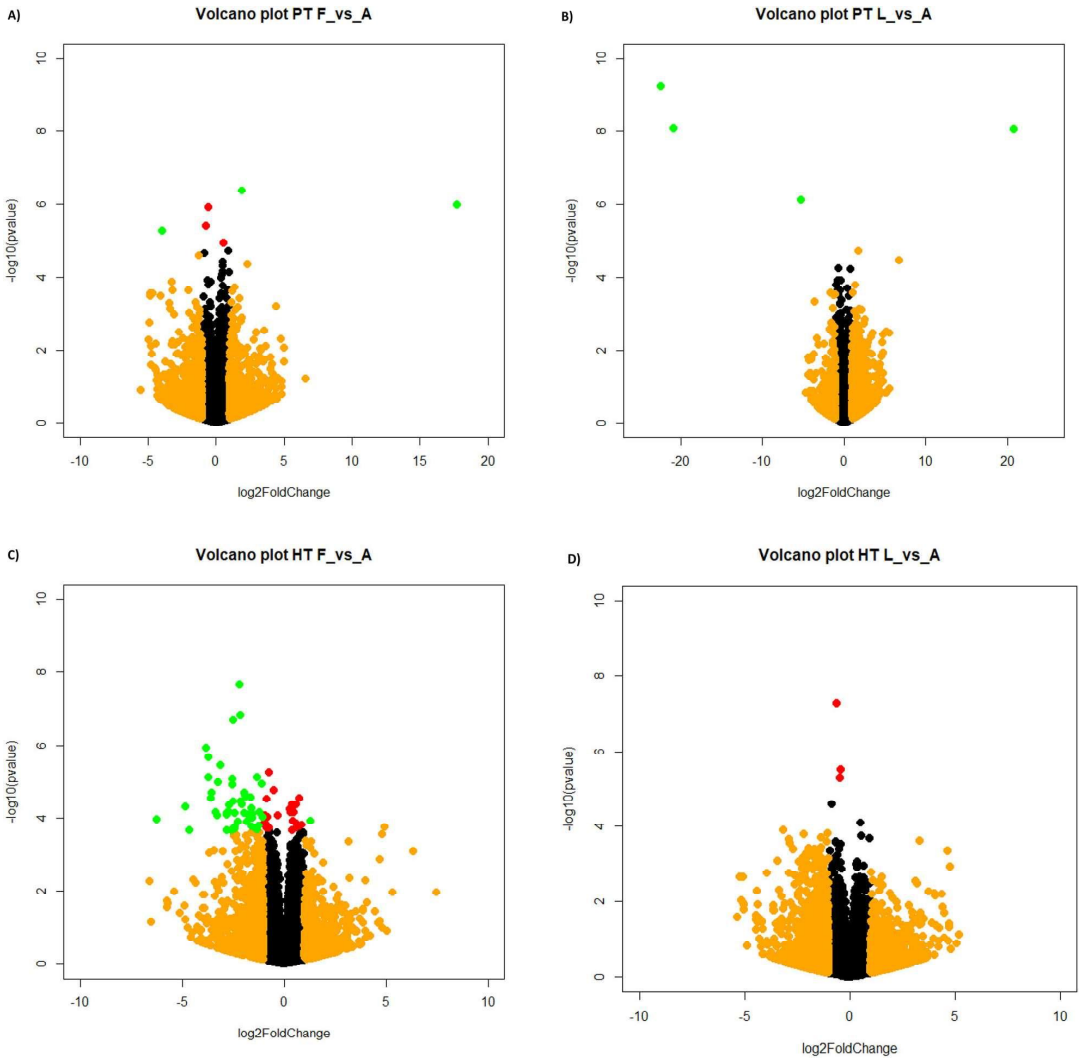

Figure 1. Volcano plots at hypothalamus and *pars tuberalis* levels between two phases comparison. The x-axis represents multiple of genes expression between two physiologic conditions and the y-axis represents the statistical significance of gene expression differences. Comparison phases at *pars tuberalis* level (A) F *vs.* A; (B) L *vs.* A Comparison phases at hypothalamus level (C) F *vs.* A; (D) L *vs.* A. A: Anestrous; F: Follicular; L: Luteal. Red: P adjusted < 0.05, Orange: log2FoldChange > 1, green if both.

understanding of molecular pathways underlying the F and L phases of the estrous cycle and the A phase could be critical for identifying key physiological processes and genes associated with spontaneous out-of-season ovulatory activity. In this research, all ewes were raised in the same conditions in the experimental farm and no significant differences were found in body condition score (A: 2.93 ± 0.31; F: 2.93 ± 0.35; L: 3.00 ± 0.14), live weight (A: 54.64 ± 8.37 kg; F: 54.00 ± 5.29 kg; L: 50.67 ± 5.53 kg) or age (A: 7.99 ± 0.45 y; F: 8.25 ± 0.08 y; L: 7.81 ± 0.78 y). Transcriptome results indicate that both tissues differed in the number of DEGs found. In fact, 10 and 75 significant DEGs were detected in the PT and HT, respectively. It can be inferred that genes from the HT may be extensively involved in sheep estrous and anestrous regulation.

Enrichment analysis of DEGs with DAVID showed 9 significantly enriched clusters in the F and anestrous comparison in the HT. These enrichment clusters included genes related to actin-binding, cytoskeleton, or calmodulin binding (Supplementary Table S4), that are related to neural plasticity modulated by gonadal steroid hormones. In this sense, it has been described

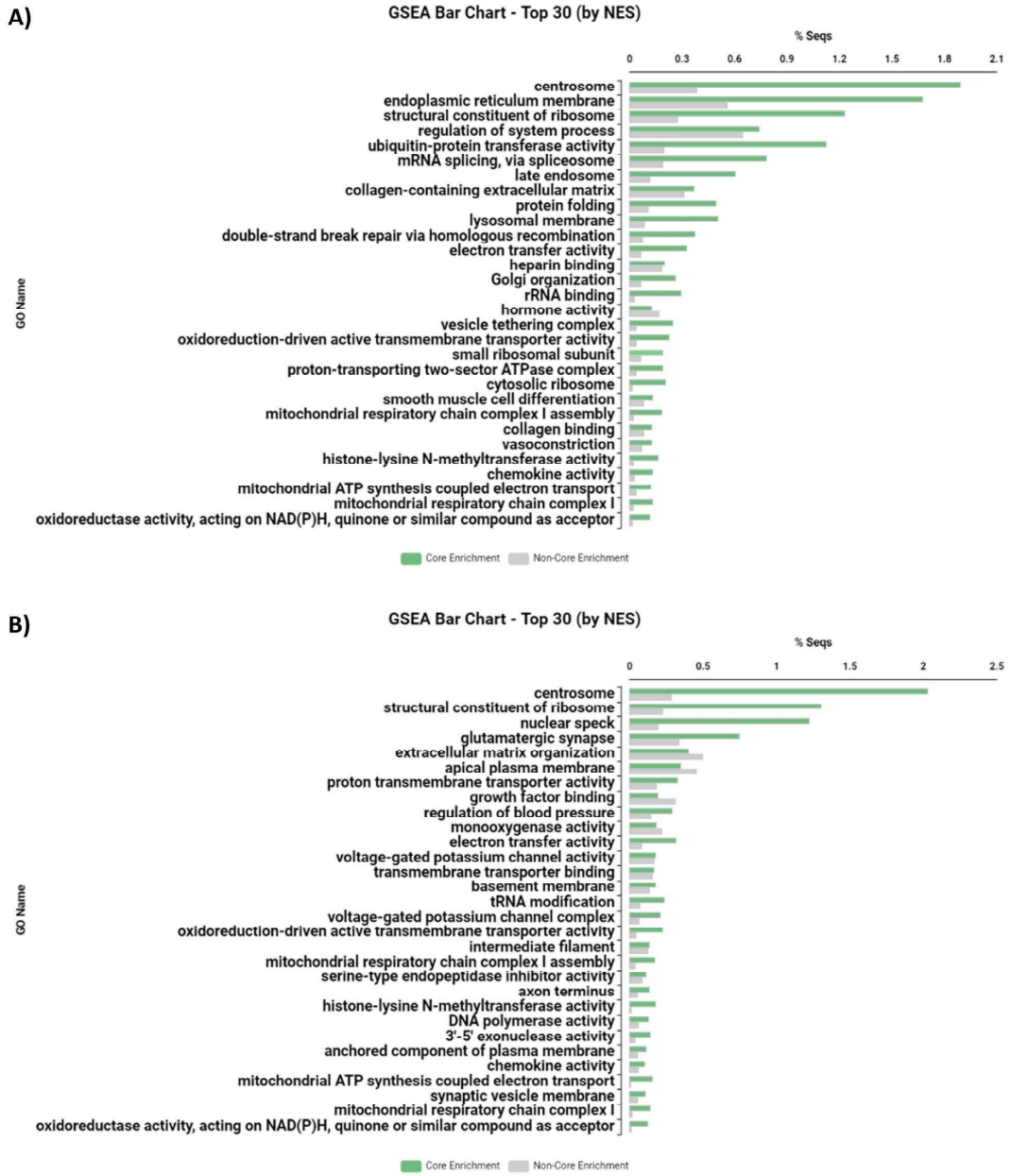

Figure 2. Top 30 most enrichment gene ontology (GO) in the hypothalamus between the alternative comparisons: (A) Follicular *vs.* Anestrus; (B) Luteal *vs.* Anestrus. The vertical axis represents the top 30 enriched pathway categories (GO) according to their normalized enrichment score (NES) at the significance threshold (false discovery rate; FDR < 0.05), and the horizontal axis represents the percentage calculated as the number of sequences (core or non-core) annotated with each GO. Core enrichment: subset of genes within the gene set that contributes most to the enrichment result. Non-core enrichment: subset of genes within the gene list that has not been enriched.

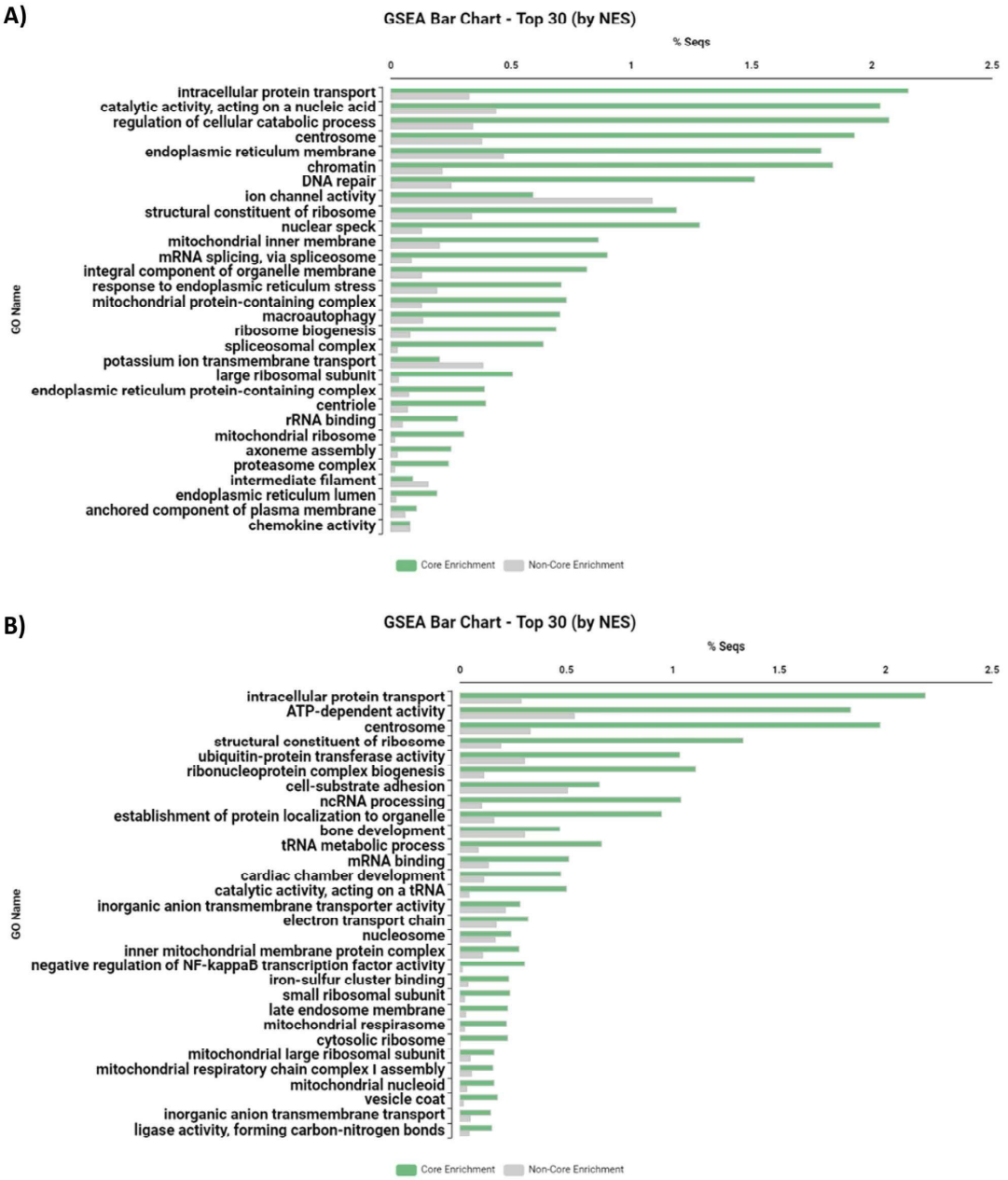

Figure 3. Top 30 most enrichment gene ontology (GO) in the *pars tuberalis* between the alternative comparisons: (A) Follicular *vs.* Anestrus; (B) Luteal *vs.* Anestrus. The vertical axis represents the top 30 enriched pathway categories (GO) according to their normalized enrichment score (NES) at the significance threshold (false discovery rate; FDR < 0.05) and the horizontal axis represents the percentage calculated as the number of sequences (core or non-core) annotated with each GO. Core enrichment: subset of genes within the gene set that contributes most to the enrichment result. Non-core enrichment: subset of genes within the gene list that has not been enriched.

Table 3. Gene expression changes in hypothalamus and *pars tuberalis* tissues in ovarian phases comparisons obtained with RNA-seq and RT-qPCR data.

			Log2FC[b]	
Tissue	Contrast[a]	Gene	RNA-seq	qPCR
Hypothalamus	F-A			
		CAVIN3	−1.56*	−0.96
		CTGF	−2.12**	−2.41***
		FBN3	0.86*	0.23
		HTR2B	−1.03*	−1.47**
		ITPR3	−2.48*	−0.88
		LTA4H	−0.30*	−0.16
		MYLK	−2.17***	−1.82***
		PTGIS	−1.56*	−1.80**
		WFDC1	−2.41*	−1.39**
	L-A			
		ASPH	−0.47*	−0.96*
Pars tuberalis	F-A			
		DDC	1.92**	0.92*
		ITLN	17.71**	1.89
		PCDH15	−3.95*	−0.96*
	L-A			
		ITLN	20.8***	2.34***
		MRPL57	−22.43***	−2.66*
		PLCXD2	−5.28**	−0.85

[a]L: luteal; A: anestrous; F: follicular; [b]Log2Fold change; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
Significant differences are shown after Benjamini and Hochberg correction.

that female sexual hormones as estradiol and progesterone promote the remodeling of the actin cytoskeleton with the implication of actin-binding proteins (ABPs) through changes in the phosphorylation state of ABPs in HT neurons.[37] For example estradiol produced in the F phase, induces structural modifications in dendritic spines, dendrite length, or the number of synapses, which have been associated with female sexual receptivity.[38] Moreover, we detected a cluster with three genes related to calmodulin binding (*CNN1*, *CALD1*, and *MYLK*). Calmodulin is a calcium ion-binding protein responsible for controlling numerous cellular processes such as the synthesis and release of neurotransmitters[39] and the regulation of transcriptional action of estrogen receptors.[40] The cyclic guanosine monophosphate (cGMP) signaling pathway, which is an intracellular nucleotide cascade involved in neuroplasticity, were also enriched.[41]

The oxytocin-signaling pathway (*ITPR3*, *KRAS*, *MYL9*, and *MYLK*) were found down-regulated in the F phase. This pathway has been implicated in hypothalamic–pituitary–adrenal axis regulation, modulating behavioral response toward stress and social behavior.[42] It is interesting that all the genes included in each significant cluster were upregulated in the A phase, and this could indicate that the upregulation of these genes may prevent the resumption of the estrous cycle in the non-breeding season.

Other interesting DEGs were also found downregulated in the F phase but were not enriched in the enrichment analysis: *HTR2B*, *ITPR3*, *LTA4H*, and *PTGIS*. These genes were reported to be linked to estrous behavior.[43] For instance, the *Leukotriene A4 Hydrolase* (*LTA4H*) was associated with estrous behavior in dairy cattle.[43] The Prostaglandin I2 Synthase (*PTGIS*) catalyzes the synthesis of prostacyclin, a prostaglandin member. It has been shown that estradiol induces prostaglandin-E2 synthesis, being implicated in neuron plasticity, and affecting neurons that synthesize and secrete GnRH.[44,45] Curiously, the *5-Hydroxytryptamine Receptor 2B* (*HTR2B*) also known as the serotonin receptor 2B, has been described to play a role in the regulation of behavior, including impulsive behavior.[46] It is outstanding that 5-hydroxytryptamine (5-HT) regulates female sexual behavior *via* its receptors.[47] Studies with agonist or antagonist drugs of 5-HT1, 5-HT2, or 5-HT3 receptors indicate that 5-HT2B receptor antagonists inhibit sexual behavior, and increase the quantity of 5-HT in brain.[48] Furthermore, Mikoshiba[49] demonstrated the involvement of the *Inositol 1,4,5-Trisphosphate Receptor Type 3* (*ITPR3*) gene in the initiation and propagation of intracellular Ca^{2+} signaling during the F phase. Finally, among the differential genes identified in the same comparison, we found the *Caveolae Associated Protein 3* (*CAVIN3*), which had been related to circadian rhythm pathways, as a regulator of circadian period length through Period and Cryptochrome proteins.[50] On the other hand, *aspartateβ-hydroxylase* (*ASPH*) gene were down-regulated in the L phase compared with the A phase in HT, being this gene associated with female fertility traits in Holstein cattle especially for days from calving to first service.[51] In this study, we speculate the involvement of these genes in relation to the A phase, specifically those related to neural plasticity and circadian rhythm. Summarizing, these results indicate that the regulation of cyclicity in ewes is a process involving comprehensive regulation of multiple biological pathways at the HT level. However, the relation between these DEGs and anestrous state needs further research.

In PT, the number for DEGs detected was lower than in HT, and no enrichment clusters of DEGs were found using DAVID. Gene expression genome-wide results suggest that a small amount of the variance in gene expression in PT is due to type of ovarian phase or ovarian phase response. However, the magnitude of gene expression differences between the alternative F and L reproductive phases was greater than in HT. In the F *vs.* A comparison, among the upregulated DEGs are *ITLN* and *DDC*. The *ITLN* is expressed in multiples tissues

including brain and reproductive tissues as placenta and ovary,[52] but we could not validate the results by RT-qPCR, possibly because its low expression level. In addition, the *Dopa Decarboxylase* (*DDC*) gene, encodes a protein that catalyzes the decarboxylation of L-3, 4-dihydroxyphenylalanine (DOPA) to dopamine, L-5-hydroxytryptophan to serotonin and L-tryptophan to tryptamine. It is related to LH regulation and plays a role in plasticity,[53] and *DDC* gene mutations impair the synthesis or metabolism of neurotransmitters leading to metabolic disturbance of dopamine and serotonin in patients.[54] Therefore, the upregulation of these genes may indicate their involvement during the F state. By contrast, *Protocadherin Related 15* (*PCDH15*) gene was upregulated in the A phase, being important for the maintenance and function of the photoreceptor cells.[55] *PCDH15* expression has also been found associated with variation in presynaptic serotonin (5-HT) transporter (SERT), and 5-HT levels in midbrain and hippocampus.[56] In the same study, a *PCDH15* functional null mouse line showed that *PCDH15* expression negatively regulates both SERT protein levels and 5-HT traits, confirming the central role of *PCDH15* in the regulation of 5-HT traits. It is also remarkable that biogenic indoleamine serotonin (5-HT) modulates central nervous system and periphery physiology including female sexual behavior. We believe that this gene could be implicated in the prevention of the resumption of the estrous cycle in the non-breeding season. Further research is required to investigate the function of this gene in relation to fertility in the out-of-breeding season.

GSEA contributed to the increasing knowledge on multiples pathways related to different ovarian phases in HT and PT. A high enrichment of genes and GO terms were identified related to the centrosome pathway (GO:0005813) in the HT F *vs.* A, and L *vs.* A comparisons (Fig. 2). Centrosomes have been involved in multiple processes during brain development, including neurogenesis, neuronal migration, and polarity.[57] Ding et al.[58] demonstrated that the loss of the centrosomal protein *Cenpj* in mice leads to abnormal development and dysfunction of the HT. Upon *Cenpj* depletion at the embryonic stages, they found that hypothalamic cells presented decreased proliferation and increased apoptosis, causing a drastic reduction in the number of Proopiomelanocortin (POMC) neurons and electrophysiological impaired of neuropeptide Y (NPY) neurons in the arcuate nucleus in adults. Furthermore, it has been shown that POMC and NPY neurons are implicated in the seasonal regulation of ewe reproductive activity.[59,60] In fact, POMC neurons make direct synaptic contact with GnRH neurons.[61] In this sense, previous studies showed that *NPY* gene is involved in the regulation of the secretion of gonadotropin-releasing hormone (GnRH),[62,63] which controls the release of LH and FSH hormones. This may explain why the centrosome pathway was highly enriched in the F and L phases compared to the A phase. On the other hand, the ER membrane (GO:0005789) was also highly enriched in the F *vs.* A comparison (Fig. 2), playing an important role in Ca^{2+} dynamics.[64] In this enrichment group, the *IP3R3* gene was included, being one of the DEGs found in the HT for the F *vs.* A comparison. The glutamatergic synapse pathway (GO:0098978) related to neuromodulation was enriched in the L *vs.* A comparison. In this sense, Ebling[65] and Gompf et al.[66] have demonstrated that the activation of glutamatergic synapses in the suprachiasmatic nucleus can induce phase shifts of circadian activity rhythms, while blockade of glutamate transmission leads to impaired photic entrainment. Furthermore, the GSEA identified the hormone activity pathway (GO:0005179) in the comparison of F *vs.* A at the level of HT with 24 enriched genes. Among these genes, the *agouti-related peptide* (*AGRP*) is expressed in neurons that are activated during starvation, and have been implicated in leptin-associated infertility.[67] Other genes such as *GNRH1*, *IGF2*, *INHBA*, *OXT*, and *PRLH*, were previously associated with the neuroendocrine control of reproduction.[6,68] The aforementioned signaling pathways work synergistically with the enriched hormone activity term, suggesting that they might play a central role in the HT during ovarian phases.

Regarding the PT, the top enriched pathway was the intracellular protein transport (GO:0006886) for both the F *vs.* A and L *vs.* A comparisons (Fig. 3). This enriched pathway plays an important role in neuronal function and survival, as nascent proteins pass through different compartments such as the lumen of the ER and the Golgi complex for post-translational processing.[69] Some enriched genes in this pathway such as *CRY2, SREBF1, NGFR, PRKAA1*, and *ARNTL* are related to circadian rhythms according to GO terms from Ensembl Biomart implemented in Omicsbox.[70] ATP-dependent activity (GO:0140657) was also highly enriched in the L *vs.* A comparison. The ATP is released in an activity-dependent manner from different cell types in the brain and acts as a synaptic neuromodulator through presynaptic regulation of neurotransmitter release, with an impact in synaptic plasticity, by postsynaptic regulation of other receptors of the intrinsic neuronal excitability.[71–73] Chromatin pathway (GO:0000785) was enriched in the F *vs.* A comparison and Jaric et al.,[74] showed that neuronal chromatin organization

fluctuates with the estrous cycle in the female ventral hippocampus of mouse, linked to changes in brain structure and behavior.

These findings suggest significant possible roles of DEGs in the regulation of F, L, and A phases involving multiple biological pathways that GSEA was able to capture. However, further studies are needed to explore the functional characterization of some selected DEGs that may contribute to a better understanding of the molecular mechanism implicated in sheep fertility in the out-of-breeding season.

Conclusion

In this study, RNA-Seq technology was used to analyze the DEGs from the HT and the PT in Rasa Aragonesa sheep in F, L, and A phases. Several new candidate genes of seasonal reproduction were predicted in the HT and PT. We found 24 and 75 significant DEGs in the PT and HT, respectively, which indicate that genes in the HT are further involved in sheep cyclicity regulation than the PT tissue. KEGG pathway analysis indicated that some of these genes are involved in oxytocin signaling pathway, smooth muscle contraction, and focal adhesion pathways. GSEA contributed to the increasing knowledge on multiples pathways related to neurotransmission and neuronal plasticity, which regulate the reproductive cycle in Rasa Aragonesa ewes. These results will afford new clues for understanding the molecular regulation of seasonal reproduction in sheep. However, further in-depth analysis is needed to gain a better understanding of these mechanisms, thus allowing new management tools to improve reproductive performance in ewes.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The datasets analyzed during this study are available in NCBI's Gene Expression Omnibus repository and are accessible through GEO Series accession number GSE191213 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE191213).

Author contributions

K.L., I.U., J.H.C., and M.S.: bioinformatics data analysis and data interpretation. B.M., B.L., and M.P.S: tissues extraction and dissection. M.P.S.: laboratory analysis. K.L.: drafted the main manuscript. K.L., M.S., J.L.A., B.L., I.U., and J.H.C.: review and editing the manuscript. J.H.C., M.S., and J.L.A.: conceptualization, and project coordination. J.L.A. and J.H.C.: funding acquisition. All authors read and approved the final manuscript.

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Supplementary Materials*

Supplementary Table S1. Characteristics of Reverse Transcription Quantitative real-time PCR (RT- qPCR) for validation of RNA-seq for each gene.

Supplementary Table S2. Descriptive statistics of RNA-seq. Results of the sequencing reads trimming and alignment to the reference genome Texel Oar_v3.1 for each of the samples in the hypothalamus (HT) and *pars tuberalis* (PT).

Supplementary Table S3. Differentially expressed genes (DEGs) in the hypothalamus through the comparison between follicular and anoestrus phases.

Supplementary Table S4. DAVID Functional Annotation Clustering of Differential Expressed genes between follicular and anoestrus phases in hypothalamus. Only clusters with an enrichment value higher than 1.3 are shown.

Supplementary Table S5. Top 30 most enriched Gene Ontology (GO) between follicular and anoestrous phases in the hypothalamus.

Supplementary Table S6. Top 30 most enriched Gene Ontology (GO) between luteal and anoestrous phases in the hypothalamus.

Supplementary Table S7. Top 30 most enriched Gene Ontology (GO) between follicular and anoestrous phases in the *pars tuberalis*.

Supplementary Table S8. Top 30 most enriched Gene Ontology (GO) between luteal and anoestrous phases in the *pars tuberalis*.

Supplementary Fig. S1. Gene stability using NormFinder in: **A)** hypothalamus; **B)** *pars tuberalis* tissues.

*Para visualizar el material suplementario, se puede acceder a través del siguiente link: https://www.tandfonline.com/doi/suppl/10.1080/10495398.2022.2155174?scroll=top&role=tab&aria-labelledby=suppl

Manuscrito 5: Exploring differentially expressed genes in hypothalamic, *pars tuberalis* and pineal gland transcriptomes in different sexual behavior phenotypes in rams using RNA-Seq

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Exploring differentially expressed genes in hypothalamic, *pars tuberalis* and pineal gland transcriptomes in different sexual behavior phenotypes in rams using RNA-Seq

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Abstract

Reproductive seasonality is a limiting factor in sheep production. Sexual behavior is a key element in reproductive efficiency, and this function is regulated by the hypothalamus-pituitary-gonadal (HPG) axis. To understand the mechanisms of sexual behavior, transcriptomic sequencing technology was used to identify differentially expressed genes (DEGs) in the hypothalamus (HT), *pars tuberalis* (PT) and pineal gland (PG) in Rasa Aragonesa rams with different sexual behavior. Bioinformatics analysis of the 16,401 identified genes by RNA-Seq revealed 103 and 12 DEGs in the HT and the PG, respectively, at a false discovery rate (FDR) of 5% with an absolute value of expression ≥ 1 (log2FC). However, no DEGs were found in the PT. Functional annotation and pathway enrichment analysis showed that DEGs of HT were enriched mainly in neuroactive ligand-receptor interactions and signaling pathways, including notable candidate genes such as *MTNR1A, CHRNA2, FSHB, LHB, GNRHR, AVP, PRL, PDYN, CGA, GABRD,* and *TSHB*, which play a crucial role in sexual behavior. The GnRH and cAMP signaling pathways were also highlighted. In addition, gene set enrichment analysis (GSEA) identified potential pathways, dominated mainly by biological process category, that could be responsible for the differences in sexual behavior observed in rams. The intracellular protein transport and pattern specification process were enriched within the PT and the transcription factor binding and protein ubiquitination pathways for the PG. Thus, these pathways together may play an important role in the regulation of the sexual behavior in Rasa Aragonesa rams through the hypothalamic-pituitary-gonadal axis. The validation of 5 DEGs using reverse transcription quantitative polymerase chain reaction (RT-qPCR) showed expression patterns like the found with RNA-Seq. Overall, these results contribute to understanding the genomic basis of sexual behavior in rams. Our study demonstrates that multiple networks and pathways orchestrate sexual behavior in sheep.

Lay Summary

Male sexual behavior is a key factor in reproduction, especially in seasonal breeders such as sheep. The identification of differentially expressed genes (DEGs) in brain regions involved in male reproduction and sexual behavior between rams with different sexual activity by RNA high-throughput sequencing can provide useful information to the sheep meat industry. This work aimed to determine the possible molecular mechanisms underlying the sexual behavior of Rasa Aragonesa rams by investigating transcriptional changes in the hypothalamus (HT), *pars tuberalis* (PT) and pineal gland (PG) between active (A) and nonactive (NA) rams. Comparative analysis revealed 103 and 12 DEGs between the A vs. NA comparison in the HT and the PG, respectively, but no DEGs were found in the PT. Gene ontology (GO) enrichment analysis of DEGs in HT samples revealed significant pathways, associated mainly with neuroactive ligand-receptor interactions, and the GnRH and cAMP signaling pathways. Furthermore, gene set enrichment analysis (GSEA) detected many overrepresented pathways related to sexual behavior via an interaction network within the hypothalamic-pituitary-gonadal axis. These data will be helpful for further investigations to look for mutations or functional single nucleotide polymorphisms (SNPs) that may be used for genetic assisted selection to improve sexual behavior in sheep.

Key words: hypothalamus, pineal gland, ram, sexual behavior, transcriptome

Abbreviations: A, active; BCS, body condition score; BP, biological process; CC, cellular component; CITA, Agrifood Research and Technology Centre of Aragon; DAVID, Database for Annotation, Visualization and Integrated Discovery; DEG, differential expressed gene; eCG, equine chorionic gonadotropin; ES, enrichment score; FDR, false discovery rate; FGA, fluorogestone acetate; GnIH, gonadotropin-inhibitory hormone; GnRH, gonadotropin-releasing hormone; GO, Gene Ontology; GSEA, gene set enrichment analysis; HPA, hypothalamic-pituitary-adrenal; HPG, hypothalamic–pituitary–gonadal; HT, hypothalamus; KEGG, Kyoto Encyclopedia of Genes and Genomes; LH, luteinizing hormone; Log2FC, Log2 Fold Change; LW, live weight; MF, molecular function; MPOA/AH, medial preoptic area/anterior hypothalamus; MRPs, mitochondrial ribosomal proteins; NA, nonactive; NES, normalized enrichment score; PG, pineal gland; PT, pars tuberalis; RFRP, RFamide-related peptide; RIN, RNA integrity number; RT-qPCR, reverse transcription quantitative real-time polymerase chain reaction; SNP, single nucleotide polymorphism

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Introduction

Reproduction is essential for the survival and evolution of species, and sexual behavior is one of the key elements in reproduction. In all species, sexual behavior includes a sequence of specific events consisting of an attraction phase followed by a precopulatory phase and finally mating (Fabre-Nys, 2010). Many investigations have been dedicated to identifying brain regions involved in male reproduction and sexual behavior, to improve our understanding of how the hypothalamic pituitary-gonadal (HPG) axis regulates reproductive functions. Schally et al. (1972) demonstrated that the pivotal hypothalamic neuropeptide gonadotropin-releasing hormone (GnRH) is the key regulator of the reproductive axis. GnRH release is controlled by other neuropeptides, neurotransmitters, and steroid hormones (Fink, 2000). Two neuropeptides appear critical in the regulation of the reproductive neuroendocrine axis. Kisspeptin stimulates GnRH release by directly acting on GnRH neurons, whereas gonadotropin-inhibitory hormone (GnIH), also named RFamide-related peptide (RFRP), inhibits gonadotropin release by inhibiting kisspeptin, GnRH neurons, or pituitary gonadotropes. These neuropeptides are also involved in sociosexual behavior by regulating the HPG axis (Marques et al., 2000; Parhar et al., 2016). However, some males in many species present a lack of sexual behavior or preference for a partner of the same sex despite having functional testes and normal blood concentrations of testosterone (Damassa et al., 1977; Perkins et al., 1992). While testosterone is often considered a predominant androgen for expressing and maintaining libido in rams (D'Occhio et al., 1985), several other studies have reported that there is no significant correlation between androgen levels and sexual behavior in rams (Schanbacher and Lunstra, 1976; Howles et al., 1980). According to Roselli et al., (2004) hormones themselves are important but not directly responsible for the control of sexual behavior. These authors demonstrated an association between natural variations in sexual partner preferences and brain structure, more specifically a region that includes a medial preoptic area/anterior hypothalamus (MPOA/AH), and where female-oriented rams had the largest ovine sexually dimorphic nucleus.

Ewes are seasonal and stop mating when the day length is increasing (anestrus) (Hafez, 1952). However, the ram effect is used to override the steroid negative feedback responsible for the anestrus state since the interactions during this period with a sexually active ram increase luteinizing hormone (LH) pulsatile secretion in anestrus ewes and eventually reinstate cyclicity (Martin et al., 1980). However, there is considerable variation in sexual behavior between rams within breeds (Lindsay, 1996). Consequently, the female response depends on the quality of the signals emitted by the male (sexual behavior, odor, vocalizations). Males with greater sexual behavior (mounts and services) lead to a higher percentage of mated ewes and higher fertility, whereas the response of the female to the male effect is low or even absent if the quality of the signals diminishes significantly (Delgadillo et al., 2008). Such patterns of behavior can decrease the reproduction rate and farm productivity, leading to economic losses (Perkins et al., 1992; Borja and Fabre-Nys, 2012). According to Simitzis et al. (2006), differences in ram sexual performance seem to be associated with libido and sexual interest rather than the capacity to efficiently perform the motor patterns of mounting and copulation. Methods such as melatonin and photoperiod treatment were proposed and adopted to manipulate the circadian rhythm to improve the fertility of ewes and to prepare the rams for breeding out of season (Chemineau et al., 1988). However, the disadvantage of using these kinds of manipulations (photoperiod or melatonin) is that animals become refractory to the stimulatory effects of light and melatonin, further limiting their use (Willard, 2002). Snowder et al. (2002) reported a moderate heritability (0.22 ± 0.04) and a high repeatability (0.72) of sexual performance scores of rams, implying that sexual performance could be improved by selection. Molecular approaches such as high-throughput RNA deep sequencing (RNA-Seq) provide a powerful means for pinpointing genes required for the expression of sexual behavior and circuits that govern behavior. RNA-Seq can also help to gain an appreciation of how behavior is encoded by genes and their regulatory sequences. Therefore, the study presented herein aims to compare and analyze transcriptomics of the hypothalamus (HT), *pars tuberalis* (PT) and pineal gland (PG) in rams with different sexual behavior using RNA-Seq techniques. The differentially expressed genes identified in this study will highlight the most important genes involved in the regulation of the biological and molecular processes of sexual behavior in rams. Genes discovered through this study could be potential molecular markers for genetic selection applications in animal breeding programs.

Materials and Methods

All experimental procedures, including care of animals and euthanasia, were performed in accordance with the guidelines of the European Union regulations for the use and care of animals in research (Directive 2010/63/EU) and approved by the Animal Ethics Committee of the Research Centre (protocol number 2017/02).

Animal selection

Fifty-nine adult Rasa Aragonesa rams aged between 1.8 and 8.3 yr old were first studied (3.7 ± 2.05 yr old). These animals belong to the Centro de Investigación y Tecnología Agroalimentaria (CITA) de Aragon herd. This herd consists of approximately 400 ewes with an annual renewal rate of 30%, reared under homogeneous management conditions (feeding, veterinary treatments, etc). All rams were sexually experienced. To classify rams as either sexually active or inactive, all rams were submitted to two individual sexual behavior pen tests, 15 d apart, performed in May on the same days. During the pen tests, each ram was exposed to three adult ewes synchronized in estrus in an area of 3 m × 3 m for 20 min to observe sexual behavior. Estrus was induced in ewes by 30 mg of fluorogestone acetate (FGA) intravaginal sponges for 14 d and 480 IU intramuscular (i.m.) of equine chorionic gonadotropin (eCG)) at sponge withdrawal. For ram sexual activity assessment, the number of mounts and services was recorded. A mount was defined as an attempt by a ram to serve an ewe, whereby his forelegs leave the ground, he straddles the ewe and contacts her with his brisket. However, a service is a mount during which the ram achieves intromission and ejaculates, characterized by a distinct pelvic thrust with the head thrown back. Sexual activity was defined as the sum of mounts and services. The day before the test, three serial blood samples were taken 1 hr apart, in lithium heparin tubes to measure testosterone. This sampling protocol was used considering that the mean value of the

three samples is a reliable estimate of the testosterone concentration of each animal since it is known that large variations in peripheral blood testosterone levels occur in short periods due to the pulsatile nature of testosterone secretion in rams (Santiago-Moreno et al., 2005). Testosterone was measured by radioimmunoassay (RIA) (Hochereau-De Reviers et al., 1990) at the Laboratoire Phénotypage-Endocrinologie, INRAE. Rams were assessed for body condition score (BCS: 3.3 ± 0.3; 1–5 scale, according to Russel et al. (1969), adapted to this breed by Paramio and Folch (1985)), live weight (LW: 81.5 ± 14.44 kg) and mean antero-posterior maximum testicular diameter, subtracting the scrotal skin thickness, using a caliper (Folch and Roca Bernaus, 1982) (6.00 ± 0.58 cm). The second individual sexual behavior pen test was performed 15 d later than the first test in only one subgroup population with similar age and BCS (N = 21) selected by a tree-based regression model (see Section 2.4.1). Body condition score (BCS: 3.4 ± 0.3), live weight (LW: 84.1 ± 10.5 kg) and testicular diameter (6.61 ± 0.54 cm) were also assessed. Three serial blood samples were also taken 1-h apart to measure testosterone. Then, animals from this group were classified as either active (A) (9.0 ± 7.5, average mounts plus services ± standard deviation (sd); N = 11) or nonactive (NA; without mounts or services; N = 10). Six rams of each group were selected for slaughtering the day after the second sexual behavior pen test to confirm that the animals were inactive or active before the slaughtering. The rams selected for RNA-Seq were born during the same lambing season of the herd and had different mothers with low relation between them according to the lambing records.

Tissues samples, RNA extraction, and sequencing

The selected rams were slaughtered and hypothalamic, *pars tuberalis* and pineal gland tissues were dissected and isolated, rapidly frozen in liquid nitrogen, and stored at −80 °C until use. Total RNA was extracted from tissue samples using an RNeasy Lipid Tissue mini kit (QIAGEN, Madrid, Spain) according to the manufacturer's instructions. RNA concentration and RIN (RNA Integrity Number) values were checked using an Agilent 2100 Bioanalyzer machine (Agilent Technologies, Palo Alto, CA, USA). For library construction, only samples with an RIN > 7 were kept for sequencing. Sequencing was carried out at the CNAG (Centro Nacional de Análisis Genómico, Spain; https://www.cnag.crg.eu/). The RNA-Seq library was prepared with a KAPA Stranded mRNA-Seq Illumina Platform Kit (Roche, Vaud, Switzerland) following the manufacturer´s recommendations using Illumina platform compatible adaptors with unique dual indices and unique molecular identifiers (Integrated DNA Technologies, Redwood City, CA, USA). The final library was validated on an Agilent 2100 Bioanalyzer with the DNA 7500 assay. The libraries were sequenced on a NovaSeq 6000 (Illumina, San Diego, CA, USA) with a read length of 2 × 151 bp following the manufacturer's protocol for dual indexing. Image analysis, base calling and quality scoring of the run were processed using the manufacturer's software Real Time Analysis (RTA v3.4.4) followed by the generation of FASTQ sequence files. The RNA-Seq sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus repository (Barrett et al., 2013) with the accession number GSE204861 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE204861).

Validation of RNA-Seq data

To confirm the differential expression of genes revealed by RNA-Seq, four genes identified to be differentially expressed in the HT and another four in the PG, were chosen for reverse transcription quantitative polymerase chain reaction (RT-qPCR) validation. These genes were: *AVP, CGA, PRL_HT* (*ENSOARG00020014542*), and *GABRD* for the HT and *CRABP1, GNG8, NPY,* and *PRL_PG* (*ENSOARG00020015585*) for the PG. Furthermore, two housekeeping genes (*RPL19* and *RPL32*), commonly used as reference genes in brain expression studies, were employed and the gene expression stability was determined using NormFinder (Andersen et al., 2004). The samples used were the 12 samples (6 A and 6 NA) used for the RNA-Seq analysis. First, we treated total RNA (1 µg) from each sample (N = 12) with DNAse (Invitrogen, Carlsbad, CA, USA), and to synthesize first-strand cDNA, we used the Super-ScriptIII Reverse Transcriptase kit (Invitrogen), following the manufacturer's recommendations. To design the primers in specific exon-spanning regions, Primer3 software was used (https://primer3.ut.ee/; last accessed on March 14, 2022). Basic alignment search tool (BLAST) was performed to confirm the specificity of the primers (NCBI: http://www.ncbi.nlm.nih.gov/BLAST/; last accessed on March 14, 2022). To test the primers and verify the amplified products, conventional PCR was achieved using standard conditions for all the genes. To confirm gene identity, the PCR products were Sanger sequenced by the company Stabvida (https://www.stabvida.com/es). Homology searches were performed with BLAST (NCBI: https://blast.ncbi.nlm.nih.gov/Blast.cgi). Quantitative real-time PCR was performed with three technical replicates on the ABI Prism 7500 platform (Applied Biosystems, Madrid, Spain) using a 10 µl PCR total reaction mixture containing SYBR Green Master Mix: SYBR Premix Ex Taq II (Tli RNase H Plus, Takara, Demlab, Zaragoza, Spain). For each gene, the correlation coefficient (R^2) and slope were estimated using standard curves through 10-fold serial dilutions of pooled cDNA. To assess the efficiency (E) of PCR amplification, the slope was employed using the formula $E = 10^{(-1/slope)}$. To remove technical variation from this source of variability, two "connector samples" were replicated in all plates. The annealing temperatures, primer concentrations, and primer sequences are described in Table 1.

Statistical analysis

Testosterone and sexual activity analysis

The plasmatic testosterone concentration phenotype was analyzed by fitting a mixed model using the SAS statistical package v. 9.3 (SAS Institute, Cary NC, USA), including age, BCS, LW, testicular diameter, and sexual activity (the sum of mounts and services) as covariates and the ram as the random effect. A second model was run including sexual activity as a factor. In this case, two levels were defined for this factor: NA without any mount or service or A with at least one mount or service.

The sexual activity phenotype was also analyzed by fitting an analysis of variance model using the "proc glm" function in SAS and including age, BCS, LW, testicular diameter, and testosterone content as covariates. The plasmatic testosterone concentration and the sexual activity phenotypes were also analyzed transforming the continuous variables to a categorical one, using the quantiles of the continuous variables.

Table 1. Characteristics of reverse transcription quantitative real-time PCR (RT-qPCR) for validation of RNA-Seq for each gene

Genes	Primers	Amplification size (bp)	AT[1]	E[2]	R[3]
RPL19	F-5′caactcccgccagcagat-3′	76	60 °C	1.99	0.99
	R-5′ccgggaatggacatgcaca-3′				
RPL32	F-5′ggcaccagtcagaccgatatg-3′	75	60 °C	1.99	0.99
	R-5′cccagacaggagaggttcag-3′				
AVP	F-5′gctacttccagaactgccca-3′	115	60 °C	2.12	0.97
	R-5′cagatgctgggcccgaag-3′				
CGA	F-5′tctccattcctttcctgatgg-3′	113	60 °C	2.03	0.99
	R-5′accccatgcactgataaattgg-3′				
PRL_HT[4]	F-5′agaggtgcggggtatgaaag-3′	134	60 °C	2.00	0.99
	R-5′cagtctctttggctccagga-3′				
GABRD	F-5′atccagtcctacatgccgtc-3′	128	60 °C	2.04	0.99
	R-5′atcagcgtggtcatggtca-3′				
CRABP1	F-5′gcacgaccgagatcaacttc-3′	126	60 °C	2.08	0.99
	R-5′ccaagagagtttgtgtgcagt-3′				
GNG8	F-5′aacaacatggccaagatcgc-3′	136	60 °C	1.99	0.99
	R-5′ggtcatctttggcgtgagtc-3′				
NPY	F-5′ctccaagcctgacaaccct-3′	82	60 °C	2.01	0.98
	R-5′attgatgtagtgtcgcagcg-3′				
PRL_PG[4]	F-5′acctctcctcggaaatgttca-3′	112	60 °C	2.05	0,99
	R-5′tcttcaggggtaggaaggga-3′				

[1]Annealing temperature.
[2]Efficiency of PCR amplification.
[3]Correlation coefficients.
[4] *PRL_HT: ENSOARG00020014542; and PRL_PG: ENSOARG00020015585.*

Finally, a tree-based regression model for the sexual activity phenotype was run using the "Party package" (Hothorn et al., 2006) in R, considering all the variables for the sexual activity phenotype (age, BCS, LW, testosterone, and testicular diameter). The tree structure represents recursive partitioning of the data to minimize residual deviance that is based on iteratively splitting the data into two subsets. Tree-structure models can be used as variable-selection procedures that inform which variables have any sort of significant relationship with the dependent variable (in our case, the sexual activity phenotype). The data are split at several points for each independent variable, organizing the dataset into groups by the response variable. The top few nodes on which the tree is split are the most important variables within the set. In this study, we used a conditional inference tree (CIT) that uses *p* values to determine splits in the data. Because factors such as age, LW, BCS, testosterone, and testicular diameter may influence the sexual activity phenotype, the tree-based regression model was applied to split the whole data into different nodes (populations subgroups) based on the independent variables. Then, we have chosen a population subgroup with similar values for the significant variables but different sexual activity phenotype for next analyses and slaughtering, avoiding confounding effects. This analysis was performed using the first individual sexual behavioral pen test data and classified the 59 rams into different subpopulation groups of rams.

A Pearson's pairwise correlation matrix between variables and principal component analysis (PCA) using the FactoMineR R package (Le et al., 2008) from the first pen test were also calculated.

RNA-Seq analysis

All transcripts were analyzed with bioinformatics tools implemented within OmicsBox v2.0.36 from BioBam (https://www.biobam.com/omicsbox). The overall quality of the sequences was inspected using FastQC tools (Andrews, 2015). The Trimmomatic program (Bolger et al., 2014) was run to trim all adapters (using the default options for adapter removal) and low-quality reads using the Sliding Window Trimming option with a window size of 40 and a required quality of 20. The filtering configuration of reads was set to 20 and 35 for the average quality and the minimum length, respectively. Clean reads were then mapped against the ovine reference genome Oar_rambouillet 1.0 (GCA_002742125.1) using STAR v2.7.8a (Dobin et al., 2013) and by providing an annotated gene file in GTF format. The mapped reads of each sample were assembled by HTSeq (Anders et al., 2015). The gene quantification levels of each sample were analyzed using the Union model, and a count table was calculated. Only reads mapping unambiguously to a single genomic feature were considered. Reads aligned to more than one position or overlapping with more than one feature were discarded from the subsequent analysis. EdgeR software (Robinson et al., 2010) was used to identify DEGs. The multifactorial design was chosen to adjust the analysis where the sexual activity (A or NA) was defined as the primary contrast condition and the tissue (PT, HT, and PG) as the secondary contrast condition. The low count genes were filtered using the threshold of count per million greater than or equal to 0.5 in at least five samples, and the trimmed mean of M-value normalization was applied to raw counts. The analysis was performed using the generalized linear model (likelihood ratio test) with a robust option against potential outlier genes. Multiple-testing corrections by Benjamini

and Hochberg false-discovery rate (FDR 5%) were applied. Genes with an absolute log2FC ≥ 1 and an adjusted *P* value ≤ 0.05 were identified as DEGs. The volcano plots were generated using the R v4.1.3 programming language (R Core Team, 2020) (https://www.R-project.org/; last accessed on 14 April, 2022).

Gene ontology (GO) enrichment and KEGG pathway analysis

To better understand the functions of DEGs, GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009) through the Functional Annotation Cluster (FAC) tool. Medium stringency EASE score parameters were selected. Only annotation clusters with an enrichment score (ES) > 1.3 are highlighted in the present study (Huang et al., 2009). As enrichment analysis of DEGs in certain comparisons was not possible due to the low number of DEGs, we decided to run the gene set enrichment analysis (GSEA) (Subramanian et al., 2005) implemented in OmicsBox (https://www.biobam.com/omicsbox) including all the expressed genes, regardless of whether they were differentially expressed. GSEA was used to characterize both modest and robust, coordinated, biologically relevant changes in molecular signaling pathways, and to explore and identify the most significant and over-represented GO terms considering as input all expressed genes. The entire unfiltered data set is used in GSEA, without fold cutoff filtering or significance test as it seeks to point out modest, coordinated transcriptomic changes. In this study, this tool was used to determine whether an a priori defined set of genes is statistically significant between A and NA rams. A normalized enrichment score (NES) was also calculated considering the number of genes in the pathway. A positive NES indicates that the list of genes is enriched at the "top" of the ordered fold change list, and a negative NES indicates the opposite, i.e., enriched "at the bottom". GSEA classifies the biological functions of the set genes based on three factors: molecular functions (MFs), biological process (BP), and cellular component (CC). The significance level was adjusted to account for multiple hypothesis testing using FDR 5%.

Gene expression validation of RNA-Seq by RT-qPCR

The differences in the expression rate were measured and analyzed by their quantification cycle (Cq) following the statistical method proposed by Steibel et al. (2009). The fitted mixed model was as follows:

$$y_{rigkm} = SG_{gi} + P_k + BC_m + A_m + LW_m + E_m + e_{rigkm}$$

where, y_{rigkm} is the Cq value (corrected for gene amplification efficiencies) of the gth gene (DEG and housekeeping genes) from the *r*th well (reactions were run in triplicate) in the *k*th plate corresponding to the *m*th animal and to the *i*th sexual activity group (active or nonactive); SG_{gi} is the fixed interaction between the *i*th sexual activity group and the *g*th gene; P_k is the fixed effect of the *k*th plate; BC_m, A_m, and LW_m are the effects of BCS, age, and the LW of the *m*th animal, respectively, included as covariates; E_m is the random effect of the *m*th animal from which samples were collected $(E_m \sim (0, \sigma^2_E))$; and e_{rigkm} is the random residual error. Gene specific residual variance (heterogeneous residual) was fitted to the gene by sexual activity interaction $(e_{rigkm} \sim N(0, \sigma^2_{egi}))$.

We carried out the approach suggested by Steibel et al. (2009) using two housekeeping genes to test differences ($diff_{DEG}$) in the expression rates for each selected gene between the two ram groups in terms of fold changes (FCs). To determine the significance of the $diff_{DEG}$ estimates ($P < 0.05$), the *T* statistic after Benjamini and Hochberg correction was used. To validate the RNA-Seq results, the Pearson correlation coefficient was calculated between *FC* values estimated in RNA-Seq analysis and qPCR expression measures.

Results

Sexual activity phenotype and animal selection

Sexual activity phenotype was defined as the sum of mounts and services. For the 59 rams, the mean and median values of sexual activity phenotype were 8.27 ± 10.09 and 6, respectively, while the 5th and 95th percentiles were 0 and 28.1. This trait was also codified as a categorical variable: NA (without any mount or service; *N* = 24) or A (with at least one mount or service; *N* = 35) (9.0 ± 7.5, average mounts and services ± sd). The Pearson's pairwise correlation matrix from the first pen test showed that age and sexual activity, testicular diameter and medium plasmatic testosterone concentration, and testicular diameter and LW had a moderate degree of correlation (Supplementary Figure S1). Statistical analysis showed that this trait was influenced by age (*P* = 0.004) and LW (*P* = 0.027). In relation to testosterone concentration, only testicular diameter was significantly associated (*P*= 0.013) with the levels of this hormone. No differences were found in testosterone concentration when sexual activity was included as a covariate (*P* = 0.77) or factor (*P* = 0.40; NA = 6.56 ± 0.70, and A = 7.30 ± 0.57 average mounts and services ± sd). Similar results were found in the analysis using the variables as categorical with the quantiles of the continuous variables.

The tree-based regression model identified three different population subgroups for the sexual activity phenotype (Supplementary Figure S2). The age and BCS were the variables that split the data into three subgroups. The group including 21 rams with similar ages (2.44 ± 0.01 years old) and BCSs (3.33 ± 0.33) (subpopulation of rams with BCS > 3 and age ≤ 2.521) was selected for further analysis and was subjected to a second individual sexual behavioral pen test. Five rams and sixteen were active and nonactive, respectively, in the first individual sexual behavioral pen test in the subgroup of 21 rams. The mean and median values of the sum of mounts and services phenotype for the second test were 5 ± 7.12 and 3, respectively, while the 5th and 95th percentiles were 0 and 16.65. Rams were also classified into A (*N* = 11) and NA (*N* = 10). No significant effects were found for the sexual activity phenotype or testosterone concentration. PCA analysis using all data showed that only the first two principal components had an eigenvalue greater than 1. Age and sexual activity phenotype were positively correlated in the second dimension (Supplementary Figure S3).

Pearson's correlation coefficient between the categorical sexual activity phenotype from the first and second sexual behavioral pen tests was 0.47 (*P* < 0.05). NA rams selected for slaughter were nonactive in the two tests, while for A rams, five were active in the two tests, and one was active in the second test.

Summary of the raw sequence reads

The statistics of the sequencing and read alignments of the 36 samples to the ovine reference genome Oar_rambouillet 1.0 (GCA_002742125.1) are shown in Table 2. Illumina sequencing produced a total of 1,052,405,770, 913,588,901, and 1,126,978,950 raw reads from tissue samples of HT, PT, and PG, respectively. PT sequences generated fewer raw reads than HT and PG tissue sequences. After filtering to remove low-quality bases and adaptor sequences, the remaining clean reads were 931,888,547, 653,499,100, and 1,056,176,441 for HT, PT, and PG, respectively, which provided abundant data for further analysis. On average, 97% of all the clean reads were mapped to the ovine reference genome, and more than 77% were uniquely mapped. According to the statistical results, there were enough reads of each sample mapped to the reference genome for subsequent analysis.

Differentially expressed genes (DEGs)

The pairwise differential expression analysis included 36 samples. In the tissues studied, 26,478 genes were expressed. After filtering, only 16,401 genes were retained for the differential expression analysis. A total of 103 genes were identified as differentially expressed for A vs. NA in HT, 78 upregulated and 25 downregulated. The top 20 DEGs are shown in Table 3. The detailed list is provided in Supplementary Table S1. In the PG only 12 DEGs were detected, of which 2 were upregulated

Table 2. Descriptive statistics of RNA-Seq

Library id.	Tissue[1]	Sexual behavior state[2]	Input raw reads	Surviving reads	Aligned concordantly exactly 1 time (%)	Reads mapped to multiple loci (%)
1	HT	A	79,797,184	71,907,386	75.98	21.80
2	HT	A	101,642,975	91,994,456	75.55	22.04
3	HT	A	66,924,466	57,272,139	69.11	28.11
4	HT	A	67,055,868	62,500,047	79.44	18.15
5	HT	A	102,094,784	93,418,875	71.36	26.14
6	HT	A	65,428,119	54,958,840	66.12	31.13
7	HT	NA	78,300,524	65,392,192	71.07	26.37
8	HT	NA	71,919,447	62,535,680	69.13	28.34
9	HT	NA	132,939,405	114,658,240	66.27	31.23
10	HT	NA	105,481,395	96,183,238	75.34	22.25
11	HT	NA	112,264,413	103,333,232	76.03	21.45
12	HT	NA	68,557,190	57,734,222	64.35	32.89
13	PT	A	75,781,333	64,561,260	79.88	17.81
14	PT	A	73,226,957	51,677,836	79.36	18.27
15	PT	A	74,967,827	48,739,917	81.51	16.00
16	PT	A	89,809,711	62,624,994	80.93	16.70
17	PT	A	82,597,893	57,435,045	81.79	15.82
18	PT	A	84,722,210	60,253,807	77.93	19.73
19	PT	NA	79,921,730	59,901,685	74.80	22.47
20	PT	NA	57,787,080	40,679,615	82.84	14.71
21	PT	NA	67,135,190	47,215,732	79.78	17.84
22	PT	NA	64,621,127	45,426,223	81.53	16.11
23	PT	NA	82,523,718	56,146,228	79.79	17.77
24	PT	NA	80,494,125	58,836,758	79.88	17.64
25	PG	A	159,369,886	150,645,392	82.81	14.41
26	PG	A	60,742,248	56,905,460	79.22	17.74
27	PG	A	82,311,456	79,085,005	82.20	15.08
28	PG	A	118,531,184	112,599,637	84.08	13.20
29	PG	A	65,429,032	59,425,250	78.33	18.63
30	PG	A	121,980,446	111,625,022	74.71	22.35
31	PG	NA	106,859,711	101,212,790	80.83	16.41
32	PG	NA	104,748,575	95,426,539	77.07	20.00
33	PG	NA	57,282,756	53,466,943	80.93	16.27
34	PG	NA	76,879,686	72,873,458	81.58	15.71
35	PG	NA	79,845,241	73,982,534	77.74	19.28
36	PG	NA	92,998,729	88,928,411	81.25	16.00

[1]HT, hypothalamus; PT, *pars tuberalis*; PG, pineal gland.
[2]A, active; NA, nonactive.

Table 3. Top 20 of differentially expressed genes (DEGs) in the hypothalamus comparison between active and nonactive rams

Genes*	Description	Log_2FC[1]	*P*-adj[2]
HEPACAM2	*HEPACAM family member 2*	−2.97	1.01E−04
CLDN9	*claudin 9*	7.00	1.09E−04
CLRN1	*clarin 1*	7.14	1.44E−04
MPZ	*myelin protein zero*	−8.93	1.88E−04
ARRDC2	*arrestin domain containing 2*	−1.49	4.70E−04
CLDN7	*Claudin 7*	5.46	0.002
SCGN	*secretagogin, EF-hand calcium binding protein*	4.57	0.002
ZNF750	*zinc finger protein 750*	6.38	0.002
PITX1	*paired like homeodomain 1*	5.15	0.002
TMPRSS9	*transmembrane serine protease 9*	4.33	0.002
REEP4	*receptor accessory protein 4*	−1.02	0.002
SPTBN5	*Spectrin Beta, NonErythrocytic 5*	−4.03	0.002
FOXL2	*forkhead box L2*	3.46	0.003
PON1	*paraoxonase 1*	4.47	0.003
ITGB6	*integrin subunit beta 6*	4.65	0.004
POU1F1	*POU class 1 homeobox 1*	5.43	0.004
PDYN	*prodynorphin*	2.12	0.004
TTC24	*Tetratricopeptide Repeat Domain 24*	3.17	0.004
LHX3	*LIM homeobox 3*	5.32	0.004
TMEM30B	*transmembrane protein 30B*	3.58	0.004

[1]Log_2 fold change.
[2]*P* adjusted.
*Only the top 20 DEGs are shown. The complete list is provided in Supplementary Table S1

Table 4. Differentially expressed genes (DEGs) in the pineal gland through the comparison between active and nonactive rams

Genes	*Description*	Log_2FC[1]	*P*-adj[2]
PRL	*Prolactin*	6.96	0.020
MCHR1	*melanin concentrating hormone receptor 1*	−4.06	0.020
NRN1	*neuritin 1*	−3.59	0.020
IRX6	*iroquois homeobox 6*	−3.74	0.020
GNG8	*G protein subunit gamma 8*	−3.08	0.043
NPY	*neuropeptide Y*	−4.24	0.043
CRABP1	*cellular retinoic acid binding protein 1*	−3.59	0.043
PITX2	*paired like homeodomain 2*	4.01	0.049
LOC101113217	*BOLA class I histocompatibility antigen, alpha chain BL3-7*	−1.89	0.049
ROBO3	*roundabout guidance receptor 3*	−3.23	0.049
TAC1	*tachykinin precursor 1*	−3.80	0.049
BVES	*blood vessel epicardial substance*	−3.38	0.049

[1]Log_2 fold change.
[2]*P* adjusted.

and 10 were downregulated (Table 4). Regarding the PT, no DEGs were detected, which suggested that the data obtained from this tissue via RNA-Seq were not able to capture DEGs. Only the *PRL* gene was found to be a DEG in common in the HT and the PG of the rams, but with different Ensembl IDs and different genome locations (*ENSOARG00020014542*: 20: g.37992053-38000561; *and ENSOARG00020015585:* 20: g.38502493-38551510). The volcano plot comparison of gene expression between A vs. NA rams in the HT and the PG is shown in Supplementary Figure S4.

Enrichment analysis

To further elucidate the functions of DEGs detected in the HT between the A and NA rams, the DAVID platform was used. Only clusters with an enrichment score above 1.3 are reported in this study (Supplementary Table S2). The analysis showed six clusters of enrichment. The first includes genes (*MTNR1A, CHRNA2, FSHB, LHB, GNRHR, AVP, PRL, PDYN, CGA, GABRD, TSHB*) related mainly to neuroactive ligand-receptor interactions. The other noteworthy KEGG pathways were the GnRH and cAMP signaling pathways. Regarding the other

clusters, terms such as glycoprotein, homeodomain, and cell adhesion molecules were also enriched. No enrichment clusters were found in the PG, as there were few DEGs (12). Therefore, further enrichment analysis was run by GSEA to explore gene networks and pathways behind the different sexual behavior in rams, mainly in PG and PT, where few/any DEGs were detected. The analysis included all the genes expressed in the three tissues studied (HT, PT, and PG) to identify the potential overrepresented pathways responsible for the sexual behavior in rams. The top 30 most enriched terms between the A vs. NA rams in each tissue are shown in Figures 1–3. The enrichment score and the gene set size details for these GO terms are described in Supplementary Table S3. The GSEA identified 119 GO terms comprising 72 BP, 29 CC, and 18 MF at the level of the HT. The nuclear speck, structural constituent of ribosome, glycoprotein biosynthetic process and angiogenesis pathways were the most enriched. The results also emphasized enrichment for regulation of peptide hormone secretion and hormone activity which might play an important role in the regulation of sexual behavior in rams at the level of the HT (Figure 1). Moreover, 64 terms were identified in the PT comprising 41 BP, 14 CC, and 9 MF. Intracellular protein transport, pattern specification process and ribosome pathways were the most enriched (Figure 2). In the PG, only 28 pathways were detected, including 12 BP, 9 CC, and 7 MF. The main enriched pathways were transcription factor binding, protein ubiquitination, and histone modification (Figure 3).

Verification of DEGs by RT-qPCR

Eight candidate genes were selected for RT-qPCR analysis. These included four genes differentially expressed in the HT (*AVP, CGA, PRL_HT*, and *GABRD*) and four genes in the PG (*CRABP1, GNG8, NPY,* and *PRL_PG*). These genes were selected because of their potential implication in the phenotype studied. The housekeeping genes *RPL19* and *RPL32* were used to normalize gene expression. As shown in Supplementary Figure S5, these two housekeeping genes were more stable than the candidate genes in both HT and PG tissues. Three of the four genes were confirmed to be differentially expressed in the HT, whereas in the PG, two genes were differentially expressed (Table 5). Although the expression patterns were slightly different from the RNA-Seq results, the qPCR analysis confirmed that some of the selected genes were differentially expressed between the A and NA rams, indicating that the RNA-Seq results were accurate. Furthermore, the RT-qPCR data were highly correlated (0.97) with the sequencing data.

Discussion

Sexual behavior is a complex trait, as many factors come into play in its engagement, including gene expression, hormone action, and multisensory information integration. In sheep, variability in sexual performance of rams within the breed (libido and sex preferences) has been studied a great deal but still not completely understood. The HPG axis is an important regulatory center of sexual activity. Therefore, the HT, PT, and PG were used as the target tissues in this study to identify the molecular mechanisms regulating the sexual behavior in Rasa Aragonesa rams through RNA-Seq. The active and nonactive rams selected for slaughtering belonged to a population subgroup with similar values for age and BCS variables but

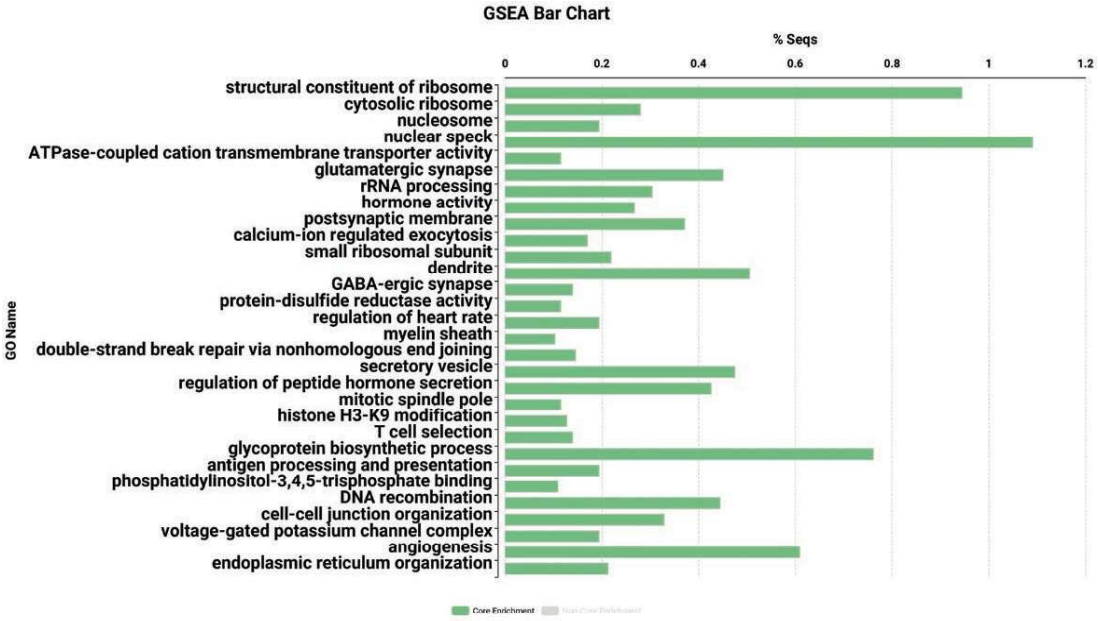

Figure 1. Top 30 enriched Gene Ontology (GO) terms of the hypothalamus between active and nonactive rams. The vertical axis represents the top 30 enriched pathway categories (GO) according to their normalized enrichment score (NES) at the significance threshold (false discovery rate; FDR < 0.05), and the horizontal axis represents the percentage calculated as the number of sequences (core or noncore) annotated with each GO. Core enrichment: subset of genes within the gene set that contributes most to the enrichment result. Noncore enrichment: subset of genes within the gene list that have not been enriched. Only core enrichment is shown in the figure.

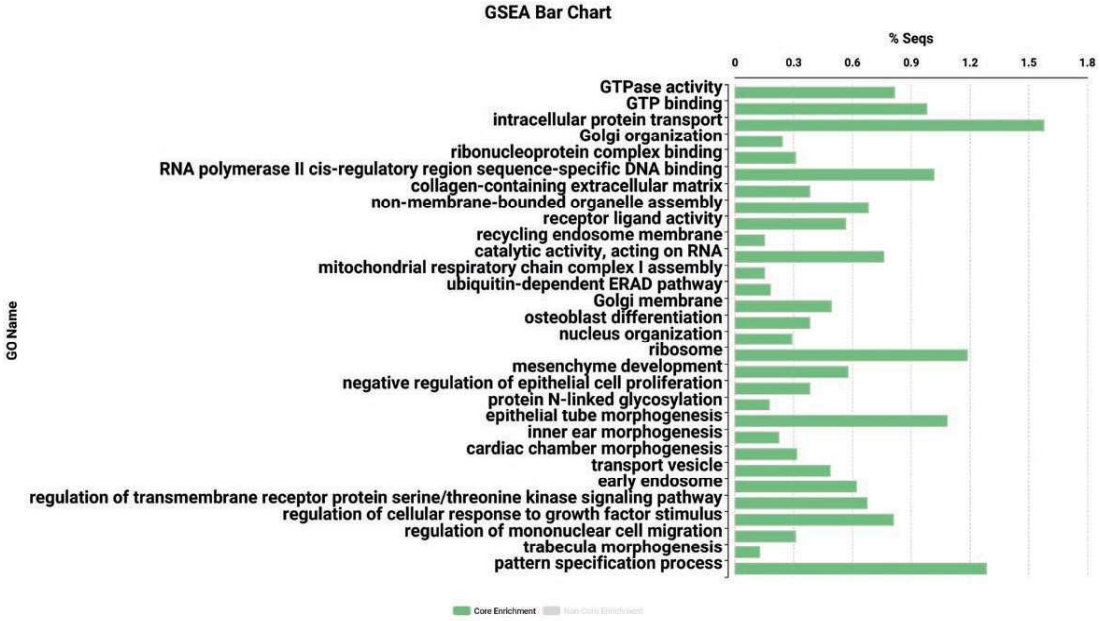

Figure 2. Top 30 enriched Gene Ontology (GO) terms of the *pars tuberalis* between active and nonactive rams. The vertical axis represents the top 30 enriched pathway categories (GO) according to their normalized enrichment score (NES) at the significance threshold (false discovery rate; FDR < 0.05), and the horizontal axis represents the percentage calculated as the number of sequences (core or noncore) annotated with each GO. Core enrichment: subset of genes within the gene set that contributes most to the enrichment result. Noncore enrichment: subset of genes within the gene list that have not been enriched. Only core enrichment is shown in the figure.

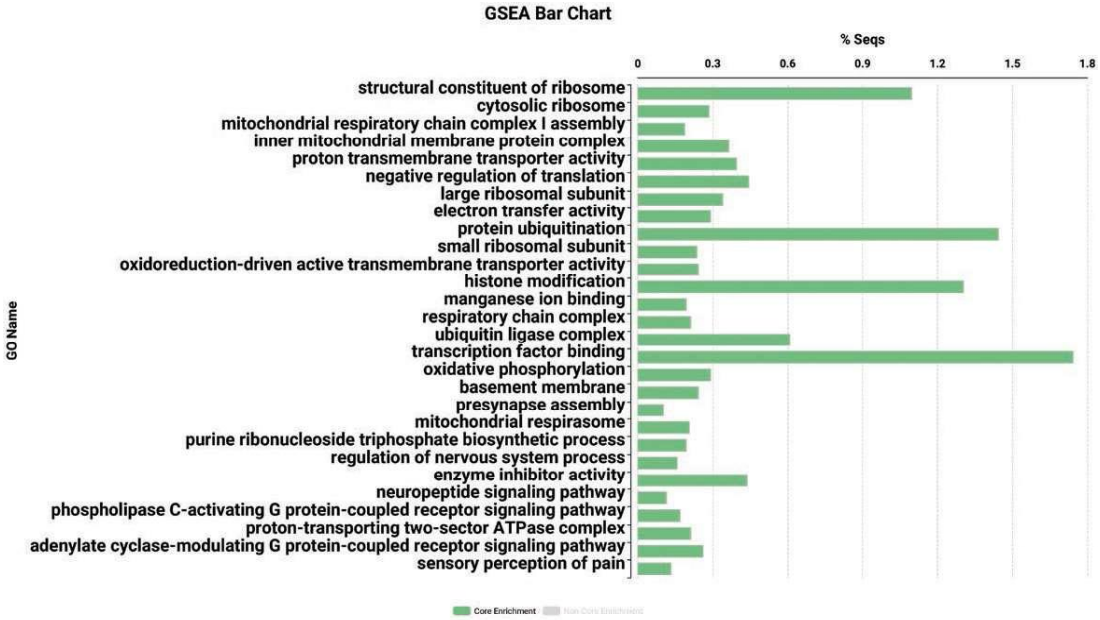

Figure 3. Top 30 enriched Gene Ontology (GO) terms of the pineal gland between active and nonactive rams. The vertical axis represents the top 30 enriched pathway categories (GO) according to their normalized enrichment score (NES) at the significance threshold (false discovery rate; FDR < 0.05), and the horizontal axis represents the percentage calculated as the number of sequences (core or noncore) annotated with each GO. Core enrichment: subset of genes within the gene set that contributes most to the enrichment result. Noncore enrichment: subset of genes within the gene list that have not been enriched. Only core enrichment is shown in the figure.

Table 5. Gene expression changes in hypothalamus and pineal gland tissues in active and nonactive rams comparisons obtained with RNA-Seq and RT-qPCR data

			Log2FC[2]	
Tissue	Contrast[1]	Gene	RNA-Seq	qPCR
Hypothalamus	A vs. NA			
		AVP	3.78*	4.50***
		CGA	6.18*	4.06***
		PRL_HT[3]	6.26*	4.47***
		GABRD	2.18*	1.58
Pineal gland	A vs. NA			
		CRABP1	−3.59*	−2.30**
		GNG8	−3.06*	−1.24
		NPY	−4.24*	−1.55
		PRL_PG[3]	6.95*	6.53***

[1]A, active; NA, nonactive.
[2]Log2 fold change.
[3]*PRL_HT: ENSOARG00020014542; and PRL_PG: ENSOARG00020015585.*
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
Significant differences are shown after Benjamini and Hochberg correction.

different sexual activity phenotype, based on pen test results and tree-based analysis. PCA confirmed that age and the sexual activity phenotype were highly correlated. The A group had an average number of mounts (± sd) of 7.93 ± 3.56, and the NA did not make any mounts.

The transcriptome analysis identified 103 and 12 DEGs in the HT and PG, respectively, whereas no DEGs were detected for the PT. These results are more likely to reflect the difference in the regulation of ram behavior in two different key brains. To investigate the biological functions of the DEGs detected in HT, we performed GO annotation and KEGG pathway analysis implemented in the DAVID platform. This analysis clearly revealed that some of the upregulated genes in the sexually active rams, including *MTNR1A, CHRNA2, FSHB, LHB, GNRHR, AVP, PRL, PDYN, CGA, GABRD,* and *TSHB,* are involved in the neuroactive ligand-receptor interaction pathway. Recently, Abecia et al. (2020) observed an increase in the sexual performance of rams in spring that carried certain mutations of the *MTNR1A* gene. Two *MTNR1A* gene loci seemed to influence the reproductive behavior of Rasa Aragonesa rams. According to the same authors, the TT genotype at position g.17355458 C> T, and the GG at position g.17355452 G > A had a positive effect on the sexual performance of rams (Abecia et al., 2022). The *neuropeptide arginine-vasopressin* (*AVP*) has long been implicated in the regulation of social behavior and communication. Rigney et al., (2021) observed that, in males, knockdown of AVP expression in the bed nucleus of the stria terminalis strongly reduced social and sexual behavior in males but not females. Another important candidate gene, the *prolactin* gene (*PRL*), has been shown to impact the activity of neuronal circuits involved in the processing of sociosexual relevant cues and thus sexual performance (Valente et al., 2021). Furthermore, *thyroid stimulating hormone subunit beta* (*TSHB*) acts locally on *TSHR*-expressing cells in the adjacent basal hypothalamus, leading to altered expression of *DIO2,* which regulates the thyroid hormone in the HT, controlling the activity of the HPG to exhibit anestrus in direct (GnRH neuron) or indirect (KISS1/RFRP system) ways (Lomet et al., 2018; Xia et al., 2021). In this study, all these genes were found to be upregulated in the active rams and downregulated in the NA rams, which may explain the passive sexual behavior of the NA rams toward the synchronized ewes. Furthermore, some of these genes were also enriched in the GnRH and cAMP signaling pathways. GnRH signal transduction in the brain has been reported to have an impact on animal sexual behavior (Foster et al., 2006; Zohar et al., 2010), whereas the cAMP signaling pathway activated by brain-derived neurotrophic factor (BDNF) has been shown to be a pivotal player in neuroplasticity (Reierson et al., 2011). However, *MPZ* was one of the highly downregulated genes in the A vs. NA comparison. The *MPZ* is involved in the myelination of the peripheral nervous system (LeBlanc et al., 2006). We hypothesized that the upregulation of this gene in the NA rams does not allow the efficient transmission of nerve impulses. Animals with brain lesions cannot combine information of a sexual nature, thus leading to defective copulatory behavior (Everitt et al., 1987). Similarly, the *GADL1* gene was also downregulated in the A vs. NA comparison, and this gene has been suggested to be involved in the biosynthesis of hypotaurine and taurine (Liu et al., 2012). The molecular mechanism underlying the effect of the *MPZ* and *GADL1* genes in relation to reproductive behavior remains to be studied in more depth.

In the PG, the DEGs detected were not enriched due to their low number. However, some of these genes are involved in the neuropeptide signaling pathway (*MCHR1, NPY,* and *TAC1*), hormone activity (*NPY* and *PRL*) and neuroactive ligand-receptor interactions (*MCHR1, NPY, PRL,* and *TAC1*). *Melanin concentrating hormone receptor 1* (*MCHR1*) is enriched in brain areas that are involved in the modulation of mood and affect in mice, and it was suggested that melanin-concentrating hormone (MCH)-dependent signaling may influence neurobiological mechanisms underlying fear and anxiety processes (Saito et al., 2001). Roy et al. (2006) showed that *MCHR1* can modulate stress and anxiety-like behaviors, suggesting that this may be due to changes in serotonergic transmission in forebrain regions. Similarly, the *neuropeptide Y* (*NPY)* gene is one of the key regulators of the HPG axis in mammals. A recent study has shown that the central injection of NPY modulates sexual behavior in

male rats via modulation of the KNDy secreting neurons as an interneural pathway to GnRH neurons (Azizi et al., 2020). Finally, *cellular retinoic acid binding protein 1* (*CRABP1)* was reported to modulate hypothalamic-pituitary-adrenal (HPA) axis homeostasis and anxiety-like behaviors by altering *FKBP5* expression. In fact, CRABP1 knockout mice exhibit reduced anxiety-like behaviors accompanied by a lowered stress-induced corticosterone level (Lin et al., 2021). In this study, we speculated that the upregulation of these genes in inactivated rams may induce the neurochemical effects implicated in behavioral responses to fear and anxiety, thus inhibiting the sexual behavior of rams.

Our study also investigated potential pathways involved in sexual behavior by running GSEA, including all the expressed genes. In HT, several pathways were enriched. The first pathway was nuclear speck (GO:0016607), also known as interchromatin granule clusters, which was reported to facilitate integrated regulation of gene expression (Galganski et al., 2017). The second enrichment pathway was the structural constituent of ribosome pathway (GO:0003735). Genes encoding this pathway are involved in the translation process, the formation of the RNA transfer that acts during protein synthesis and ribosome biogenesis (Xue et al., 2020). Among these genes, we found that *MRPL35, RPL15, RPS27A, RPS27,* and *RPL35A* were all associated with ribosome functions. Malfunction of ribosomes will affect the translation of mRNAs. In this context, any possible dysfunction of ribosomes may compromise the sexual behavior of rams. Among various GO terms enriched in the PT, the intracellular protein transport (GO:0006886) and pattern specification process (GO:0007389) pathways were the most enriched, including 259 and 211 genes, respectively. The intracellular transport of proteins involves a network of membranous compartments connected by transport vesicles and other transport structures, such as the lumen of the endoplasmic reticulum and the Golgi complex, for posttranslational processing (Popescu, 2012). Regarding PG, transcription factor binding (GO:0008134) was highly enriched. This pathway is important for the control of neuronal and neuroendocrine functions. Transcription factors modulate gene expression by binding to gene promoter regions or to distal regions called enhancers (Boeva, 2016) involved in regulating transcriptional events of melatonin synthesis, the hormone of the PG (Von Gall et al., 2000). The protein ubiquitination (GO:0016567) pathways were also highly enriched. This pathway has a large variety of functions, including cell signaling, apoptosis, protein processing, immune response, and DNA repair (Guo and Tadi, 2020). Klein's research group (Klein and Moore, 1979; Klein, 2007) provided evidence of the involvement of the ubiquitin proteasome system in the regulation of the rate limiting enzyme for melatonin synthesis, arylalkylamine N-acetyltransferase (AANAT).

Conclusion

The sexual performance of the ram affects flock breeding efficiency, so its control constitutes a key for improving sheep efficiency. In this study, we investigated the DEGs in ram brains with different sexual behavior by RNA-Seq. Our results contribute to understanding the genomic basis of sexual behavior in rams. We demonstrated that multiple gene networks and pathways orchestrate sexual behavior and identified the major genes affecting sexual behavior of rams. Genes discovered through this study could be potential molecular markers to be used for genetic-assisted selection to improve sexual behavior in the ovine species.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

Acknowledgments

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Data Availability

The datasets analyzed during the current study are available in NCBI's Gene Expression Omnibus repository and are accessible through GEO Series accession number GSE204861 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE204861).

Conflict of Interest Statement

The authors declare no real or perceived conflicts of interest.

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Supplementary Materials*

Supplementary Figure 1. Pearson's pairwise correlation matrix between variables (Age; live weight, LW; Body condition score, BCS; testicular diameter, TD; sexual activity phenotype as the sum of mounts and services; and medium plasmatic testosterone) from the first sexual behavior pen test. Color intensity and the size of the circle are proportional to the correlation coefficients. Negative/positive correlations: red/blue.

Supplementary Figure 2. Tree-based regression plot for the mounts and services phenotype using the "Party package" taking into account all the variables (Age, BCS, LW, testosterone and testicular diameter). Node 3: subpopulation of rams with BCS ≤ 3 and age ≤ 2.521; node 4: subpopulation of rams with BCS > 3 and age ≤ 2.521; node 5: subpopulation of rams with age > 2.521

Supplementary Figure 3. Principal component analysis (PCA). Representation of the variables (Body condition score, BCS; live weight, LW; testicular diameter, TD; Age; sexual activity phenotype as the sum of mounts and services; and medium plasmatic testosterone) on the correlation circle with contributions of variables to Dim 1 and Dim 2.

Supplementary Figure 4. Volcano plots in which the x-axis represents multiple of genes expression between the two conditions and the y-axis represents the statistical significance of gene expression differences. a) Hypothalamus; b) Pineal gland. Red: padj<0.05, Orange: |log2FC |>1, green if both.

Supplementary Figure 5. Gene stability using NormFinder in **a)** Pineal gland; **b)** hypothalamus tissues.

Supplementary Table 1. Differentially expressed genes (DEG) in the hypothalamus through the comparison between active and nonactive rams

Supplementary Table 2. DAVID Functional Annotation Clustering of Differential Expressed genes in active vs nonactive rams in hypothalamus

Supplementary Table S3 Top 30 most enriched terms between the active *vs* nonactive rams for each tissue.

* el material suplementario está disponible en línea en Journal of Animal Science via el siguiente link: https://academic.oup.com/jas/article-abstract/doi/10.1093/jas/skac365/6795963

Manuscrito 6: Blood transcriptome of Rasa Aragonesa rams with different sexual behavior phenotype reveals *CRYL1* and *SORCS2* as genes associated with this trait

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Blood transcriptome of Rasa Aragonesa rams with different sexual behavior phenotype reveals *CRYL1* and *SORCS2* as genes associated with this trait

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Abstract

Reproductive fitness of rams is seasonal, showing the highest *libido* during short days coinciding with the ovarian cyclicity resumption in the ewe. However, the remarkable variation in sexual behavior between rams impair farm efficiency and profitability. Intending to identify in vivo sexual behavior biomarkers that may aid farmers to select active rams, transcriptome profiling of blood was carried out by analyzing samples from 6 sexually active (A) and 6 nonactive (NA) Rasa Aragonesa rams using RNA-Seq technique. A total of 14,078 genes were expressed in blood but only four genes were differentially expressed (FDR < 0.10) in the A vs. NA rams comparison. The genes, *acrosin inhibitor 1* (*ENSOARG00020023278*) and *SORCS2*, were upregulated (log2FC > 1) in active rams, whereas the *CRYL1* and *immunoglobulin lambda-1 light chain isoform X47* (*ENSOARG00020025518*) genes were downregulated (log2FC < -1) in this same group. Gene set Enrichment Analysis (GSEA) identified 428 signaling pathways, predominantly related to biological processes. The lysosome pathway (GO:0005764) was the most enriched, and may affect fertility and sexual behavior, given the crucial role played by lysosomes in steroidogenesis, being the *SORCS2* gene related to this signaling pathway. Furthermore, the enriched positive regulation of ERK1 and ERK2 cascade (GO:0070374) pathway is associated with reproductive phenotypes such as fertility via modulation of hypothalamic regulation and GnRH-mediated production of pituitary gonadotropins. Furthermore, external side of plasma membrane (GO:0009897), fibrillar center (GO:0001650), focal adhesion (GO:0005925), and lamellipodium (GO:0030027) pathways were also enriched, suggesting that some molecules of these pathways might also be involved in rams' sexual behavior. These results provide new clues for understanding the molecular regulation of sexual behavior in rams. Further investigations will be needed to confirm the functions of *SORCS2* and *CRYL1* in relation to sexual behavior.

Lay Summary

Analyzing ram sexual behavior via blood transcriptome profiling can help to identify in vivo sexual behavior biomarkers as an innovative alternative to invasive and time-consuming methods in farms. Using RNA-sequencing technique, we compared 12 Rasa Aragonesa rams with different sexual behavior (6 sexually active and 6 nonactive) to identify differentially expressed genes (DEGs) in peripheral blood putatively responsible of *libido* differences between rams. Comparative analysis revealed four candidate genes and several signaling pathways related to sexual behavior such as lysosome, and positive regulation of the extracellular signal-regulated kinase 1/2 (ERK1 and ERK2) cascade. This data will be helpful for further investigations to understand the differences of sheep sexual behavior.

Keywords: sexual behavior, transcriptome, rams, blood, *CRYL1*, *SORCS2*.

Abbreviations: A, active; BCS, body condition score; BDNF, brain-derived neurotrophic factor; BP, biological process; CC, cellular component; CITA, Agrifood Research and Technology Centre of Aragon; CPM, count per million; DEG, differential expressed gene; ES, enrichment score; FDR, false discovery rate; GEO, Gene Expression Omnibus; GnRH, gonadotropin-releasing hormone; GO, gene ontology; GSEA, gene set enrichment analysis; LH, luteinizing hormone; Log2FC, log2 fold change; LW, live weight; MF, molecular function; NA, not active; NES, normalized enrichment score; PCA, principal component analysis; RIN, RNA integrity number; RT-qPCR, reverse transcription quantitative real-time PCR; SNP, single-nucleotide polymorphism; TMM, trimmed mean of M-values; trkB, tyrosine receptor kinase B

Introduction

In livestock production systems, fertility is a key factor of farms efficiency. In Mediterranean sheep, this is particularly important since the animals are seasonal breeding and mate in autumn and winter, hence the appellation "short-day breeders". This seasonality impedes the balanced supply throughout the year (Hazard, 2010) and consequently induces major variation in lamb production as well as disparity in the market price. To improve flock sustainability and profitability, several methods have been developed to avoid the seasonality such as the use of hormonal and/or photoperiodic treatments (Chemineau et al., 1988). For ethical reasons, breeders are forced to reduce the use of these treatments (Martin and

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Kadokawa, 2006). The male effect is used in sheep as a strategy to improve flock fertility during seasonal anestrus period. The ram emits a considerable range of different sensory stimuli (courtship, odor and vocalizations) that may be responsible for inducing a reproductive neuroendocrine response in ewes by stimulating luteinizing hormone (LH) secretion (Fabre-Nys et al., 2015). However, differences in sexual behavior among individuals have been reported (Lindsay, 1996). Rams expressing high sexual behavior produce a greater stimulus during seasonal anestrus, which lead into a higher percentage of mated ewes and higher fertility (Delgadillo et al., 2008). On the other hand, rams with low sexual behavior extend the lambing season, and decrease the number of lambs born per ewe lambing, leading to an increase of farm costs (Alexander et al., 2012). This is why ram selection is fundamental to increase the efficiency of sheep farming (Simitzis et al., 2006), and sexual behavior is one of the most important traits to be considered during the selection of replacement rams and ewes. Yet, selection processes rarely include an evaluation of sexual behavior, such as sexual behavior pen tests, because of time, labor and facilities constraints (Alexander et al., 2012). Hence, alternative solutions must be sought, such as the identification of sexual behavior biomarkers. In a previous study, aiming to determine the possible molecular mechanisms underlying the sexual behavior of Rasa Aragonesa rams, our research group investigated transcriptional changes in the hypothalamus, *pars tuberalis*, and pineal gland in rams with different sexual behavior, identifying important differential expressed genes related to sexual behavior such as *MTNR1A, CHRNA2, FSHB, LHB, GNRHR, AVP, PRL, PDYN, CGA, GABRD*, and *TSHB* (Lakhssassi et al., 2023). Nevertheless, brain gene expression studies are highly invasive because they are based on *post mortem* tissue samples. Intending to identify in vivo sexual behavior biomarkers that may aid farmers in breeding decisions, this study investigate the differentially expressed genes in peripheral blood between active (A) and nonactive (NA) rams of Rasa Aragonesa sheep breed using RNA-Seq technique.

Material and Methods

All experimental procedures, including care of animals and euthanasia, were performed in accordance with the guidelines of the European Union regulations for the use and care of animals in research (Directive 2010/63/EU) and approved by the Animal Ethics Committee of the Research Centre (protocol number 2017/02).

Animals' selection

Animal selection, sexual behavior test and classification into sexual active/nonactive rams are described in Lakhssassi et al. (2023). Briefly, 49 previously exposed to ewes during breeding season Rasa Aragonesa rams (1.8 to 8.3 years of age) were submitted to individual sexual behavior pen tests. These rams were exposed to three adult ewes synchronized in estrus, and their sexual behavior was recorded. Sexual activity phenotype was defined as the sum of mounts and services (Lakhssassi et al., 2023). Blood samples for testosterone determination and RNA analyses (validation studies) were taken from all rams. Body condition score (BCS: 3.3 ± 0.3; 1 to 5 scale, according to Russel et al. (1969)), live weight (LW: 81.5 ± 14.44 kg) and mean antero-posterior maximum testicular diameter, subtracting the scrotal skin thickness, using a caliper (Folch and Roca Bernaus, 1982) (6.00 ± 0.58 cm) were also measured. A tree-based regression model for the sexual activity phenotype and a principal component analysis (PCA) were performed, considering all the variables for the sexual activity phenotype (age, BCS, LW, testosterone, and testicular diameter), for choosing a population subgroup with similar values for the significant variables, but different sexual activity phenotype for next analyses (Lakhssassi et al., 2023). Then, we could choose a subgroup of 21 rams with similar age and BCS (significant variables) that were submitted to a second individual sexual behavior pen test fifteen days later than the first one to confirm the results from the first pen test. Rams from this group (n = 21) were classified as either active (A) (9.0 ± 7.5, average mounts plus services ± standard deviation (sd); n = 11) or nonactive (NA; without mounts or services; n = 10). Six rams of each group were selected the day after the second sexual behavior pen test for taking the blood sample for RNA-Seq (6 A and 6 NA rams). Blood samples taken from the total ram population (n = 59) the day of the first pen test were used for validation of RNA-Seq data by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Blood samples, RNA extraction, and sequencing

The day after the second pen test, blood samples were taken in Tempus tubes (Applied Biosystems, Thermofisher, UK) and stored at – 80 °C until total RNA extraction. The Tempus Spin RNA Isolation Reagent Kit was used to extract the total RNA from peripheral blood (Applied Biosystems, Thermofisher, UK) according to the manufacturer´s instructions. The Agilent 2100 Bioanalyzer machine (Agilent Technologies, Palo Alto, CA) was used to check for RNA concentration and RNA Integrity Number (RIN) values. For library construction, only samples with a RIN > 7 were maintained for sequencing. The RNA-Seq library was prepared at the CNAG (Centro Nacional de Análisis Genómico, Spain; https://www.cnag.crg.eu/) as described by Lakhssassi et al. (2023), and sequencing was carried out with Novaseq 6000 (Illumina) generating paired-end reads of 151 bp. The RNA-Seq sequencing data have been deposited in the NCBI's Gene Expression Omnibus repository (Barrett et al., 2013) with the accession number GSE218667 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE218667).

Validation of RNA-Seq data by RT-qPCR

The validation of the RNA-Seq results was done in the 59 rams sexually characterized in the first pen test (15 days before taking blood samples for RNA-Seq study). Blood samples from the total ram population were collected the day of the first pen test in Tempus tubes and stored at – 80 °C until total RNA extraction. RNA extraction was performed as described above. Three genes identified to be differentially expressed in the 12 blood samples by RNA-Seq were chosen for RT-qPCR validation. These genes were: *acrosin inhibitor 1*, *CYRL1* and *SORCS2*. *Acrosin inhibitor 1*, and *CYRL1* genes were DEGs in the two scenarios, while *SORCS2* was differential expressed only in the first scenario, but its function was related to sexual behavior. Furthermore, four housekeeping genes (*B2M*, *GAPDH*, *RPL19*, *RPL32*) commonly used as reference genes in expression studies, were used to normalize the gene expression of *acrosin inhibitor 1*, *CYRL1*, and *SORCS2*. The gene expression stability was studied using NormFinder (Andersen et al., 2004). Initially, we treated total RNA (1 µg) from each sample with DNAse (Invitrogen, Carlsbad, CA, USA). Then,

the Super-ScriptIII Reverse Transcriptase kit (Invitrogen) was used following the manufacturer's recommendations, to synthesize first-strand cDNA. Primer3 software was used (https://primer3.ut.ee/; last accessed on November 11, 2022), to design the primers in specific exon-spanning regions. Conventional PCR was achieved using standard conditions for all the genes to confirm the specificity of the primers. Gene identity was confirmed by Sanger sequencing of the PCR products by the company Stabvida (https://www.stabvida.com/es). Homology searches were performed with BLAST (National Center for Biotechnology Information: https://blast.ncbi.nlm.nih.gov/Blast.cgi; last accessed on November 11, 2022). The quantitative real-time PCR was carried out in a 10 µl PCR total reaction mixture containing SYBR Green Master Mix: SYBR Premix Ex Taq II (Tli RNase H Plus, Takara, Demlab, Zaragoza, Spain). Reactions were run in triplicate on an ABI Prism 7500 platform (Applied Biosystem, Madrid, Spain) following the manufacturer's cycling parameters. Table 1 shows the annealing temperatures, primer concentrations, and primer sequences. The efficiency (E) and correlation coefficient (R^2) of PCR amplification for each gene were assessed using the standard curve method using the formula $E = 10^{(-1/\text{slope})}$, through 10-fold serial dilutions of pooled cDNA. Two "connector samples" were replicated in all plates to remove technical variation from this source of variability.

Statistical analysis

RNA-Seq analysis

All transcriptome analyses were carried out within OmicsBox platform v2.0.36 from BioBam's (https://www.biobam.com/omicsbox; last accessed on November 11, 2022). The quality of reads was assessed using FastQC tools (Andrews, 2015). The adapters sequences and low-quality reads were trimmed running the Trimmomatic program (Bolger et al., 2014) using the Sliding Window Trimming option with a window size of 40, and a required quality of 20. The filtering configuration of reads was set to 20 and 35 for the average quality and the minimum length, respectively. We mapped the obtained clean reads to the ovine reference genome Oar_rambouillet v1.0 (GCA_002742125.1) using STAR v2.7.8a (Dobin et al., 2013) supported by an annotated gene file in GTF format. Gene-level quantification was estimated by HTSeq (Anders et al., 2015) using the Union model as overlapping mode, gene as feature and strand specific forward. For subsequent analysis, only reads mapping unambiguously to a single genomic feature were considered. EdgeR software (Robinson et al., 2010) was run to detect the differentially expressed genes (DEGs) using a simple design where A rams was defined as the primary contrast condition and NA rams as primary reference condition. A threshold of count per million (CPM) greater than or equal to 0.5 in at least two samples and the trimmed mean of M-values (TMM) with Zero Pairing normalization method were applied to filter the low count genes. The statistical analysis was achieved using the exact test with robust option to avoid potential outlier genes. Multiple-testing corrections were performed using the Benjamini and Hochberg step-up false discovery rate (FDR) procedure to calculate adjusted P-values. To judge the significance of gene expression difference, the absolute log2FC ≥ 1 and FDR ≤ 0.10 were used as the threshold. The choice of relaxed FDR (<10%) was based on that our objective was mainly exploratory, so it is convenient to retain some information that we could lose being more restrictive.

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) (Subramanian et al., 2005) implemented in OmicsBox (https://www.biobam.com/omicsbox) were applied to identify potential pathway responsible to cause differences in sexual behavior between rams. GSEA includes in the analyses all the expressed genes, regardless of whether they were differentially expressed, and determines both modest and robust, coordinated, biologically relevant changes in molecular signaling pathways, and explores and identifies the most significant and over-represented gene ontology (GO) terms. In this study, GSEA was performed to determine whether an a priori defined set of genes is statistically significant between A and NA rams in blood, classifying the biological functions of the set genes on molecular functions (MFs), biological process (BP), and cellular component (CC). GSEA Enrichment scores (ES) were calculated according to the rank-ordered gene list and GO terms containing a minimum of 15 genes. A normalized enrichment

Table 1. Characteristics of reverse transcription quantitative real-time PCR (RT-qPCR) for validation of RNA-Seq for each gene

Genes	Primers	Amplification Size (bp)	AT[1]	E[2]	R^2[2*]
RPL19	F-5′caactcccgccagcagat-3′	76	60°C	1.99	0.99
	R-5′ccgggaatggacatgcaca-3′				
RPL32	F-5′ggcaccagtcagaccgatatg-3′	75	60°C	1.99	0.99
	R-5′cccagacaggagaggttcag-3′				
GAPDH	F-5′tccatgaccactttggcatcgt-3′	80	60°C	2.05	0.99
	R-5′gtcttctgggtggcagtga-3′				
B2M	F-5′ggtgctgcttagaggtctcg-3′	109	60°C	1.99	0.99
	R-5′acgctgagttcactcccaac-3′				
CRYL1	F-5′ tgttcgacattgagcctcgg-3′	124	60°C	2.05	0.99
	R-5′atgagtgacagctgctcctc-3′				
SORCS2	F-5′agatcagcttcctcctgcg-3′	97	60°C	2.06	0.99
	R-5′ cagtagccgcctccacttc-3′				

[1]Annealing temperature.
[2]Efficiency of PCR amplification
*Correlation coefficient.

score (*NES*) was also calculated considering the number of genes in the pathway. Genes enriched at the top of the ordered fold change list had a positive *NES*, while a negative *NES* indicates the opposite, i.e., enriched at the bottom. Pathways with an FDR 5% were considered significant.

Gene expression validation of RNA-Seq by RT-qPCR

The statistical analysis was done in the 59 rams sexually characterized in the first pen test. Furthermore, a second analysis considering only the 47 rams not used for RNA-seq was run. The corresponding mRNA levels were measured and analyzed by their quantification cycle (Cq), following the statistical method proposed by Steibel et al. (2009). The mixed model fitted was as follows:

$$y_{rigkm} = SG_{gi} + P_k + BC_m + A_m + LW_m + E_m + e_{rigkm}$$

where y_{rigkm} is the Cq value (transformed data taking into account gene amplification efficiencies) of the *g*th gene (DEGs and housekeeping genes) from the *r*th well (reactions were run in triplicate) in the *k*th plate corresponding to the *m*th animal and to the *i*th sexual activity group (NA without any mount or service or A with at least one mount or service in the first ram pen test); SG_{gi} is the fixed interaction between the *i*th sexual activity group and the *g*th gene; P_k is the fixed effect of the *k*th plate; BC_m, A_m, and LW_m are the effects of body condition score, age and the live weight of the *m*th animal, respectively, included as covariates; E_m is the random effect of the *m*th animal from which samples were collected $(E_m : (0, \sigma^2{}_E))$; and e_{rigkm} is the random residual. Gene-specific residual variance (heterogeneous residual) was fitted to the gene by sexual behavior $(e_{rigkm} : N(0, \sigma^2{}_{egi}))$.

To test differences in the expression rate of the genes of interest ($diff_{DEGs}$) between treatments in terms of fold change (FC), the approach suggested by Steibel et al. (2009) was used using the three housekeeping genes. The significance of the of the $diff_{DEGs}$ estimates was determined with the *t*-test ($P < 0.05$) after Bonferroni correction.

Results

Summary of the raw sequence reads

RNA-Seq data were obtained for 12 blood samples (6 A and 6 NA rams). The statistics of the sequencing and read alignments against the ovine reference genome Oar_rambouillet v1.0 (GCA_002742125.1) are listed in Supplementary Table S1. Illumina sequencing produced a total of 1,024,395,857 raw reads from blood samples. After removing low-quality bases and adaptor sequences, the remained clean reads were 905,265,453 which provided abundant data for further analysis. On average, 97.7% of all the clean reads were mapped to the ovine reference genome of which 90.1 % were uniquely mapped. These results demonstrated good data quality suitable for subsequent research analysis (scenario 1). However, we observed that for sample 1, more than a half of input raw reads were trimmed. In total, 76% of all read bases included in library 1 were trimmed off, while it was less than 10% for the other libraries (Supplementary Table 1). So, we performed a second analysis without this sample to check a putative effect on downstream analyses (scenario 2).

Differentially expressed genes (DEGs)

We next investigated the differences in gene expression data between A and NA rams. As a result, 26,478 genes were expressed in the blood samples. After filtering, a total of 14,078 were retained for the differential expression analysis. Only four differentially expressed genes (FDR = 0.1) were identified of which two were significant with a FDR < 0.05 (*CRYL1* and *acrosin inhibitor 1*) between A and NA rams (Table 2) in the scenario 1. The *acrosin inhibitor 1* and *SORCS2* genes were upregulated (log2FC > 1) in the A rams whereas *CRYL1* and *immunoglobulin lambda-1 light chain isoform X47* (*ENSOARG00020025518*) genes were downregulated (log2FC < -1) in this same group of rams. The results of the second analysis (5 A and 6 NA rams) revealed that the results of the study do not change greatly in relation to DEGs. A total of 14,021 genes remained after filtering. Similarly, only four DEGs were detected (FDR = 0.1). Three genes were upregulated, and one were downregulated (Table 2). The results of this analysis also showed *CRYL1* and *Acrosin inhibitor 1* genes in common between the two analyses. On the other hand, the *GPC3* and *OSBP2* genes were found upregulated under this scenario. The FC were similar between the two analyses. In this sense, the correlation coefficient between the four significant DEGs in the first analysis with respect to the results of the same genes in the second analysis was 0.99 and vice versa. The FCs for *CRYL1*, *LOC121819654*, *SORCS2*, and *ENSOARG00020025518* were −5.6, 36.30, 2.72, and

Table 2. Differentially expressed genes (DEGs) in the blood through the comparison between active and nonactive rams in scenario 1 (*n* = 12; 6 active vs 6 nonactive rams) and 2 (*n* = 11; 5 active vs 6 nonactive rams)

	Genes	Description	FC	Log2FC[1]	*P*-adj[2]
Scenario 1	*CRYL1*	*Crystallin lambda 1*	−5.60	−2.48	2.19E−08
	LOC121819654	*Acrosin inhibitor 1*	36.30	5.18	0.006
	SORCS2	*Sortilin related VPS10 domain containing receptor 2*	2.73	1.45	0.095
	ENSOARG00020025518	*Immunoglobulin lambda-1 light chain isoform X47*	−2.48	−1.30	0.099
Scenario 2	*CRYL1*	*Crystallin lambda 1*	−5.46	−2.45	0
	GPC3	*Glypican 3*	3.28	1.71	0.0041
	LOC121819654	*Acrosin inhibitor 1*	42.17	5.40	0.0002
	OSBP2	*Oxysterol binding protein 2*	41.70	5.38	0.0932

[1]Log2Fold Change.
[2]*P* adjusted.

–2.48, respectively, while in the second analysis were –5.46, 42.17, 2.71, and –2.39.

Enrichment analysis

The GSEA revealed 428 signaling pathways, mainly dominated by biological processes (291 BP, 74 MF, and 63 CC). The top 30 most enriched terms between the A and NA rams are shown in Figure 1. The enrichment score and the gene set size details for these GOs are described in Supplementary Table S2. The most enriched pathways were the lysosome (GO:0005764), external side of plasma membrane (GO:0009897), fibrillar center (GO:0001650), and focal adhesion (GO:0005925).

Verification of DEGs by RT-qPCR

To validate the RNA-Seq results, the expression fold changes of three genes (*acrosin inhibitor 1*, *CRYL1*, and *SORCS2*) were tested by using RT-qPCR in RNA samples of 59 rams of the first pen test. Unfortunately, the *acrosin inhibitor 1* could not be validated due to its very low expression in blood samples, with an average of 12.7 counts (number of reads aligned to each genomic feature). The gene stability analysis with NormFinder program revealed that the housekeeping genes were more stable than the DEGs (Supplementary Figure S1). Therefore, the RT-qPCR results were normalized with the three more stable housekeeping genes: *B2M*, *GAPDH*, and *RPL32*. The comparison A vs. NA rams was performed considering active those that had at least 1 mount or service. The active ones presented 14.15 ± 9.61 mounts + service (n = 35), whereas the NA rams did not have any mount or services (0, n = 24). The two genes *CRYL1* and *SORCS2* were significant as in RNA-Seq but in lower magnitude (FC of –1.48 and 1.64 vs –5.60 and 2.73 for *CRYL1* and *SORCS2*, in RT-qPCR vs RNA-Seq) considering the 59 rams (Figure 2). The analysis considering only the 47 rams not used for RNA-seq also confirmed that the expression of both genes was significant affected (FC of –1.29 and 1.99 for *CRYL1* and *SORCS2*, respectively). In general, the expression patterns of these two genes were consistent with those obtained with the RNA-Seq technique.

Discussion

Understanding the molecular mechanisms underlying the sexual behavior of rams is crucial to improve flock sustainability and profitability. RNA-Seq technology is a valuable tool allowing large scale analysis of the transcriptional changes related to biological conditions of interest (Crow et al., 2019). Ram's blood transcriptome is expected to provide complete

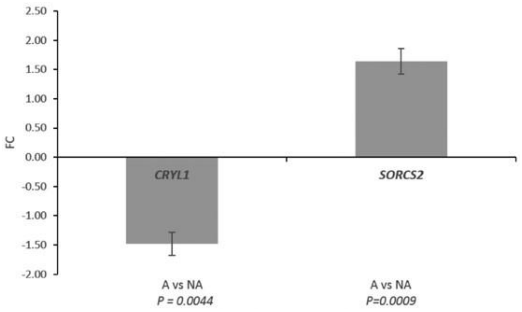

Figure 2. Differences in the *CRYL1* and *SORCS2* expression rate (measure as a fold change, FC) between active (A; n = 35) and nonactive (NA; n = 24) rams using RT-qPCR. Segments indicate the error standard of the estimate.

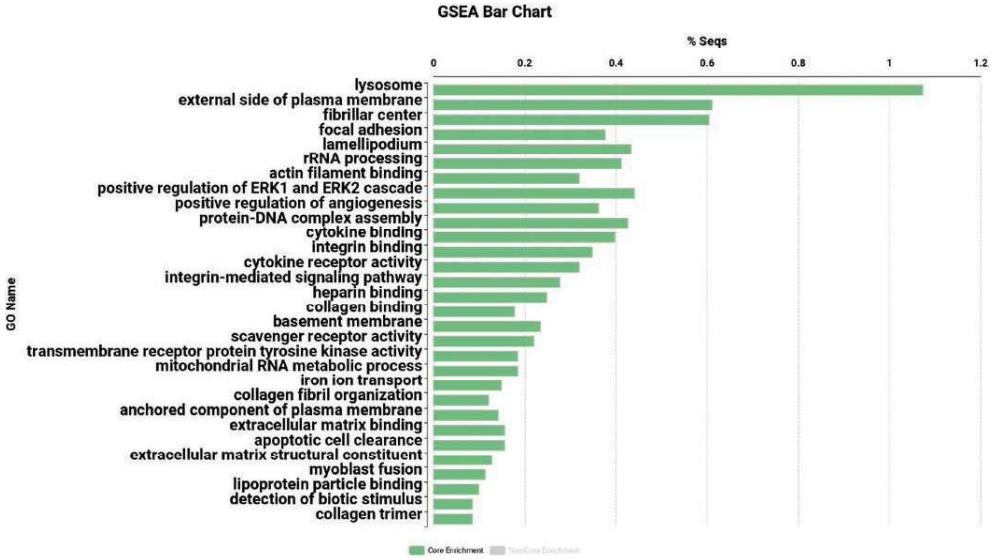

Figure 1. Top 30 enriched Gene Ontology (GO) terms of the blood between active and nonactive rams. The vertical axis represents the top 30 enriched pathway categories (GO) according to their normalized enrichment score (NES) at the significance threshold (false discovery rate; FDR < 0.05), and the horizontal axis represents the percentage calculated as the number of sequences (core or noncore) annotated with each GO. Core enrichment: subset of genes within the gene set that contributes most to the enrichment result. Noncore enrichment: subset of genes within the gene list that have not been enriched. Only core enrichment is shown in the figure.

information on physiological differences about the state of sexual behavior with potential outcomes for applications in animal breeding such as biomarkers.

In this study, we investigated transcriptional differences in peripheral blood samples from rams of Rasa Aragonesa breed with different sexual behavior using RNA-Seq technology. In a previous study, we analyzed the factors influencing the sexual activity phenotype for these 59 rams (Lakhssassi et al., 2023). Testosterone is considered as the predominant androgen for expressing and maintaining libido in rams in numerous studies (Perkins and Roselli, 2007). However, the differences observed in sexual behavior between rams were not due to testosterone in the current study, as no significant effect between testosterone concentration and rams sexual behavior in the pen test was found (Lakhssassi et al., 2023). In that study, statistical analysis showed that rams sexual behavior was influenced by age ($P < 0.01$) and LW ($P < 0.05$), and these results were also confirmed by a tree-based regression model analysis for the sexual activity phenotype (Lakhssassi et al., 2023). For this reason, for the RNA-Seq analysis we choose a population subgroup from the total population with similar values for these significant variables but different sexual activity phenotype.

Unfortunately, as evidenced by read trimming, more than 50% of sample 1 was trimmed (Supplementary Table S1). Best practices for ENCODE2 RNA-Seq experiments indicate that experiments whose purpose is to look for gene expression profiling, require 20 to 25M mappable reads/sample to the genome or known transcriptome (https://genome.ucsc.edu/ENCODE/protocols/dataStandards/ENCODE_RNAseq_Standards_V1.0.pdf). In our case, surviving reads for sample 1 reached 22,708,884. Therefore, we decided to perform the transcriptomic analysis including this sample ($n = 12$) (scenario 1). Despite that, we also wanted to determine whether similar results (DEGs) would be obtained if we had excluded sample 1 from the analyses (scenario 2). Likewise, analyses with the 11 samples (scenario 2) identified also 4 DEGs (FDR < 0.10) where three were upregulated, and one was downregulated (Supplementary Table S2). *CRYL1* and *Acrosin inhibitor 1* genes appears in common between the two analyses in addition to two new upregulated genes appeared in scenario 2, *GPC3* and *OSBP2*. *GPC3* and *oxysterol binding protein 2* (*OSBP2)* genes do not seem to be related to our phenotype according to literature. In this case, the *SORCS2* gene did not reach the statistical significance (*P-adj* = 0.11). Therefore, only common DEGs to the two transcriptomic analyses were chosen for RNA-Seq validation (*CRYL1* and *acrosin inhibitor 1*). Besides, *SORCS2* gene, that was only significant in scenario 1, was also chosen for validation due to its putative involvement in sexual behavior according to literature. It is outstanding that samples used for validation of the RNA-Seq ($n = 59$) were taken for the total population the day of the first individual sexual behavior pen test (sexual behavior phenotype from the first pen test), while the RNA-Seq samples correspond to the animals that were submitted to two pen tests (sexual behavior phenotype from the two-pen test), and were taken the day after the second pen tests. In this sense, population validation samples ($n = 59$) and RNA-seq samples ($n = 12$) were collected 15 days apart. With the second individual sexual behavior pen test performed fifteen days later than the first one in subpopulation of selected rams with similar BCS and age ($n = 21$; BCS > 3 and age ≤ 2.521; Lakhssassi et al., 2023), we confirmed that the animals selected for RNA-seq studies were kept A or NA rams. The genes *acrosin inhibitor 1* (*ENSOARG00020023278*) and *SORCS2* were upregulated (log2FC > 1) in active rams. According to the literature, acrosin is considered to play a critical role in the reproduction process. However, this gene could not be validated in the total ram population ($n = 59$) by RT-qPCR. This gene was characterized by relatively low expression levels, as indicated by lower RNA-Seq read counts, with an average of 12.7 (number of reads aligned to each genomic feature). *SORCS2* which encodes the sortilin-related VPS10 domain containing receptor 2 was reported to be involved in neural plasticity, development and regeneration of neuronal circuits in mammals (Glerup et al., 2014, 2016). Furthermore, the *SORCS2* gene is mainly expressed in central nervous system but also in peripheral tissues (https://www.proteinatlas.org/ENSG00000184985-SORCS2/tissue; last accessed on 14 November 2022), including bone marrow tissue. Glerup et al. (2014) showed that *SORCS2* regulated dopaminergic axon guidance and peripheral sensory neuron apoptosis, respectively, beside interacting with pro-BDNF/p75NTR, and controlling the activity of Trk receptors. Knowing that the brain-derived neurotrophic factor (BDNF) and its receptor, tyrosine receptor kinase B (trkB), related to synaptic plasticity, are implicated in sexual behavior in male rats (Sanna et al., 2019), the gene expression changes in *SORCS2* gene in peripheral blood cells could be a reflection of those changes occurring in the brain and a marker for sexual activity phenotype. On the other hand, the *CRYL1* and *immunoglobulin lambda-1 light chain isoform X47* (*ENSOARG00020025518*) genes were downregulated. The *crystallin, lambda 1* (*CRYL1*) belongs to the family of crystallins, many of which function as small heat-shock proteins involved in stress-protection (Wistow, 2012). A genome-wide association study analyzing coping behaviors in humans identified the *CRYL1* gene as associated with the emotional support seeking behavior (Shimanoe et al., 2019). Biological roles for activin have been proposed in a number of reproductive organs including the testis, where it regulates spermatogenesis (Welt et al., 2002). Moreover, *CRYL1* expression levels decreased after administration of activin A in testis in a study of Sertoli cell maturation (Itman et al., 2009). In that study, Activin modulates the number of GnRH receptors and hence, response to GnRH (Gregory and Kaiser, 2004), as well as a number of other genes in LβT2 cells including *inhbb*, *inha*, *gdf9*, and the *17β-HSD* gene (Zhang et al., 2006). Thus, activin has a major role in modulating neuroendocrine reproductive control (Xia and Schneyer, 2009). In our study, this gene was downregulated in A rams, both in RNA-Seq ($n = 12$) and the validation study ($n = 59$). Thus, we can hypothesize that *CRYL1* gene expression levels could be a biomarker of low mating behavior.

DEGs detected in this study were not found in our previous research on hypothalamus, *pars tuberalis* and pineal gland transcriptome using the same animals (Lakhssassi et al., 2023), even if some of them were also expressed in brain (*CRYL1* and *SORCS2*).

Gene enrichment of DEGs was not allowed since we detected fewer DEGs ($n = 4$) in 12 blood samples. To identify overrepresented pathways related to rams' sexual behavior, GSEA was performed including all the expressed genes. Therefore, the lysosome pathway (GO:0005764) comprising

218 genes and considered as a cellular component, was the most enriched (Supplementary Table S2). According to Xie et al. (2006), lysosomes play a crucial role in steroidogenesis. The genes comprised in this pathway might be an indirect or direct effect on steroid hormone biosynthesis. In fact, infertility and sub-infertility due to some lysosome storage disorder in mice was reported, particularly those that present a shortage in lipid catabolism, which interfere in steroidogenesis since steroidal hormones are synthesized from cholesterol (Butler et al., 2002; Xu et al., 2011). In addition, Almeida et al. (2021) described that *SORCS2* deletion leads to altered neuronal lysosome activity in mouse thus leading to many neurodegenerative diseases. It is to be reminded that *SORCS2* was a DEG upregulated in A rams. Therefore, it is tempting to speculate that any disorders of lysosomal hydrolases could damage hormone production and affect consequently sexual behavior and fertility. Another interesting pathway was the positive regulation of ERK1 and ERK2 cascade (GO:0070374) considered as biological process category. Many studies indicated that ERK1 and ERK2 are important modulators of hypothalamic GnRH-mediated regulation of pituitary gonadotropin production and fertility (Liu et al., 2002; Saba-El-Leil et al., 2003; Nekrasova et al., 2005; Bliss et al., 2009). Furthermore, Bliss et al. (2009) reported that ERK signaling is required in females for ovulation and fertility, whereas male reproductive function is unaffected by this signaling deficiency. Additionally, external side of plasma membrane (GO:0009897), fibrillar center (GO:0001650), focal adhesion (GO:0005925), and lamellipodium (GO:0030027) pathways have also been identified, suggesting that some molecules of these pathways might also be involved in rams' sexual behavior.

The DEGs detected in this study may act as factors controlling sexual behavior through the regulation of dopaminergic pathways and BDNF and also modulating activin expression levels. The mechanism and the regulatory functions of these candidate genes found in this work related to sexual behavior is still unclear and should be investigated in future studies.

Conclusion

Blood transcriptome investigation offers an accessible and less invasive alternative to study behavioral change in rams. Few genes differentially expressed were identified throughout blood transcriptome comparison between active and non-active rams. These results will provide new clues for understanding the molecular regulation of sexual behavior in rams. Nonetheless, it is recommended to study more in depth how the expression variability of these genes affects the sexual behavior in rams. Extending this approach to other breeds and a much larger data set will help to get a detailed picture on physiological change in sexual behavior between rams.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Data Availability

The datasets analyzed during the current study are available in NCBI's Gene Expression Omnibus repository and are accessible through GEO Series accession number GSE218667 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE218667).

Conflict of Interest Statement

The authors declare that they have no competing interests.

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Supplementary Materials*

Supplementary Figure 1. Gene stability using NormFinder in blood samples

Supplementary Table 1. Descriptive statistics of RNA-Seq.

Supplementary Table 2. Top 30 most enriched terms between the active *vs* nonactive rams in the blood

* el material suplementario está disponible en línea en Journal of Animal Science vía el siguiente link: https://academic.oup.com/jas/article-abstract/doi/10.1093/jas/skad098/7095688

IV- Discusión general

La gestión de la estacionalidad reproductiva es una cuestión prioritaria para el sector ganadero del ovino de aptitud cárnica. La estacionalidad de la reproducción provoca limitaciones en la industria cárnica, siendo la principal de ellas la irregularidad del suministro de canales a lo largo del año lo que provoca una importante oscilación de los precios de venta. Por tanto, el control de la estacionalidad reproductiva en ovino de carne se constituye como uno de los pilares básicos para fortalecer y mejorar la eficiencia de este sector. Sin embargo, en el contexto socioeconómico actual, y el concepto de producción animal "limpia, verde y ética" ("clean, green and ethical") (Martin y Kadokawa, 2006), las prácticas de control de la reproducción de los animales deben evolucionar teniendo en cuenta no solo los objetivos de eficiencia y rentabilidad económica de los productores, sino también, cuestiones medioambientales, de bienestar animal y de seguridad alimentaria, relacionadas éstas últimas, con la protección de la salud humana y las crecientes exigencias de calidad por parte de los consumidores. Es necesario por lo tanto encontrar un compromiso entre los objetivos de producción de los ganaderos, las exigencias de los consumidores y las directrices actuales de preservación del medio ambiente, que requerirá líneas de actuación tanto sobre los sistemas de producción como sobre los criterios de selección de los animales de los Programas de Mejora Genética. Es en esta última línea, donde se enmarcan los objetivos de esta Tesis Doctoral, en la que la caracterización de la base genética de caracteres reproductivos es el principal objetivo, que surge como alternativa a la selección basada exclusivamente en criterios de producción. La mejora genética de la aptitud reproductiva de los animales en el contexto de la producción ovina de carne, puede lograr ambos objetivos, por un lado, incrementando la eficiencia de producción de las explotaciones ganaderas asegurando una producción de carne sostenida a lo largo del año y por otro proporcionando al consumidor un producto de calidad nutricional y sanitaria, libre de aditivos y de residuos de tratamientos hormonales.

En este trabajo, el análisis de la base genética de los fenotipos de estacionalidad reproductiva en hembras, y de otras variables que influyen en la misma (CC, PV y TC), así como la capacidad de cubrición de los machos en la especie ovina, se ha abordado haciendo uso de las más modernas técnicas moleculares (genotipado de SNPs y secuenciación masiva) que permiten explorar el genoma e identificar genes y polimorfismos responsables de caracteres de interés. Estas herramientas genómicas,

podrían permitir una detección más precisa de los animales más fértiles en época de anestro estacional que son los que contribuirán a incrementar la eficiencia y rentabilidad de las explotaciones ganaderas de ovino de carne.

En primer lugar, los estudios de asociación mediante la aproximación de genes candidatos permitieron asociar los polimorfismos encontrados en el gen candidato *LEPR* con los caracteres de estacionalidad reproductiva (DTA, CiP4 y CiC) en Rasa Aragonesa. Concretamente, los SNPs rs403578195 (predicho con un efecto deletéreo sobre la funcionalidad de la proteína) y rs405459906, localizados en los exones 8 y 20, respectivamente, se asociaron a una disminución del CiC, que osciló entre un 10 y 12% en los animales portadores del alelo G. Estos resultados confirman por primera vez la implicación del gen *LEPR* en la estacionalidad reproductiva de las ovejas. Hasta ahora, este gen se había asociado únicamente a la edad al primer parto en ovino (Juengel et al., 2016b), que está relacionada con la primera activación del ovario. Por lo tanto, nuestros resultados apuntan a que las rutas genéticas implicadas en la entrada en pubertad y en la reactivación del ovario tras el anestro estacional podrían estar relacionadas. Por otra parte, el gen *LEPR* se relaciona con las reservas corporales y el PV (Paczoska-Eliasiewicz et al., 2006), siendo ambos muy importantes para la activación del ovario en la pubertad, así como para la duración del anestro. En este sentido, varios estudios sugieren que el gen *LEPR* influye en la actividad de las neuronas GnRH y en la secreción de GnRH mediante una interacción cruzada con la kisspeptina (Elias, 2012), cuya expresión en el ARC se reduce notablemente durante el período no reproductivo y aumenta en la fase F del ciclo estral en ovejas expuestas a un fotoperíodo corto, lo que sugiere la participación de las neuronas kisspeptina en esta activación (Clarke et al., 2009).

Por otra parte, un estudio GWAS, permitió detectar con un umbral de significación a nivel genómico, una asociación entre una región del genoma que contiene el gen *CD226* y los caracteres de estacionalidad reproductiva. Este gen codifica la proteína CD226 también conocida como DNAX accessory molecule-1 (DNAM-1), que es una glicoproteína de la superficie de las células natural killer (NK). Dicha proteína actúa como receptora y tiene la función de controlar la citotoxicidad de las células NK y la producción de interferón-γ contra una amplia gama de células cancerosas e infectadas por virus (De Andrade et al., 2014). Según la bibliografía, este

gen podría estar relacionado con la angiogénesis folicular y el desarrollo de ovocitos en humanos (Fainaru et al., 2010; Křížan et al., 2009; Lukassen et al., 2003). Se puede inferir, por tanto, que mutaciones en el gen *CD226* podrían producir una alteración de la función de esta glicoproteína y, como consecuencia, alterar funcionalmente las células NK, produciendo una inhibición de la angiogénesis folicular. Por ello, se decidió confirmar los resultados del análisis GWAS utilizando un enfoque de gen candidato. Tras el aislamiento de los SNPs no sinónimos en la región codante del gen *CD226* y su posterior genotipado, los resultados mostraron que el SNP rs404360094, localizado en el exón 3, presentó un gran efecto sobre los caracteres estudiados: un incremento entre un 42 y 50% de los DTA, una disminución entre un 15 y 19% del CiP4 y una disminución entre un 17 y 21% del CiC, en los animales homocigotos para el alelo A. Es importante señalar que Martinez-Royo et al., (2017) encontraron una correlación negativa entre DTA y CiP4 ($r = -0,92$; $P < 0,0001$), ambos fenotipos basados en los niveles de P4 en la sangre y relacionados con la función ovárica, mientras que las correlaciones con el carácter CiC, relacionado con actividad ovárica incluido el comportamiento estral fueron -0,72 y 0,61 con DTA y CiP4, respectivamente. Es necesario remarcar que los genotipos asociados a una menor CiC (0,047 y 0,06 para los genotipos AA y CG, para los genes *CD226* y *LEPR*, respectivamente), y mayor DTA (AA para *CD226*) se encontraron en baja frecuencia en la población. Por lo tanto, una estrategia para incrementar la productividad del ovino de carne a través de programas de selección genética sería evitar dejar como reproductores a aquellos animales que presentan el genotipo/alelo asociado a una disminución del CiC y un incremento de los DTA. Otro punto a tener en cuenta es que en la población analizada no se utilizaron tratamientos hormonales, mientras que en las granjas comerciales sí que se utilizan rutinariamente para romper el anestro, sincronizar los partos y activar los machos de las explotaciones. Estos tratamientos hormonales o incluso de manejo, como el efecto macho, podrían producir interacciones y por lo tanto modificar los resultados de los genes analizados. En este sentido, sería interesante validar los resultados en poblaciones comerciales con registros de los tratamientos hormonales o de manejo que se lleven a cabo.

En un estudio previo en Rasa Aragonesa con la misma población genotipada en este trabajo, se encontró que el gen *MTNR1A* se encontraba asociado a los fenotipos

DTA, CiP4 y CiC (Calvo et al., 2018). En este caso el alelo que estaba en menor frecuencia (T), se encontraba asociado a una disminución de DTA e incremento de la CiC. Con el objetivo de estudiar la posible interacción entre los SNPs rs403212791 (*MTNR1A*), rs403578195 (*LEPR*) y rs404360094 (*CD226*), y corregir los efectos de los distintos genotipos de los SNPs sobre los fenotipos en el cálculo de las estimas de las LSMeans, se llevó a cabo un estudio de asociación para los fenotipos DTA y CiC, incluyendo los genotipos de los tres SNPs simultáneamente. Los tres SNPs mostraron asociación significativa con el fenotipo DTA, aunque el SNP rs403212791 (*MTNR1A*) no fue significativo para la CiC (p = 0,054) (Tabla 2), a diferencia de lo encontrado en el estudio de Calvo et al. (2018). Por otra parte, el SNP rs403578195 (*LEPR*) resultó significativo para el fenotipo DTA, mientras que en el estudio de Lakhssassi et al. (2020; manuscrito 1) sólo se encontró significativo para el fenotipo CiC. Estos resultados confirmaron los resultados obtenidos para estos tres marcadores (Calvo et al., 2018; Lakhssassi et al., 2021, 2020), aunque con pequeñas diferencias debido a la utilización de un modelo que incluye los 3 SNPs analizados.

Es destacable que ningún animal con genotipo AA (asociado a un incremento de DTA y disminución de CiC) para el rs404360094 (*CD226*) presentó el genotipo GC para el SNP del gen *LEPR* (asociado a un incremento de DTA y disminución de CiC). En el caso del gen *MTNR1A*, de los 17 animales con el genotipo TT (asociado a la disminución de DTA y aumento de CiC), sólo una oveja presentó el genotipo AA para el rs404360094. Sin embargo, de los animales con el genotipo heterocigoto para el SNP del gen *LEPR* (n = 29) 20 presentaron el genotipo CT para el gen *MTNR1A*, que se asoció a un fenotipo intermedio para DTA. Por otra parte, únicamente la interacción de los tres SNPs fue significativa para DTA (p = 0,033), mientras que las interacciones por pares no resultaron significativas en ninguno de los modelos analizados. En la tabla siguiente se indican los contrastes que resultaron significativos en el efecto fijo de la interacción de los tres SNPs (Tabla 3).

Tabla 2. Prueba tipo III para los efectos de condición corporal (CC), peso vivo (PV), edad (E), y SNPs rs403212791 (*MTNR1A*), rs403578195 (*LEPR*) y rs404360094 (*CD226*) utilizando los datos de los fenotipos de estacionalidad días totales de anestro (DTA) y ciclicidad por progesterona (CiC) de las ovejas Rasa Aragonesa. También se muestran las medias de mínimos cuadrados (LSMeans) y los errores estándar de los polimorfismos de los 3 SNPs en los datos del fenotipo (F) de estacionalidad en ovejas Rasa Aragonesa. Letras distintas indican diferencias significativas a, b: p < 0,05; c,d: p < 0,01

SNP	F	P valor				LSMeans SNP		
		CC	PV	E	SNP			
						CC	*TC*	*TT*
rs403212791 (*MTNR1A*)	DTA	0,249	<0,001	0,547	<0,001	100,7±6,1c	83,4±6,09c	64±11,94d
	CiC	0,282	<0,001	0,129	0,054	0,37±0,03	0,42±0.03	0,47±0,06
						CC	*GC*	*GG*
rs403578195 (*LEPR*)	DTA	0,249	<0,001	0,547	0,018	72,4±5,1a	93±5,56b	-
	CiC	0,282	<0,001	0,129	0,015	0,47±0,02a	0,37±0,04b	-
						GG	*AG*	*AA*
rs404360094 (*CD226*)	DTA	0,249	<0,001	0,547	0,002	70,4±6,02c	68,6±6,08c	109,1±12,16d
	CiC	0,282	<0,001	0,129	0,003	0,47±0,03c	0,48±0.03c	0,30±0,06d

Tabla 3. Diferencias de las medias de mínimos cuadrados (LSMeans) y los errores estándar de los contrastes que resultaron significativos para la interacción de los SNPs rs403212791 (*MTNR1A*), rs403578195 (*LEPR*) y rs404360094 (*CD226*) con el fenotipo de días totales de anestro (DTA).

Contraste[1]	LSMeans DTA	ES	p - valor
CC-CC-AA vs CC-CC-AG	55,051	14,38	0,019
CC-CC-AA vs CT-CC-AG	58,635	14,46	0,008
CC-CC-AA vs CT-CC-GG	66,921	14,59	0,001
CC-CC-AA vs TT-CC-GG	89,821	20,33	0,002
TT-CC-GG vs CC-GC-AG	-86,974	24,52	0,047

[1]El orden de los genotipos corresponden a los SNPs rs403212791 (*MTNR1A*), rs403578195 (*LEPR*) y rs404360094 (*CD226*)

En los contrastes significativos se puede observar que el genotipo AA del gen *CD226* incrementa los días en anestro, con respecto a los otros dos genotipos del mismo, y a los diferentes genotipos del gen *MTNR1A*, por lo que este genotipo del gen *CD226* podría tener un efecto dominante sobre los diferentes genotipos del gen *MTNR1A*. Por otra parte, en el último contraste en el que no aparece el genotipo AA del gen *CD226*, se observa que la combinación del genotipo CC del *MTNR1A* junto con el GC del *LEPR* incrementa los DTA. Estos análisis conjuntos de los 3 SNPs que afectan a la estacionalidad reproductiva son preliminares, ya que la población analizada es pequeña, y no se encuentran representadas todas las combinaciones de genotipos posibles. Sin embargo, estos resultados sugieren el interés de validar los mismos en una población diferente y con un mayor número de individuos. Estos SNPs podrían utilizarse junto con otros SNPs ya caracterizados en Rasa Aragonesa asociados a caracteres relacionados con la eficiencia reproductiva, como la prolificidad o la estacionalidad reproductiva (Calvo et al., 2020b). En este sentido, en Rasa Aragonesa y otras razas explotadas en Aragón, se está utilizando un panel de 192 SNPs para asignación de paternidad de la reposición: 159 SNPs para la asignación de paternidad (Calvo et al., 2020b), y 33 funcionales. Entre los SNPs funcionales se incluyen los de resistencia al scrapie (codones clásicos del gen *Prnp*), los alelos de prolificidad del gen *BMP15*, el SNP del gen *MTNR1A*, así como los SNPs del gen *LEPR*, incluyendo desde el año pasado el SNP localizado en el gen *CD226*. Recientemente, un análisis de asociación GWAS con los 192 SNPS en 4.010 ovejas, pertenecientes a 16 explotaciones de Rasa Aragonesa con fenotipos registrados para caracteres reproductivos, se encontró el SNP rs596133197 localizado en el exón 7 del gen *LEPR*, y que producía un cambio aminoacídico predicho como deletéreo, asociado a un incremento de la prolificidad (Lakhssassi et al., 2023). El efecto del SNP rs596133197 sobre la prolificidad fue modesto en heterocigosis +0,09 ± 0,03 ($p = 0,011$; $n = 300$), mientras que en homocigosis su efecto fue de +0,60 ± 0,2 ($p = 0,006$), aunque se debe puntualizar que sólo hay 18 partos de 8 animales. Sin embargo, antes de su utilización para la preselección de reproductores (SAM) habría que considerar si el alelo asociado al incremento de la prolificidad se asocia a una mayor estacionalidad reproductiva ya que, aunque este SNP no resultó significativo para los fenotipos de estacionalidad reproductiva (manuscrito 1), presento un MAF 0,02, con

sólo 11 animales en heterocigosis con 10,2 días más de anestro que los homocigotos CC. Estos resultados confirman la implicación del gen *LEPR* en caracteres reproductivos.

Uno de los factores que más inciden en la estacionalidad reproductiva es la CC. Como se ha comentado previamente, ovejas de Rasa Aragonesa, con una CC inferior o igual a 2,5 presentaron 113 días de anestro, frente a los 64 días de anestro de aquellas de ovejas con una CC igual o superior 2,75 (Forcada et al., 1992). En la población utilizada para los estudios con caracteres de estacionalidad reproductiva (Grupo experimental 1), se analizó la influencia de la CC, el PV y la edad sobre la estacionalidad reproductiva, tomando como fenotipo los DTA, siendo la CC y el PV por orden de importancia, las variables que más se correlacionan con los DTA, y por lo tanto con la estacionalidad. Un análisis de regresión entre la variación de la CC y DTA, mostró una reducción de unos 56 días por cada punto que se gana en CC. Es decir, unos 14 días menos de anestro por cada cuarto de punto más de CC (Figura 14; Alabart, comunicación personal).

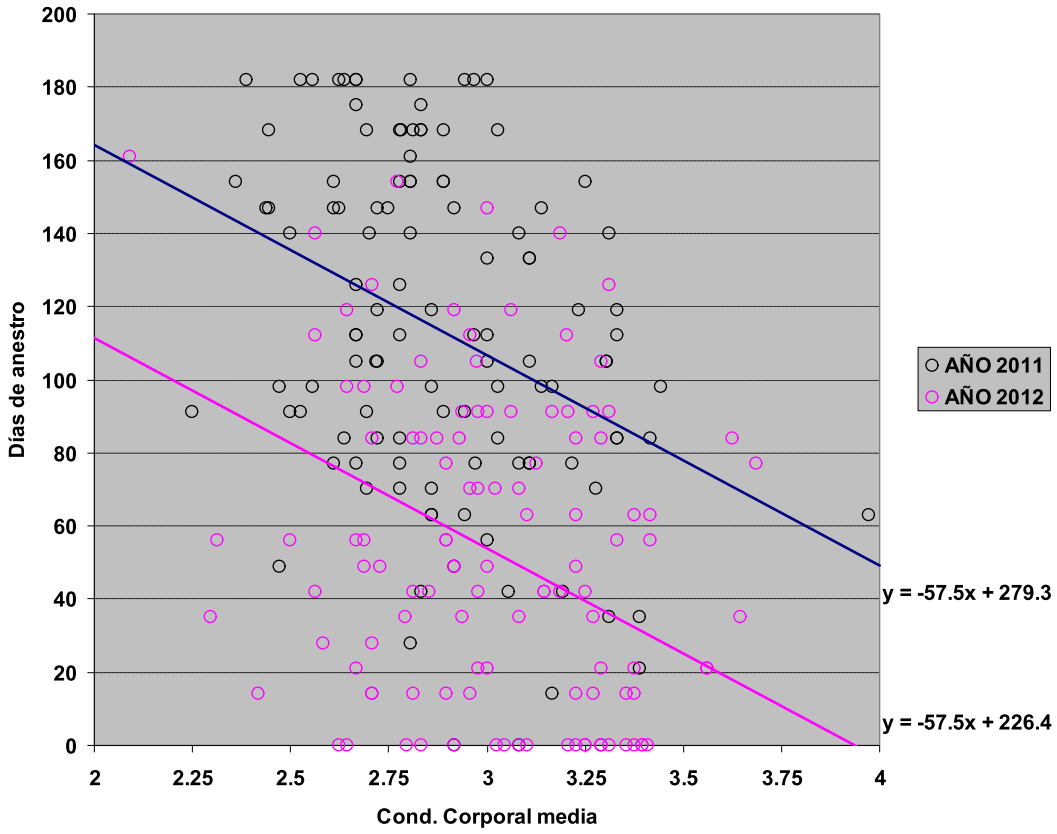

Figura 14. Días totales de anestro (DTA) en ovejas de diferente condición corporal media (CC) durante los años 2011 y 2012.

Debido a la implicación de los caracteres de crecimiento sobre la estacionalidad reproductiva, se llevó a cabo un estudio GWAS para buscar en el genoma variantes asociadas a los caracteres PV, CC y TC. En el cromosoma 9 ovino y próximo al gen *CYP7B1*, se encontró un SNP, OAR9_42035112, con significación a nivel genómico para el carácter TC. *CYP7B1* es un gen relacionado con el metabolismo de los oxisteroles, y concretamente con su inactivación y posterior conversión en ácidos biliares. Los oxisteroles son mediadores clave en la homeostasis lipídica y del colesterol (Guillemot-Legris et al., 2016). En individuos obesos, se han encontrado bajos niveles de expresión de este gen (Worthmann et al., 2017). Este gen era el único candidato con función conocida que pudiese estar relacionada con el carácter TC contenido en un intervalo de 0,5 Mb a ambos lados del SNP asociado significativamente con el carácter. Para profundizar más en el posible papel de las variantes de este gen sobre el carácter TC, se aisló y secuenció la totalidad del gen, encontrando una inserción de 22 pares de bases y otros polimorfismos en la región promotora del mismo que producía una modificación de los sitios de unión de factores de transcripción, lo cual podría tener una influencia directa sobre la tasa de expresión del gen.

Los estudios de asociación confirmaron los resultados de GWAS, relacionándose los genotipos de la inserción de 22 pb (localizada a -58 nucleótidos del sitio de inicio de la transcripción; indel (-58)), un polyC (-25), y dos SNP A/G (SNP3 (-114) y SNP5 (-63)), asociados con el fenotipo TC, mientras que sólo el indel (-58) se asoció con la CC. Los análisis de expresión génica mostraron que el SNP5 (-63) y el polyC (-25) localizados en las posiciones del genoma Rambouillet 1.0 OAR9: OAR9: g. 45801539 y OAR9: g.45801510, respectivamente, afectaban a la tasa de expresión del gen, de manera que los niveles de expresión de *CYP7B1* en los corderos AA (n = 13) y GG (n = 17) fueron 2,9 y 3,2 veces mayores que los de los corderos AG (n = 17) para el SNP5 (-63). El alelo A del SNP5 (-63) produce en una modificación del sitio de unión de factores de transcripción SP1 y el sitio consenso de unión de Adf-1, pudiendo por tanto explicar los cambios observados en la tasa de expresión del gen. Sin embargo, no se encontró efecto significativo para la indel (-58) sobre la expresión del gen *CYP7B1* a pesar de ser el polimorfismo que mayor impacto mostró *in silico* respecto a la modificación de sitios de unión de factores de transcripción, quizás debido a que no todos los genotipos estaban igualmente representados en la población analizada. En futuros trabajos, será necesario

incrementar el número de animales en las poblaciones de estudio, para validar los resultados encontrados de expresión génica, e incrementar también los genotipos de SNPs en baja frecuencia que en la población estudiada se encontraron muy descompensados para la indel (-58): 28 ovejas homocigotas sin la inserción, 15 heterocigotas con la inserción y 4 homocigotas con la inserción.

Cabe destacar que los estudios de expresión génica se hicieron con corderos sacrificados a los 75,20 ± 1,84 días, mientras que los estudios de asociación incluyeron ovejas adultas manejadas según el sistema tradicional local. A pesar de estas posibles limitaciones, los resultados demostraron la implicación de los polimorfismos del promotor del gen *CYP7B1* en la variabilidad observada para los caracteres TC y CC en las ovejas Rasa Aragonesa. Aunque el efecto encontrado es pequeño, y se trata de caracteres poligénicos, regulados por un número considerable de genes, estos SNPs podrían servir como marcadores genéticos potenciales en Programas de Mejora Genética en ovino para la mejora de características de crecimiento que podrían tener una influencia significativa sobre los caracteres reproductivos.

Con el objetivo de identificar nuevos genes y rutas metabólicas implicadas en la estacionalidad reproductiva en hembras, y la capacidad de cubrición en machos, se llevaron a cabo estudios de secuenciación masiva del transcriptoma. En el caso de las hembras, el análisis de RNA-Seq se realizó en animales en diferente fase ovárica (F, L o AR) y en dos tejidos clave en la estacionalidad reproductiva y los ritmos circadianos, el HT y el PT. El objetivo de este análisis fue detectar genes y rutas moleculares que pudiesen estar implicadas en las diferentes fases ováricas y que por lo tanto influyeran en la activación o inactivación del ovario, y en consecuencia estar relacionados con la estacionalidad reproductiva. Estos estudios permitieron detectar 72 y 3 GDEs en las comparaciones entre las fases F *vs.* AR y L *vs.* AR, respectivamente, en el HT. Los genes *HTR2B, ITPR3, LTA4H y PTGIS* mostraron una subexpresión en la fase F comparada con la AR en el HT. La relación de estos genes con el comportamiento estral ya había sido descrita en bovino lechero (Kommadath et al., 2011). El análisis de enriquecimiento funcional de la comparación F *vs.* AR mostró un enriquecimiento significativo de la ruta KEEG de señalización de la oxitocina (*ITPR3*, *KRAS*, *MYL9*, y *MYLK*). La oxitocina regula el eje hipotálamo-pituitaria-adrenal relacionado con la modulación del comportamiento en respuesta al estrés y al comportamiento social (Neumann, 2002). En

cuanto al PT, 6 y 4 genes fueron GDEs en las comparaciones de las fases F *vs.* AR y L *vs.* AR, respectivamente. En la comparación F *vs.* AR, entre los GDEs sobreexpresados se encuentran los genes *ITLN*, *DDC o PCDH15*. El *ITLN* se expresa en múltiples tejidos, incluido el cerebro y los tejidos reproductivos, como la placenta y el ovario (French et al., 2009). El gen *DDC* se ha relacionado con la regulación de la LH (necesaria para el crecimiento folicular) y juega un papel importante en la plasticidad neuronal (Veilleux et al., 2018). *PCDH15* estuvo sobreexpresado en anestro, siendo importante para el mantenimiento y la función de las células fotorreceptoras (Ahmed et al., 2003). Igualmente, la expresión de *PCDH15* también se ha encontrado asociada a variaciones en el transportador presináptico de serotonina (SERT) y a los niveles de serotonina en el mesencéfalo y el hipocampo (Ye et al., 2014). En este sentido, la serotonina es un modulador del sistema nervioso central y la fisiología periférica, incluido el comportamiento sexual femenino. Recientemente, en un estudio GWAS llevado a cabo en 147 ovejas de Rasa Aragonesa que tenía como objetivo identificar regiones genómicas y/o genes asociados con la activación ovárica de ovejas en las que coincidía el anestro de lactación y el estacional, mediante el chip de alta densidad de Illumina HD Ovine Beadchip (600K), se anotó este gen en una de las regiones significativas para el carácter AIM (en anestro a la introducción del macho) (datos no publicados). Este fenotipo consistió en medir el nivel de P4 en sangre durante 5 tomas, muestreadas en un intervalo semanal desde el destete (46,1 ± 4,9 días) en primavera hasta la introducción de machos, y se categorizaron los animales como 0 (anestro total) o 1 (al menos una medida de P4 superior al umbral de 0,5 ng/ml).

En el mismo sentido, el análisis de enriquecimiento con GSEA reveló múltiples vías relacionadas con la neurotransmisión y la plasticidad neuronal. Estos resultados muestran que algunos de estos genes podrían ser claves en la entrada o salida del anestro estacional de las ovejas, constituyéndose en genes candidatos interesantes para la búsqueda de polimorfismos que pudieran estar relacionados con la estacionalidad reproductiva. Los resultados del estudio masivo del transcriptoma sugieren también que una pequeña parte de la variación en la expresión génica en el PT se debe al tipo de fase ovárica o a la respuesta de fase ovárica. Sin embargo, la magnitud de las diferencias de expresión génica entre las fases reproductivas alternativas F y L fue mayor que en HT.

No obstante, este estudio revela nuevos genes candidatos implicados en las transiciones de las etapas reproductivas en ovejas estacionales.

Como se ha indicado previamente, la actividad sexual del macho juega un papel decisivo en el desarrollo de la actividad sexual anual de las hembras (Delgadillo et al., 2008). En este sentido resulta interesante profundizar en la base genética de esta activación sexual en los machos, mediante análisis de expresión diferencial del transcriptoma con el fin de detectar genes sobre o subexpresados asociados al comportamiento sexual de los moruecos. Este trabajo tuvo como objetivo determinar los posibles mecanismos moleculares que subyacen al comportamiento sexual de los moruecos de Rasa Aragonesa, investigando los cambios transcripcionales en el HT, PT, GP y sangre entre machos con diferente comportamiento sexual o capacidad de cubrición. Mediante los test de aprisco, se categorizaron los animales en función de su capacidad de cubrición (A y NA) de forma natural sin inducción de la actividad sexual mediante fotoperiodos largos de luz o melatonina, siendo la edad y la CC factores clave en esta actividad. Cabe destacar que, aunque la testosterona se ha considerado en muchos estudios como andrógeno predominante para expresar y mantener la libido en machos (Perkins y Roselli, 2007), en este estudio, las diferencias observadas en el comportamiento sexual entre moruecos no se debieron a diferencias en la concentración de esta hormona. Los resultados de RNA-Seq revelaron 103 y 12 GDEs en el HT y el GP, respectivamente, de machos con diferente comportamiento sexual. Sin embargo, no se encontraron GDEs en el PT. La anotación funcional y el análisis de enriquecimiento de vías metabólicas mostraron que los GDEs de HT se enriquecieron principalmente en interacciones neuroactivas de ligando-receptor y vías de señalización, incluidos genes candidatos notables como *MTNR1A, CHRNA2, FSHB, LHB, GNRHR, AVP, PRL, PDYN, CGA, GABRD y TSHB*, que juegan un papel crucial en el comportamiento sexual. Todos estos genes estaban sobreexpresados en los machos A y subexpresados en los machos NA, lo que puede explicar el comportamiento sexual pasivo de los animales NA hacia las ovejas sincronizadas. Además, algunos de estos genes estaban enriquecidos en las vías de señalización de GnRH y cAMP, sabiendo que la transducción de señales de GnRH en el cerebro influye en el comportamiento sexual de los animales (Foster et al., 2006; Zohar et al., 2010), mientras que la vía de señalización de cAMP activada por el factor neurotrófico derivado del cerebro (BDNF) ha demostrado ser un actor

fundamental en la neuroplasticidad (Reierson et al., 2011). En cuanto al gen *MTNR1A*, recientemente Abecia et al. (2020) observaron un aumento del rendimiento sexual en primavera de moruecos de Rasa Aragonesa que portaban ciertas mutaciones en los SNPs clásicos del gen (*RsaI* y *MnlI*, en las posiciones g.17355458 C > T y g.17355452 G > A, respectivamente). Según los mismos autores, el genotipo TT en la posición g.17355458 C > T, y el GG en la posición g.17355452 G > A, tenían un efecto positivo sobre el rendimiento sexual de los moruecos (Abecia et al., 2022). En nuestra población de machos, no se han observado variaciones de la frecuencia de estos dos genotipos y otros del gen *MTNR1A* entre grupos de machos A y NA, por lo que la variación de la expresión encontrada no parece ser debida a SNPs que pudiesen variar la tasa de expresión. En cuanto a los GDEs en la GP están implicados en la vía de señalización de neuropéptidos (*MCHR1*, *NPY* y *TAC1*), la actividad hormonal (*NPY* y *PRL*) y las interacciones neuronales (*MCHR1*, *NPY*, *PRL* y *TAC1*). Por lo tanto, estas vías en conjunto pueden jugar un papel importante en la regulación del comportamiento sexual en machos de Rasa Aragonesa a través del eje hipotálamo-pituitario-gonadal.

Por otro lado, los análisis mediante GSEA en HT, PT y GP mostraron vías potenciales, dominadas principalmente por la categoría de procesos biológicos, que podrían ser responsables de las diferencias en el comportamiento sexual observado en los moruecos. En la HT, la vía del "nuclear speck" (GO:0016607), relacionada con grupos de gránulos intercromáticos, y que facilita la regulación integrada de la expresión génica fue enriquecida (Galganski et al., 2017). La segunda vía de enriquecimiento fue la vía del constituyente estructural del ribosoma (GO:0003735). Los genes que codifican esta vía están implicados en el proceso de traducción, la formación del ARN de transferencia que actúa durante la síntesis de proteínas y la biogénesis del ribosoma (Xue et al., 2020). Entre estos genes, encontramos que *MRPL35, RPL15, RPS27A, RPS27* y *RPL35A* estaban todos asociados con las funciones de los ribosomas. El mal funcionamiento de los ribosomas afectará a la traducción de los ARNm. En este contexto, cualquier posible disfunción de los ribosomas puede comprometer el comportamiento sexual de los moruecos. El transporte de proteínas intracelulares (GO:0006886) y el proceso de especificación de patrones (GO:0007389) se enriquecieron dentro del PT. El transporte intracelular de proteínas implica una red de compartimentos membranosos conectados por vesículas de transporte y otras estructuras

de transporte, como el lumen del retículo endoplásmico y el complejo de Golgi, para el procesamiento postraduccional (Popescu, 2012). En cuanto a la GP, la vía de unión de factores de transcripción (GO:0008134) estaba muy enriquecida. Esta vía es importante para el control de las funciones neuronales y neuroendocrinas. Los factores de transcripción modulan la expresión génica uniéndose a regiones promotoras de genes o a regiones distales denominadas potenciadores (Boeva, 2016) implicadas en la regulación de eventos transcripcionales por ejemplo de la síntesis de melatonina, la hormona de la GP. Las vías de ubiquitinación de proteínas (GO:0016567) también estaban muy enriquecidas. El grupo de investigación de Klein (Klein, 2007; Klein y Moore, 1979) aportó pruebas de la implicación del sistema ubiquitina proteasoma en la regulación de la enzima limitante de la tasa de síntesis de melatonina, la AANAT.

Los procesos de selección rara vez incluyen la evaluación del comportamiento sexual, como son las pruebas de capacidad de cubrición, debido a limitaciones de tiempo, mano de obra e instalaciones (Alexander et al., 2012). Como una alternativa innovadora a los métodos invasivos y laboriosos de las granjas, se estudió el transcriptoma de la sangre periférica, con el fin de identificar biomarcadores de comportamiento sexual *in vivo* que pudiesen ayudar a los ganaderos a detectar machos sexualmente activos, y también determinar la existencia de GDEs comunes en la sangre y en los tejidos del sistema nervioso analizados, asociados al comportamiento sexual de los machos, que de ser así, permitiría detectar en sangre aquellos GDEs claves para la activación sexual sin necesidad de sacrificar a los animales. Los animales incluidos en este estudio fueron los mismos que se utilizaron en el estudio del transcriptoma en el cerebro de los moruecos de Rasa Aragonesa.

El análisis del transcriptoma de células sanguíneas reveló cuatro genes candidatos que mostraron una expresión diferencial entre machos A y NA. Los genes *inhibidor de acrosina 1* y *SORCS2* estaban sobreexpresados en machos A, mientras que los genes *CRYL1* y la *isoforma X47 de la cadena ligera lambda-1 de inmunoglobulina* estaban subexpresados en estos mismos machos. No se encontraron GDEs comunes entre las muestras de sangre y las de los tejidos de HT, PT y la GP, de los mismos animales, a pesar de que algunos de los genes detectados en sangre (*CRYL1* y *SORCS2*) también se expresan en el cerebro. Estos genes fueron validados en la población total de machos (n = 59) mediante RT-qPCR, en una muestra de sangre tomada en un momento

diferente a la utilizada para el estudio de RNA-Seq (n = 12). El gen *SORCS2* está implicado en la regulación de la guía de axones dopaminérgicos y la apoptosis de neuronas sensoriales periféricas, además de interactuar con pro-BDNF/p75NTR y controlar la actividad de los receptores Trk. Sabiendo que el BDNF y su receptor, el receptor tirosina quinasa B (trkB), están relacionados con la plasticidad sináptica y están implicados en el comportamiento sexual en ratas macho (Sanna et al., 2019), los cambios en la expresión génica del gen *SORCS2* en células de sangre periférica podrían reflejar cambios que ocurren en el cerebro y actuar como marcador del fenotipo de actividad sexual. El gen *CRYL1* se asocia al comportamiento de búsqueda de apoyo emocional (Shimanoe et al., 2019) y a la activina A, que desempeña un papel importante en la modulación del control reproductivo neuroendocrino (Itman et al., 2009; Xia y Schneyer, 2009). Este gen resultó subexpresados en los machos A. Por lo tanto, los niveles de expresión del gen *CRYL1* podrían constituirse como un biomarcador de un comportamiento sexual pobre.

Debido a que se detectaron pocos GDEs en la sangre, resultó imposible su enriquecimiento vía DAVID. Sin embargo, el análisis GSEA reveló 428 vías de señalización, dominadas principalmente por procesos biológicos. El componente celular lisosoma (GO:0005764) fue el más enriquecido pudiendo estar implicado en la fertilidad y el comportamiento sexual, dado el papel crucial que juegan los lisosomas en la esteroidogénesis (Xie et al., 2006) y estando el gen *SORCS2* relacionado con esta vía de señalización. También resultó enriquecida la vía de la regulación positiva de la cascada ERK1 y ERK2 (GO:0070374), que se han descrito en varios estudios como moduladores esenciales de la regulación hipotalámica y de la producción de gonadotropinas hipofisarias mediada por GnRH, y asociada a fenotipos reproductivos como la fertilidad (Bliss et al., 2009; Liu et al., 2002; Nekrasova et al., 2005; Saba-El-Leil et al., 2003). Estos resultados proporcionan nuevas pistas para comprender la regulación molecular del comportamiento sexual de los machos. Nuestro estudio demuestra que múltiples redes y vías orquestan el comportamiento sexual en los moruecos. Se necesitarán más investigaciones para confirmar las funciones de *SORCS2* y *CRYL1* en relación con el comportamiento sexual, en poblaciones diferentes e incluso en otras razas ovinas con diferente comportamiento sexual. Por otra parte, aprovechando la información de secuencia que proporciona la técnica RNA-Seq se puede llevar a cabo una búsqueda de

polimorfismos y variantes estructurales en estos genes, *SORCS2* y *CRYL1*, o bien en todos los genes expresados en los diferentes tejidos, que tras un análisis de su impacto sobre la proteína o bien de modificación de posibles sitios funcionales en regiones reguladoras *in silico*, podrían ser seleccionados para futuros estudios relacionados con la capacidad de cubrición de los machos.

En conjunto, la presente Tesis Doctoral proporciona información básica y aplicada sobre los factores genéticos que influyen en la estacionalidad reproductiva de las hembras, y la actividad sexual de los machos. Los resultados sugieren que los SNPs rs403212791 (*MTNR1A*), rs403578195 (*LEPR*) y rs404360094 (*CD226*) influyen en los caracteres de estacionalidad reproductiva en hembras de Rasa Aragonesa y que podrían ser utilizados en SAM, para incrementar la frecuencia de los alelos favorables del gen *MTNR1A*, y disminuir la de los alelos desfavorables encontrados en los genes *LEPR* y *CD226*. La utilización de marcadores genéticos en los Programas de Mejora Genética, hacen que estos sean más eficientes y sobre todo para aquellos caracteres que presentan una baja heredabilidad y una difícil y costosa medida, como son los estudiados en esta Tesis Doctoral. Por otra parte, hay que tener en cuenta, que habría que validar sus efectos en poblaciones comerciales que usan tratamientos hormonales o incluso de manejo, como el efecto macho, que podrían producir interacciones y por lo tanto modificar los resultados de los genes analizados. Además, se han identificado SNPs en la región promotora del gen *CYP7B1* asociados a caracteres de crecimiento en hembras adultas (manejadas según el sistema tradicional local), que indirectamente se asocian a la estacionalidad reproductiva, y que contribuyen al conocimiento de la base genética de la TC y CC. Sin embargo, el pequeño efecto encontrado sobre la variabilidad del carácter hace difícil su aplicación directa en SAM, teniendo en cuenta que estos caracteres presentan una heredabilidad moderada.

Finalmente, hay que destacar que el abordaje transcriptómico empleado, ha generado un enorme volumen de datos de secuencias codificantes, y de splicing alternativo que deberán ser analizados en el futuro. En este sentido, el estudio de posibles polimorfismos en GDEs y su impacto en regiones reguladoras y regiones codificantes podrían asociarse a los fenotipos analizados o a las diferencias de expresión encontradas. Además, estos resultados pueden servir de base para el estudio de otros ARNs reguladores como los lncRNAs, o para integrar toda la información transcriptómica con

el genotipado de los mismos animales mediante chips de SNPs, que nos permitan detectar eQTLs, es decir genes reguladores de la expresión génica. Por otra parte, el material animal generado en esta Tesis presenta gran interés para futuros estudios relacionados con los caracteres reproductivos objeto de estudio en esta Tesis Doctoral.

VII- Conclusiones generales

1. El SNP rs403578195 ubicado en el dominio extracelular del exón 8 del gen *LEPR,* que produje un cambio aminoacídico en la posición 284 de la secuencia aminoacídica de alanina por glicina y predicho como deletéreo *in silico*, se asoció con el carácter CiC en ovejas de Rasa Aragonesa, confirmando por primera vez la implicación del gen *LEPR* en la estacionalidad reproductiva en rumiantes.

2. Los estudios de asociación haplotípica del gen *LEPR* permitieron detectar la implicación del SNP no sinónimo rs405459906 localizado en el exón 20 con el carácter CiC. Este SNP también produce un cambio de aminoácido (Lys1069Glu) en el dominio intracelular de la proteína y segrega independientemente de rs403578195.

3. El estudio GWAS sobre caracteres de estacionalidad reproductiva mostró una asociación a nivel genómico del SNP rs404991855 situado en el intrón 2 del gen *CD226* con el fenotipo CiP4, y mostró una tendencia también a nivel genómico para el carácter DTA, lo que indica su papel en la estacionalidad reproductiva.

4. Los estudios de validación mostraron que el genotipo AA del SNP rs404360094 ubicado en el exón 3 del gen *CD226*, y que produce un cambio aminoacídico (Asn243Asp), se asocia con un DTA más alto y un menor CiP4, ambos caracteres relacionados con la función ovárica basados en los niveles de P4 en sangre**.** Este genotipo se asoció también con valores menores de CiC comparados con los otros dos genotipos, indicador del comportamiento estral en las ovejas. Estos resultados confirman por primera vez la implicación del gen *CD226* en la estacionalidad reproductiva en ovino.

5. El estudio GWAS realizado con el objetivo de identificar regiones genómicas asociadas con los caracteres de crecimiento, así como los estudios de asociación génica con el gen *CYP7B1*, situado a 83 kb del SNP asociado con la TC a nivel genómico en el GWAS, reveló a este gen como gen candidato relacionado con los caracteres de crecimiento en ovejas de Rasa Aragonesa. Este es el primer

estudio que demuestra una asociación entre algunos polimorfismos del promotor del gen *CYP7B1* y caracteres de crecimiento en ovino.

6. Los polimorfismos de la región promotora del gen *CYP7B1* influyen en la expresión génica del mismo, indicando que el efecto de los genotipos sobre los fenotipos observados podría ser debido a las diferencias en la tasa de transcripción del mismo.

7. El estudio del transcriptoma en ovejas de Rasa Aragonesa en diferentes fases reproductivas mediante RNA-Seq reveló GDEs involucrados en las vías de señalización de la oxitocina (*ITPR3, KRAS, MYL9* y *MYLK*), la contracción del músculo liso y las vías de adhesión focal. El análisis GSEA reveló funciones relacionadas con la neurotransmisión y la plasticidad neuronal como posibles reguladores del ciclo reproductivo en ovejas de Rasa Aragonesa.

8. Los resultados del estudio del transcriptoma en tejidos cerebrales de machos con diferente comportamiento sexual mediante RNA-Seq contribuyen a la comprensión de la base genómica que subyace al comportamiento sexual en moruecos. Se han identificado múltiples redes de genes y vías que orquestan el comportamiento sexual de los mismos, como la vía de interacción neuroactiva ligando-receptor, que incluye genes como *MTNR1A, FSHB, GNRHR, AVP, PRL, o TSHB*, o las vías de señalización de GnRH y cAMP.

9. El estudio del transcriptoma en sangre periférica de machos con diferente capacidad de cubrición detectó muy pocos GDEs, validándose los genes candidatos *CRYL1* y *SORCS2*, ambos relacionados funcionalmente con el fenotipo. Estos resultados juntos con los del análisis GSEA proporcionan nuevas pistas para entender la regulación molecular del comportamiento sexual en los moruecos, revelando las vías del componente celular lisosoma y de la regulación positiva de la cascada ERK1 y ERK2 importantes para el fenotipo estudiado.

VIII- Referencias bibliográficas

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