DEVELOPMENT OF NEW ARTIFICIAL INSEMINATION EXTENDERS SUPPLEMENTED WITH GnRH ANALOGUES TO INDUCE OVULATION AND PROTEOMIC CHARACTERIZATION OF RABBIT SEMEN

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A thesis submitted to the Polytechnic University of Valencia in partial fulfilment of the requirements for the degree of doctor of philosophy

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ABSTRACT

The general objectives of this thesis were to develop new artificial insemination extenders supplemented with a GnRH analogue in order to induce doe ovulation and to characterise the proteomic profile of rabbit semen.

In chapter I, the inclusion of a protease inhibitors cocktail in the insemination extender to avoid part of the rabbit seminal plasma protease activity was evaluated. Seminal quality and fertility rate were not affected by the cocktail, having similar values between experimental and control groups. However, prolificacy rate was significantly lower in experimental group compared to positive and negative control groups (8.2 ± 0.22 *vs.* 9.3± 0.23 and 9.2± 0.26 total born per litter, respectively). From this chapter, it may be concluded that the addition of a wide variety of protease inhibitors in the rabbit semen extender negatively affects prolificacy rate and it in the future it would be advisable to test specific aminopeptidase inhibitors.

Therefore, in chapter II, we supplemented the insemination extender with specific aminopeptidase inhibitors (bestatin and EDTA), and we studied their effect on rabbit seminal quality and reproductive performance. Again, the values of motility, acrosome integrity and sperm viability were not significantly different between the experimental and the control group. Regarding reproductive performance, the inclusion of bestatin and EDTA, did not affect fertility (85.3 *vs*. 88.6 %), nor the prolificacy rate (10.12 *vs*. 10.51 kits per delivery) in comparison with control group. Thus, we concluded that bestatin and EDTA can be used in rabbit insemination extenders to inhibit part of the seminal plasma aminopeptidase activity.

In the light of previous results, in chapter III, we prove new rabbit insemination extenders containing aminopeptidase inhibitors (AMIs) with or without chitosan (CS)-dextran sulfate (DS) nanoparticles entrapping the GnRH analogue. Besides, different hormone concentrations were tested in these extenders, evaluating their *in vivo* effect on rabbit reproductive performance after artificial insemination. The following experimental extenders were studied: C4 group (4 µg buserelin/doe in control medium: Tris-citric acid-glucose supplemented with bestatin 10 µM and EDTA 20 mM), C5 group (5 µg of buserelin/doe in control medium), Q4 group (4 µg of buserelin/doe into CS-DS nanoparticles in control medium) and Q5 group (5 µg of busereline/doe into CS-DS nanoparticles in control medium). Results showed that fertility was significantly lower in C4 group compared to C5, Q5 and Q4 groups (0.7 *versus* 0.85, 0.85 and 0.82, respectively). On the contrary, prolificacy was similar in the four experimental groups studied ($P>0.05$). Thus, we concluded that the CS-DS nanoparticles prepared by a coacervation process as carrier for buserelin acetate allow to reduce the concentration of hormone used in extenders supplemented with bestatin and EDTA without affecting the fertility and prolificacy of rabbit females. Therefore, nanoencapsulation seems to be a promising system to protect the GnRH analogue in order to decrease the hormone concentration in rabbit artificial insemination extenders.

On the other hand, the aim of the last three chapters of the present thesis was to characterize rabbit seminal plasma and sperm proteome. In chapters IV and V, we characterised rabbit seminal plasma proteins (SP proteins) focusing on the influence of the genetic origin and seasonality. Semen samples were recovered from January to December 2014 using 6 males belonging to genotype A and 6 from genotype R. For each genotype, one pooled sample at the beginning, middle and end of each season

was selected to develop the experiment. A total of 24 pools (3 for each season and genetic line) were analysed. In chapter IV, we used a 1D polyacrylamide gel electrophoresis approach and we observed that seven protein bands were significantly different between genetic lines and among these, three protein bands were significantly different between seasons. On the contrary, in chapter V, SP was subjected to in-solution digestion nano LC-MS/MS and bioinformatics analysis. The resulting library included 402 identified and quantified proteins. Only 6 proteins were specifically implicated in reproductive processes according to Gene Ontology annotation. Twenty-three proteins were differentially expressed between genotypes, 11 over-expressed in genotype A and 12 in genotype R. Regarding the effect of season on rabbit SP proteome, results showed that there was no clear pattern of protein variation throughout the year. The results obtained in both chapters provide evidence that genotype is related to a specific abundance of SP proteins.

The final chapter of this thesis was conducted to characterise rabbit sperm proteins through LC-MS/MS technique. Six samples were recovered during two months from five males from genotype A and five from genotype R. Sperm proteins were extracted and subjected to in-gel digestion nano LC-MS/MS and bioinformatics analysis. The resulting library included 487 identified and quantified proteins. Regarding the comparison of the sperm proteins' abundance between genotypes, forty proteins were differentially expressed. Among them, 25 proteins were over-expressed in genotype A, while 15 proteins were over-expressed in genotype R. In conclusion, this last chapter characterizes for the first time rabbit sperm proteins and shows that genotype has also a huge impact on protein abundance in rabbit sperm. Furthermore, the differential presence of these proteins could be one of the causes explaining the differences

observed in fertility and seminal parameters between these two genetic lines in earlier studies. Finally, with the results obtained in chapters V and VI, the first publicly accessible database of the rabbit seminal plasma and sperm proteome was created.

RESUMEN

Los objetivos generales de esta tesis fueron desarrollar nuevos diluyentes de inseminación artificial suplementados con un análogo de la GnRH para inducir la ovulación de la coneja y caracterizar el perfil proteómico del semen de conejo. En el capítulo I, se evaluó la inclusión de un cóctel de inhibidores de proteasas en el diluyente de inseminación para evitar parte de la actividad proteasa del plasma seminal de conejo. La calidad seminal y la tasa de fertilidad no se vieron afectadas por el cóctel, teniendo valores similares entre los grupos experimentales y el control. Sin embargo, la tasa de prolificidad fue significativamente menor en el grupo experimental en comparación con los grupos de control positivo y negativo (8,2 ± 0,22 frente a 9,3 ± 0,23 y 9,2 ± 0,26 nacidos totales por camada, respectivamente). A partir de este capítulo, se puede concluir que la adición de una amplia variedad de inhibidores de proteasas en el diluyente de semen de conejo afecta negativamente la tasa de prolificidad y que en el futuro sería aconsejable probar inhibidores específicos de aminopeptidasas.

Por lo tanto, en el capítulo II, suplementamos el diluyente de inseminación con inhibidores específicos de aminopeptidasas (bestatina y EDTA), y estudiamos su efecto sobre la calidad seminal del conejo y el rendimiento reproductivo. Nuevamente, los valores de motilidad, integridad del acrosoma y la viabilidad del esperma no fueron significativamente diferentes entre el grupo experimental y el control. Con respecto al desempeño reproductivo, la inclusión de bestatina y EDTA, no afectó la fertilidad (85,3 *vs.* 88,6%), ni la tasa de prolificidad (10,12 *vs.* 10,51 gazapos por parto) en comparación con el grupo control. Por lo tanto, llegamos a la conclusión de que la

bestatina y el EDTA se pueden usar en los diluyentes de inseminación de conejos para inhibir parte de la actividad aminopeptidasa del plasma seminal.

A la luz de los resultados previos, en el capítulo III, probamos nuevos diluyentes de inseminación de conejo, los cuales contenían inhibidores de aminopeptidasas con o sin nanopartículas de quitosano (CS)-sulfato de dextrano (DS) que atrapan el análogo de la GnRH. Además, se probaron diferentes concentraciones de hormona en estos diluyentes, evaluando su efecto *in vivo* sobre el rendimiento reproductivo del conejo después de la inseminación artificial. Se estudiaron los siguientes diluyentes experimentales: grupo C4 (4 μg de buserelina/coneja en medio de control: tris-ácido cítrico-glucosa suplementado con 10 μM de bestatina y EDTA 20 mM), grupo C5 (5 μg de buserelina/coneja en medio de control), Grupo Q4 (4 μg de buserelina/coneja en nanopartículas CS-DS en medio de control) y grupo Q5 (5 μg de buserelina/coneja en nanopartículas CS-DS en medio de control). Los resultados mostraron que la fertilidad fue significativamente menor en el grupo C4 en comparación con los grupos C5, Q5 y Q4 (0,7 frente a 0,85, 0,85 y 0,82, respectivamente). Por el contrario, la prolificidad fue similar en los cuatro grupos experimentales estudiados ($P>0,05$). Por lo tanto, llegamos a la conclusión de que las nanopartículas de CS-DS preparadas mediante un proceso de coacervación como transportador de acetato de buserelina permiten reducir la concentración de hormona utilizada en diluyentes suplementados con bestatina y EDTA sin afectar la fertilidad y prolificidad de las hembras de conejo. Por ello, la nanoencapsulación parece ser un sistema prometedor para proteger el análogo de la GnRH a fin de disminuir la concentración de la hormona en los diluyentes de inseminación artificial de conejos.

Por otro lado, el objetivo de los últimos tres capítulos de esta tesis fue caracterizar el proteoma del plasma seminal y del espermatozoide de conejo. En los capítulos IV y V, caracterizamos las proteínas del plasma seminal del conejo centrándonos en la influencia del origen genético y la estacionalidad. Las muestras de semen se recuperaron de enero a diciembre de 2014 utilizando 6 machos pertenecientes al genotipo A y 6 del genotipo R. Para cada genotipo, se seleccionó una muestra heteroespérmica del comienzo, del medio y del final de cada estación para desarrollar el experimento. Se analizaron un total de 24 muestras (3 para cada estación y línea genética). En el capítulo IV, utilizamos un enfoque de electroforesis en gel de poliacrilamida 1D y observamos que siete bandas de proteínas eran significativamente diferentes entre las líneas genéticas y de éstas, tres bandas de proteínas eran significativamente diferentes entre las estaciones. Por el contrario, en el capítulo V, el plasma seminal se sometió a una digestión en solución, nano LC-MS/MS y análisis bioinformático. La biblioteca resultante incluía 402 proteínas identificadas y cuantificadas. Sólo 6 proteínas estuvieron específicamente implicadas en procesos reproductivos de acuerdo con la anotación funcional. Veintitrés proteínas se expresaron diferencialmente entre genotipos, 11 sobreexpresadas en el genotipo A y 12 en el genotipo R. Con respecto al efecto de la estación en el proteoma del plasma seminal del conejo, los resultados mostraron que no hubo un patrón claro de variación proteica a lo largo del año. Los resultados obtenidos en ambos capítulos evidencian que el genotipo está relacionado con una abundancia específica de proteínas del plasma seminal.

El último capítulo de la tesis se realizó para caracterizar las proteínas del espermatozoide de conejo a través de la técnica LC-MS/MS. Seis muestras se

recuperaron durante dos meses de cinco machos del genotipo A y cinco del genotipo R. Las proteínas del espermatozoide se extrajeron y se sometieron a digestión en gel, nano LC-MS / MS y análisis bioinformático. La biblioteca resultante incluyó 487 proteínas identificadas y cuantificadas. En cuanto a la comparación de la abundancia de proteínas del esperma entre genotipos, cuarenta proteínas se expresaron diferencialmente. Entre ellas, 25 proteínas se sobreexpresaron en el genotipo A, mientras que 15 proteínas se sobreexpresaron en el genotipo R. En conclusión, este último capítulo caracteriza por primera vez las proteínas del espermatozoide del conejo y muestra que el genotipo también tiene un gran impacto en la abundancia de proteínas del espermatozoide de conejo. Además, la presencia diferencial de estas proteínas podría ser una de las causas que explican las diferencias observadas en la fertilidad y los parámetros seminales entre estas dos líneas genéticas en estudios anteriores. Finalmente, con los resultados obtenidos en los capítulos V y VI, se creó la primera base de datos de acceso público del proteoma del plasma seminal y del espermatozoide de conejo.

RESUM

Els objectius generals d'aquesta tesi van ser desenvolupar nous diluents d'inseminació artificial suplementats amb un anàleg de la GnRH per induir l'ovulació de la conilla i caracteritzar el perfil proteòmic del semen de conill. En el capítol I, es va avaluar la inclusió d'un còctel d'inhibidors de proteases en el diluent d'inseminació per evitar part de l'activitat proteasa del plasma seminal de conill. La qualitat seminal i la taxa de fertilitat no es van veure afectades pel còctel, tenint valors similars entre els grups experimentals i el control. No obstant això, la taxa de prolificitat va ser significativament menor en el grup experimental en comparació amb els grups de control positiu i negatiu (8,2 ± 0,22 enfront de 9,3 ± 0,23 i 9,2 ± 0,26 nascuts totals per ventrada, respectivament). A partir d'aquest capítol, es pot concloure que l'addició d'una àmplia varietat d'inhibidors de proteases en el diluent de semen de conill afecta negativament la taxa de prolificitat i que en el futur seria aconsellable provar inhibidors específics de aminopeptidasas.

Per tant, en el capítol II, suplementàrem el diluent d'inseminació amb inhibidors específics de aminopeptidasas (bestatina i EDTA), i vam estudiar el seu efecte sobre la qualitat seminal del conill i el rendiment reproductiu. Novament, els valors de motilitat, integritat de l'acrosoma i la viabilitat de l'esperma no van ser significativament diferents entre el grup experimental i el control. Pel que fa al rendiment reproductiu, la inclusió de bestatina i EDTA, no va afectar la fertilitat (85,3 *vs*. 88,6%), ni la taxa de prolificitat (10,12 *vs.* 10,51 catxaps per part) en comparació amb el grup control. Per tant, arribem a la conclusió que la bestatina i l'EDTA es poden

usar en els diluents d'inseminació de conills per inhibir part de l'activitat aminopeptidasa del plasma seminal.

A la llum dels resultats previs, en el capítol III, vam provar nous diluents d'inseminació de conill, els quals contenien inhibidors de aminopeptidasas amb o sense nanopartícules de quitosà (CS)-sulfat de dextrà (DS) que atrapen l'anàleg de la GnRH. A més, es van provar diferents concentracions d'hormona en aquests diluents, avaluant el seu efecte *in vivo* sobre el rendiment reproductiu del conill després de la inseminació artificial. Es van estudiar els següents diluents experimentals: grup C4 (4 µg de buserelina/conilla en medi control: tris-àcid cítric-glucosa suplementat amb 10 µL de bestatina i 20 mM d'EDTA), grup C5 (5 µg de buserelina/conilla en medi control), grup Q4 (4 µg de buserelina/conilla en nanopartícules CS-DS en medi control) i grup Q5 (5 µg de buserelina/conilla en nanopartícules CS-DS en medi control). Els resultats van mostrar que la fertilitat va ser significativament menor en el grup C4 en comparació amb els grups C5, Q5 i Q4 (0,7 enfront de 0,85, 0,85 i 0,82, respectivament). Per contra, la prolificitat va ser similar en els quatre grups experimentals estudiats ($P>0,05$). Per tant, arribem a la conclusió que les nanopartícules de CS-DS preparades mitjançant un procés de coacervació com a transportador d'acetat de buserelina permeten reduir la concentració d'hormona utilitzada en diluents suplementats amb bestatina i EDTA sense afectar la fertilitat ni la prolificitat de les femelles de conill. Per tant, la nanoencapsulació sembla ser un sistema prometedor per protegir l'anàleg de la GnRH per tal de disminuir la concentració de l'hormona en els diluents d'inseminació artificial de conills.

D'altra banda, l'objectiu dels últims tres capítols d'aquesta tesi va ser caracteritzar el proteoma del plasma seminal i de l'espermatozoide de conill. En els capítols IV i V,

caracteritzàrem les proteïnes del plasma seminal del conill centrant-nos en la influència de l'origen genètic i l'estacionalitat. Les mostres de semen es van recuperar de gener a desembre de 2014 utilitzant 6 mascles pertanyents al genotip A i 6 del genotip R. Per a cada genotip, es va seleccionar una mostra heteroespérmica del començament, del mitjan i del final de cada estació per desenvolupar l'experiment. Es van analitzar un total de 24 mostres (3 per a cada estació i línia genètica). En el capítol IV, ferem servir un enfocament d'electroforesi en gel de poliacrilamida 1D i observàrem que set bandes de proteïnes eren significativament diferents entre les línies genètiques i entre aquestes, tres bandes de proteïnes eren significativament diferents entre les estacions. Per contra, en el capítol V, el plasma seminal es va sotmetre a una digestió en solució, nano LC-MS / MS i anàlisi bioinformàtica. La biblioteca resultant incloïa 402 proteïnes identificades i quantificades. Només 6 proteïnes van estar específicament implicades en processos reproductius d'acord amb l'anotació funcional. Vint-i-tres proteïnes es van expressar diferencialment entre genotips, 11 sobreexpressades en el genotip A i 12 en el genotip R. Pel que fa a l'efecte de l'estació en el proteoma del plasma seminal del conill, els resultats van mostrar que no hi havia un patró clar de variació proteica a llarg de l'any. Els resultats obtinguts en els dos capítols evidencien que el genotip està relacionat amb una abundància específica de proteïnes del plasma seminal.

L'últim capítol de la tesi es va realitzar per caracteritzar proteïnes de l'espermatozoide de conill a través de la tècnica LC-MS/MS. Sis mostres es van recuperar durant dos mesos de cinc mascles del genotip A i cinc del genotip R. Les proteïnes de l'espermatozoide es van extreure i es van sotmetre a digestió en gel, nano LC-MS/MS i anàlisi bioinformàtica. La biblioteca resultant va incloure 487 proteïnes identificades i

quantificades. Pel que fa a la comparació de l'abundància de proteïnes de l'espermatozoide entre genotips, quaranta proteïnes es van expressar diferencialment. Entre elles, 25 proteïnes es sobreexpresaren en el genotip A, mentre que 15 proteïnes es sobreexpresaren en el genotip R. En conclusió, aquest últim capítol caracteritza per primera vegada les proteïnes de l'espermatozoide del conill i mostra que el genotip també té un gran impacte en l'abundància de proteïnes de l'esperma de conill. A més, la presència diferencial d'aquestes proteïnes podria ser una de les causes que expliquen les diferències observades en la fertilitat i els paràmetres seminals entre aquestes dues línies genètiques en estudis anteriors. Finalment, amb els resultats obtinguts en els capítols V i VI, es va crear la primera base de dades d'accés públic del proteoma del plasma seminal i de l'espermatozoide de conill.

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ABREVIATIONS

AB	Alive kits born per litter
ABC	Ammonium bicarbonate
ACN	Acetonitrile
AI	Artificial insemination
ALH	Mean amplitude of lateral head displacement
AMIs	Aminopeptidase inhibitors
ANOVA	Analysis of variance
APN	Aminopeptidase activity
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
BCA	Bicinchoninic acid method
BCF	Frequency of head displacement
BSA	Bovine serum albumin
ºC	Celsius degrees
CL	Corpus lutea
cm	Centimetre
cps	Counts per second
CS	Chitosan
eCG	Equine chorionic gonadotropin
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
DA	Discriminant Analysis
DE	Digestible energy
DM	Dry matter
DS	Dextrane sulfate
DTT	Dithiothreitol
FA	Formic acid
FDR	False discovery rate
FITC-PNA	Fluorescein labeled lectin from the peanut plant *Arachis hypogaea*
fmol	Femtomole

FSH	Follicle stimulating hormone
g	Grams
GLM	General linear model procedure
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
h	Hours
IAM	Iodoacetamide
i.m.	Intramuscular
IU	International unit
i.v.	Intravaginally
kcal	Kilocalories
kDa	Kilodalton
kg	Kilograms
kV	Kilovolts
LC-MS/MS	Liquid chromatography- mass spectrometry
LH	Luteinizing hormone
LIN	Linearity coefficient
LSD	Least significant difference
LSM	Least square means values
M	Molar
m	Mass
MALDI TOF/TOF	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight
mg	Milligrams
min	Minutes
mm	Millimetres
mL	Millilitres
mM	Millimolar
MSMS	Tandem mass spectrometry
mW	Milliwatt
NAR	Normal apical ridge
NCBI	National center for biotechnology

nL	Nanolitre
nm	Nanometre
OIF	Ovulation-inducing factor
PAGE	Polyacrylamide gel electrophoresis
PANTHER	Protein Analysis THrough Evolutionary Relationships
PI	Propidium iodide
pg	Picogram
PLS	Partial Least Squares
pmol	Picomole
ppm	Parts per million
PRIDE	Proteomics Identifications
PSPEP	Proteomics System Performance Evaluation Pipeline
rpm	Revolutions per minute
s	Seconds
SDS	Sodium dodecyl sulfate
SE	Standard error
SP	Seminal plasma
spz	Spermatozoa
STR	Straightness index
TB	Total number of kits born per litter
TCG	Tris-citric acid-glucose
TFA	Trifluoroacetic acid
x g	G force
V	Volt
VAP	Average path velocity
VCL	Average of curvilinear velocity
vol	Volume
z	Charge number of ions
β-NGF	Beta-nerve growth factor
1D	One-dimension
µ	Micro

µg	Micrograms
µL	Microlitres
µM	Micromolar
µm	Micrometre
µmol	Micromol

I.GENERAL INTRODUCTION

I.GENERAL INTRODUCTION

Rabbit (*Oryctolagus cuniculus*) is an important mammalian species worldwide, being at the same time of commercial interest and a research animal model. European rabbit meat production is approximately 500 thousand tons, corresponding to a 30% share of world production (Petracci et al., 2009). Besides, rabbits account for the seventh highest number of animals slaughtered per year in the European Union-27, with 347,603 × 1000 heads in 2014 (FAOSTAT, 2014).

1. RABBIT ARTIFICIAL INSEMINATION

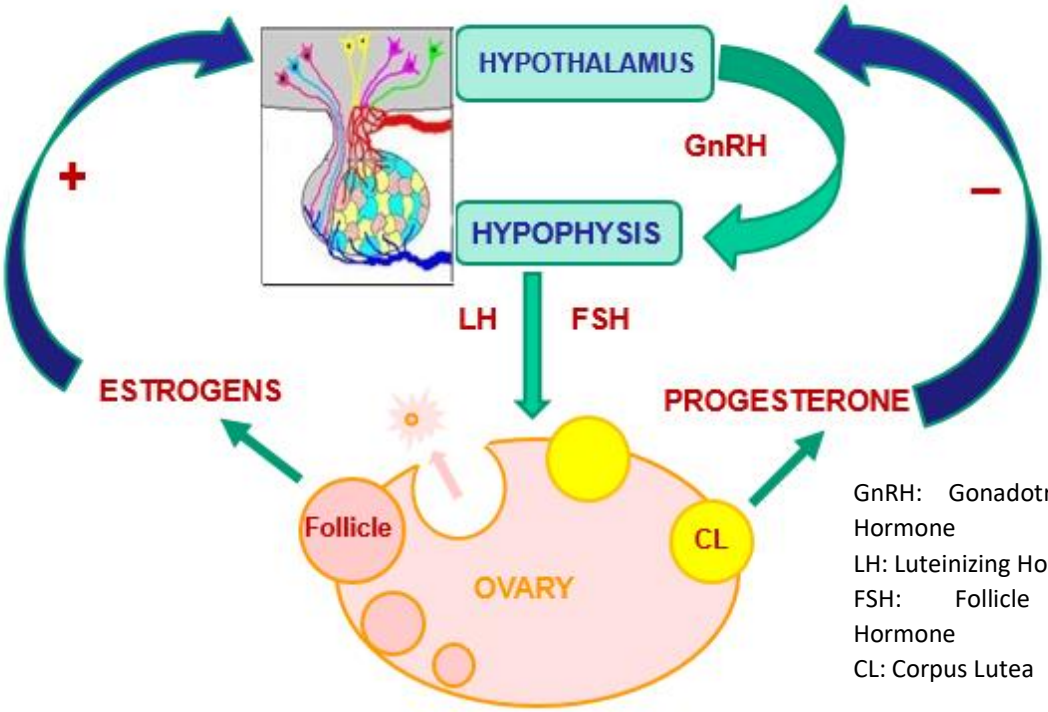
Artificial insemination (AI) in rabbit dates from the late 80's (Vega et al., 2012), and as a consequence of its commercial development, reproductive control has experienced a great change in this species. The use of AI in intensive meat rabbit production is currently a common practice in many European countries, such as Italy, France and Spain. AI offers the same benefits for rabbit breeding as in other species in the control of genetic diversity, rapid upgrading of stock, establishment of pregnancies in females which refuse to mate, and avoidance of the spread of diseases (Morrell, 1995). In addition, AI permitted to develop a new system of production which consists in executing breeding operations on fixed days of the week allowing better production planning and reducing the needed of man power (Castellini, 1996). Furthermore, using AI, an ejaculate from one male can be used to inseminate a large number of females and it has been proved that conception rates after AI with fresh semen are equivalent

to or even better than those obtained by natural mating (Daniel and Renard, 2010; Kitajima, 2009).

2. OVULATION INDUCTION IN RABBIT DOES

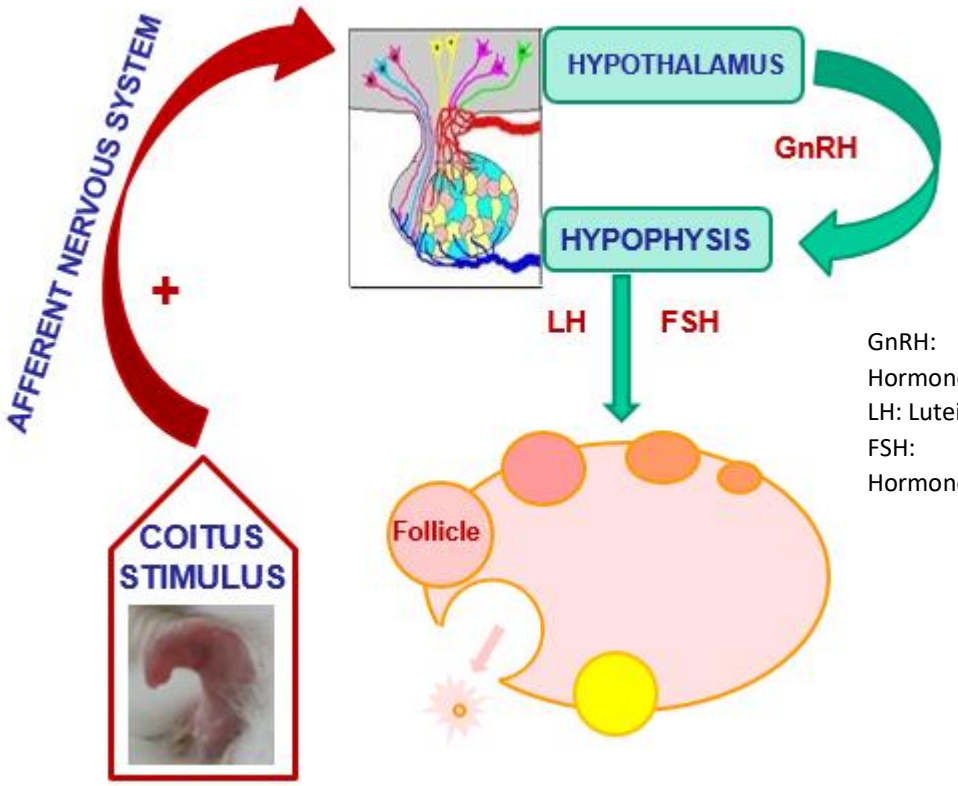
Mammalian species have been classified as either spontaneous or induced ovulators based on the type of stimulus responsible for eliciting GnRH (gonadotropin-releasing hormone) release from the hypothalamus (Bakker and Baum, 2000; Spies et al., 1997). In animals considered as spontaneous ovulators (e.g., women, cattle, horses, sheep, goat, pigs, monkeys and most rodents), ovulation is induced by ovarian steroid hormones at regular intervals (Bakker and Baum, 2000) (Figure I.1). Briefly, the ovarian follicular dynamic leads to the emergence of one or more dominant follicles (depending of species been monoparous or multiparous), which increases the systemic concentration of estradiol (El Allali et al., 2017). High estradiol concentration induces a positive feedback, which is permissive for the activation of GnRH release into the portal blood. GnRH in turn triggers a strong and transitory release of luteinizing hormone (LH) (LH surge) from the gonadotrophs of the pituitary gland (Bakker and Baum, 2000). This increase in circulating LH activates a whole cascade of inflammatory and proteolytic responses that leads to the rupture of the dominant follicular boundary wall and the ovulation of the oocyte.

Figure I.1. Ovulation cycle of spontaneous ovulators.



On the other hand, in induced or reflex ovulators (e.g., rabbit, Bactrian camel, llama, alpaca, cat, ferret), physical stimulation of the genitalia during copulation is the primary trigger for inducing ovulation (Adams et al., 2016), while the positive feedback action of estradiol is reduced or absent (El Allali et al., 2017) (Fig. I.2). It has been suggested that genital-somatosensory signals generated by penile intromission during copulation activate neural circuitries, mainly noradrenergic neurons, in the midbrain and brainstem to promote GnRH release (Bakker and Baum, 2000). In rabbits, a short mating bout including ejaculation induces genital somatosensory cues that contribute to the activation on GnRH neurons and the consequential generation of a preovulatory LH surge from the pituitary gland (Rebollar et al., 2012). Plasma LH levels start to rise within 3 minutes after mating and reach a plateau within 15 to 75 minutes (Jones et al., 1976).

Figure I.2. Ovulation cycle of reflex ovulators.



GnRH: Gonadotropin-releasing Hormone
LH: Luteinizing Hormone
FSH: Follicle Stimulating Hormone

Recent llama and alpaca studies have clearly showed that ovulation in these reflex ovulators species can be also induced by a protein factor present in seminal plasma (Silva et al., 2015). The first evidence of an ovulation-inducing factor (OIF) in semen came from workers in China who concluded that some factor in the semen was responsible for eliciting ovulation in Bactrian camels, rather than the mechanical stimulation of copulation (Adams et al., 2016). Recently, the OIF has been identified as βeta-nerve growth factor protein (β-NGF) in the seminal plasma of alpaca (Kershaw-Young et al., 2012), llama (Ratto et al., 2012) and camel (Kumar et al., 2013). This nerve growth factor is a highly conserved protein of about 14 kDa (Fig. I.3), capable of inducing ovulation when administered intramuscularly to alpaca, llama and camel

females (Kershaw-Young et al., 2012; Silva et al., 2015; Berland et al., 2016; Adams et al., 2016; El Allali et al., 2017).

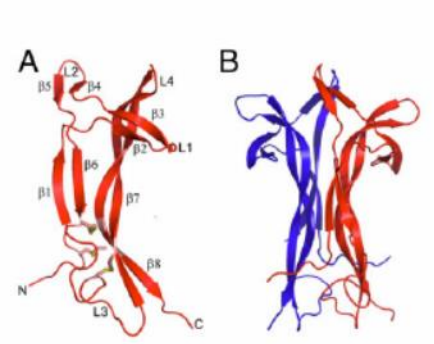


Figure I.3. Protein structure of ovulation-inducing factor (OIF) from llama seminal plasma. (A) Monomer of OIF. (B) Biological dimer of OIF. (Ratto et al., 2012)

The mechanism and sites of action of β-NGF in the hypothalamus–pituitary–gonadal axis are as yet unclear, but the abundance of NGF in seminal plasma and the effects of seminal plasma on ovarian function strongly support the idea of an endocrine mode of action after being absorbed by the genital mucosa into circulation (Adams et al., 2016). β-NGF quantity in reflexively ovulating species such as llamas and alpacas is abundant and represents the 30% of the total seminal plasma protein content (20 mg/ejaculate) (Berland et al., 2016). The semen of most mammals such as cattle, sheep, pig and horse also contains β-NGF (Bogle et al., 2011; Druart et al., 2013), however, β-NGF concentration is greater in induced as compared to spontaneous ovulators (El Allali et al., 2017). In rabbits, the intramuscular administration of seminal plasma does not provoke ovulation in rabbit does (Silva et al., 2011), but the study of this protein is interesting because it may exert a role in promoting the formation and development of the testis and the differentiation, maturation, and movement of the spermatozoa (Li et al., 2010). Moreover, NGF and its two receptors were reported to be expressed in the testis, prostate gland and seminal vesicle of rabbit, suggesting a possible role of this factor in the testicular development and spermatogenesis (Maranesi et al., 2015).

3. NEW EXTENDERS FOR RABBIT ARTIFICIAL INSEMINATION

For over a century, it has been well known that ovulation is induced by coitus in the rabbit (Heape, 1905). Therefore, when using artificial insemination, the administration of a GnRH analogue is mandatory to induce doe ovulation due to the lack of nervous stimuli evoked by the male. This treatment requires an intramuscular injection, which can result in stress to the animal and additional work for the farm operators (Dal Bosco et al., 2011). Moreover, in most rabbit farms, GnRH injection is usually performed by the farmer, with a certain risk of misuse, and increasing the time needed for the artificial insemination of each doe (Quintela et al., 2009). Recent studies have investigated the possibility of ovulation induction in rabbits by vaginal absorption after supplementation of the seminal dose with GnRH synthetic analogues and tested their reproductive performance (Table I.1).

There are clear breeding advantages of intravaginal administration of GnRH analogues, such as less treatment distress, labor for the operators and operating time, but unfortunately, the concentration of GnRH analogue to be added to the seminal dose to achieve fertility results similar to those of intramuscular administration is much higher than the amount administered intramuscularly (Viudes-de-Castro et al., 2014). For this reason, in order to obtain fertility rates comparable with those obtained with the usual intramuscular injection, the intravaginal buserelin dose has to be at least 15-fold higher (Quintela et al., 2009), becoming a potential health risk for farmers.

Table I.1. Fertility and prolificacy rates of rabbits does induced to ovulate with different GnRH analogues added to the seminal dose.

GnRH analogue (µg/doe)	Sperm concentration (x10^6 spz/doe)	Fertility rate (%)	Prolificacy rate (Mean)	References
Buserelin (8, 12, 16)	30	79.2, 87.0, 87.5 %	9.9, 9.7, 11.7	Quintela et al., 2004
Control i.m. (0.8)		91.7 %	9.4	
Triptorelin (5)	6	68.4 %	9.6	Viudes-de-Castro et al., 2007
Buserelin (5)		74.8 %	10.1	
Control i.m. (1)		78.6 %	9.9	
Buserelin (5)	-	76.0 %	10.3	Vicente et al., 2008
Control i.m. (1)		86.4 %	10.4	
GnRH-Lecirelinum (2.5, 5, 7.5, 15)	24-95.2	42.99, 59.97, 72.09, 52.77 %	8.79, 8.82, 9.25, 9.10	Ondruška et al., 2008
Control i.m. (2.5)		62.74 %	9.03	
[des-Gly 10, $_D$-Ala6]-LHRH ethylamide (25, 30)	30	82.8, 73.3 %	10.29, 10.41	Quintela et al., 2009
Control i.m. (20)		80.6 %	10.96	
Buserelin (5)	12	74 %	9.0	Vicente et al., 2011
Lecilerin (5)	10	80 %	-	Dal Bosco et al., 2014
Control i.m. (5)		80 %		
Buserelin (5)	8	58 %	8.7	Viudes-de-Castro et al., 2014
Control i.m. (1)		85 %	10.4	
[des-Gly 10, $_D$-Ala6]-LHRH ethylamide (5, 10, 15)	25	29.4, 68.8, 66.7 %	10.1, 9.2, 9.7	Gogol, 2016a
Control i.m. (1)		72.2 %	9.8	
Goserelin (5, 10)	25	60.0, 80.9 %	11.7, 10.9	Gogol, 2016b
Leuprolide (5, 10)		54.2, 75.0 %	10.7, 10.9	
Control i.m. (1)		85.9 %	11	

Spz: spermatozoa; i.m.: intramuscular.

GnRH synthetic analogues can be absorbed via mucosa less efficiently than via parenteral (about five times less) (Viudes-de-Castro et al., 2007). The absorption of GnRH by vaginal mucosa is influenced by the state of the mucosa, the mucosal and sperm peptidase activity, the extender composition and the formulation of the analogue. Various approaches to improve protein delivery by vaginal route include: use of enzyme inhibitors, absorption enhancers, mucoadhesive polymers and/or novel carrier systems such as nanoparticles.

In rabbit, results from Vicente et al. (2011) showed that when buserelin acetate was added to seminal plasma diluted 1:5, a more marked decrease in ovulation frequency occurred than if it was diluted 1:20. This was due to the increased availability of GnRH analogue as a consequence of the reduction of the existing aminopeptidases. Recently, Viudes-de-Castro et al. (2014) showed that the bioavailability of buserelin acetate when added to the seminal dose appears to be determined by the activity of the existing seminal plasma aminopeptidases. This fact suggests that a possible solution to avoid using high hormone levels to induce ovulation effectively in rabbit could be the addition of protease or aminopeptidase inhibitors to semen extenders. This way, part of the enzyme activity that degrades the GnRH analogue would be inhibited and therefore, the bioavailability of the hormone would be higher.

Another possible approach in order to protect the hormone from enzyme degradation would be to encapsulate the GnRH analogue inside nanoparticles. Recently, proteins such as lutein, insulin, rhodamine 6G and bovine serum albumin (BSA) have been entrapped in nanoparticles of chitosan (CS) and dextran sulfate (DS) for their delivery in oral or ocular mucosa (Chen et al., 2007; Pechenkin et al., 2011; Chaiyasan et al., 2015). CS and DS are biodegradable, biocompatible and non-toxic polymers of natural

origin with high adsorption capacity, which are widely used in pharmaceutical formulations (Chen et al., 2003, Domínguez-Delgado et al., 2014). In addition, they are polymers with multiple substituted negatively charged sulfate groups (in DS), or positively charged amine groups (deacetylated CS), and when mixed in aqueous solution, they form polyelectrolyte complexes through electrostatic interactions, better known as nanoparticles (Bader et al., 2015, Valente et al., 2013). CS-DS nanoparticles prepared by a coacervation process show promise as a system for controlled delivery of small and large molecules, including proteins (Chen et al., 2007), and they reduce the likelihood of their inactivation (Bader et al., 2015). In rabbits, an *in vitro* recent experience showed that the incubation of CS-DS nanoparticles with rabbit semen did not affect semen motility, viability nor membrane functionality, but improved acrosome integrity (Fernández-Serrano et al., 2017). Moreover, in the same study, a 40-50% of GnRH entrapment efficiency was achieved with CS-DS nanoparticles, meaning that the hormone was encapsulated and therefore protected.

4. RABBIT GENETICS IN PRODUCTION

Efficiency of rabbit meat production depends on several traits, which can be divided into production and reproduction related traits (Baselga and Blasco, 1989; Baselga, 2004). Production traits (growth, feed efficiency, carcass and meat traits) are relevant during fattening period and attributed to paternal lines, while reproduction traits (kindling interval, number of weaning kits, number of live young rabbits and longevity) are important for maternal lines (Naturil-Alfonso, 2016). Industrial rabbit production is based on a three-way crossbreeding scheme to exploit the complementarity of different synthetic breeds (lines) and makes use of heterosis or hybrid vigour (Simm,

1998). In rabbit, a first cross involves two maternal lines generating crossbred does. And a second cross consists of mating males of a third line, commonly selected for growth rate or weight at a given age, with the crossbred does to produce the rabbits to be slaughtered for meat (Baselga and Blasco, 1989). The final aim of the cross is to capitalize on the complementarity between reproductive and growth traits to produce large number of rabbits with fast growth and high feed efficiency (Baselga, 2004).

There are different rabbit genetic lines or breeds used in production such as A and R lines. Rabbit genetic line A is based on New Zealand White rabbits selected since 1980 by a family index for litter size at weaning over 45 generations (Fig. I.4 right). Whereas line R comes from the fusion of two lines, one founded in 1976 with Californian rabbits reared by Valencian farmers and another founded in 1981 with rabbits belonging to specialised paternal lines (Fig. I.1 left). The selection method was individual selection on post-weaning daily gain, with weaning taking place at 28 days and the end of the fattening at 63 days.

Figure I.4. Picture of rabbit genetic lines R (left) and A (right).

In rabbit, as in other species, selection for growth traits shows negative correlated responses in reproductive performance (Mgheni et al., 1985; Gómez et al., 1999; Ragab and Baselga, 2010). Several authors have compared the seminal characteristics

and reproductive performance between these two genetic lines and have found differences (Vicente et al., 2000; Mocé et al., 2003; Safaa et al., 2008; Vicente et al., 2012). For instance, Vicente et al. (2000) found lower sperm production, less motility and more acrosomal defects in a paternal line selected for growth (line R) than in 3 maternal ones (V, A and H). Mocé et al. (2003) showed a significantly lower fertilizing ability of line R sperm after being frozen by the same protocol than two maternal lines (A and V). In addition, Safaa et al. (2008) observed greater acrosome integrity, viability and percentage units for hypo-osmotic-swelling test in line A compared to line R. And finally, Vicente et al. (2012) found that the paternal line (R) had lowest ovulation frequency, number of implanted embryos, total born and live born than the maternal one (A).

5. IMPORTANCE OF RABBIT BUCK IN REPRODUCTION

In the rabbit industry, a single ejaculate can be divided into 20-50 insemination doses for artificial insemination (Lavara et al., 2005). With AI, the impact of reproductive performance (i.e. male contribution to fertility and prolificacy) of individual males is vital (Piles et al., 2013).

Rabbit ejaculates present some peculiarities that should be taken into account, for instance, they present occasionally gel plug or gelatinous mass (Fig. I.5). Besides, rabbit seminal plasma contains several droplets and vesicles similar in size to spermatozoa that have been related to modulate different sperm functions such as motility, capacitation and acrosome reaction (Castellini et al., 2006, 2012, 2013; Collodel et al., 2012).

Figure I.5. Gel plug of rabbit's ejaculate.

On the male side, the efficiency of AI depends on the efficient production of potentially fertile doses, which in turn, depends on quantitative semen production traits such as the sperm number per ejaculate and on quality characteristics that are potentially linked to the fertilising ability of the semen (Brun et al., 2016). In addition, the male genetic value should be considered because the maternal or growth traits have a direct effect on the productive traits of the offspring (Safaa et al., 2008). Many factors influence the production and quality of rabbit semen such as the genetic origin (growth lines have worse seminal qualities and fertility rates than maternal lines) (Vicente et al., 2000; Mocé et al., 2003; Safaa et al., 2008), the season (Marai et al., 2002; Pascual et al., 2004; Schneidgenová et al., 2011; Theau-Clément et al., 2015), the photoperiod (Roca et al., 2005; Ain-Baziz et al., 2012; Sabés-Alsina et al., 2015) and the collection frequency (Nizza et al., 2003). The production of fertile doses is determined by several components: i) male libido and characteristics of the ejaculate which form part of the criterion for ejaculate rejection; ii) volume and sperm concentration of the ejaculate (determining the amount of doses that can be obtained); and iii) the quality of sperm (determining the minimum sperm dosage required to ensure fertilization) (Piles et al., 2013). Subjective estimation of motility and evaluation of sperm morphology are the two laboratory assays most widely used for the rabbit semen evaluation in insemination centers (Lavara et al., 2005). However, the ability of these

seminal characteristics to predict reproductive performance is very low (Piles et al., 2013). Consequently, new criteria for rabbit ejaculate selection are being explored, starting to focus on the sperm cell and on seminal plasma composition.

5.1. Rabbit spermatozoa proteins

New advances in proteomics are having a major impact on our understanding of how spermatozoa acquire their capacity for fertilization (Aitken and Baker, 2008). Sperm proteomics aims at the identification of the proteins that compose the sperm cell and the study of their function (de Mateo et al., 2013). The sperm cell is one of the most highly differentiated cells and is composed of a head with a highly compacted chromatin structure and a large flagellum with midpiece that contains the required machinery for movement and therefore to deliver the paternal genetic and epigenetic content to the oocyte (Codina et al., 2015). By being so highly differentiated, spermatozoa are advantageous cells to study proteomics of specific compartments such as the membrane, which basically is the area of major importance for its role in interacting with the surroundings and the oocyte (Rodríguez-Martínez et al., 2011). The fusion of a sperm and an oocyte is a sophisticated process that must be preceded by suitable changes in the sperm's membrane composition (Nowicka-Bauer and Kurpisz, 2013). Recent studies of spermatozoa from the proteomic point of view have allowed the identification of different proteins in spermatozoa that are responsible for the regulation of normal/defective sperm functions (Rahman et al., 2013).

While several techniques are available in proteomics, LC-MS (liquid chromatography-mass spectrometry) based analysis of complex protein/peptide mixtures has turned

out to be a mainstream analytical technique for quantitative proteomics (Tuli et al., 2009). These advanced proteomics-related techniques constitute a powerful arsenal to study protein composition as well as their biological functions in cells, tissues and their secretions (Souza et al., 2012). Indeed, mass spectrometry-based proteomics has become the tool of choice for identifying and quantifying the proteome of an organism (Karpievitch et al., 2010), because of its exceptional sensitivity and resolving power that allow for the detection of proteins and peptides at low femtomole quantities (Wither et al., 2016).

Using LC-MS/MS, detailed proteomic data are now available for human (Baker et al., 2007), macaque (Skerget et al., 2013; Kawase et al., 2015), mouse (Baker et al., 2008a), rat (Baker et al., 2008b), ram (Pini et al., 2016), honeybee (Zareie et al., 2013), bull (Byrne et al., 2012; Ashrafzadeh et al., 2013; Somashekar et al., 2015), stallion (Swegen et al., 2015), fruit fly (Wasbrough et al., 2010), *Caenorhabditis elegans* (Ma et al., 2014), carp (Dietrich et al., 2016), chicken (Labas et al., 2015), rainbow trout (Nynca et al., 2014) and mussel (Zhang et al., 2015) sperm proteins. On the contrary, rabbit sperm proteome is not yet characterised, missing important information of potential proteins of interest which could improve breeding success in this agriculturally important animal.

5.2. Rabbit seminal plasma proteins

The general role of seminal plasma is to create an optimal environment for the storage of spermatozoa (Dietrich et al., 2014). Seminal plasma contributes to the safe environment for sperm maturation, sperm viability and fertilization in mammals (Kumar et al., 2013). In rabbits, seminal plasma has a positive effect in maintaining

sperm motility and viability during *in vitro* storage (Castellini et al., 2000). Moreover, seminal plasma is a promising source for the study of potential reproductive biomarkers, because it is a complex mixture of secretions from testis, epididymis and male accessory sex glands (González-Cadavid et al., 2014). Species of mammals differ regarding the presence and size of accessory sexual glands, which obviously lead to variations in their relative contribution to semen composition and volume, particularly regarding seminal plasma (Rodríguez-Martínez et al., 2011).

A greater understanding of the proteins involved in reproduction can benefit human fertility and animal production (Bayram et al., 2016). The protein composition of mammalian seminal plasma varies among species, and has important effects on sperm function (Rodríguez-Martínez et al., 2011). These proteins participate in various events related to sperm function such as epididymal sperm maturation, sperm capacitation, sperm membrane stabilization and even the interaction with the oviduct and oocyte (Topfer-Petersen et al., 1998; Gwathmey et al., 2006). The diversity in the seminal plasma protein composition of different species of mammals may explain the variation in reproductive capacity and function (Druart et al., 2013).

In some species, seminal plasma proteins related to fertility (Killian et al., 1993; Brandon et al., 1999; Novak et al., 2010a, 2010b; Ashrafzadeh et al., 2013; Aslam et al., 2014), cold-shock damage sperm membrane resistance (Barrios et al., 2000; Pérez-Pé et al., 2001; Muiño-Blanco et al., 2008; Colás et al., 2009), sperm tolerance to freezing (Goularte et al., 2014; Rickard et al., 2015), freezability (Jobim et al., 2004, 2011; Zahn et al., 2005; Asadpour et al., 2007; Rego et al., 2016; Magalhães et al., 2016; Dietrich et al., 2017; Valencia et al., 2017) and semen quality (Cardozo et al., 2006; de Souza et al.,

2007; Wang et al., 2009; Arruda-Alencar et al., 2012; Nandre et al., 2013; Kiso et al., 2013; Sarsaifi et al., 2015) have been identified.

Seminal plasma proteome of several animal species has been published during the past years (bull: Moura et al., 2010; ram: Souza et al., 2012; Pini et al., 2016; boar: Pérez-Patiño et al., 2016; carp: Dietrich et al., 2014; human: Pilch et al., 2006; Batruch et al., 2011, 2012; salmon: Gombar et al., 2017; honeybee: Baer et al., 2009; rainbow trout: Nynca et al., 2017). In the case of rabbit, seminal plasma proteins have been studied with a traditional 1D polyacrylamide gel (Viudes-de-Castro et al., 2004; Safaa et al., 2008) and with a 2D gel approach (Arruda-Alencar et al., 2012). In these previous studies, only two proteins were identified (Annexin 5 and Zeta globin-like). Therefore, a lack of information is missing regarding rabbit seminal plasma complete proteome identification.

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II.OBJECTIVES

II.OBJECTIVES

The general aims of this thesis were to develop new artificial insemination extenders supplemented with a GnRH analogue in order to induce doe ovulation and to characterise the proteomic profile of rabbit semen. To this end, the specific objectives of the thesis were as follows:

The first three chapters of the current thesis aimed to develop new artificial insemination extenders supplemented with different substances to increase GnRH analogue's bioavailability without affecting reproductive performance. Chapter I focused on supplementing the extenders with a protease inhibitors cocktail, whereas chapter II was conducted to study the effect of specific aminopeptidase inhibitors addition to insemination extenders. Finally, in chapter III, the objective was to test different GnRH concentrations in the extenders to check if the aminopeptidase inhibitors protected the hormone and to study the efficacy of new extenders with the hormone encapsulated in chitosan-dextran sulfate nanoparticles.

The objectives in chapters IV, V and VI were to identify and quantify for the first time the complete seminal plasma and sperm's protein profile of rabbit species. First by using a one-dimension (1D) polyacrylamide gel approach (chapter IV) and later with nano LC-MS/MS technique (chapters V and VI).

III.CHAPTER I

III.CHAPTER I

Does the inclusion of protease inhibitors in the insemination extender affect rabbit reproductive performance?

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ABSTRACT

The bioavailability of buserelin acetate when added to the seminal dose appears to be determined by the activity of the existing aminopeptidases. Thus, the addition of aminopeptidase inhibitors to rabbit semen extenders could be a solution to decrease the hormone degradation. This study was conducted to evaluate the effect of the protease activity inhibition on rabbit semen quality parameters and reproductive performance after artificial insemination. Seminal quality was not affected by the incubation with protease inhibitors, being the values of motility, viability and acrosome integrity not significantly different between the protease inhibitors and the control group. In addition, seminal plasma aminopeptidase activity was inhibited in a 55.1% by the protease inhibitors. On the other hand, regarding the effect of protease inhibitors on reproductive performance, our results showed that the presence of protease inhibitors affected the prolificacy rate (9.2 ± 0.26 and 9.3 ± 0.23 *vs.* 8.2 ± 0.22 total born per litter for negative control, positive control and aminopeptidase inhibitors group, respectively; $p<0.05$), having this group one kit less per delivery. We conclude that the addition of a wide variety of protease inhibitors in the rabbit semen extender negatively affects prolificacy rate. Therefore, the development of new extenders with specific aminopeptidase inhibitors would be one of the strategies to increase the bioavailability of GnRH analogues without affecting the litter size.

INTRODUCTION

The addition of the GnRH synthetic analogues to the seminal dose is a welfare-orientated method to induce ovulation in rabbits and in addition reduces the time spent by farmers (Dal Bosco et al., 2011). The success of this method depends on the enzymes present in the seminal plasma (Viudes-de-Castro et al., 2014), the status of the vaginal mucosa and on the extender composition (Dal Bosco et al., 2014). Consequently, to achieve similar fertility results, when the GnRH analogue is applied intravaginally, the required concentration is much higher than the one used intramuscularly. Results from Vicente et al. (2011) showed that when buserelin acetate was added to seminal plasma diluted 1:5, a more marked decrease in ovulation frequency occurred than if it was diluted 1:20. This was due to the increased availability of GnRH analogue as a consequence of the reduction of the existing aminopeptidases. Recently, Viudes-de-Castro et al. (2014) showed that the bioavailability of buserelin acetate when added to the seminal dose appears to be determined by the activity of the existing seminal plasma aminopeptidases. In addition, it has been observed that males selected for maternal characteristics showed significantly lower aminopeptidase activity than males selected for growth rate, suggesting that the genetic origin of rabbit male could determine the aminopeptidase concentration present in the seminal plasma (Viudes-de-Castro and Mocé, 2013). These facts suggest that a possible solution to avoid using high hormone levels to induce ovulation effectively in rabbit could be the addition of aminopeptidase inhibitors to semen extenders. This way, part of the enzyme activity that degrades the GnRH analogue would be inhibited and therefore, the bioavailability of the hormone would be higher.

The aim of this study was to evaluate the effect of the inclusion of protease inhibitors in semen extender on *in vitro* rabbit semen quality parameters (motility, viability and acrosome status) and *in vivo* reproductive performance (fertility and prolificacy) after artificial insemination.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals in this study were purchased from Sigma-Aldrich Química S.A (Madrid, Spain).

1. Animals

All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013.

To study the effect of protease inhibitors on semen quality parameters, a total of 12 adult bucks belonging to a paternal rabbit line (Line R) were used. All males were kept individually in flat deck cages under 16 h light/8 h dark conditions at the experimental farm of the Animal Technology and Research Centre (CITA-IVIA, Segorbe, Castellón, Spain) and fed *ad libitum* with the same commercial diet (17.5% crude protein, 2.3% ether extract, 16.8% crude fiber, 2.600 kcal DE/Kg) and had free access to water.

To study the effect of protease inhibitors on reproductive performance, commercial crossbreed does were inseminated on one commercial farm (Altura, Castellón, Spain) with semen from the 12 Line R adult males. A total of 709 artificial inseminations were performed. Receptive females were classified by physiological status as multiparous lactating does (more than two delivered births and eight or nine young rabbits suckled), multiparous non-lactating females (females with more than one delivery

without suckling any young) and nulliparous females (females who have never given birth). Multiparous lactating does were inseminated 10 to 12 days after delivery. The sexual receptivity in multiparous does was obtained by closing the nest during 36 h.

2. *In vitro* effect of protease inhibitors on seminal quality

2.1. Semen collection and evaluation

Two ejaculates per male were collected each week during 4 weeks using an artificial vagina, with a minimum of 30 minutes between ejaculate collections, on a single day. Sperm evaluation was performed to assess the initial seminal quality. Only ejaculates exhibiting a white colour and possessing motility rate higher than 70% were used in the experiment. Finally, the ejaculates were pooled. In total, four pools were used.

In order to evaluate seminal quality aliquots of pooled semen were taken. A 20 µL aliquot was diluted 1:50 with 0.25% glutaraldehyde solution to calculate the concentration and rate of abnormal sperm in a Thoma chamber by phase contrast at a magnification of 400X.

The motility characteristics of sperm (percentage of total and progressively motile sperm, evaluated using a computer-assisted sperm analysis system) were determined as described by Viudes-de-Castro et al. (2014). A spermatozoon was defined as non-motile if the average path velocity (VAP) was <10 µm s^{-1} and a spermatozoon was considered to be progressively motile when VAP was >50 µm s^{-1} and the straightness index (STR) was ≥70%.

Flow cytometric analyses to assess viability and acrosome integrity, were performed using a Coulter Epics XL cytometer (Beckman Coulter, IZASA, Barcelona, Spain). The fluorophores were excited by a 15 mW argon ion laser operating at 488 nm. A total of

10,000 gated events (based on the forward scatter and side scatter of the sperm population recorded in the linear mode) were collected per sample. Flow cytometric data were analyzed with the software Expo32ADC (Beckman Coulter Inc.). Samples were diluted to 30 x 10^6 sperm/mL with TCG extender (Viudes-de-Castro and Vicente, 1997) supplemented with 2g/L BSA. All the dilutions were performed at 22 ºC. The percentage of viable sperm was determined using a dual fluorescent staining with SYBR-14/PI according to Viudes-de-Castro et al. (2014). The status of the acrosome in each sample was determined using a dual fluorescent staining with FITC-PNA/PI. Diluted samples were stained by transferring 0.1 mL aliquots into tubes containing 0.45 mL of TCG, 1.5 µl of fluorescein labeled lectin from the peanut plant *Arachis hypogaea* (FITC-PNA, 1 mg/mL solution in saline solution) and 2.5 µL of PI (1.5 mM solution in purified water). They were incubated (10 minutes, 22ºC), filtered through a 40-µm nylon mesh to remove large clumps of cells and debris. Fluorescence was measured using a FL-1 sensor, a 525 nm band-pass filter to detect FITC-PNA, and a FL-2 sensor and a 575 nm band-pass filter to detect PI. Four sperm sub-populations were detected: live acrosome intact, live acrosome damaged, dead acrosome intact and dead acrosome damaged. Percentage of normal apical ridge (NAR) was calculated as the proportion of acrosome intact sperm.

2.2. Protease inhibitor activity evaluation

In this experiment, two different extenders were tested: TCG (control) and an experimental extender containing TCG supplemented with Protease Inhibitor Cocktail (P2714, Sigma) diluted 1:100. The protease inhibitor cocktail used contains 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), aprotinin, bestatin, E-64, Ethylenediaminetetraacetic acid (EDTA) and leupeptin. Sperm samples were split into

two equal fractions and diluted 1:20 with TCG (control) and supplemented TCG respectively. Fractions were stored two hours at room temperature (20-25°C). Then, three aliquots of each sample were taken to measure the motility, the viability and the status of the acrosome again. The remaining pooled semen was used to measure alanyl peptidase (APN) activity.

2.3. Seminal plasma preparation and measurement of APN activity

Semen samples were centrifuged at 10.000 x g for 10 min at 22 ºC. The resulting supernatants were collected and centrifuged again (10.000 x g for 10 min) to remove residual spermatozoa and cell debris. The resulting pellets were discarded, whereas the supernatants were stored at -80ºC until use.

APN activity in seminal plasma was determined according to Viudes-de-Castro et al. (2014). Briefly, samples were incubated with the substrate (alanine-β-naphthylamide) for 30 min at 37 ° C, after which the reaction was stopped with 0.1 M sodium acetate buffer (pH 4.2). The release of β-naphthylamide as a result of enzyme activity was determined by measuring the fluorescence intensity at 460 nm with excitation at 355 nm. Protein concentration of semen samples was measured using the bicinchoninic acid (BCA) method, using BSA as the standard (Smith et al., 1985). APN activity and protein concentration were measured in triplicate. The peptidase activity was expressed as pmol of β-naphthylamide released per milligram of protein per minute. In order to calculate the percentage of APN activity inhibition, the APN activity of the control group was used as reference in each case.

3. *In vivo* effect of protease inhibitors on reproductive performance

3.1. Semen collection and evaluation

Each week, two ejaculates per male were collected, with a minimum of 30 minutes between ejaculate collections, on a single day using an artificial vagina. Sperm evaluation was performed to assess the initial seminal quality. Only ejaculates exhibiting a white colour and possessing more than 70% of motility rate, 85% of normal intact acrosome, and less than 15% of abnormal sperm were used in this experiment. All other ejaculates were discarded. The pools used in the experiment presented an average sperm concentration of 296 spermatozoa mL^{-1}.

The remaining pooled semen was split into three aliquots and diluted 1:20 respectively with 1) TCG extender supplemented with 10 µg of buserelin acetate/mL 2) TCG extender supplemented with the protease inhibitor (protease inhibitor cocktail dilution rate 1:100) and 10 µg of buserelin acetate/mL and 3) TCG extender (non GnRH-supplemented).

3.2. Insemination procedure

A total of 709 inseminations were performed in six different days. Only receptive females (red colour of vulvar lips) were inseminated with 0.5 mL of semen using standard curved pipettes (22 cm). Each female was randomly assigned to one of the three experimental groups:

-Positive control group: does inseminated with 0.5 mL diluted semen in TCG. At the time of insemination, each female received an intramuscular injection of buserelin acetate to induce ovulation (1 µg per doe).

-Negative control group: does inseminated with 0.5 mL diluted semen in supplemented extender with 10 µg/mL of buserelin acetate.

-Protease inhibitors group: does inseminated with 0.5 mL diluted semen in supplemented extender with protease inhibitor cocktail (dilution rate 1:100) and 10 µg/mL of buserelin acetate.

Fertility rate at birth (number of does giving birth/number of inseminated does) and prolificacy (number of total kits born) were reproductive performances considered.

4. Statistical analysis

Data were statistically evaluated with SPSS 16.0 library procedures (SPSS Inc., Chicago, Illinois, USA, 2002). To analyse the effect of the extender on motility, viability and acrosome integrity, an analysis of variance (ANOVA) was used. A probit link with binomial error distribution was used to analyse the fertility rate at birth, including as fixed effects the extender used and the physiological state of the females and their interactions. For total number of kits born per litter, a general linear model procedure (GLM) was performed, including as fixed effects the extender used and the physiological state of the females and their interactions. Means were separated using Fishers Least Significant Difference (LSD) test at a fixed 5% error level and the results are presented as least square means values (LSM) ± standard error of the mean (SE).

RESULTS

1. Effect of protease inhibitors on seminal quality

Results showed that the presence of protease inhibitors did not affect the motility, neither the viability nor the acrosome integrity of the seminal samples. The percentage of total motile sperm, percentage of progressively motile sperm, average of curvilinear velocity (VCL), mean amplitude of lateral head displacement (ALH), frequency of head

displacement (BCF), linearity coefficient (LIN) and VAP were 90.4±2.1%, 50.4±5.8%, 52.06±4.18µm/s, 2.42±0.10µ, 10.0±0.28%, 48.71±2.14% and 35.52±3.35µm/s respectively (data not shown in tables). The values of viability and acrosome integrity after two hours of incubation with the two extenders were similar (82.5±2.03 of viability and 98.7±0.4 % of normal apical ridge). On the other hand, the APN activity was inhibited in the extender containing the protease inhibitors cocktail. The APN activity was a 55.1% lower than the control extender.

2. Effect of protease inhibitors in fertility and prolificacy

Fertility rate at birth and prolificacy are presented in Table 1.1. These parameters were affected by the experimental group. Positive control group showed the highest fertility rate at birth while the fertility of females from groups with buserelin acetate in the seminal dose was reduced 19 and 15% (negative control group and protease inhibitors group, respectively).

Regarding prolificacy, the total number of kits born per litter was affected by treatment ($p<0.05$; Table 1.1). Does from the positive and the negative control group showed similar prolificacy rate. On the other hand, females from protease inhibitors group showed a significantly lower prolificacy than the other two groups.

Multiparous non-lactating and nulliparous does showed significantly higher fertility rate than the multiparous lactating does group (0.77 ±0.02 and 0.79 ± 0.07 *vs*. 0.65 ± 0.03, respectively; $p<0.05$).

No interaction between experimental group and physiological status at the moment of insemination was observed.

Table 1.1. Reproductive performance of inseminated does. (Least square means ± standard error).

Groups	Inseminated does	Fertility rate at birth	Total Born per litter
Positive control	166	0.84 ± 0.03^{a}	9.2 ± 0.26^{a}
Negative control	273	0.65 ± 0.04^{b}	9.3 ± 0.23^{a}
Protease inhibitors	269	0.69 ± 0.04^{b}	8.2 ± 0.22^{b}

Positive control: does induced to ovulate with buserelin acetate applied intramuscularly, Negative control: does induced to ovulate with buserelin acetate added in the insemination extender; Protease inhibitors: does induced to ovulate with buserelin acetate added in the insemination extender containing protease inhibitors. Values within a column with different superscripts in the same column differ significantly at $p<0.05$.

DISCUSSION

In this article, we have shown that rabbit semen extender affects neither semen quality nor fertility rate, but affects the prolificacy rate by decreasing the total number of kits born per litter.

In mammals, seminal plasma is a complex mixture of secretions from the epididymis and from the various accessory sex glands (La Falci et al., 2002). Its composition is designed to assure the successful fertilization of the oocyte and is characterized by a high abundance of proteins which play important roles in sperm survival and are involved in various events such as epididymal sperm maturation, sperm capacitation, sperm membrane stabilization, modulation of the uterine immune response, sperm transport in the female genital tract, gamete interaction and fusion and even

pregnancy establishment (Okabe et al., 1993; Topfer-Petersen et al., 1998; Gwathmey et al., 2006; Rodríguez-Martínez et al., 2011; Laflamme et al., 2013). In human, the 60% of the seminal plasma proteome has enzymatic activity. The abundance of proteases and protease inhibitors in seminal plasma show the importance of this system in this body fluid (Pilch et al., 2006). Many enzymes, hormones and other physiologically active proteins are synthesized as inactive precursors that are converted to the active form by the action of specific proteases.

Because the protease inhibitor cocktail tested in this work contained a broad enzyme inhibition spectrum, it was predictable that the seminal quality, the fertilization process, or both, were affected.

Several studies have tested the effect of different protease inhibitors on mammal sperm *in vitro* quality and fertilization process. For instance, leupeptin had no effect in rabbit sperm motility (de Lamirande et al., 1986). In pig, spermatozoa incubated in medium with AEBSF were less motile after 6 h of incubation, yet progressive motility, VAP, VSL, acrosome status and mitochondrial potential remained unaltered (Beek et al., 2015). These results are consistent with ours, as seminal quality parameters were similar between control and protease inhibitors group after 2 h of incubation.

In the present work, the inclusion of protease inhibitors in semen extender affected only the prolificacy rate, having this group one kit less per delivery. This fact could be explained because proteases play an important role during mammalian fertilization, so the inhibition of a wide variety of proteases in this work, could have negatively affected this process. The part of the fecundation process affected by protease inhibitors seems to be species-specific. In guinea pig, the incubation of sperm with leupeptin inhibited the completion of the acrosomal reaction, but bestatin had no

effect (Flaherty et al., 1993). In bovine sperm, the use of trypsin and chymotrypsin inhibitors, but not metalloproteases, were effective in inhibiting the acrosomal reaction process and in addition, sperm incubation with these proteases did not reveal statistically differences in the sperm zona pellucida binding capacity in relation to control sperm (Deppe et al., 2008). In pig, the effect of AEBSF on *in vitro* fertilization and polyspermy rates were a decreased by at least 50% (Beek et al., 2015). In mouse and human, the use of specific serine proteases inhibitors decreases the fertilization rate (Fraser, 1982; Llanos et al., 1993). Finally, studies in sea urchin provide evidence for the involvement of metalloproteases in membrane fusion during the acrosome reaction (Farach et al., 1987).

Considering the results of the present study, we can conclude that the addition of a wide variety of protease inhibitors in the rabbit semen extender negatively affects the prolificacy rate. Therefore, the development of new extenders with specific aminopeptidase inhibitors would be one of the strategies to increase the bioavailability of GnRH analogues without affecting the litter size. In the future, the study of inhibitors individually will be necessary to define how to increase the GnRH bioavailability without affecting the fertilizing capacity of spermatozoa.

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IV.CHAPTER II

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Insemination extender supplementation with bestatin and EDTA has no effect on rabbit reproductive performance

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ABSTRACT

The addition of aminopeptidase inhibitors (AMIs) to rabbit semen extenders could be a solution to decrease the hormone degradation (GnRH) by the aminopeptidases existing in the seminal plasma. Therefore, the quantity of GnRH needed to induce ovulation in doe would be comparable with the amount administered intramuscularly (i.m.). This study was conducted to evaluate the effects of two AMIs (bestatin and EDTA) on rabbit semen quality parameters, β nerve growth factor (β-NGF) degradation and reproductive performance after artificial insemination. Results showed that seminal quality was not affected by the incubation with AMIs; the values of motility, acrosome integrity and sperm viability were not significantly different between the AMIs and the control groups (positive i.m. and negative intravaginally without AMIs). In addition, the aminopeptidase activity of seminal plasma was inhibited in a 55.5% by the AMIs as well as β-NGF degradation. On the other hand, regarding the effect of AMIs on reproductive performance, our results showed that the presence of bestatin and EDTA did neither affect the fertility (85.3 *vs*. 88.6 %), nor the prolificacy rate (10.12 *vs*. 10.51 kits per delivery), comparing AMIs group to positive control group, respectively. We conclude that the addition of specific AMIs in the rabbit semen extender has no effect on reproductive performance. Therefore, due to the fact that AMIs inhibit part of the aminopeptidase activity that degrades the GnRH analogue and β-NGF, they could be used to develop new extenders with less hormone concentration.

INTRODUCTION

The use of artificial insemination (AI) in rabbit farms has become a common practice in European countries, being currently used in more than 80% of the Spanish and EU rabbit farms (Quintela et al., 2009). The rabbit is considered a reflexively ovulating species in which ovulation is induced by sensory stimulation associated with mating. On the other hand, seminal plasma contains a protein, β-NGF, which is able to provoke the ovulation induction in females of other ovulating species such as camelids (Kershaw-Young et al., 2012). Although β-NGF has been identified in seminal plasma of rabbits (Casares-Crespo et al., 2016a), the genital somatosensory stimulus during coitus seems to be the main factor in the ovulation induction. Indeed, Silva et al. (2011) administered rabbit seminal plasma intramuscularly (i.m.) but it did not provoke ovulation in rabbit does. β-NGF in rabbit's seminal plasma only represents 1.5% of the total protein content of seminal plasma (results not published) and its amount is very low (1984 ± 277 pg/mL) (Maranesi et al., 2015) in comparison to the llama, another reflex ovulating species, where it represents 30% of the total seminal plasma protein content (20 mg/ejaculate) (Berland et al., 2016). Nevertheless, this protein has an important role in promoting the formation and development of the testis and the differentiation, maturation, and movement of the spermatozoa (Li et al., 2010).

Therefore, when artificial insemination (AI) is used in rabbits, it is necessary to induce ovulation with GnRH synthetic analogues. In most rabbit farms, GnRH administration is usually done by the farmer himself, with a certain risk of misuse, and an increase in the time needed for each AI (Quintela et al., 2004). GnRH analogues administration in rabbit could be performed i.m. or intravaginally (i.v.). The addition of the GnRH to the seminal dose reduces the time spent by farmers in AI procedures (Dal Bosco et al.,

2011) and it is also a welfare-orientated method to induce ovulation in rabbits. The success of this method depends on the enzymes present in the seminal plasma (Viudes-de-Castro et al., 2014), the status of the vaginal mucosa, the extender composition (Dal Bosco et al., 2014) and the GnRH analogue used. Unfortunately, to achieve fertility results similar to those with GnRH intramuscular injection, the hormone concentration intra-vaginally is much higher than the amount administered intramuscularly (Viudes-de-Castro et al., 2014).

In previous works, the bioavailability of buserelin acetate when added to the seminal dose appeared to be determined by the seminal plasma aminopeptidase activity (APN) (Viudes-de-Castro et al., 2014) and the addition of a protease inhibitor cocktail to the semen extender negatively affected the prolificacy rate (Casares-Crespo et al., 2016b). Therefore, in order to reduce the amount of hormone needed to induce ovulation without affecting the litter size, new semen extenders with specific Aminopeptidase Inhibitors (AMIs) should be developed.

APN activity has been inhibited in animal sperm with different substances such as bestatin (Togo and Morisawa, 2004; Kubo et al., 2008; Yasuhara et al., 1983; Flaherty et al., 1993), Ethylenediaminetetraacetic acid (EDTA) (Nag Das et al., 1984, 1988; Farach et al., 1987; Deppe et al., 2008), or both (Huang et al., 1997; Marinho et al., 2008). This inhibition can affect different fertilization steps depending on the species considered. To our knowledge, no previous study of the effect of these inhibitors on rabbit semen and fertilization processes has been done. In addition, no previous data are available regarding the effect of AMIs on seminal β-NGF.

The aim of this study was to evaluate the effect of the inclusion of bestatin and EDTA in semen extender on aminopeptidase activity and β-NGF protection in semen.

Moreover, the effect of these inhibitors was evaluated on *in vitro* rabbit semen traits (motility, acrosome status and viability) and on *in vivo* reproductive performance (fertility and prolificacy) after artificial insemination.

MATERIALS AND METHODS

The chemicals used in this study were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain), except for busereline acetate, which was purchased from Hoechst Marion Roussel, S.A. (Madrid, Spain); SYBR-14, propidium iodide (PI) and fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA), were purchased from Invitrogen (Barcelona, Spain).

1. *In vitro* effect of aminopeptidase inhibitors on seminal quality

1.1. Animals

All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013.

To study the effect of AMIs (bestatin and EDTA) on semen quality parameters, 12 adult bucks belonging to a paternal rabbit line (Line R, Estany et al., 1992) were used. All males were kept individually in flat deck cages under 16 h light/8 h dark conditions at the experimental farm of the Animal Technology and Research Centre (CITA-IVIA, Segorbe, Castellón, Spain) and fed *ad libitum* with the same commercial diet (17.5% crude protein, 2.3% ether extract, 16.8% crude fiber, 2.600 kcal DE/Kg) and had free access to water.

Seminal samples were collected using an artificial vagina over twelve weeks. Each week, two ejaculates per male/day were collected with a minimum of 30 minutes between ejaculate collections.

1.2. Semen evaluation

Sperm evaluation was performed to assess the initial seminal quality. Only ejaculates exhibiting a white colour and possessing motility rate higher than 70% were used in the experiment. Finally, the ejaculates were pooled. In total, twelve pools were used.

Seminal quality was evaluated on aliquots of pooled semen. A 20 µL aliquot was diluted 1:50 with 0.25% glutaraldehyde solution to calculate the concentration and rate of abnormal sperm in a Thoma chamber by phase contrast at a magnification of 400X.

The motility characteristics of sperm (percentage of total motile sperm, evaluated using a computer-assisted sperm analysis system) were determined as described by Viudes de Castro et al. (2014). Briefly, sperm samples were adjusted to 7 x 10^6 sperm/mL with TCG (Tris-Citric acid-Glucose) extender (Viudes-de-Castro and Vicente, 1997) supplemented with 2 g/L BSA and motility was assessed at 37ºC. A spermatozoa was defined as non-motile if the average path velocity (VAP) was <10 µm s^{-1} and a spermatozoon was considered to be progressively motile when VAP was >50 µm s^{-1} and the straightness index (STR) was ≥70%.

Flow cytometric analyses to assess acrosome integrity and viability were performed using a Coulter Epics XL cytometer (Beckman Coulter, IZASA, Barcelona, Spain). The fluorophores were excited by a 15 mW argon ion laser operating at 488 nm. A total of 10,000 gated events (based on the forward scatter and side scatter of the sperm population recorded in the linear mode) were collected per sample. Flow cytometric

data were analyzed with the software Expo32ADC (Beckman Coulter Inc.). Samples were diluted to 30 x 10^6 sperm/mL with TCG extender supplemented with 2 g/L BSA. All the dilutions were performed at 22 ºC. The percentage of viable sperm was determined using a dual fluorescent staining with SYBR-14/PI according to Viudes-de-Castro et al. (2014). Only the percentages of live sperm were considered in the results (SYBR-14-positive and PI-negative). The status of the acrosome was determined using a dual fluorescent staining with FITC-PNA/PI according to Casares-Crespo et al. (2016a). Four sperm sub-populations were detected: live sperm with intact acrosome, live sperm with damaged acrosome, dead sperm with intact acrosome and dead sperm with damaged acrosome. Percentage of normal apical ridge (NAR) was calculated as the proportion of acrosome intact sperm.

1.3. Experimental design

Three different extenders were tested:

-TCG (control).

-TCG supplemented with busereline acetate (10 µg/mL).

-TCG supplemented with busereline acetate (10 µg/mL), bestatin (10 µM) and EDTA (20 mM).

Sperm samples were split in three equal fractions and diluted with the appropriate extender (dilution 1:20; v: v). Fractions were stored two hours at room temperature (20-25°C). Then, three aliquots of each sample were taken again to measure the motility, the viability and the status of the acrosome. The remaining pooled semen was used to measure seminal plasma aminopeptidase activity (APN).

1.4. Measurement of aminopeptidase activity on seminal plasma (APN)

Semen samples were centrifuged at 7400 x g for 10 min at 22 ºC. The resulting supernatants were collected and centrifuged again (7400 x g for 10 min) to remove residual spermatozoa and cell debris. The resulting pellets were discarded, whereas the supernatants were stored at -80 ºC until use.

APN activity in seminal plasma was determined according to Viudes-de-Castro et al. (2014). Briefly, samples were incubated with the substrate (alanine-β-naphthylamide) for 30 min at 37 °C, after which the reaction was stopped with 0.1 M sodium acetate buffer (pH 4.2). The release of β-naphthylamide as a result of enzyme activity was determined by measuring the fluorescence intensity at 460 nm with excitation at 355 nm. Fluorescence values obtained by the experimental samples were transformed into pmol of released β-naphthylamide by comparison with a standard curve previously obtained. Protein concentration of semen samples was measured using the bicinchoninic acid (BCA) method, using BSA as the standard (Smith et al., 1985). APN activity and protein concentration were measured in triplicate. The peptidase activity was expressed as pmol of β-naphthylamide released per mg of protein per minute. In order to calculate the percentage of APN activity inhibition, the APN activity of the control group was used as reference in each case.

1.5. Evaluation of β-NGF on seminal plasma

B-NGF concentration in rabbit's seminal plasma was detected by ELISA according to the manufacturer's instructions of the Duo Set ELISA (R&D System, Milan, Italy), on ten sperm samples. Seminal samples were split in two equal fractions and diluted with the TCG extender or TCG extender supplemented with bestatin (10 µM) and EDTA (20 mM)

(dilution 1:20; v:v) and stored at room temperature (20-25 °C). Then, one aliquot of each sample was taken at 4, 8 and 12 hours to measure β-NGF amount.

2. *In vivo* effect of aminopeptidase inhibitors on reproductive performance

2.1. Animals

To study the effect of AMIs on reproductive performance, commercial crossbreed does from a commercial farm (Altura, Castellón, Spain) were inseminated using semen from 50 Line R adult males. In order to have the same high receptivity rate, nulliparous and multiparous non-lactating does (females with more than one delivery without suckling rabbits) received an i.m. injection of 15 and 20 IU of eCG respectively, two days before insemination.

The trial lasted from July to December 2016. Animals were housed in flat deck cages, under a 16-h light: 8-h darkness photoperiod, fed a standard diet (17.5% crude protein, 2.3% ether extract, 16.8 % crude fibre, 2600 Kcal DE/Kg) and had free access to water.

2.2. Semen collection and evaluation

Two ejaculates per male were collected with a minimum of 30 minutes between ejaculate collections, on a single day using an artificial vagina. Sperm evaluation was performed to assess the initial seminal quality. Only ejaculates exhibiting a white colour and possessing more than 70% of motility rate, 85% of normal intact acrosome, and less than 15% of abnormal sperm were used for this experiment. All other ejaculates were discarded.

The remaining pooled semen was split into three aliquots and diluted 1:20, respectively with: (1) TCG extender supplemented with 10 µg of buserelin acetate/mL; (2) TCG extender supplemented with bestatin (10 µM), EDTA (20 mM) and 10 µg of buserelin acetate/mL; and (3) TCG extender (non GnRH - supplemented extender).

2.3. Insemination procedure

A total of 887 inseminations were performed along three different days. Females were inseminated with 0.5 mL of semen using standard curved cannulas (24 cm). Each female was randomly assigned to one of the three experimental groups:

- Positive control group: does inseminated with 0.5 mL diluted semen in TCG. At the time of insemination, females were treated intramuscularly with 1 µg of buserelin acetate to induce ovulation.

- Negative control group: does inseminated with 0.5 mL diluted semen in TCG extender supplemented with 10 µg/mL of buserelin acetate.

- Aminopeptidase inhibitors group: does inseminated with 0.5 mL diluted semen in TCG extender supplemented with bestatin (10 µM), EDTA (20 mM) and 10 µg/mL of buserelin acetate.

After diluting the semen in the three experimental extenders, the insemination was initiated immediately. About two hours elapsed between the first and the last inseminated female.

Pregnancy rate at birth (number of does giving birth/number of inseminated does) and prolificacy (number of total kits born) were the reproductive performances indicators considered.

3. Statistical analysis

The effect of the aminopeptidase activity inhibitors on motility, viability acrosome integrity, APN activity and β-NGF quantity was analysed by ANOVA using the general linear models procedure. A chi-square test was used to test differences in pregnancy rate at birth between groups and female reproductive status. A Kruskal-Wallis test was

performed to analyze the effect of the extender used on the total number of kits born per litter and a Mann-Whitney U test was used to analyse the interaction between the physiological state of the females and the total number of kits born per litter. All analyses were performed with SPSS 20.0 software package (SPSS Inc., Chicago, Illinois, USA). Values were considered statistically different at $p<0.05$. Results are presented as least square means (LSM) ± standard error of the mean (SE).

RESULTS

1. Effect of aminopeptidase inhibitors on seminal quality

Seminal quality parameters after the incubation of semen samples with the experimental extenders are shown in Table 2.1. The presence of AMIs had no effect on the total motility, either on the acrosome integrity, or on the viability of the spermatozoa. On the other hand, the APN activity was inhibited in the extender containing the AMIs (10 μM bestatin and 20 mM EDTA). The average APN activity in this group was 322.88 *versus* 725.58 in the control group (pmol of β-naphthylamide released per mg of protein per minute). Therefore, the APN activity in AMIs extender was 55.5% lower than in the control extender.

Table 2.1. Seminal quality after two hours' incubation at room temperature with the experimental extenders (%; least square means ± standard error). (n=35)

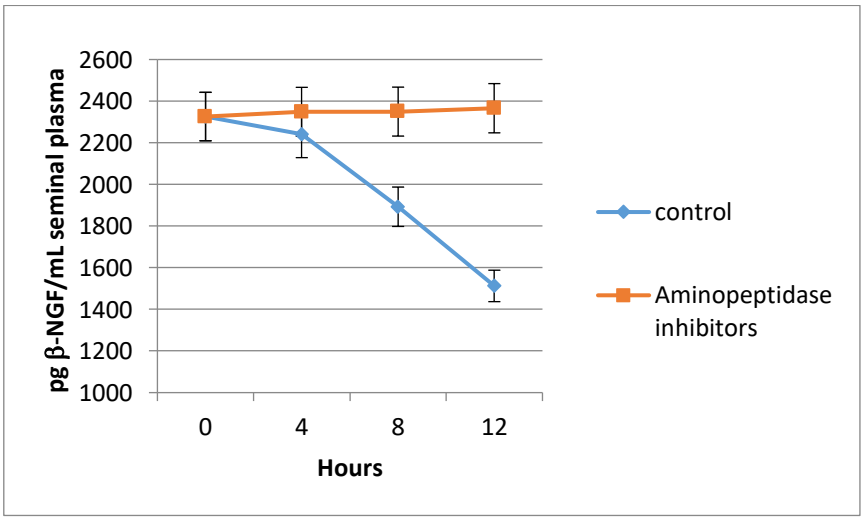
Extenders	Total Motility (%)	Acrosome integrity (%)	Viability (%)
TCG	75.00±4.47	86.25±4.31	64.33±5.83
TCG+GnRH analogue	78.83±4.28	86.53±4.11	68.55±5.83
TCG+GnRH analogue+AMIs	67.92±4.28	84.44±4.11	64.24±5.83

TCG: Tris-Citric acid-Glucose extender; GnRH analogue: 10 µg/mL busereline acetate; AMIs: Aminopeptidase inhibitors (10 µM bestatin and 20 mM EDTA).

2. Effect of aminopeptidase inhibitors on β-NGF quantity

The effect of the addition of AMIs to rabbit semen samples is represented in Figure 2.1. The results showed that the presence of AMIs improved the availability of β-NGF in the semen up to 12 hours of storage compared to control group (2350 *versus* 1550 pg β-NGF/mL seminal plasma, $p<0.05$).

Figure 2.1. Time-dependent effect of aminopeptidase inhibitors (10 µM bestatin and 20 mM EDTA) on seminal β-NGF (means ± standard error).



3. Effect of aminopeptidase inhibitors on fertility and prolificacy

Fertility rate at birth and prolificacy values are presented in Table 2.2. A total of 39 does died in the period from the insemination to kindling, 13 in the control group, 7 in the negative control and 19 in the AMIs group. Neither fertility nor prolificacy were affected by the experimental group, being both parameters similar between groups.

Table 2.2. Reproductive performance of inseminated does induced to ovulate with buserelin acetate applied intramuscularly (Positive Control) or intravaginally with buserelin acetate supplemented extender without (Negative control) or with AMIs.

Groups	Inseminated does (N)	Pregnancy rate at birth (%)	Total Born per litter (LSM±SE)
Positive Control	263	88.6 (233/263)	10.51 ± 0.19
Negative control	286	86.7 (248/286)	10.21 ± 0.19
AMIs	299	85.3 (255/299)	10.12 ± 0.19

Positive Control: inseminated females treated intramuscularly with 1 µg of buserelin acetate to induce ovulation. Negative control: females inseminated with 0.5 mL diluted semen in extender supplemented with 10 µg/mL of buserelin acetate. AMIs: females inseminated with 0.5 mL diluted semen in extender with 10 µM bestatin and 20 mM EDTA, and supplemented with 10 µg/mL of buserelin acetate. LSM±SE: Least square means ± standard error.

Regarding physiological status, nulliparous does showed significantly higher pregnancy rate at birth than the multiparous non-lactating does (91.3 % *vs*. 82.1 %; $p<0.05$). On the contrary, multiparous non-lactating does showed significant higher prolificacy than nulliparous does (10.69 ±0.18 *vs*. 9.91 ±0.14 kits per delivery; $p<0.05$).

DISCUSSION

The addition of the GnRH synthetic analogues to the seminal dose is a welfare-orientated method to induce ovulation in rabbits but the success of this method depends on the enzymes present in the seminal plasma and unfortunately, the concentration of GnRH analogue to be added to the seminal dose to achieve fertility

results similar to those of intramuscular administration is much higher than the amount administered intramuscularly. In a previous study we observed that the bioavailability of buserelin acetate when added to the seminal dose was determined by the activity of the existing seminal plasma aminopeptidases (Viudes-de-Castro et al., 2014) and in a latter work we showed that fertility rate was not affected by the addition of a protease inhibitor cocktail to the semen extender, but decreased the total number of kits born per litter (Casares-Crespo et al., 2016b). In the present work, the addition of specific aminopeptidase inhibitors such as bestatin and EDTA has shown no effects on fertility or prolificacy. Bestatin is a highly effective inhibitor of rabbit seminal plasma aminopeptidase activity (Casares-Crespo et al., 2015). In agreement with our results, the addition of bestatin to guinea pig sperm had no effect on membrane fusion (Flaherty et al., 1993) and the incubation of bovine sperm with EDTA did not affect the acrosome reaction (Deppe et al., 2008).

On the contrary, in non-mammal species, several authors reported that AMIs affect seminal quality and/or different fertilization steps. For instance, acrosome reaction was suppressed in the mussel in the presence of the bestatin (Togo and Morisawa, 2004), sperm binding to the vitelline envelope was inhibited in the frog (Kubo et al., 2008) and the fertilization process was inhibited in the sea urchin (Yasuhara et al., 1983). Similarly, the sperm incubation with EDTA inhibited the acrosome reaction in sea urchin sperm (Farach et al., 1987). In addition, puromycin-sensitive aminopeptidase-deficient mice are infertile, lack copulatory behavior, and have impaired spermatogenesis (Osada et al., 2001), suggesting that aminopeptidase activity is necessary for the fertilization in this species. Therefore, it seems that the effect of AMIs on semen and fertilization is species-specific.

The present results showed that the addition of bestatin and EDTA in the rabbit semen extender has neither effect on semen quality nor on the fertilizing capacity of spermatozoa. In contrast with previous paper (Casares-Crespo et al., 2016b) where fertility rate of group intramuscularly treated with the GnRH analogue was significantly higher than intravaginal treated groups, in the present experiment, all groups showed the same pregnancy rate. The lack of fertility differences between intramuscularly and intravaginal GnRH administration could be addressed to the reproductive status of does used. It should be underlined that in the current paper all does were non-lactating, which is assumed to increase the fertility rate. On the other hand, previous paper showed (Casares-Crespo et al., 2016b) that the prolificacy of semen extender containing the same dilution rate (1:20) and GnRH amount (5 µg/AI) but with a wide variety of AMIs, was lower than semen extender without AMIs or control group with GnRH administered i.m. (8.2 *vs.* 9.3 and 9.2 total born per litter, respectively). The fecundation process damaged in our previous work by protease inhibition seems to not be affected by bestatin and EDTA, showing a similar prolificacy rate in groups with or without AMIs. In addition, the largest amounts of prostasomes in rabbit seminal plasma, which affect sperm kinetics traits and reactiveness of sperm to undergo capacitation and acrosome reaction (Castellini et al., 2013) seems responsible of a time-dependent modulation between ovulation and fertilization. Maranesi et al. (2015) hypothesized a mediator role of β-NGF on the modulation of ovulation/fertilization events. Furthermore, it is possible that a broad AMIs differently affected ovulation and fertilization processes, and the behavior of spermatozoa and their response to these inhibitors agents might alter the delicate equilibrium involved in capacitation and acrosome reaction processes. In the present study there is a huge

degradation of β-NGF starting from 8h until 12h. Considering the prolificacy results of the present work, the possible ovulation/fertilization modulator role of β-NGF is assured over this time by the presence of bestatin and EDTA in the extender, being able to protect β-NGF from enzyme degradation.

Since the bioavailability of buserelin acetate when added to the rabbit seminal dose appears to be partly determined by the activity of the seminal plasma aminopeptidases (Viudes-de-Castro et al., 2014), with bestatin and EDTA added to the semen extender, the hormone concentration could be reduced. Therefore, when using sperm extenders supplemented with the GnRH analogue, in order to reduce the GnRH concentration used, with the present results, the co-administration of EDTA and bestatin seems appropriate.

In the future, the next step would be to decrease the GnRH analogue concentration in semen extender with AMIs and to study its effect on fertility and prolificacy and the role of β-NGF on synchrony of the ovulation/fertilization process.

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V.CHAPTER III

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Protection of GnRH analogue by chitosan-dextran sulfate nanoparticles for intravaginal application in rabbit artificial insemination

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ABSTRACT

The present study was designed to prove new rabbit insemination extenders containing aminopeptidase inhibitors (AMIs) with or without chitosan (CS)-dextran sulfate (DS) nanoparticles entrapping the GnRH analogue. In addition, different hormone concentrations were tested in these extenders, evaluating their *in vivo* effect on rabbit reproductive performance after artificial insemination. A total of 911 females were inseminated with semen diluted with the four experimental extenders (C4 group: 4 µg buserelin/doe in control medium (Tris-citric acid-glucose supplemented with bestatin 10 µM and EDTA 20 mM), C5 group: 5 µg of buserelin/doe in control medium, Q4 group: 4 µg of buserelin/doe into CS-DS nanoparticles in control medium, Q5 group: 5 µg of busereline/doe into CS-DS nanoparticles in control medium). Results showed that fertility was significantly lower in C4 group compared to C5, Q5 and Q4 groups (0.7 *versus* 0.85, 0.85 and 0.82, respectively). On the contrary, prolificacy was similar in the four experimental groups studied (P>0.05). We conclude that the CS-DS nanoparticles prepared by a coacervation process as carrier for buserelin acetate allow to reduce the concentration of hormone used in extenders supplemented with bestatin and EDTA without affecting the fertility and prolificacy of rabbit females.

INTRODUCTION

The vagina has been rediscovered as a potential route for systemic delivery of peptides and proteins (Hussain and Ahsan, 2005; Jitendra et al., 2011). The rich blood supply and the large surface area of the vaginal mucosa enable rapid absorption of low molecular weight drugs (Gupta et al., 2011; Jitendra et al., 2011). Artificial insemination with GnRH supplemented extenders is a welfare-orientated method to induce ovulation in rabbits. There are clear breeding advantages of intravaginal administration of GnRH analogue (noninvasive route, less treatment distress, labor for the farmers, and operating time), but unfortunately, to achieve fertility results similar to those with GnRH intramuscular injection, the intravaginally hormone concentration should be much higher than the amount administered intramuscularly (Viudes-de-Castro et al., 2014), being a potential health risk for the farmers. The absorption of GnRH by vaginal mucosa is influenced by several factors. The main barrier is mucosal permeation, but another factor that limits the bioavailability of GnRH analogue is the proteolytic activity found in the seminal plasma as well as in the female vagina. Various approaches to improve protein delivery by vaginal route include: use of enzyme inhibitors, absorption enhancers, mucoadhesive polymers and/or novel carrier systems such as nanoparticles. In previous works, we have proved that rabbit's seminal plasma aminopeptidase activity affects the bioavailability of GnRH analogues added to the insemination extenders (Viudes-de-Castro et al., 2014). As a consequence, we have been trying to develop new extenders supplemented with protease and aminopeptidase inhibitors in order to protect the hormone from being degraded without affecting reproductive performance (Casares-Crespo et al., 2016, 2018). We have observed that extender supplementation with aminopeptidase inhibitors (AMIs)

as bestatin and EDTA did not affect rabbit seminal quality nor reproductive performance (Casares-Crespo et al., 2018), but inhibited part of the seminal plasma aminopeptidase activity. Another possible approach in order to protect the hormone from enzyme degradation would be to encapsulate the GnRH analogue. Nanoparticles of biodegradable polymers have extensively been studied over last few decades in pharmaceutical research for controlled drug delivery. Recently, proteins such as lutein, insulin, rhodamine 6G and bovine serum albumin (BSA) have been entrapped in nanoparticles of chitosan (CS) and dextran sulfate (DS) for their delivery in oral or ocular mucosa (Chen et al., 2007; Pechenkin et al., 2011; Chaiyasan et al., 2015). CS and DS are biodegradable, biocompatible and non-toxic polymers of natural origin with high adsorption capacity, which are widely used in pharmaceutical formulations (Chen et al., 2003; Domínguez-Delgado et al., 2014). CS-DS nanoparticles containing buserelin acetate have been developed and *in vitro* tested (Fernández-Serrano et al., 2017). In this study, we achieved a hormone entrapment efficiency of 40-50% and showed that these nanoparticles did not affect rabbit seminal quality parameters and, in addition, significantly increased the acrosome integrity of spermatozoa. Therefore, the next step would be to reduce hormone concentration in the insemination extender to check if these systems are able to protect the hormone from seminal plasma enzyme degradation.

Hence, the current study aims to evaluate the effect of a 20% reduction of hormone concentration in extenders supplemented with AMIs and with the GnRH analogue free or entrapped in CS-DS nanoparticles on rabbit reproductive performance.

MATERIALS AND METHODS

Busereline acetate was purchased from Hoechst Marion Roussel, S.A. (Madrid, Spain); DS was purchased from Thermofisher Acros Organics (Geel, Belgium) and SYBR-14 and propidium iodide (PI) were purchased from Invitrogen (Barcelona, Spain). All other chemicals and reagents were purchased from Sigma-Aldrich Química S.A. (Madrid, Spain).

1. Animals

All animals were handled according to the principles of animal care published by the Directive 2010/63/EU. The trial lasted from January to October 2017. Commercial crossbreed does from a commercial farm (Altura, Castellón, Spain), were inseminated using semen from 50 Line R adult males. Animals were housed in flat deck cages, under a 16-h light: 8-h darkness photoperiod, fed a standard diet (17.5% crude protein, 2.3% ether extract, 16.8 % crude fibre, 2600 Kcal DE/Kg) and had free access to water.

2. Semen collection and evaluation

Two ejaculates per male were collected with a minimum of 30 minutes between ejaculate collections, on a single day using an artificial vagina. A subjective sperm evaluation was performed to assess the initial seminal quality. Only ejaculates exhibiting a white color and possessing more than 70% of motility rate, 85% of normal intact acrosome, and less than 15% of abnormal sperm were used in this experiment. All other ejaculates were discarded.

After the insemination procedure, the seminal quality of an aliquot of each experimental extender was evaluated. A 20 µL aliquot was diluted 1:50 with 0.25%

glutaraldehyde solution to calculate the concentration and the percentage of spermatozoa with normal apical ridge (NAR, percentage of acrosome integrity), in a Thoma chamber by phase contrast at a magnification of 400X.

The motility characteristics of sperm (percentage of total motile sperm, evaluated using a computer-assisted sperm analysis system) were determined as described by Viudes de Castro et al. (2014). Briefly, sperm samples were adjusted to 7.5 x 10^6 sperm/mL with TCG extender supplemented with 2 g/L BSA and motility was assessed at 37ºC. A spermatozoa was defined as non-motile if the average path velocity (VAP) was <10 µm s-1 and a spermatozoon was considered to be progressively motile when VAP was >50 µm s-1 and the straightness index (STR) was ≥70%.

Flow cytometry analyses to assess viability were performed using a Coulter Epics XL cytometer (Beckman Coulter, IZASA, Barcelona, Spain). The fluorophores were excited by a 15 mW argon ion laser operating at 488 nm. A total of 10,000 gated events (based on the forward scatter and side scatter of the sperm population recorded in the linear mode) were collected per sample. Flow cytometry data were analyzed with the software Expo32ADC (Beckman Coulter Inc.). Samples were diluted to 30 x 10^6 sperm/mL with TCG extender supplemented with 2 g/L BSA. All the dilutions were performed at 22 ºC. The percentage of viable sperm was determined using a dual fluorescent staining with SYBR-14/PI according to Viudes-de-Castro et al. (2014). Only the percentages of live sperm were considered in the results (SYBR-14-positive and PI-negative).

3. Preparation of GnRH-loaded CS-DS nanoparticles

CS and DS were dissolved (0.05%) in the Control medium, which consisted in Tris-citric acid-glucose (TCG), supplemented with bestatin 10 µM and EDTA 20 mM (Casares-Crespo et al., 2018). Incorporation of buserelin acetate into nanoparticles was achieved by dissolving the hormone in DS solution in order to obtain the desired final GnRH concentration in the diluted semen (8 and 10 µg/mL for Q4 and Q5 extenders, respectively). Nanoparticles were spontaneously formed on incorporation of CS solution into DS solution (4:1) through magnetic stirring (~600 rpm) during 30 minutes at room temperature.

4. Semen preparation

The seminal pools were first diluted 1:4 (vol:vol) with Control medium and then were split into four equal fractions, which were diluted 1:5 with one of the four experimental extenders, respectively, in order to obtain the desired final GnRH concentration in the diluted semen:

- C5 fraction: diluted with control medium supplemented with busereline acetate to obtain a final concentration of 10 µg/mL busereline acetate.
- C4 fraction: diluted with control medium supplemented with busereline acetate to obtain a final concentration of 8 µg/mL busereline acetate.
- Q5 fraction: diluted with Q5 extender to obtain a final concentration of 10 µg/mL of busereline acetate-loaded into CS-DS nanoparticles.
- Q4 fraction: diluted with Q4 extender to obtain a final concentration of 8 µg/mL of busereline acetate-loaded into CS-DS nanoparticles.

5. Insemination procedure

In order to achieve the same high receptivity rate, nulliparous and multiparous non-lactating does (females with more than one delivery without suckling rabbits) received an intramuscular injection of 15 and 20 IU of eCG respectively, two days before insemination. To induce ovulation, the GnRH analogue buserelin acetate was used. A total of 911 inseminations were performed in three different days. Females were inseminated with 0.5 mL of diluted semen using standard curved cannulas (24 cm). Each female was randomly assigned to one of the four experimental extender groups:

C4 group: 4 µg buserelin/doe in control medium.

C5 group: 5 µg of buserelin/doe in control medium.

Q4 group: 4 µg of buserelin/doe into CS-DS nanoparticles in control medium.

Q5 group: 5 µg of busereline/doe into CS-DS nanoparticles in control medium.

Pregnancy rate at birth (number of does giving birth/number of inseminated does) and prolificacy (number of total and alive kits born) were the reproductive performances considered.

6. Statistical analysis

The effect of AMIs and CS-DS nanoparticles on total motility, acrosome integrity and viability was analysed by ANOVA using the general linear model procedure. A probit link with binomial error distribution was used to analyze the fertility rate at birth, including as fixed effects the extender group and the reproductive status of the females (nulliparous and multiparous) and their interactions. For total number of kits born per litter, an ANOVA was performed, including as fixed effects the extender group

and physiological state and their interaction. For the number of live born kits per litter, a covariance analysis was used, including the total number of kits born per litter as covariable. All analyses were performed with SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA). Values were considered statistically different at $P<0.05$. Results are presented as least square means (LSM) ± standard error of the mean (SE).

RESULTS

1. Seminal quality after insemination with experimental extenders

Seminal quality parameters of samples from the experimental extenders are shown in Table 3.1. The presence of AMIs and CS-DS nanoparticles had no effect on the total motility, either on the acrosome integrity, or on the viability of the spermatozoa.

Table 3.1. Seminal quality after insemination procedure with the experimental extenders (%; Least square means ± standard error) (n=3).

Extenders	Total Motility (%)	Acrosome integrity (%)	Viability (%)
C4	68.5±10.6	89.5±6.3	76.5±2.7
C5	63.0±10.6	80.9±6.3	73.3±2.7
Q4	59.0±10.6	83.9±6.3	73.1±2.7
Q5	68.5±10.6	87.7±6.3	69.5±2.7

C4: 4 µg busereline/doe in control medium (Tris-citric acid-glucose supplemented with bestatin 10 µM and EDTA 20 mM); C5: 5 µg of busereline/doe in control medium; Q4: 4 µg of busereline/doe into chitosan-dextran sulfate (CS-DS) nanoparticles in control medium; Q5: 5 µg of busereline/doe into CS-DS nanoparticles in control medium.

2. Reproductive performance of experimental extenders

Fertility rate at birth and prolificacy values are presented in Table 3.2. An interaction was found between extender group and reproductive status on fertility rate. Fertility was significantly lower in the C4 group and without differences between nanoparticles groups Q4 and Q5 and control group C5. Regarding physiological status, nulliparous does showed significantly higher fertility than multiparous non-lactating does (Table 3.2). The results of the interaction indicated that nulliparous does from Q5 group showed significantly higher fertility than multiparous non-lactating does, while in the other groups, no significant difference was observed between females with different reproductive status.

No interactions were found between extender group and reproductive status on the total number of kits born per litter and number of alive kits born per litter. Prolificacy was similar in all experimental groups. The physiological status significantly affected the prolificacy. Multiparous non-lactating does showed significantly higher prolificacy of total number of kits born per litter than nulliparous does (Table 3.2).

Table 3.2. Reproductive performance of inseminated does.

Group	**N**	**Fertility at birth**	**TB**	**AB**
C4	294	0.70±0.03^{a}	10.69±0.20	10.27±0.12
C5	343	0.85±0.02^{b}	10.68±0.19	10.16±0.11
Q4	112	0.85±0.04^{b}	11.12±0.33	10.23±0.20
Q5	162	0.82±0.03^{b}	11.22±0.29	9.94±0.18
Reproductive Status	**N**	**Fertility at birth**	**TB**	**AB**
MNL	496	0.77±0.03^{a}	11.53±0.21^{a}	10.22±0.13
N	415	0.84±0.02^{b}	10.31±0.16^{b}	10.07±0.10

Group*Reproductive status				
C4*MNL	173	0.66±0.04[a]	11.21±0.27	10.36±0.16
C4*N	121	0.74±0.04[ac]	10.17±0.30	10.19±0.18
C5*MNL	244	0.88±0.02[bd]	11.14±0.20	10.17±0.12
C5*N	99	0.81±0.04[bce]	10.21±0.32	10.15±0.19
Q4*MNL	32	0.84±0.06[bcdf]	11.59±0.55	10.33±0.33
Q4*N	80	0.86±0.04[bg]	10.64±0.35	10.12±0.21
Q5*MNL	47	0.66±0.07[aef]	12.19±0.52	10.04±0.31
Q5*N	115	0.92±0.03[dg]	10.24±0.28	9.84±0.17
Total	911	0.81±0.02	10.92±0.13	10.15±0.08

TB: total number of kits born per litter; AB: number of alive kits born per litter; C4: 4 µg busereline/doe in control medium (Tris-citric acid-glucose supplemented with bestatin 10 µM and EDTA 20 mM); C5: 5 µg of busereline/doe in control medium; Q4: 4 µg of busereline /doe into chitosan-dextran sulfate (CS-DS) nanoparticles in control medium; Q5: 5 µg of busereline /doe into CS-DS nanoparticles in control medium; MNL: females with more than one delivery without suckling rabbits; N: nulliparous does; Values within a column with different superscripts in the same column differ significantly at $P<0.05$.

DISCUSSION

In rabbit artificial insemination, the administration of GnRH analogues in the seminal dose presents clear advantages *versus* intramuscular administration. However, due to degradation by aminopeptidases and the low absorption in the vagina mucosa, there is a decrease in the analogues' bioavailability and large doses are required for ovulation induction following vaginal administration. According to a previous work (Casares-Crespo et al., 2018), the employment of bestatin and EDTA in the rabbit insemination

extenders inhibited part of the seminal aminopeptidase activity without affecting reproductive performance. On the other hand, we have developed CS-DS nanoparticles to entrap the GnRH analogue and a previous *in vitro* characterization showed that these nanoparticles did not affect rabbit seminal quality (Fernández-Serrano et al., 2017).

Therefore, to increase the bioavailability of GnRH when intravaginally route is used, in the present study a double approach was used to protect the GnRH analogue against enzymatic degradation, the use of aminopeptidases inhibitors as bestatin and EDTA or/and the use of polymers as chitosan and dextran sulfate to encapsulate the GnRH analogue. Our hypothesis was that these strategies were able to protect the GnRH analogue and in consequence it would be possible to reduce the quantity of hormone used in the extender to induce ovulation. According to our results, when the buserelin acetate was non encapsulated, although the extenders were supplemented with bestatin and EDTA, the utilization of 4 μg hormone/doe significantly reduced fertility rate compared to group with 5 μg hormone/doe. This fact shows that even though part of the enzymatic activity of seminal plasma is inhibited, the bioavailability of GnRH is not enough to allow a 20% reduction in the concentration of hormone in the extender without compromising fertility. It is possible that we are working with a limiting hormone concentration (5 μg/doe) and even a small hormone reduction could affect fertility. In this sense, there is only another work in which a GnRH analogue concentration lower than 5 μg/doe has been used in rabbit ovulation induction, and the results were the same as ours, with fertility rate significantly lower and similar prolificacy rate (2.5 μg/doe GnRH-Lecirelinum in seminal dose) (Ondruška et al., 2008).

On the other hand, when buserelin acetate was encapsulated in CS-DS nanoparticles, no differences in fertility and prolificacy were observed between 4 µg hormone/doe or 5 µg hormone/doe, showing similar values than C5 group. Thus, with the use of nanoparticles, the GnRH analogue seems to be protected against degradation and a 20% hormone reduction does not affect fertility. In resemblance with our results, Trapani et al. (2010) employed CS based nanoparticles in oral administration of a small peptide (glutathione), and they achieved to protect the drug from the enzymatic gastric degradation and induce permeabilization of the intestinal epithelia. In addition, Han et al. (1995), in an *in vitro* study in rabbit, observed that the permeability of the vaginal membrane to GnRH increased twice when EDTA was used, suggesting that enzyme inhibition effect of EDTA resulted in substantial enhancement of vaginal absorption. Therefore, the enzyme inhibitor role of bestatin and EDTA besides the absorption enhancement effect of EDTA and the protection role of chitosan and dextran sulfate nanoparticles and their mucoadhesive function, all together, could explain the fertility rate improvement of Q4 group compared to C4 group.

In conclusion, the CS-DS nanoparticles prepared by coacervation process as carrier for buserelin acetate overcome some of the limitations associated with the vaginal application of the hormone in rabbit artificial insemination and allow to reduce the concentration of hormone used in an extender supplemented with bestatin and EDTA without affecting the fertility and prolificacy of rabbit females. Therefore, nanoencapsulation seems to be a promising system to protect the GnRH analogue in order to decrease the hormone concentration in rabbit artificial insemination extenders.

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VI.CHAPTER IV

VI.CHAPTER IV

Can the genetic origin affect rabbit seminal plasma protein profile along the year?

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ABSTRACT

The study was designed to evaluate the influence of genetic origin on rabbit seminal plasma protein profile variation along the year. Seminal plasma of rabbits from line A (maternal line) and R (paternal line) collected during a natural year was subjected to polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoretic profile of rabbit seminal plasma resulted in multiple protein bands of different intensity ranging from 9 to 240 kDa. Results showed that seven protein bands were significantly different between genetic lines and among these three protein bands were significantly different between seasons. The differentially expressed proteins were identified by MALDI TOF/TOF or LC-MS/MS analysis and were the following ones: FAM115E-like (220, 113 and 59 kDa), ectonucleoside triphosphate diphosphohydrolase 3 isoform X2 (72 kDa), annexin A5 (32 kDa), lipocalin allergen Ory c 4 precursor (19 kDa), and hemoglobin subunit zeta-like (13 kDa) between genetic lines and FAM115E-like (113 kDa), hemoglobin subunit zeta-like (13 kDa) and β-Nerve growth factor (12 kDa) between seasons. These results indicate that proteins from rabbit seminal plasma are under both seasonal and genetic control. Furthermore, the differential presence of these proteins could be one of the causes explaining the differences observed in fertility and seminal parameters between these two lines in earlier studies.

INTRODUCTION

Seminal plasma is a promising source for the study of potential reproductive biomarkers, because it is a complex mixture of secretions from testis, epididymis and male accessory sex glands (González-Cadavid et al., 2014). Species of mammals differ regarding the presence and size of accessory sexual glands, which obviously lead to variations in their relative contribution to semen composition and volume, particularly regarding seminal plasma (Rodríguez-Martínez et al., 2011). Seminal fluid is very complex and plays an important role in the fertilizing ability of sperm (La Falci et al., 2002). The protein composition of mammalian seminal plasma varies among species, and has important effects on sperm function (Mortarino et al., 1998). These proteins participate in various events related to sperm function such as epididymal sperm maturation, sperm capacitation, sperm membrane stabilization and even the interaction with the oviduct and oocyte (Topfer-Petersen et al., 1998; Gwathmey et al., 2006). The diversity in the seminal plasma protein composition of different species of mammals may explain the variation in reproductive capacity and function (Druart et al., 2013). In rabbits, seminal plasma has a positive effect in maintaining sperm motility and viability during *in vitro* storage (Castellini et al., 2000). This effect has been associated with the antioxidant properties of seminal plasma (Arruda-Alencar et al., 2012).

In some species, seminal plasma proteins related to fertility (Killian et al., 1993; Brandon et al., 1999; Ashrafzadeh et al., 2013), cold-shock damage sperm membrane resistance (Barrios et al., 2000; Pérez-Pé et al., 2001), sperm tolerance to freezing (Goularte et al., 2014), freezability (Jobim et al., 2011) and semen quality (Cardozo et al., 2006; De Souza et al., 2007; Arruda-Alencar et al., 2012; Nandre et al., 2013; Kiso et

al., 2013) have been identified. In addition, differences in seminal plasma protein composition among seasons have been examined in several species (goat: La Falci et al., 2002; Teixeira et al., 2009; ram: Cardozo et al., 2006; Domínguez et al., 2008; buffalo: Nandre et al., 2013; Sharma et al., 2014). In an earlier study, Viudes-de-Castro et al. (2004) studied the protein composition of rabbit A and R lines seminal plasma and observed two line-specific protein bands (line A 41 kDa; line R 13 kDa). On the contrary, in a recent study with the same rabbit lines, Safaa et al. (2008) found no differences in the protein profile. Both studies were conducted in different seasons, the first one in winter and the second in spring, which could indicate the possible existence of seasonal variations in the seminal plasma protein composition in this species.

On the other hand, rabbit semen characteristics vary from one season to another (Marai et al., 2002). Several studies have found variations of rabbit sperm parameters depending on the environmental conditions (Marai et al., 2002; Nizza et al., 2003; Pascual et al., 2004; Roca et al., 2005; Schneidgenová et al., 2011; Ain-Baziz et al., 2012; Sabés-Alsina et al., 2015; Theau-Clément et al., 2015). For instance, Theau-Clément et al. (2015) observed that except for pH, all rabbit semen characteristics were influenced by the season, being sperm production higher in autumn. In addition, Schneidgenová et al. (2011) showed the seasonal effect on the fertility traits of rabbit ejaculates and concluded that is necessary to evaluate the semen quality of bucks throughout the year. The aim of the present study was to evaluate the effect of genetic line and season on rabbit seminal plasma protein profile along the year.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals in this study were purchased from Sigma-Aldrich Química S.A (Madrid, Spain).

1. Animals

All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013.

To study the seasonal variation of seminal plasma proteins, a total of 24 adult bucks, 12 belonging to a paternal rabbit line (Line R) and 12 from a maternal rabbit line (Line A) were used. All bucks were of proven fertility and subjected to a weekly pattern of ejaculate collection. Males were kept individually in flat deck cages under 16 h light/8 h dark conditions at the experimental farm of the Animal Technology and Research Centre (CITA-IVIA, Segorbe, Castellón, Spain) and fed *ad libitum* with the same commercial diet (17.5% crude protein, 2.3% ether extract, 16.8% crude fiber, 2.600 kcal DE/Kg) and had free access to water.

2. Semen collection

One ejaculate per male was collected each week using an artificial vagina. Sperm evaluation was performed to assess the initial seminal quality. Only ejaculates exhibiting a white colour and possessing more than 70 of motility rate, 85% of normal intact acrosome, and less than 15% of abnormal sperm were used in this experiment. All ejaculates from the same line were pooled (12 pools from Line R and 12 pools from Line A, 3 pools for each season and line). The sperm recovery was performed from January to December 2014. Table 4.1 reports the minimum, maximum and mean temperatures recorded during the whole period of study.

Table 4.1. Temperatures registered during the trial (year 2014) in Segorbe (mean ± SD).

Season	Temperature (ºC)		
	Minimum	Maximum	Average
Winter (21st December-20th March)	4.36 ± 0.11	16.87 ± 1.58	10.69 ± 0.63
Spring (21st March-20th June)	10.77 ± 2.76	25.15 ± 2.56	18.14 ± 2.80
Summer (21st June-20th September)	16.56 ± 1.28	29.62 ± 0.97	22.98 ± 0.87
Autumn (21st September-20th December)	7.47 ± 4.04	20.02 ± 5.74	13.43 ± 4.89

3. Preparation of seminal plasma samples

Semen samples were centrifuged at 7400 x g for 10 min at 22 ºC. The resulting supernatants were collected and centrifuged again (7400 x g for 10 min) to remove residual spermatozoa and cell debris. The resulting pellets were discarded, whereas the supernatants were collected, supplemented with a protease inhibitor cocktail (P2714, Sigma) diluted 1:100 and stored at -80ºC until use.

After thawing, total protein concentration was quantified by the bicinchoninic acid method (BCA) using BSA as standard protein (Smith et al., 1985). To perform electrophoresis, samples were diluted in saline at a concentration of 5 µg/µL. After this, three pools of each season were pooled resulting in one sample per line and season (8 samples in total). Later, samples were mixed with 2x Laemmli buffer and boiled at 95ºC for 5 minutes.

4. Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were electrophoresed using two polyacrylamide concentrations, 13 and 22%. A 5% stacking gel was used in all runs. The total loaded quantity of protein in each well was 25 µg in 10 µL. Gels were run in a PROTEAN® II XL Cell (Bio-Rad Laboratories, Hercules, CA, USA) with constant voltage of 180 V for 3h and 250V for 5h. The molecular weight was estimated using Precision Plus Protein™ Dual Xtra Standards (Bio-Rad). At the end of the electrophoresis, the gels were fixed with a fixation solution (50 % water, 40 % absolute ethanol, 20% acetic acid) during 20 minutes, stained with Coomassie Colloidal Blue (Bio-Rad) overnight and then destained with three changes of destaining solution (water) at room temperature.

The 1D gel images were processed for analysis with Quantity One 1-D software (Bio-Rad) to determine the molecular weight and relative quantity of each protein band.

5. Protein identification

The eight protein bands that resulted significantly different between genetic lines and seasons were carefully excised and places into micro tubes. The proteomic analysis was carried out in the SCSIE University of Valencia Proteomics Unit, a member of the PRB2-ISCIII ProteoRed Proteomics Platform.

6. MALDI TOF/TOF analysis

6.1. Sample preparation

Samples were digested with sequencing grade trypsin (Promega) as described elsewhere (Shevchenko et al., 1996). The digestion was stopped with Trifluoroacetic

acid (1% final concentration); the digested peptides were concentrated to 15 µL. A BSA plug was analysed in the same way to control the digestion process.

6.2. MALDI TOF/TOF analysis

Previously, the plate and the acquisition methods were calibrated with 0.5 µL the CM5 calibration mixture (AB Sciex), in 13 positions. The resulting mixtures were analyzed in a 5800 MALDI TOF/TOF (AB Sciex) in positive reflectron mode (3000 shots every position). Five of the most intense precursors (according to the threshold criteria: minimum signal-to-noise: 10, minimum cluster area: 500, maximum precursor gap: 200 ppm, maximum fraction gap: 4, were selected for every position for the MSMS analysis. And, MS/MS data was acquired using the default 1kV MS/MS method.

6.3. Protein identification

The MS-MSMS information was sent to MASCOT search engine v 4.0 (Matrix-Science) via the Protein Pilot (AB Sciex). Database search was performed on NCBI database. Searches were done with tryptic specificity allowing one missed cleavage and a tolerance on the mass measurement of 100 ppm in MS mode and 0.8 Da in MSMS mode. Carbamidomethylation of Cys was used as a fixed modification and oxidation of Met and deamidation of Asn and Gln as variable modifications.

7. LC MS/MS analysis

Two protein bands (220 and 19 kDa) could not be identified by MALDI TOF/TOF analysis because they were a mixture of different proteins. Therefore, a liquid chromatography and tandem mass spectrometry (LC MS/MS) analysis was performed.

7.1. Sample preparation

Samples were digested as described previously. The digestion was stopped with Trifluoroacetic acid (1% final concentration); the digested peptides were concentrated to 20 µL (band 220 kDa) or 10 µL (band 19 kDa).

7.2. LC MS/MS analysis

5 µL of every sample (except the main bands) were loaded onto a trap column (nanoLC Column, 3µm C18-CL, 350µm diameter x 0.5mm; Eksigen Technologies) and desalted with 0.1% TFA at 3µL/min during 5 min. The peptides were then loaded onto an analytical column (LC Column, 3 µm particles size C18-CL, 75µm diameter x 12cm long, Nikkyo) equilibrated in 5% acetonitrile (ACN) 0.1% FA (formic acid). Elution was carried out with a linear gradient of 5 to 45% solvent B in A for 45 min. (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 300nL/min. Peptides were analysed in a mass spectrometer nano ESI qQTOF (5600 TripleTOF, AB Sciex). The triple TOF was operated in information-dependent acquisition mode, in which a 0.25-s TOF MS scan from 350–1250 m/z was performed, followed by 0.05-s product ion scans from 100–1500 m/z on the 50 most intense 2-5 charged ions.

7.3. Protein identification

ProteinPilot search engine v 4.5. (AB Sciex) default parameters were used to generate peak list directly from 5600 Triple TOF wiff files. The Paragon algorithm of Protein Pilot was used to search NCBI protein database with the following parameters: trypsin specificity, iodoacetamide cys-alkylation, no taxonomy restriction except for some particular spots, and the search effort set to rapid. To avoid using the same spectral evidence in more than one protein, the identified proteins are grouped based on MS/MS spectra by the Protein-Pilot Progroup algorithm. Thus, proteins sharing MS/MS

spectra are grouped, regardless of the peptide sequence assigned. The protein within each group that can explain more spectral data with confidence is shown as the primary protein of the group. Only the proteins of the group for which there is individual evidence (unique peptides with enough confidence) are also listed, usually toward the end of the protein list.

8. Statistical analysis

Data were statistically evaluated with Statgraphics® Plus 5.1 library procedures (Statistical Graphics Corp., Rockville, MO, USA). To analyze the differences in relative quantity of each protein band based on the genetic line and season, a two-way analysis of variance (ANOVA) was used. Means were separated using the Fishers Least Significant Difference (LSD) test at a fixed 5% error level and the results are presented as the least square mean values (LSM) ± the standard error (SE).

RESULTS

Gel images are shown in Figure 4.1 and 4.2. The electrophoretic profile of rabbit seminal plasma resulted in multiple bands of different intensity ranging from 9 to 240 kDa. A total of 30 protein bands were identified, 21 and 13 in the 13 and 22% gels, respectively, with four bands in common between both gels. In the 13% gel, molecular weights ranged from 241 to 25 kDa and in the 22% gel, from 37 to 9.7 kDa.

Figure 4.1. 5-13% SDS-PAGE of rabbit seminal plasma proteins. Lane 1 and 6 are molecular weight markers (250 to 25 kDa). Lane 2 and 3 are A and R line in winter, respectively. Lane 4 and 5 are A and R line in spring. Lane 7 and 8 are A and R line in summer. And lane 9 and 10 are A and R line in autumn.

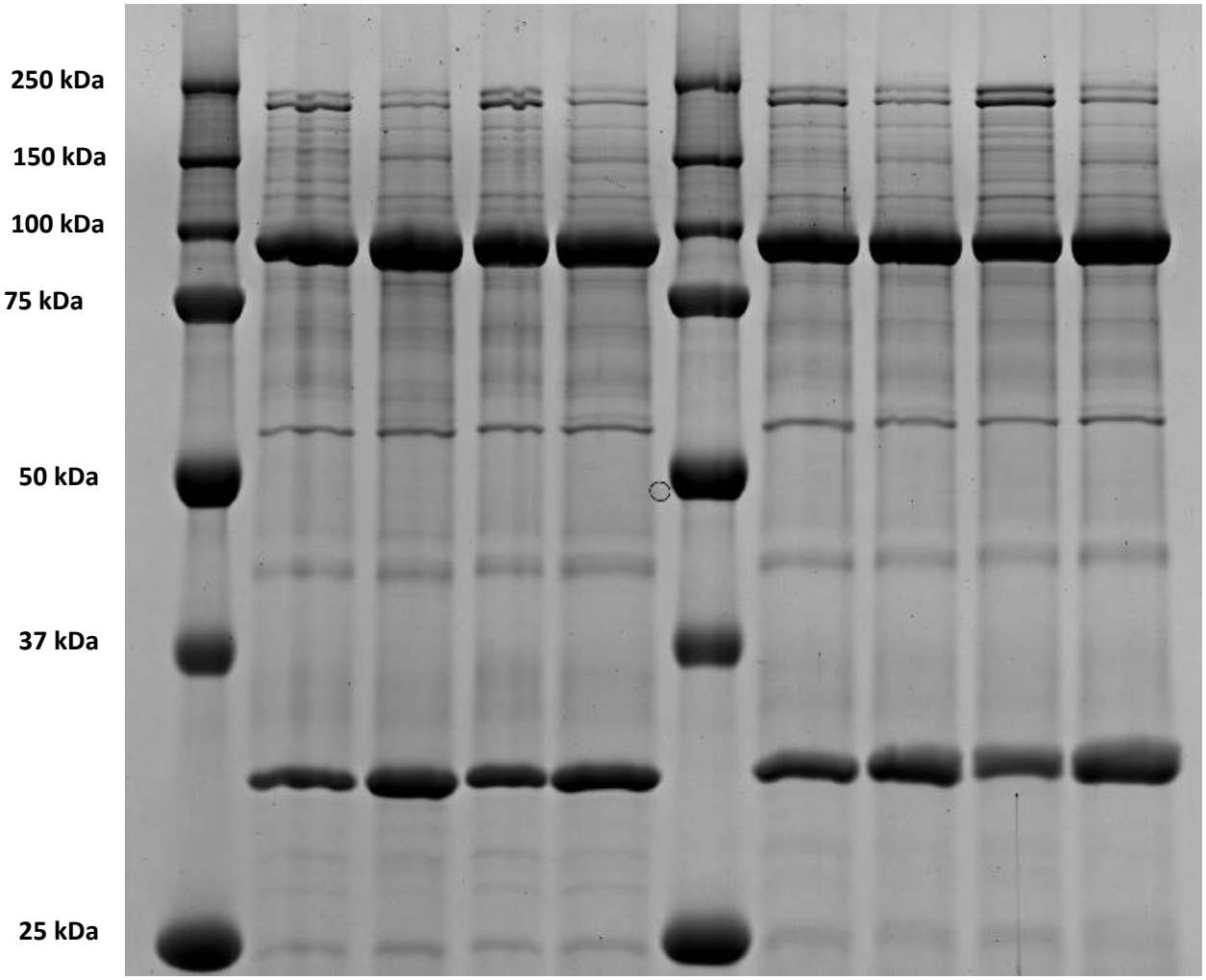
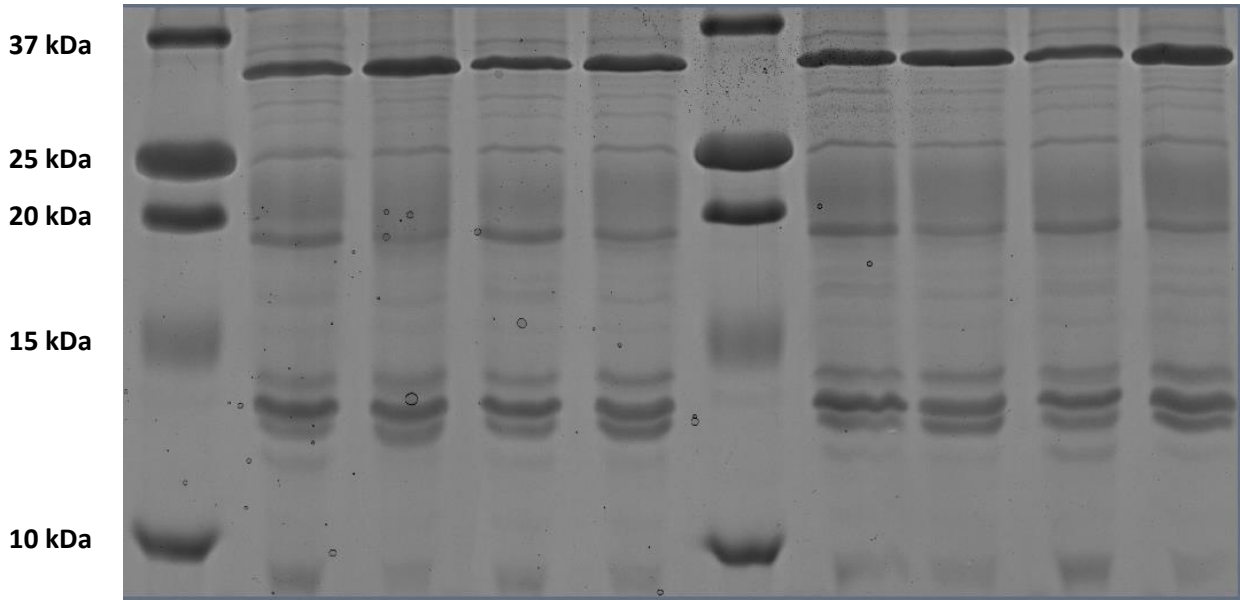


Figure 4.2. 5-22% SDS-PAGE of rabbit seminal plasma proteins. Lane 1 and 6 are molecular weight markers (37 to 10 kDa). Lane 2 and 3 are A and R line in winter, respectively. Lane 4 and 5 are A and R line in spring. Lane 7 and 8 are A and R line in summer. And lane 9 and 10 are A and R line in autumn.



Significantly different protein bands between genetic lines and seasons are shown in Table 4.2. Seven proteins bands were significantly different between genetic lines ($p<0.05$).The relative quantity of FAM115E-like (220, 113 and 59 kDa), ectonucleoside triphosphate diphosphohydrolase 3 isoform X2 (72 kDa), annexin A5 (32 kDa), lipocalin allergen Ory c 4 precursor (19 kDa), and hemoglobin subunit zeta-like (13 kDa) was significantly different between lines A and R. Of those seven protein bands, A line exhibited significantly higher values in four of them. Regarding the effect of season on the protein profile, three protein bands were significantly different between seasons ($p<0.05$). FAM115E-like (113 kDa) and β-Nerve growth factor (NGF, 12 kDa) protein bands were significantly decreased in winter, whereas hemoglobin subunit zeta-like protein band (13 kDa) was significantly increased in winter and spring.

Table 4.2. Relative quantity of significantly different protein bands from rabbit seminal plasma according to genetic line and season (LSM ± SE).

Molecular weight (kDa)	Protein	Line			Season				
		A	R	SE	Winter	Spring	Summer	Autumn	SE
220	FAM115E-like	6.35[a]	1.66[b]	±0.58	3.46	3.60	3.65	5.29	±0.83
113	FAM115E-like	0.58[a]	0.64[b]	±0.01	0.53[a]	0.61[b]	0.60[b]	0.70[c]	±0.01
72	Ectonucleoside triphosphate diphosphohydrolase 3 isoform X2	0.55[a]	0.33[b]	±0.05	0.44	0.60	0.38	0.34	±0.07
59	FAM115E-like	0.02[a]	0.19[b]	±0.03	0.15	0.11	0.09	0.08	±0.04
32	Annexin A5	21.09[a]	28.71[b]	±0.80	24.89	23.86	26.70	23.90	±1.13
19	Lipocalin allergen Ory c 4 precursor	7.72[a]	2.76[b]	±0.96	6.34	6.40	5.16	3.03	±1.36
13	Hemoglobin subunit zeta-like	16.89[a]	14.99[b]	±0.22	17.09[b]	16.89[b]	14.64[a]	15.13[a]	±0.31
12	B-Nerve growth factor (NGF)	6.35	6.97	±0.60	2.5[a]	8.51[b]	7.81[b]	7.81[b]	±0.84

Values with different superscript in the same row and factor (line or season) differ significantly at 5% level ($p<0.05$).

DISCUSSION

Seminal plasma composition is characterized by a high abundance of proteins which play important roles in sperm survival and are involved in various events related with the sperm physiology, the gamete interaction and fusion and even pregnancy establishment (Okabe et al., 1993, Topfer-Petersen et al., 1998, Gwathmey et al., 2006, Rodríguez-Martínez et al., 2011). In the present work, our results show the existence of differences in the relative quantity of many seminal plasma proteins belonging to male genetic A line against R line along the year.

On the contrary to the previous findings of Viudes-de-Castro et al. (2004), we did not found the presence of line-specific protein bands. This difference may be due to the different protein analysis and the higher resolution protein electrophoresis used in the present study.

FAM115E protein is also known as experimental autoimmune prostatitis antigen 2 (http://www.uniprot.org/uniprot/D3ZRI3). In this work, this protein was present in three different sizes (220, 113 and 59 kDa), what suggests that rabbit seminal plasma FAM115E protein has several subunits. The biological role of FAM115E in rabbit semen remains unknown and we have not been able to find any reference of the presence of this protein in the seminal plasma of other species.

The function of ectonucleoside triphosphate diphosphohydrolase 3 isoform X2 and its relationship with semen function remains unknown to date. In a previous study by Taha et al. (2006) in rabbit seminal plasma, they found an association between the decreasing content of a 70 kDa peptide band and the increased percentage of altered acrosome. The protein band of ectonucleoside triphosphate diphosphohydrolase 3 isoform X2 (72 kDa) found in the present study could be the same protein that these authors found. Therefore, the greater quantity of 72 kDa protein in A line *versus* R line could explain the better acrosome integrity of spermatozoa from A line found in earlier studies (Vicente et al., 2000; Safaa et al., 2008).

The lipocalins are a diverse family of extracellular transport proteins that show high binding affinity for small hydrophobic ligands and specific cell surface receptors (Gerena et al., 1998). Lipocalins have also been found in boar seminal plasma (González-Cadavid et al., 2014). In this work, A line had significantly more quantity of lipocalin allergen Ory c 4 precursor than R line. If this protein is related to fertility like

in other species such as the bull (Gerena et al., 1998), this could explain the better fertility of A line compared to R line found in previous works (Vicente et al., 2000; Mocé et al., 2003; Vicente et al., 2012).

Zeta globin-like protein in rabbit seminal plasma has been correlated with the percentage of cells with both membrane and acrosome damage (Arruda-Alencar et al., 2012). This finding contradicts the results found in this work because A line contains more hemoglobin subunit zeta-like protein but does not have more percentage of sperm with acrosome damage (Vicente et al., 2000; Safaa et al., 2008).

Annexin 5 (32 kDa) was found to be one of the most abundant seminal plasma proteins in rabbit, which agrees with previous works (Taha et al., 2011; Arruda-Alencar et al., 2012). This protein is an anticoagulant that appears to have a beneficial effect in maintaining rabbit sperm viability, given its positive correlation with several semen parameters such as sperm vigor, concentration and percentage of morphologically normal sperm (Arruda-Alencar et al., 2012). In this work, R line has more quantity of Annexin 5 protein but this finding does not match with better sperm vigor or higher sperm concentration of R line (Lavara et al., 2013) compared with A line (Sabés-Alsina et al., 2015).

Season is one of the main causes of sperm quality traits variation in rabbits (Marai et al., 2002; Nizza et al., 2003; Pascual et al., 2004; Roca et al., 2005; Schneidgenová et al., 2011; Ain-Baziz et al., 2012; Sabés-Alsina et al., 2015; Theau-Clément et al., 2015). This variation could be due to differences in seminal plasma or sperm membrane composition (Lavara et al., 2013). In the present work, FAM115E-like (113 kDa) and NGF (12 kDa) were significantly decreased in winter, whereas hemoglobin subunit zeta-like (13 kDa) was significantly increased in winter and spring. The NGF protein,

neurotropin primarily involved in the development and maintenance of the nervous system, has also been recognized to influence the reproductive system. Regarding the male, NGF and its two receptors were reported to be expressed in the testis, prostate gland and seminal vesicle of rabbit, suggesting a possible role of this factor in the testicular development and spermatogenesis (Maranesi et al., 2015). Furthermore, there are some evidences of the involvement of this protein in sperm motility since it has been observed that exogenous NGF stimulate the motility in a time and dose-dependent manner (Jin et al., 2010; Shi et al., 2012). The wild rabbit is a seasonal breeder with the peak of reproductive activity occurring in the spring and early summer. With domestic rabbits, genetic selection and environmental control have resulted in sexual activity throughout the year, with periods of reduced fertility. An autumn fertility drop is also often noticed as a result of high summer temperatures. Results from this work showed that the production of NGF in rabbit seminal plasma during winter decreases four times compared to the other seasons. This may be related to the natural reluctance of rabbits to breed in the early winter. This result is in agreement with Schneidgenová et al. (2011) findings which showed less sperm motility and concentration during winter season. Besides, this also accords with Zhang et al. (2015) findings in wild ground squirrels, which showed that the production of NGF in testes was decreased during the non-breeding season and increased in the breeding season.

In conclusion, the results of the present work indicate that the protein composition of rabbit seminal plasma varies both in terms of the male genetic line and the season, although more protein variation was found between genetic lines than between seasons. However, further studies are needed to elucidate the exact role of these

seminal plasma proteins and identify those related to changes in fertility in this species.

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VII.CHAPTER V

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Rabbit seminal plasma proteome: the importance of the genetic origin

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ABSTRACT

The present study was conducted to characterise rabbit seminal plasma proteins (SP proteins) focusing on the influence of the genetic origin and seasonality. In addition, β-NGF protein quantity in SP was determined. Semen samples were recovered from January to December 2014 using 6 males belonging to genotype A and six from genotype R. For each genotype, one pooled sample at the beginning, middle and end of each season was selected to develop the experiment. A total of 24 pools (3 for each season and genetic line) were analysed. SP proteins of the two experimental groups were recovered and subjected to in-solution digestion nano LC-MS/MS and bioinformatics analysis. The resulting library included 402 identified proteins validated with ≥ 95% Confidence (unused Score ≥ 1.3). These data are available via ProteomeXchange with identifier PXD006308. Only 6 proteins were specifically implicated in reproductive processes according to Gene Ontology annotation. Twenty-three proteins were differentially expressed between genotypes, 11 over-expressed in genotype A and 12 in genotype R. Regarding the effect of season on rabbit SP proteome, results showed that there is no clear pattern of protein variation throughout the year. Similar β-NGF relative quantity was observed between seasons and genotypes. In conclusion, this study generates the largest library of SP proteins reported to date in rabbits and provide evidence that genotype is related to a specific abundance of SP proteins.

INTRODUCTION

The control of rabbit reproduction has experienced great changes in the last decade, mainly as a consequence of the development of new techniques such as commercially applicable artificial insemination (AI) (Safaa et al., 2008). The use of AI in intensive meat rabbit production is currently a common practice (Piles et al., 2013), like in the vast majority of livestock (Hansen, 2014), and it utilisation has contributed to improve the knowledge of rabbit spermatozoa and bucks' management (Boiti et al., 2005; Castellini et al., 2008; Lavara et al., 2005; Pascual et al., 2016; Safaa et al., 2008; Theau-Clément et al., 2015, 2016; Viudes-de-Castro et al., 2014). Rabbit ejaculates present some peculiarities that should be taken into account, for instance, they present occasionally gel plug or gelatinous mass and contain several vesicles that have been related to modulate different sperm functions such as motility, capacitation and acrosome reaction (Castellini et al., 2006, 2012, 2013; Collodel et al., 2012). In addition, rabbit belongs to the few species in which ovulation is induced by copulation (Fisher et al., 2012), like cats, camelids, koala, voles and sumatran rhinos (McGraw et al., 2015). In these species, a specific protein named β-NGF has been studied in seminal plasma because of its potential role in inducing ovulation in camelids (Adams and Ratto, 2013; Berland et al., 2016; Druart et al., 2013; Kershaw-Young et al., 2012; Li et al., 2010; Silva et al., 2011). Nevertheless, in rabbits, the intramuscular administration of seminal plasma did not provoke ovulation (Silva et al., 2011), but plays a role in promoting the formation and development of the testis and the differentiation, maturation, and movement of the spermatozoa (Li et al., 2010).

Many factors influence the production and quality of rabbit semen such as the genetic origin (growth lines have worse seminal qualities and fertility rates than maternal

lines) (Mocé et al., 2003; Vicente et al., 2000), the season (Marai et al., 2002; Pascual et al., 2004; Schneidgenová et al., 2011; Theau-Clément et al., 2015), the photoperiod (Ain-Baziz et al., 2012; Roca et al., 2005; Sabés-Alsina et al., 2015) and the collection frequency (Nizza et al., 2003). The production of fertile doses is determined by several components: i) male libido and characteristics of the ejaculate which form part of the criterion for ejaculate rejection; ii) volume and sperm concentration of the ejaculate (determining the amount of doses that can be obtained); and iii) the quality of sperm (determining the minimum sperm dosage required to ensure fertilization) (Piles et al., 2013). Subjective estimation of motility and evaluation of sperm morphology are the two laboratory assays most widely used for the rabbit semen evaluation in insemination centers (Lavara et al., 2005). However, the ability of these seminal characteristics to predict reproductive performance is very low (Piles et al., 2013). In line with the greater number of livestock species, the prediction of ejaculates of high fertility or good cryopreservation remains unresolved. However, while most of these previous studies have been focused on the sperm cell, little attention has been paid to the seminal plasma in rabbit. To date, a limited number of studies have performed an analysis of rabbit seminal plasma proteins (Arruda-Alencar et al., 2012; Casares-Crespo et al., 2016a; Davis and Davis, 1983; de Lamirande et al., 1983; Lavon, 1972; Minelli et al., 2001; Okabe et al., 1993; Thomas et al., 1986; Viudes-de-Castro et al., 2004) in comparison to the main commercially relevant domestic mammalian species (Rodríguez-Martínez et al., 2011; Druart et al., 2013; Bromfield, 2016).

Seminal plasma contributes to the safe environment for sperm maturation, sperm viability and fertilization in mammals (Muiño-Blanco et al., 2008; Rodríguez-Martínez et al., 2011; Manjunath et al., 2007; Bromfield, 2016). Moreover, seminal plasma is a

promising source for the study of potential reproductive biomarkers, because it is a complex mixture of secretions from testis, epididymis and male accessory sex glands (González-Cadavid et al., 2014). Sperm maturation is acquired during the transit of the spermatozoa through the epididymis, where its plasma membrane undergoes intense changes in protein composition and in localization of their components (Dacheux et al. 2003). The protein composition of mammalian seminal plasma varies among species, and has important effects on sperm function (Rodríguez-Martínez et al., 2011). Even though seminal plasma contains hundreds of proteins, their functions are not completely understood. In rabbits, seminal plasma has a positive effect in maintaining sperm motility and viability during *in vitro* storage (Castellini et al., 2000).

Against this background, the present study was conducted to characterise rabbit seminal plasma proteins through nano LC-MS/MS analysis, focusing on the influence of the genetic origin and seasonality. In addition, β-NGF protein quantification was done.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals in this study were purchased from Sigma-Aldrich Química S.A (Madrid, Spain). All the experimental procedures used in this study were performed in accordance with Directive 2010/63/EU EEC for animal experiments.

1. Localization and animals

The experiment was carried out with 24 males from two Spanish commercial rabbit genetic lines (genotypes A and R) from January to December 2014. All bucks were of proven fertility and subjected to a weekly pattern of ejaculate collection. Line A is based on New Zealand White rabbits selected since 1980 by a family index for litter

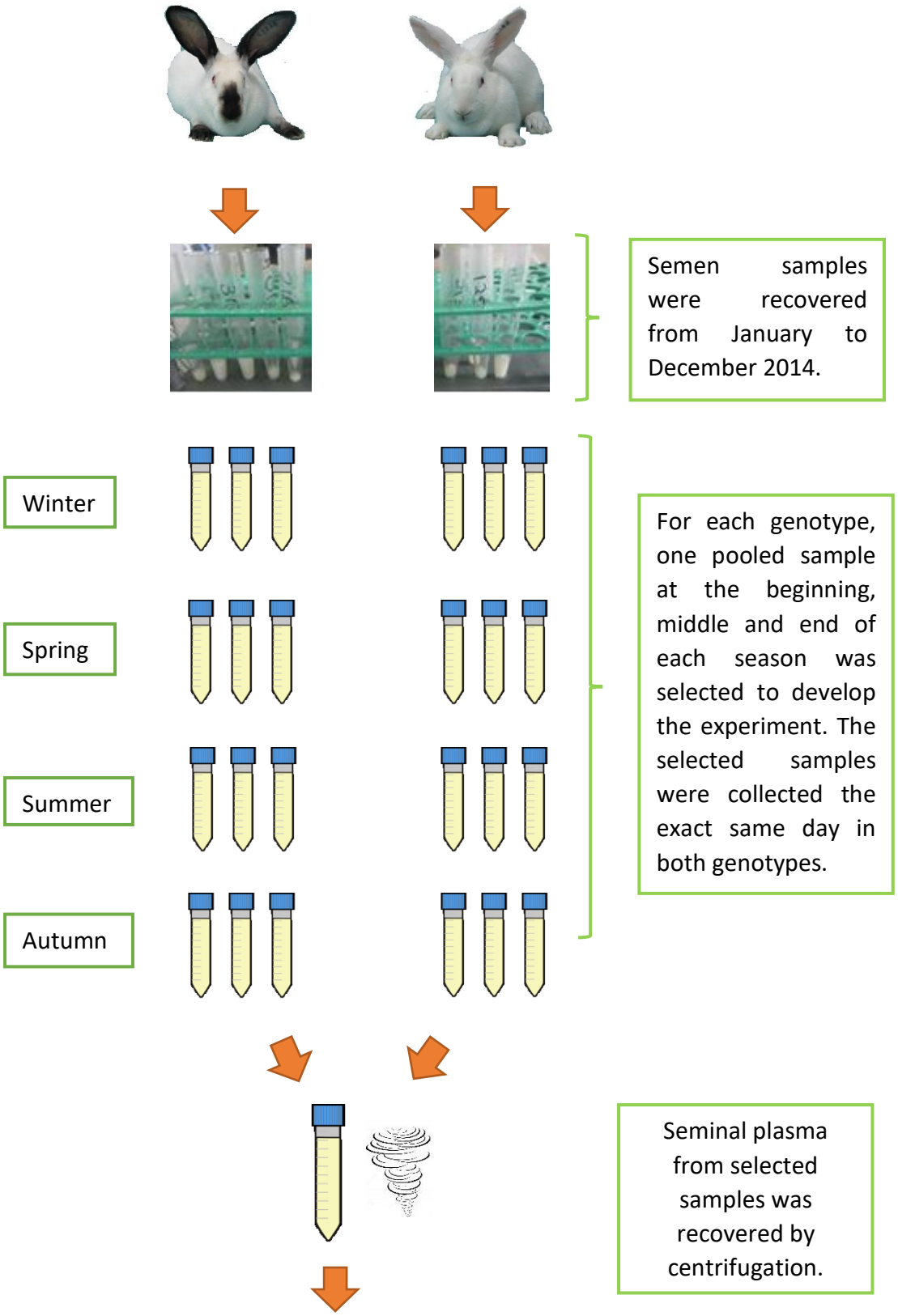
size at weaning over 45 generations. Line R comes from the fusion of two lines, one founded in 1976 with Californian rabbits reared by Valencian farmers and another founded in 1981 with rabbits belonging to specialised paternal lines. The selection method was individual selection on post-weaning daily gain, with weaning taking place at 28 days and the end of the fattening at 63 days. All animals were housed at the Animal Technology and Research Centre (CITA-IVIA, Segorbe, Castellón, Spain) experimental farm in flat deck indoor cages (75×50×30 cm), with free access to water and commercial pelleted diets (minimum of 15 g of crude protein per kg of dry matter (DM), 15 g of crude fibre per kg of DM, and 10.2 MJ of digestible energy per kg of DM). The photoperiod was set to provide 16 h of light and 8 h of dark, and the room temperature was regulated to keep temperatures between 14°C and 28°C.

2. Semen collection and preparation of seminal plasma samples

Semen samples were obtained by artificial vagina and collected into a sterile tube. One ejaculate was collected per male and week. Collections were performed on the same day of the week during 1 year. Routine diagnostic semen analyses were performed to assess the initial seminal quality. Only ejaculates exhibiting a white colour and possessing more than 70% of motility, 85% of intact apical ridge (acrosomal status), and less than 15% of abnormal sperm were used in this experiment. Then, ejaculates from the same genotype were pooled each day as a single sample. Each sample was centrifuged at 7400 x g for 10 min at 22 ºC. The resulting supernatants were collected and centrifuged again (7400 x g for 10 min) to remove residual spermatozoa and cell debris. The supernatants were collected, supplemented with a 1% v/v protease inhibitor cocktail (P2714, Sigma) and stored at -80ºC until use.

For each genotype, one pooled sample at the beginning, middle and end of each season was selected to develop the experiment (Fig. 5.1). A total of 24 pools (3 for each season and genetic line) were analysed. Total protein concentration was quantified in duplicate by the bicinchoninic acid method (BCA) using BSA as standard protein (Smith et al., 1985) and seminal samples were adjusted to 5 μg/μL in saline solution.

Figure 5.1. Experimental design scheme.



3. In-solution digestion

The proteomic analysis was performed in the proteomics facility of SCSIE University of Valencia that belongs to ProteoRed, PRB2-ISCIII, supported by grant PT13/0001. Forty µg of every sample were taken and the volume was set to 22.5 µL with 50 mM ammonium bicarbonate (ABC). Samples were digested with 800 ng of sequencing grade trypsin (Promega) according the following steps: (1) the proteins were reduced using 2 mM Dithiothreitol (DTT) reducing agent in 50 mM NH4HCO3 to a final volume of 25 µL, being the incubation at 60ºC during 20 minutes; (2) the proteins were alkylated at room temperature using 5.5 mM iodoacetamide (IAM) in 50 mM NH4HCO3 to a final volume of 30 µL, being the incubation during 30 minutes in the dark; (3) trypsin was added (800 ng) to a final volume of 38 µL, the sample was carefully mixed and digestion was carried overnight at 37ºC. The digestion was stopped with 4 µL of trifluoroacetic acid (Fisher Scientific; 10% final concentration). Final tryptic peptides were at 0.9 µg/µL.

4. Nano LC-MS/MS analysis

Two µL of each sample were loaded onto a trap column (nano LC Column, 3µm particles size C18-CL, 350 µm diameter x 0.5mm long; Eksigent Technologies) and desalted with 0.1% TFA at 3µL/min during 5 min. The peptides were then loaded onto an analytical column (LC Column, 3 µm particles size C18-CL, 75µm diameter x 12cm long, Nikkyo) equilibrated in 5% acetonitrile (ACN) 0.1% formic acid (FA). Peptide elution was carried out with a linear gradient of 5% to 35% of solvent B in A for 120 min. (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 300 nL/min. Peptides were analysed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSCIEX).

Eluted peptides were ionized applying 2.8 kV to the spray emitter. The mass spectrometric analysis was carried out in a data-dependent mode. Survey MS1 scans were acquired from 350–1250 m/z for 250 ms. The quadrupole resolution was set to 'UNIT' for MS2 experiments, which were acquired from 100–1500 m/z for 25 ms in 'high sensitivity' mode. Following switch criteria were used: charge: 2+ to 5+; minimum intensity; 70 counts per second (cps). Up to 25 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s. The system sensitivity was controlled with 2 fmol of 6 proteins mixture (LC Packings). Samples were injected in a random order.

The proteomics data and result-files from the analysis have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository, with the dataset identifier PXD006308 and 10.6019/PXD006308X.

5. Protein identification

The SCIEX.wiff data-files were processed using ProteinPilot v5.0 search engine (AB SCIEX). ProteinPilot default parameters were used to generate peak list directly from 5600 TripleTof wiff files. The Paragon algorithm of ProteinPilot v 5.0 was used to search Swiss-Prot (07/01/2017) database with the following parameters: trypsin specificity, cys-alkylation, no taxonomy restriction, and the search effort set to through.

To avoid using the same spectral evidence in more than one protein, the identified proteins are grouped based on MS/MS spectra by the Protein-Pilot Progroup algorithm. A protein group in a Pro Group Report is a set of proteins that share some physical evidence. Unlike sequence alignment analyses where full length theoretical

sequences are compared, the formation of protein groups in Pro Group is guided entirely by observed peptides only. Since the observed peptides are actually determined from experimentally acquired spectra, the grouping can be considered to be guided by usage of spectra. Then, unobserved regions of protein sequence play no role in explaining the data. Only peptide and protein identifications with ≥ 95% Confidence (unused Score ≥ 1.3) were validated. Protein identifications were accepted if they contained at least two identified peptides.

6. Label-free protein quantification using Chromatographic Areas

For quantification, the group file generated by Protein Pilot was used. The ions areas were extracted from the wiff files obtained from LC-MS/MS experiment by Peak View® v1.1. Only peptides assigned with confidence ≥ 95%, among those without modifications or shared by different proteins were extracted. A total of 24 samples were analysed and 402 proteins were quantified.

7. Bioinformatics analysis

Gene ontology terms for biological process, molecular function and cellular component were obtained using UniProt database (http://www.uniprot.org/ accessed on 07/08/2017) in order to retrieve the gene names in combination with PANTHER v11.1 (http://www.pantherdb.org/ accessed on 07/08/2017, Mi et al., 2017), with *Homo sapiens* as the organism to maximise classifications.

8. Statistical analysis

The quantitative data obtained by PeakView® were analysed by Marker View® v1.3 (AB Sciex). First, areas were normalized by total areas summa. A t-test was used to identify the differentially expressed proteins between genotypes. Proteins were considered differentially expressed if the adjusted p-value < 0.05. Mean quantity of proteins were calculated and the fold-changes between the two groups were estimated. No multiple corrections were performed. The standard deviation was pooled out by calculating a separate t value for each peak. Group comparison is performed by calculating the square of t according to the following equation:

$$t^2 = (<R_1> - <R_2>)^2 / (\sigma^2 / n_1 + \sigma^2 / n_2)$$

$$\text{where } \sigma^2 = [(n_1 - 1)\,\sigma_1^2 + (n_2 - 1)\,\sigma_2^2\,] / (n_1 + n_2 - 2)$$

Finally, an estimation of the β-NGF proportion as percentage of the total seminal plasma protein was calculated by comparing the peak area of β-NGF protein with the total area of each sample and an ANOVA comparing the β-NGF proportion between genotypes and seasons was done (STATGRAPHICS®). Partial Least Squares Discriminant Analysis (PLS-DA) was performed to evaluate the classification of the samples using mixOmics R package and proteins with a vip score > 1.5 were selected and represented in a heat map.

RESULTS

1. Rabbit seminal plasma proteome

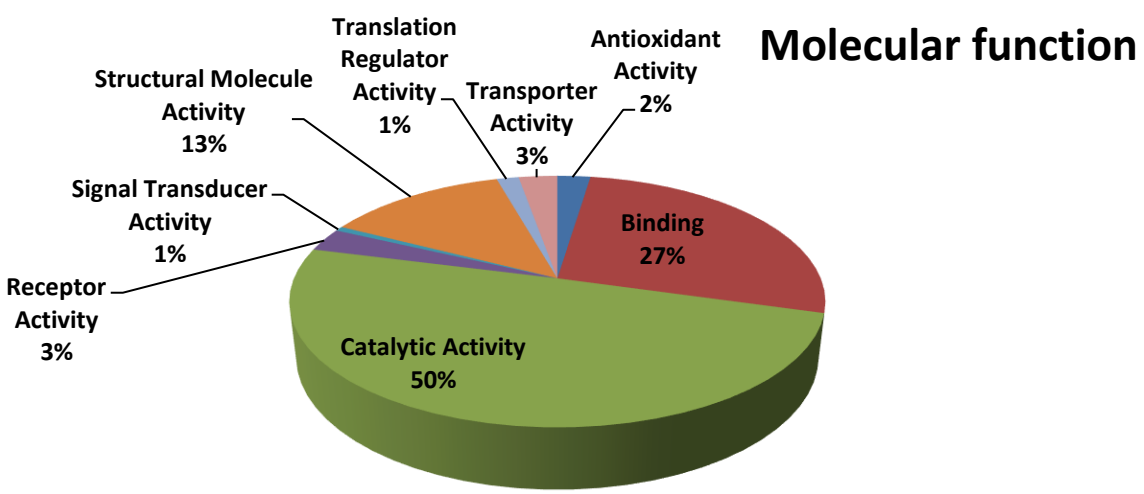
The Proteomics System Performance Evaluation Pipeline (PSPEP) Software was used to perform a false discovery rate analysis on Paragon™ algorithm results. The complete spectral library included 88,385 spectral corresponding to 4,600 peptides and 402

proteins validated with ≥ 95% Confidence (unused Score ≥ 1.3) when using at least 2 peptides for identification (Table S5.1). These 402 proteins were quantified based on their chromatographic or peak areas (Table S5.2).

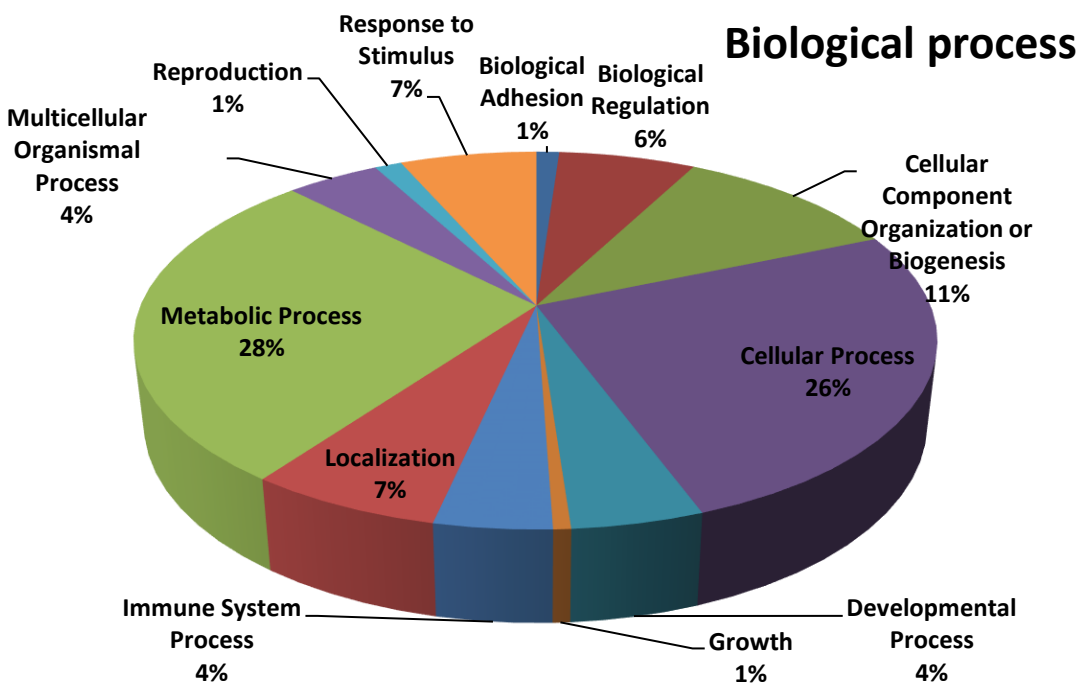
The complete rabbit seminal plasma proteome was classified under different categories based on their molecular function, biological process and cellular components (PANTHER analysis). The results are shown in Figure 5.2. For molecular function (Fig. 5.2.a), a total of 251 hits were found. The catalytic activity was the predominant function (50%), followed by binding (27%) and structural molecule activity (13%). Regarding biological process (Fig. 5.2.b), a total of 471 hits were found. The metabolic (28%) and the cellular process (26%) were the most abundant categories, but it is worth mentioning that 6 hits (1%) were classified in the category of reproduction specifically to gamete generation and fertilization functions. Finally, a total of 195 hits were found for cellular component category (Fig. 5.2.c). Cell part (52%), organelle (27%), macromolecular complex (9%) and extracellular region (7%) were the most abundant cellular components of the studied proteins.

Figure 5.2. Pie charts showing the distribution of rabbit seminal plasma proteins based on their a) molecular function, b) biological process and c) cellular component, using UniProt database in combination with PANTHER.

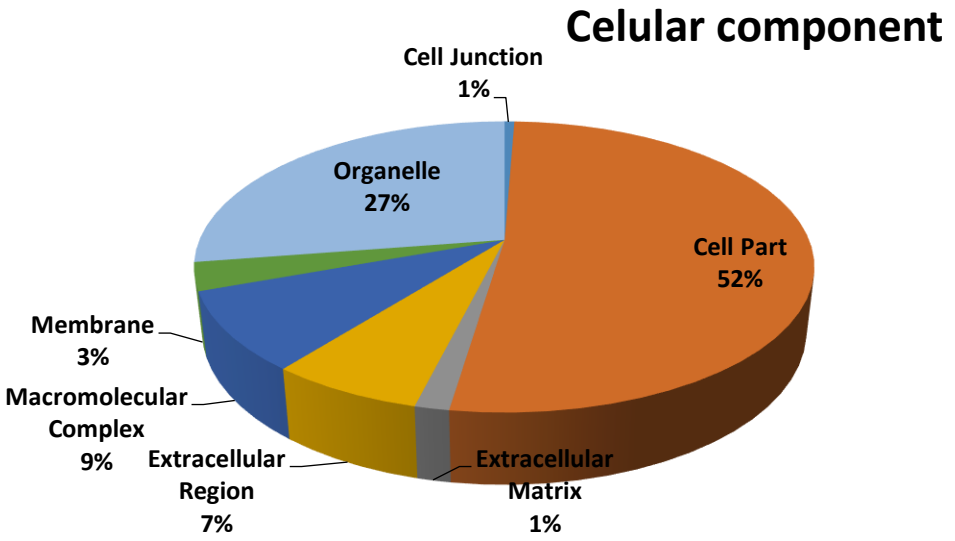
a)



b)



c)



2. Effect of genetic origin on seminal plasma proteome

The results of the seminal plasma proteome comparison between both genetic lines (A and R) are shown in Fig. 5.3. PLS-DA analysis showed a clear effect of the genetic

origin. Proteins with a vip score > 1.5 (high influence in the response variable) were selected and a heat map was generated (Fig. 5.4). The hierarchical clustering of seminal plasma proteins separated the twenty-four seminal samples into two different main clusters, differentiating between genotypes.

Figure 5.3. Partial Least Squares Discriminant Analysis (PLS-DA) showing the classification of seminal samples from genotypes A and R, based on relative protein amount.

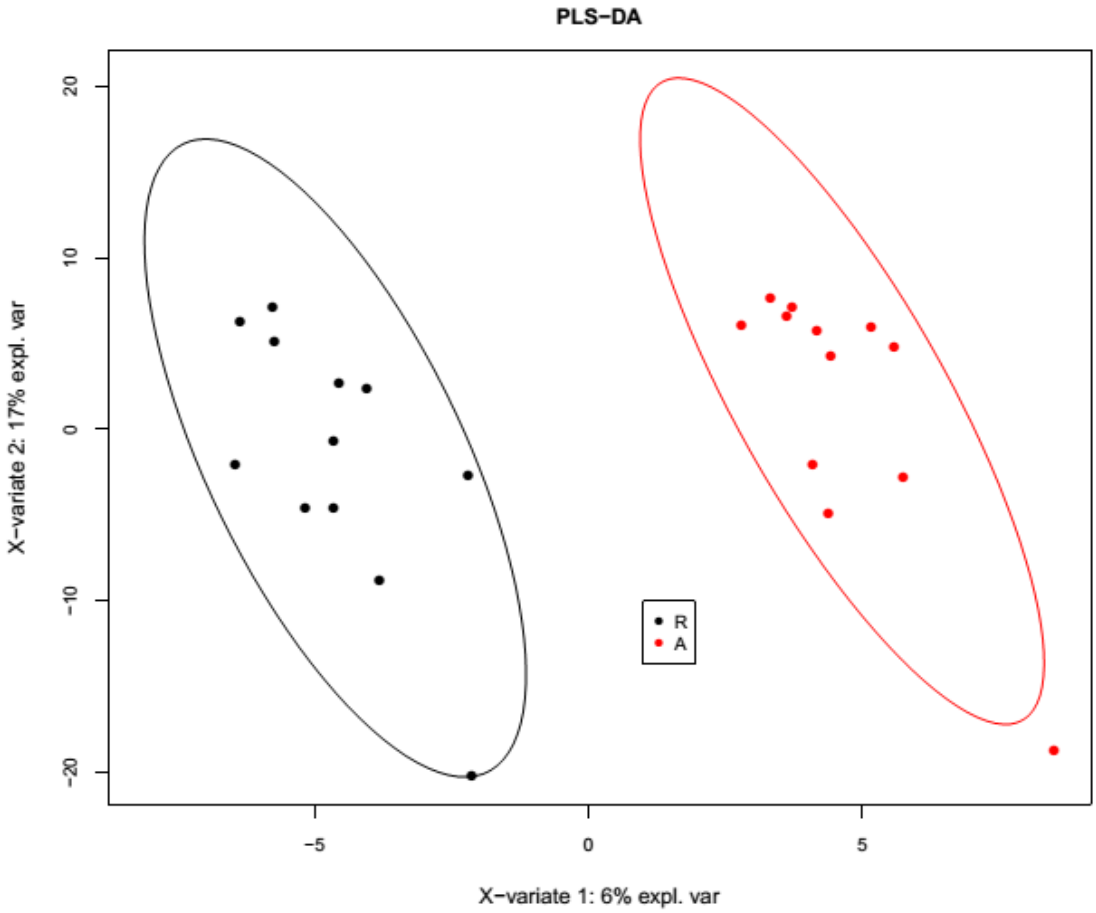
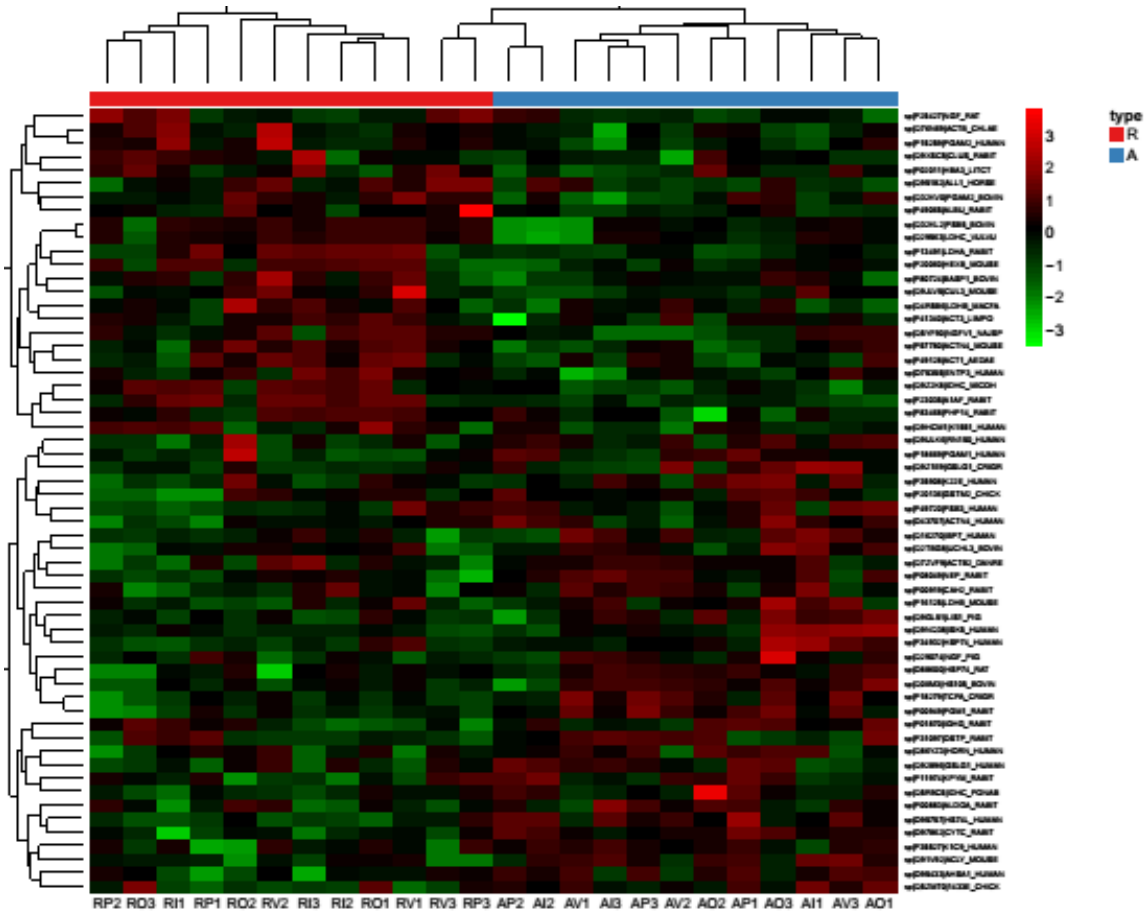


Figure 5.4. Heat map representing levels of differentially expressed seminal plasma proteins between genetic origins A and R and hierarchical clustering, showing two main clusters comprising genotype A and R.



Given that the proteome between both genotypes presented high variability, a t-test was done. Results showed a total of 23 differentially expressed proteins ($p < 0.05$) between genotypes (Table S5.3). Of the differentially expressed proteins, 11 proteins were over-expressed in genetic line A and 12 proteins over-expressed in line R (Table 5.1).

Table 5.1. List of differentially expressed proteins in rabbit seminal plasma between genotypes A and R.

Protein name	Gene name	Mean protein amount		Log (Fold Change)	p-value
		Line A	Line R		
Elongation factor 4	lepA	5971106.95	185466.34	-1.508	0.041
Uteroglobin	SCGB1A1	5041496.51	1589062.14	-0.501	0.004
Zonadhesin	ZAN	27743.01	8848.46	-0.496	0.022
Peptidyl-prolyl cis-trans isomerase	Fkbp1a	912341.90	343914.90	-0.424	0.043
Plastin-1	Pls1	5001378.19	2121482.19	-0.372	0.025
Ubiquitin carboxyl-terminal hydrolase isozyme L3	UCHL3	421244.19	179445.24	-0.371	0.001
CD109 antigen	CD109	163095.41	76843.68	-0.327	0.033
Catalase	CAT	1185007.71	610573.83	-0.288	0.006
Ectonucleoside triphosphate diphosphohydrolase 3	ENTPD3	7966888.56	4244516.56	-0.273	0.002
Carbonic anhydrase 2	CA2	9004455.75	5035722.69	-0.252	0.000
Aspartate aminotransferase	GOT1	181617.72	112819.89	-0.207	0.047
Heat shock 70 kDa protein 1-like	HSPA1L	1600299.91	2195440.94	0.137	0.045
Fructose-1,6-bisphosphatase 1	FBP1	999212.40	1371171.41	0.137	0.029
Polyubiquitin-C	UBC	3011220.72	4814226.51	0.204	0.043
Peptidyl-glycine alpha-amidating monooxygenase	PAM	8091199.72	13912767.58	0.235	0.048
Aldehyde oxidase 3	Aox3	716956.23	1488232.49	0.317	0.002
Insulin-like growth	IGFBP7	32046.86	68416.81	0.329	0.049

factor-binding protein 7					
Heme-binding protein 2	HEBP2	53370.24	144433.36	0.432	0.038
Destrin	DSTN	32058.12	87093.22	0.434	0.045
Calumenin	CALU	219223.020	1137831.85	0.715	0.038
Carboxypeptidase Q	CPQ	583281.624	3847442.484	0.819	0.002
ATP-dependent 6-phosphofructokinase	PFKP	1529350.190	23963532.689	1.195	0.002
Hemoglobin subunit alpha-3	HBA3	114877.044	5472291.847	1.678	0.008

3. Effect of season on seminal plasma proteome

The results of the comparison between seasons are shown in Fig. 5.6. PLS-DA analysis showed unclear separation between seasons, existing overlaps between winter, spring, summer and autumn samples. Seminal samples from autumn seem to be the most differential ones compared with the others; however, half of them overlap with other seasons samples. After applying vip score function to the previous PLS-DA results and only selecting the proteins with a vip score > 1.5 (high influence in the response variable), a heat map was generated (Fig. 5.7). Predictably, in the heat map, the hierarchical clustering of seminal plasma proteins separated the 24 seminal samples into four different main clusters, but these clusters did not match the four seasons but a mixture of them.

Figure 5.6. Partial Least Squares Discriminant Analysis (PLS-DA) showing the classification of seminal samples belonging to the four seasons, based on relative protein amount.

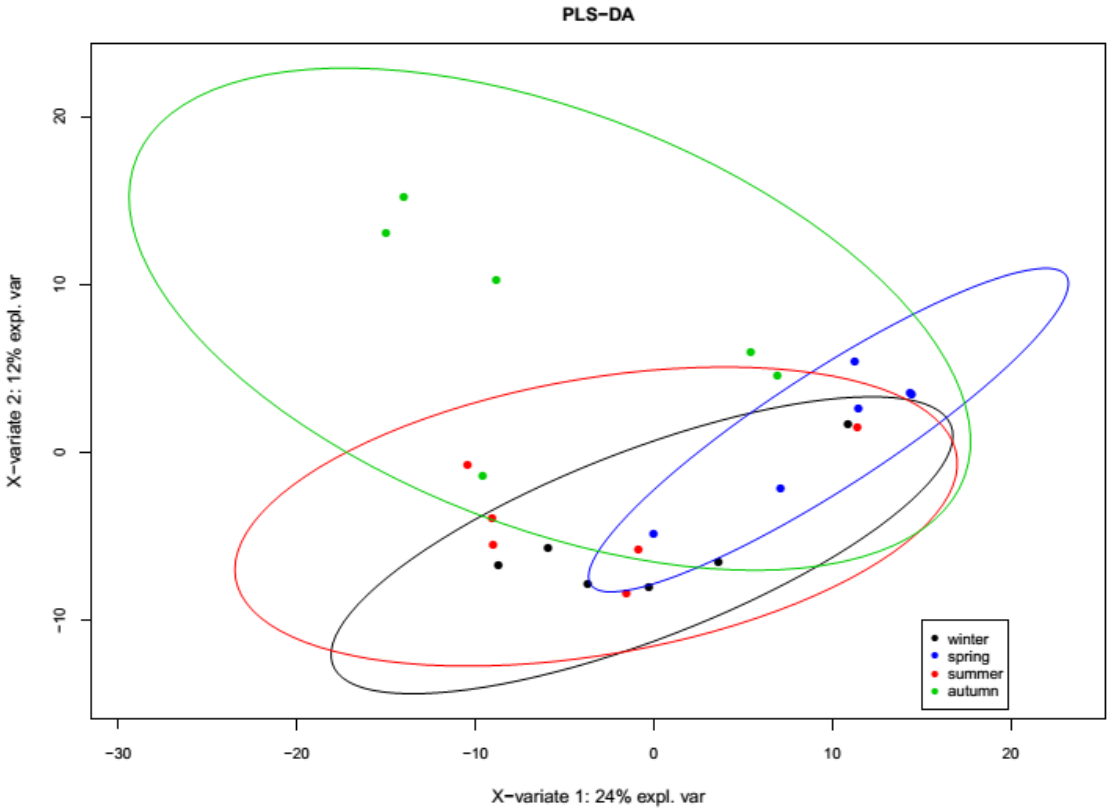
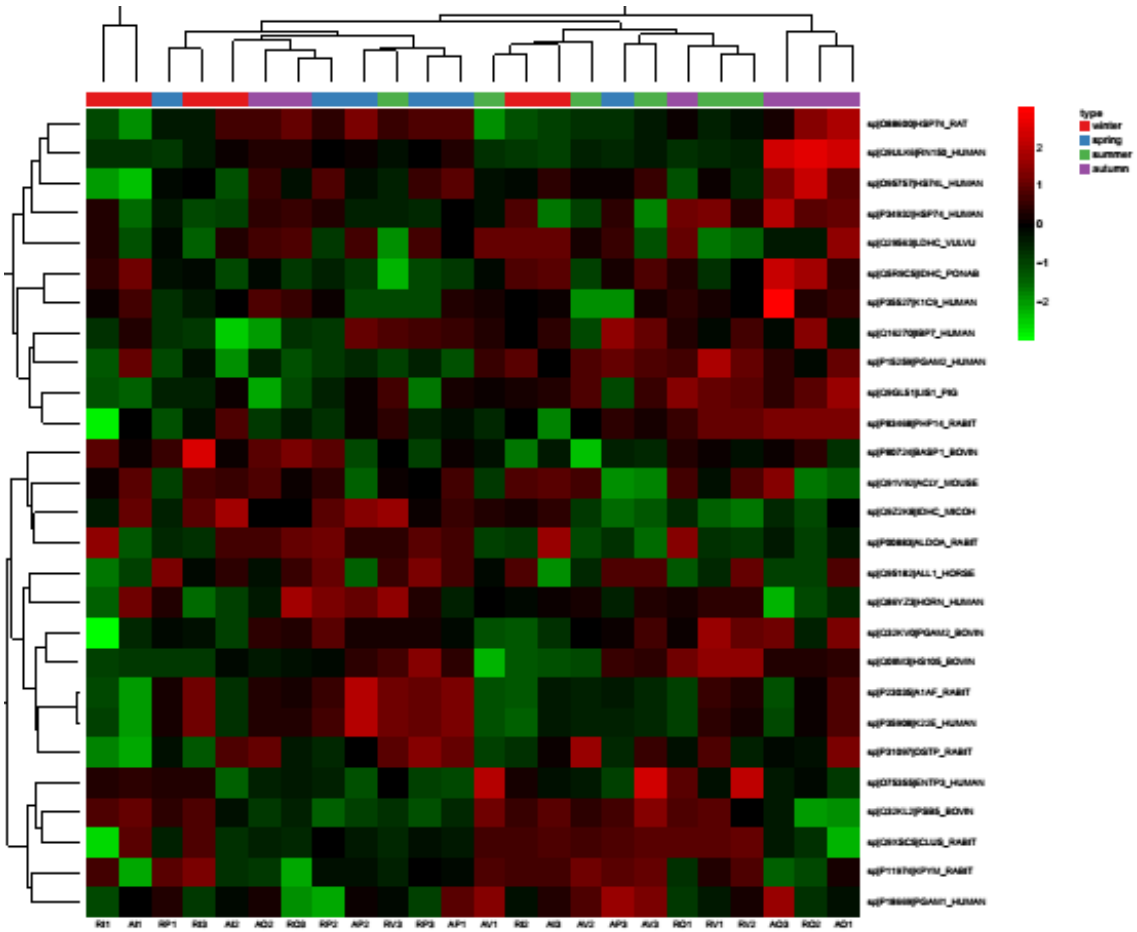


Figure 5.7. Heat map representing levels of differentially expressed seminal plasma proteins between seasons and hierarchical clustering, showing four main clusters comprising a mixture of samples from different seasons.



4. β-NGF relative quantification in rabbit seminal plasma

The proportion of β-NGF in rabbit seminal plasma in each season and genotype was the following: winter (0.96%), spring (2.34%), summer (1.34%), autumn (1.16%), genotype A (1.41%) and genotype R (1.49%). The β-NGF quantity detected in seminal plasma indicated that neither genetic origin (p=0.74) nor season (p=0.08) have influence on this protein abundance.

DISCUSSION

To the best of our knowledge this study generates the largest library of seminal plasma (SP) proteins reported to date in rabbits. Moreover, one of the most important contributions of this study is the significant relationship found between genetic origin and SP proteins in rabbit. In previous studies, rabbit seminal plasma proteome has already been proved different between rabbit genotype A and R (Casares-Crespo et al., 2016a; Safaa et al., 2008; Viudes-de-Castro et al., 2004). In these previous studies a traditional 1-D polyacrylamide gel was done, identifying only major proteins visible after Coomassie Colloidal Blue staining and obtaining the relative quantity of these protein bands through scanning and analysing the gel with 1D software. The fact that in the present study a more accurate technique such as LC-MS/MS was used in order to identified the differentially expressed proteins, could explain the differences found between previous (Casares-Crespo et al., 2016a; Safaa et al., 2008; Viudes-de-Castro et al., 2004) and current results. Indeed, the exceptional sensitivity and resolving power of today's mass spectrometers allow for the detection of proteins and peptides at low femtomole quantities (Wither et al., 2016). That is why, in the current study, with the application of LC-MS/MS, we identified and quantified 402 rabbit SP proteins, compared to the seven rabbit seminal plasma proteins identified previously (Casares-Crespo et al., 2016a).

Bioinformatics analysis of rabbit SP proteome revealed that 50% of identified proteins were related to catalytic activity and the second dominant group of proteins was assigned to a binding function (27%). These proportions agree with previous proteomic studies of human, ram, carp and boar seminal plasma (Dietrich et al., 2014; Pérez-Patiño et al., 2016; Pilch and Mann, 2006; Souza et al., 2012). Inside the category of

catalytic activity, the aminopeptidase B protein is included. This enzyme has an important role in rabbit AI because it is responsible for degrading the GnRH analogue when it is added to the seminal dose to induce doe ovulation. In previous works, we have demonstrated that aminopeptidase activity in rabbit seminal plasma reduces the bioavailability of the GnRH analogue (Viudes-de-Castro et al., 2014) and new extenders with aminopeptidase inhibitors are being developed (Casares-Crespo et al., 2016b, 2017). Regarding biological process, the metabolic (28%) was the most abundant category in rabbit SP, which coincides with human, carp and boar SP proteins (Dietrich et al., 2014; Pérez-Patiño et al., 2016; Souza et al., 2012). It is also noticeable that only 6 of the 402 proteins identified in rabbit SP are to date recorded in GO as being directly associated with reproductive processes. Similar results were found in boar SP, where only 20 of the 374 proteins identified were annotated as related to reproduction (Pérez-Patiño et al., 2016).

To date, recent research on seminal plasma of major domestic mammalian species (Aquino-Cortez et al., 2017; Druart et al., 2013; Pérez-Patiño et al., 2016; Pini et al., 2016; Souza et al., 2012), human (Pilch and Mann, 2006) and fish (Dietrich et al., 2014; Gombar et al., 2017; Nynca et al., 2017), including semen quality (Sarsaifi et al., 2015), fertilizing markers (Kwon et al., 2015) and freezability (Dietrich et al., 2017; Vilagran et al., 2015) have been reported. Nevertheless, it is unknown at present if there is a variation in seminal plasma protein composition among genotypes within the same species. Our results clearly indicate that SP proteins abundance in rabbit seems to be related to a specific genotype, which in several previous studies have demonstrate differences in sperm quality, fertility and prolificacy (Safaa et al., 2008; Vicente et al., 2000). As stated, we identified a higher abundance of 11 proteins in genotype A

seminal plasma, while another 12 proteins were more abundant in genotype R seminal plasma.

Among the over-expressed proteins in genotype A, we find uteroglobin and zonadhesin. Uteroglobin has been identified in rabbit seminal plasma and in rabbit uterus secretions (Kirchner and Schroer, 1976; Müller, 1983) and zonadhesin in spermatozoa (Lea et al., 2001), but their role remains unknown to date. While uteroglobin, also present in the prostate, may be responsible for suppressing sperm antigenicity in the rabbit (Mukherjee et al., 1983), zonadhesin is located exclusively in the anterior acrosome and may be one of the proteins that anchors the acrosomal shroud to the zona pellucida (http://www.uniprot.org/uniprot/P57999), thereby allowing the spermatozoa to continue penetration and fertilization to proceed spermatozoa (Lea et al., 2001). In addition, we also observed a greater amount of ectonucleoside triphosphate diphosphohydrolase 3 protein, which agrees with the results of a previous study (Casares-Crespo et al., 2016a) and has been related with acrosome alteration when it concentration decreased (Taha et al., 2011). All of these findings, especially the increased amount of these proteins observed in genotype A in comparison with genotype R could explain in part the better acrosome integrity of spermatozoa and the enhanced fertility and prolificacy previously described in genotype A (Lavara et al., 2005; Safaa et al., 2008).

Other over-expressed proteins in line A such as plastin 1 and ubiquitin carboxyl-terminal hydrolase have been found related to spermatogenesis in other species (Kwon et al., 2004; Li et al., 2015). Plastins are a family of actin binding proteins known to cross-link actin microfilaments in mammalian cells, creating actin microfilament bundles necessary to confer cell polarity and cell shape (Li et al., 2016). There are three

types of plastins: plastin 1, 2, and 3. All three are expressed in Sertoli cells and plastin 1 and 2 in testes germ cells (Li et al., 2016). Plastin protein has been found in boar seminal plasma exosomes (Piehl et al., 2013) and in rat testis (Li et al., 2015). Plastin 1 deficient mice were fertile and displayed a normal reproductive rate (Grimm-Gunter et al., 2009), what suggests an additional role of plastin far from the fertility process. On the other hand, ubiquitin carboxyl-terminal hydrolase isozyme 3 may function in the meiotic differentiation of spermatocytes into spermatids (Kwon et al., 2004).

Seminal plasma contains antioxidants that are free radical scavengers that protect sperm cells against oxidative stress (Bousnane et al., 2017). For instance, catalase serves to protect cells from the toxics effects of hydrogen peroxide (http://www.uniprot.org/uniprot/Q64405). In bulls, the levels of catalase in seminal plasma have been found higher in high-fertile males than in subfertile bulls (Kumar et al., 2016). In addition, the supplementation of post-thawed rooster semen with 100 µg/mL of catalase has beneficial effects on semen quality (Amini et al., 2015). In line with this, the protein named elongation factor 4 is required for accurate and efficient protein synthesis under certain stress conditions (http://www.uniprot.org/uniprot/Q5KWZ3). Therefore, the over-expression of catalase and elongation factor 4 proteins in seminal plasma of genotype A supports the better recovery and performance of thawed semen from genotype A compared to R (Mocé et al., 2003).

The rest of the over-expressed proteins in line A were enzymes such as carbonic anhydrase 2, which has been found to have a role in the regulation of bicarbonate concentration in horse seminal plasma and accordingly regulate seminal plasma pH (Asari et al., 1996), aspartate aminotransferase (AST) which is an important regulator

of glutamate (http://www.uniprot.org/uniprot/P33097) and peptidyl-prolyl cis-trans isomerise which keeps in an inactive conformation of the TGF-beta type I serine/threonine kinase receptor, preventing TGF-beta receptor activation in absence of ligand (http://www.uniprot.org/uniprot/P26883).

On the other hand, genotype R presents higher abundance in several proteins related with reproductive function such as insulin-like growth factor-binding protein 7 which is important for correct spermatogenesis (Belardin et al., 2016) and polyubiquitin C which is involved in sperm-zona pellucida interactions and antipolyspermy defense in pig (Yi et al., 2007). Besides, genotype R seminal plasma has more quantity of Heat shock 70 kDa 1-like protein. Heat shock proteins (70 and 90 kDa) are chaperones implicated in a wide variety of cellular processes, including protection of the proteome from stress, folding and transport of newly synthesized polypeptides, activation of proteolysis of misfolded proteins and the formation and dissociation of protein complexes (http://www.uniprot.org/uniprot/P0CB32). In several species like porcine, ovine and bovine, heat shock 70 kDa protein 8 was found to prolong the survival of spermatozoa at body temperature *in vitro* (Elliot et al., 2009; Lloyd et al., 2009). The greater abundance of this heat shock protein in line R could explain the better performance of line R spermatozoon when they are stored *in vitro* during several days (unpublished work, ICTA, 2016). Based on the foregoing, our results provide evidence that genotype has a clear effect on seminal plasma protein abundance.

Regarding the effect of the season on the rabbit seminal plasma proteome, a previous study showed a season effect on the abundance of three proteins (FAM115E-like, haemoglobin subunit zetalike and nerve growth factor) (Casares-Crespo et al., 2016a), but again, these relative quantity protein differences were obtained with a less

resolute proteomic technique. In the current work, results showed that there are slight protein differences between seasons but it does not exist a clear pattern of protein variation between genotypes. This lack of variation could be explained by the controlled environmental conditions used in our study where animals were kept under 16 h light/8 h dark and maintained between 14ºC and 28ºC using cooling and heating systems over the year.

Finally, we determined the variation of β-NGF in rabbit seminal plasma. β-NGF quantity in other reflexively ovulating species such as llama represents 30% of the total seminal plasma protein content (20 mg/ejaculate) (Berland et al., 2016), whereas in rabbit seminal plasma it only represents about 1.4%, independently of the genotype. Kershaw-Young et al. (2012) observed that intramuscular administration of llama seminal plasma (equivalent to <1/4 of an ejaculate) resulted in high rate ovulation induction of females (94% compared to 0% when saline was administered). Interestingly, in other works, the intramuscular administration of rabbit seminal plasma induced ovulation in llamas, but not in rabbits (Silva et al., 2011). The low proportion of β-NGF protein in rabbit seminal plasma and the fact that this protein is also present in a relatively low proportion in the seminal plasma of the majority of spontaneous ovulators (Druart et al., 2013), could lead us to think that β-NGF may have different function. In cows, Stewart et al. (2018) have shown that β-NGF from bull seminal plasma enhances corpus luteum formation and conceptus development. On the other hand, Maranesi et al. (2015) hypothetized that the role of β-NGF protein in rabbit seminal plasma may be related to the modulation of the ovulation/fertilization events. Moreover, β-NGF concentration in rabbit seminal plasma decreased in winter compared to the other seasons, which agrees with the results of a previous study

(Casares-Crespo et al., 2016a). This may be related to the natural reluctance of rabbits to breed in the early winter and it accords with Zhang et al. (2015) findings in wild ground squirrels, which showed that the production of NGF in testes was decreased during the non-breeding season and increased in the breeding season.

CONCLUSIONS

In summary, the present study provides the largest catalogue of rabbit seminal plasma proteins to date and generates a public accessible database of rabbit seminal plasma proteome. Gene ontology analysis of the rabbit complete proteome showed the functional diversity of seminal plasma proteins, with only six of them known to be involved in reproduction processes. Additionally, our data provide evidence that genotype is related to a specific abundance of seminal plasma proteins in rabbit. Thus, upon further validation in other species, the results of the present study intend to be a starting point in the development of specifics extenders for each genotype preventing sperm premature oxidation or selecting GnRH analogues with different amino acid composition less sensitive to enzyme degradation of rabbit seminal plasma proteins. In addition, the comparison of seminal plasma proteins between fertile and subfertile rabbit males, could lead to the identification of fertility biomarkers which could be used to detect subfertile males in commercial rabbit farming. Furthermore, a study of rabbit sperm membrane proteome would be interesting in the future to complete the proteomic information about rabbit sperm.

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VIII.CHAPTER VI

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Characterization of rabbit (*Oryctolagus cuniculus*) sperm proteins

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ABSTRACT

The present study was conducted to characterise rabbit sperm proteins focusing on the influence of the genetic origin. Six samples were recovered during two months from five males from genotype A (New Zealand White origin) and five from genotype R (California origin). Sperm proteins were extracted and subjected to in-gel digestion nano LC-MS/MS and bioinformatics analysis. The resulting library included 487 identified proteins validated with ≥ 95% Confidence (unused Score ≥ 1.3). All the identified proteins belonged to *Oryctolagus cuniculus* taxonomy. These data are available via ProteomeXchange with identifier PXD007989. Only 7 proteins were specifically implicated in reproductive processes according to Gene Ontology annotation. Regarding the comparison of the sperm proteins abundance between genotypes, forty proteins were differentially expressed. Among them, 25 proteins were over-expressed in genotype A, while 15 proteins were over-expressed in genotype R. In conclusion, this study characterizes for the first time rabbit sperm proteins and provides evidence that genotype is related to a specific abundance of spermatozoa proteins.

INTRODUCTION

A greater understanding of the proteins involved in reproduction can benefit animal production. New advances in proteomics are having a major impact on our understanding of how spermatozoa acquire their capacity for fertilization (Aitken and Baker, 2008). Sperm proteomics aims at the identification of the proteins that compose the sperm cell and the study of their function (de Mateo et al., 2013). The sperm cell is one of the most highly differentiated cells and is composed of a head with a highly compacted chromatin structure and a large flagellum with midpiece that contains the required machinery for movement and therefore to deliver the paternal genetic and epigenetic content to the oocyte (Codina et al., 2015). By being so highly differentiated, spermatozoa are advantageous cells to study proteomics of specific compartments such as the membrane, which basically is the area of major importance for its role in interacting with the surroundings and the oocyte (Rodríguez-Martínez et al., 2011). The fusion of a sperm and an oocyte is a sophisticated process that must be preceded by suitable changes in the sperm's membrane composition (Nowicka-Bauer et al., 2013). Recent studies of spermatozoa from the proteomic point of view have allowed the identification of different proteins in spermatozoa that are responsible for the regulation of normal/defective sperm functions (Rahman et al., 2013).

While several techniques are available in proteomics, LC-MS based analysis of complex protein/peptide mixtures has turned out to be a mainstream analytical technique for quantitative proteomics (Tuli and Ressom, 2009). Using this method, detailed proteomic data are now available for human (Baker et al., 2007), macaque (Skerget et al., 2013; Kawase et al., 2015), mouse (Baker et al., 2008a), rat (Baker et al., 2008b), bull (Byrne et al., 2012; Ashrafzadeh et al., 2013; Somashekar et al., 2015), stallion

(Swegen et al., 2015), fruit fly (Wasbrough et al., 2010), *Caenorhabditis elegans* (Ma et al., 2014), carp (Dietrich et al., 2016), rainbow trout (Nynca et al., 2014), mussel (Zhang et al., 2015), ram (Pini et al., 2016), honeybee (Zareie et al., 2013), rooster (Labas et al., 2015) and sika deer (Kawase et al., 2015) sperm proteins.

Rabbit (*Oryctolagus cuniculus*) is an important mammalian species worldwide, being at the same time of commercial interest and a research model animal. European rabbit meat production is approximately 500 thousand tons, corresponding to a 30% share of world production (Petracci et al., 2009). Besides, rabbits account for the seventh highest number of animals slaughtered per year in the European Union-27, with 347,603 × 1000 head in 2014 (FAOSTAT 2014). In a previous work, we identified and quantified rabbit seminal plasma proteins between two different genotypes (Casares-Crespo et al., 2018), concluding the clear effect of genotype in the abundance of certain seminal plasma proteins. However, it is unknown at present whether these differences also exist at sperm proteome level. Therefore, the aim of the present study was to characterise rabbit sperm membrane proteins through nano LC-MS/MS analysis focusing on the influence of the genetic origin.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals in this study were purchased from Sigma-Aldrich Química S.A (Madrid, Spain). All the experimental procedures used in this study were performed in accordance with Directive 2010/63/EU EEC for animal experiments.

1. Localization and animals

The experiment was carried out with 10 males from two Spanish commercial rabbit genetic lines (genotypes A and R) from March to April 2017. Line A is based on New Zealand White rabbits selected since 1980 by a family index for litter size at weaning over 45 generations. Line R comes from the fusion of two lines, one founded in 1976 with Californian rabbits reared by Valencian farmers and another founded in 1981 with rabbits belonging to specialised paternal lines. All bucks were of proven fertility and subjected to a weekly pattern of ejaculate collection. All animals were housed at the Animal Technology and Research Centre (CITA-IVIA, Segorbe, Castellón, Spain) experimental farm in flat deck indoor cages (75×50×30 cm), with free access to water and commercial pelleted diets (minimum of 15 g of crude protein per kg of dry matter (DM), 15 g of crude fibre per kg of DM, and 10.2 MJ of digestible energy (DE) per kg of DM). The photoperiod was set to provide 16 h of light and 8 h of dark, and the room temperature was regulated to keep temperatures between 14°C and 28°C.

2. Semen collection, evaluation and sperm samples preparation

Semen samples were obtained by artificial vagina and collected into a sterile tube. One ejaculate was collected per male and week. Collections were performed on the same day of the week during two months. For each genotype, one pooled sample each week was selected to develop the experiment. A total of 6 pools (3 for each genetic line) were analysed. Routine diagnostic semen analyses were performed to assess the initial seminal quality. Only ejaculates exhibiting a white colour and possessing motility rate higher than 70% were used in the experiment. The volume (mL) of each ejaculate was measured in a graduated conical tube. A 20 µL aliquot was diluted 1:50 with 0.25%

glutaraldehyde solution to calculate the concentration in a Thoma chamber, the percentage of sperm abnormality and spermatozoa with normal apical ridge (NAR) by phase contrast at a magnification of 400X. The motility characteristics of sperm (percentage of total and progressive motile sperm, evaluated using a computer-assisted sperm analysis system) were determined as described by Viudes-de-Castro et al. (2014). Then, ejaculates from the same genotype were pooled each day as a single sample. Each sperm sample was centrifuged at 7,400 x g for 10 min at 22 ºC. The resulting pellets were washed twice by centrifugation at 900 x g for 10 min in PBS. Sperm proteins were extracted according to Casares-Crespo et al. (2017) protocol with few modifications. Briefly, sperm pellets were resuspended in 1% SDS (w/v) in TCG (Tris-citrate-glucose supplemented with a 1% v/v protease inhibitors cocktail) and sonicated on ice 6 times for 5 s at 30% amplitude using an Ultrasonic Lab Homogenizer UP 100 H (Hielscher Ultrasonics GmbH). After sonication, the solution was kept in ice for 15 min and centrifuged for 10 min at 15,000x g at 4ºC. Protein lysates were stored at -80ºC until analysis. Before the proteomic analysis, total protein concentration was quantified in triplicate by the bicinchoninic acid method (BCA) using BSA as standard protein (Smith et al., 1985) and seminal samples were adjusted to 5 µg/µL in saline.

3. In-gel digestion

The proteomic analysis was performed in the proteomics facility of SCSIE University of Valencia that belongs to ProteoRed, PRB2-ISCIII, supported by grant PT13/0001.Thirty µg of total protein was loaded onto a 1-D SDS PAGE gel but not resolved. The entire sample was cut and analyzed as a single band. Samples were digested with sequencing grade trypsin (Promega) as described elsewhere (Shevchenko et al., 1996). Six hundred

ng of trypsin in 100 µL of ammonium bicarbonate (ABC) solution was used for each sample. The digestion was stopped with trifluoroacetic acid (Fisher Scientific; 1% final concentration); a double extraction with acetonitrile (ACN) (Fisher Scientific) was done and all the peptide solutions were dried in a rotatory evaporator. The final mixture was resuspended with 30 µL of 2% ACN; 0.1% trifluoroacetic acid (TFA).

4. Nano LC-MS/MS analysis

Three µL of each sample were loaded onto a trap column (nano LC Column, 3µm particles size C18-CL, 350 µm diameter x0.5mm long; Eksigent Technologies) and desalted with 0.1% TFA at 3µL/min during 5 min. The peptides were then loaded onto an analytical column (LC Column, 3 µm particles size C18-CL, 75µm diameter x12cm long, Nikkyo) equilibrated in 5% acetonitrile (ACN) 0.1% formic acid (FA). Peptide elution was carried out with a linear gradient of 5% to 35% of solvent B in A for 60 min. (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 300nL/min. Peptides were analysed in a mass spectrometer nano ESIqQTOF (5600 TripleTOF, ABSCIEX).

Eluted peptides were ionized applying 2.8 kV to the spray emitter. The mass spectrometric analysis was carried out in a data-dependent mode. Survey MS1 scans were acquired from 350–1250 m/z for 250 ms. The quadrupole resolution was set to 'UNIT' for MS2 experiments, which were acquired from 100–1500 m/z for 25 ms in 'high sensitivity' mode. Following switch criteria were used: charge: 2+ to 5+; minimum intensity; 70 counts per second (cps). Up to 25 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s. The system sensitivity was controlled with 2 fmol of 6 proteins mixture (LC Packings). Samples were injected in a random order.

The proteomics data and result-files from the analysis have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository, with the dataset identifier PXD007989 and 10.6019/PXD007989.

5. Protein identification

The SCIEX.wiff data-files were processed using ProteinPilotv5.0search engine (AB SCIEX). ProteinPilot default parameters were used to generate peak list directly from 5600 TripleTofwiff files. The Paragon algorithm of ProteinPilot v5.0 was used to search UniProt_mammals' protein database (22/06/2017) with the following parameters: trypsin specificity, cys-alkylation, taxonomy restricted, FDR (False Discovery Rate) calculation and the search effort set to through. For building the spectra library all the files combined were searched with the parameters previously used.

To avoid using the same spectral evidence in more than one protein, the identified proteins are grouped based on MS/MS spectra by the Protein-Pilot Progroup algorithm. A protein group in a Pro Group Report is a set of proteins that share some physical evidence. Unlike sequence alignment analyses where full length theoretical sequences are compared, the formation of protein groups in Pro Group is guided entirely by observed peptides only. Since the observed peptides are actually determined from experimentally acquired spectra, the grouping can be considered to be guided by usage of spectra. Then, unobserved regions of protein sequence play no role in explaining the data. Only peptide and protein identifications with ≥ 95% Confidence (unused Score ≥ 1.3) were validated. Protein identifications were accepted if they contained at least two identified peptides.

6. Label-free protein quantification using Chromatographic Areas

For quantification, the group file generated by Protein Pilot was used. The ions areas were extracted from the wiff files obtained from LC-MS/MS experiment by Peak View® v1.1. Only peptides assigned with confidence ≥ 95%, among those without modifications or shared by different proteins were extracted. A total of 6 samples were analysed and 487 proteins were quantified.

7. Bioinformatics analysis

Gene names of the proteins were obtained from UniProt database by running a Retrieve/ID mapping tool of the protein accession numbers. Gene ontology terms for biological process, molecular function and cellular component were obtained using PANTHER v12.0 (http://www.pantherdb.org/ accessed on 16/08/2017) (Mi et al., 2017). Gene names of all sperm proteins were used to search against the panther database with *Homo sapiens* as the organism to maximise classifications, and a PANTHER™ Go Slim analysis was performed for each category.

8. Statistical analysis

The quantitative data obtained by PeakView® were analysed by Marker View®v1.3 (AB Sciex). First, areas were normalized by total areas summa, and then a Discriminant Analysis (DA) and a T-Test were done. Proteins were considered differentially expressed between genotypes if the adjusted p-value < 0.05. Mean quantity of proteins were calculated and the fold-changes between the two groups were estimated.

RESULTS

1. Sperm quality parameters

Mean values of volume, concentration, percentage of abnormal spermatozoa, total and progressive sperm motility and spermatozoa with normal apical ridge of the samples used in the study are shown in Table 6.1.

Table 6.1. Rabbit sperm characteristics (n=4).

Parameters	
Concentration (N x 10^6 spermatozoa/ml)	447.5 ± 108.3
Volume (mL)	1.5 ± 0.3
Sperm abnormality (%)	26.2 ± 3.3
Acrosome integrity (%)	86.5 ± 2.6
Total sperm motility (%)	83.3 ± 9.1
Progressive sperm motility (%)	47.7 ± 5.5

2. Rabbit sperm proteome

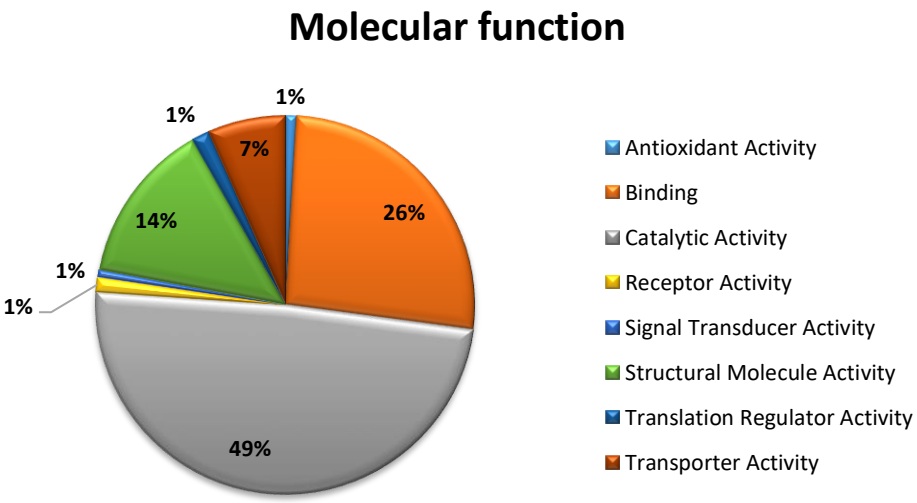
The complete spectral library included 487 proteins validated with ≥ 95% Confidence (unused Score ≥ 1.3) when using at least 2 peptides for identification (Table S6.1). All the identified proteins belonged to *Oryctolagus cuniculus* taxonomy. These 487 proteins were quantified based on their chromatographic or peak areas (Table S6.2).

The complete rabbit sperm proteome was classified under different categories based on their molecular function, biological process and cellular components (PANTHER analysis). The results are shown in Figure 6.1. For molecular function (Fig. 6.1a), a total of 343 hits were found. The catalytic activity was the predominant function (49%), followed by binding (26%) and structural molecule activity (14%). Regarding biological process (Fig. 6.1b), a total of 669 hits were found. The cellular process (29%) and the

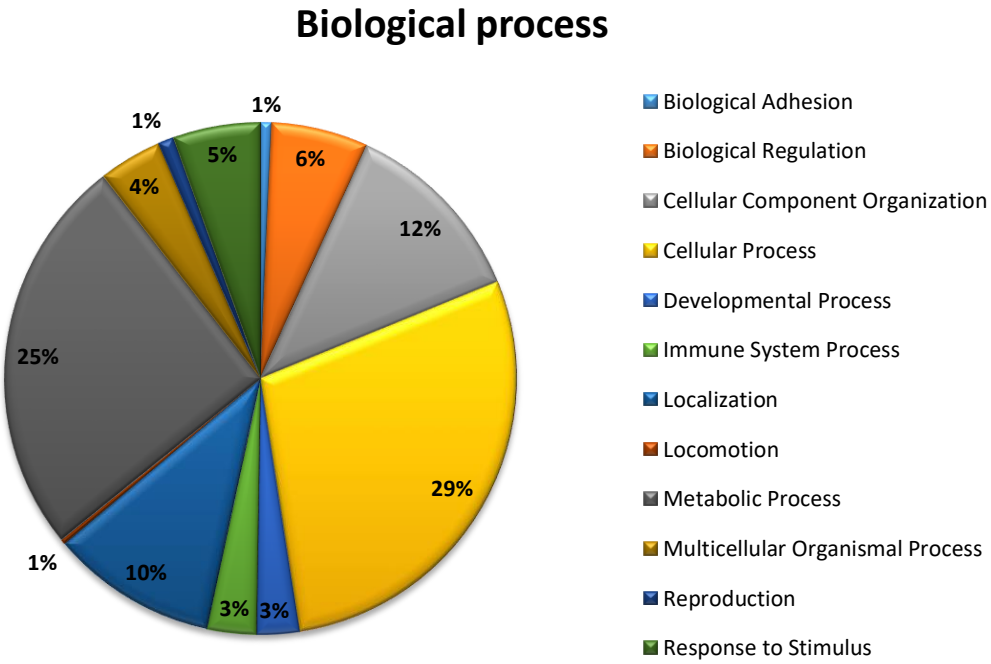
metabolic (25%) were the most abundant categories, but it is worth mentioning that 8 hits (1%) were classified in the category of reproduction, 4 in the fertilization and the other 4 in gamete generation process. Finally, a total of 336 hits were found for cellular component category (Fig. 6.1c). Cell part (47%), organelle (27%), macromolecular complex (15%) and membrane (6%) were the most abundant cellular components of the studied proteins.

Figure 6.1. Pie charts showing the distribution of rabbit sperm proteins based on their a) molecular function, b) biological process and c) cellular component, using UniProt KB database in combination with PANTHER.

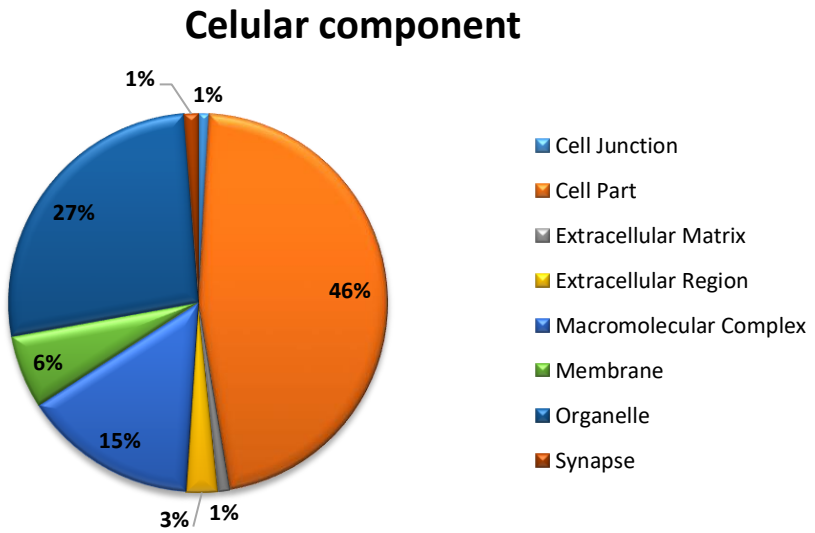
a)



b)



c)



3. Effect of genetic origin on spermatozoa proteome

The results of the sperm proteome comparison between both synthetic lines (A and R) are shown in Fig. 6.2. Discriminant Analysis (DA) showed a clear separation between samples from different genetic origin, classifying the six sperm samples into two different main clusters corresponding to both genotypes. Given that the proteome between both genotypes presented high variability, a t-test was done. Results showed a total of 40 differentially expressed proteins ($p < 0.05$) between genetic lines. Of the differentially expressed proteins, 25 proteins were over-expressed in genetic line A and 15 proteins over-expressed in line R (Table 6.2).

Figure 6.2. Discriminant Analysis (DA) showing the classification of spermatozoa protein samples from genotypes A and R, based on relative protein amount.

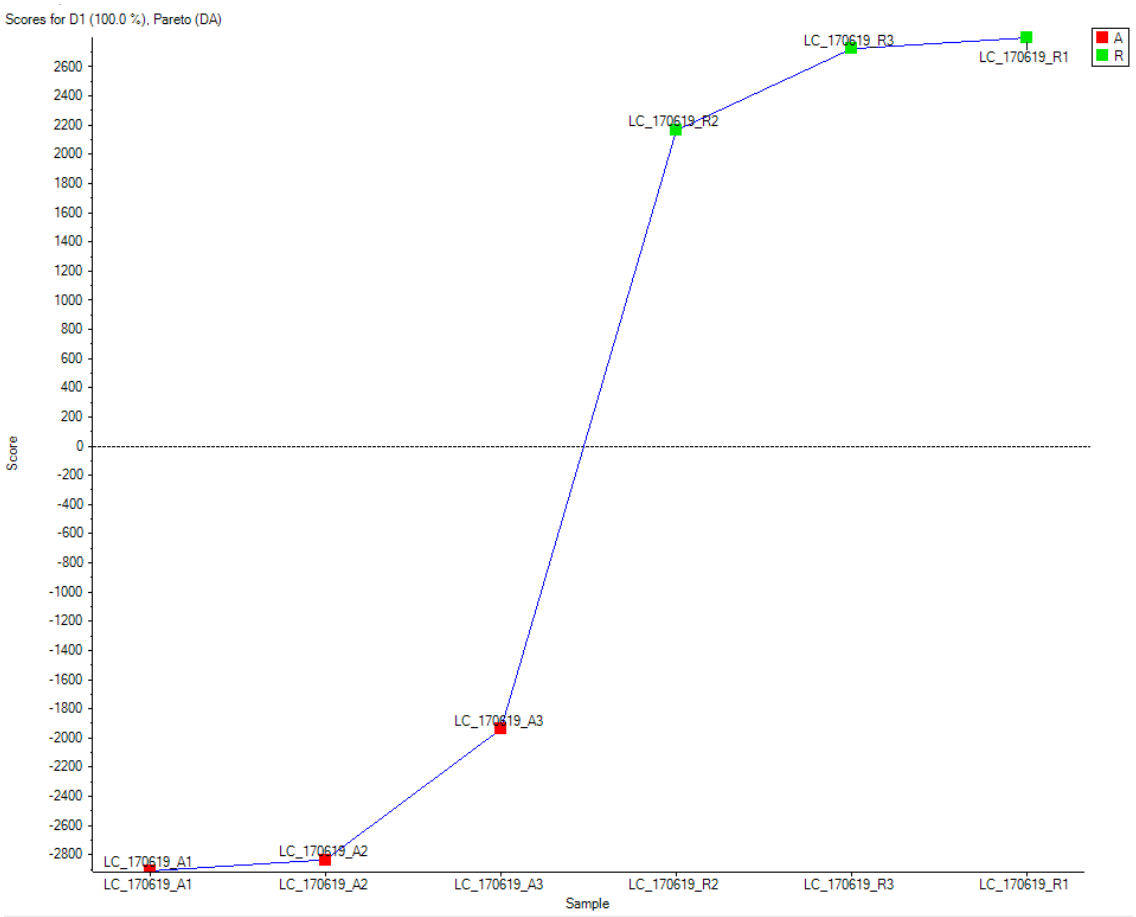


Table 6.2. List of differentially expressed rabbit spermatozoa proteins, included in *Oryctolagus cuniculus* taxonomy, between genotypes A and R.

Gene ID	Gene name	Mean protein amount		Log (FoldChange)	p-value	Biological Process
		Line A	Line R			
BASP1	Brain acid soluble protein 1	24770,351	88227,532	-0.552	0.00256	
A0A0G2JH23	Uncharacterized protein	14742,829	39547,488	-0.429	0.00648	
USP5	Ubiquitin carboxyl-terminal hydrolase 5	19155,099	7991,209	0.380	0.00775	
CCT8	T-complex protein 1 subunit theta	289998,762	197226,072	0.167	0.00824	Protein folding, protein complex assembly
AKR1B1	Aldose reductase	2042126,661	684791,411	0.475	0.00885	
PGK2	Phosphoglycerate kinase 2	1853797,274	1018697,679	0.260	0.00950	Glycolysis
FLNB	Filamin-B	80934,073	142157,789	-0.245	0.01146	Cellular component morphogenesis
PSMA1	Proteasome subunit alpha type-1	43475,764	19905,528	0.339	0.01313	
SORT1	Sortilin	34590,467	16905,899	0.311	0.01353	Intracellular protein transport, lipid metabolic process, lipid

						transport, receptor-mediated endocytosis
ATP11A	Probable phospholipid-transporting ATPase IH	7586,766	13297,619	-0.244	0.01413	Anion transport, catabolic and cellular process
S100A8	Protein S100-A8	3106,240	42277,814	-1.134	0.01579	DNA replication, cell cycle, macrophage activation
HSPB1	Heat shock protein beta-1	122006,550	270497,552	-0.346	0.01613	Protein folding, immune system process, response to stress
CCT3	T-complex protein 1 subunit gamma	190302,157	113244,540	0.225	0.01738	Protein folding, protein complex assembly
PRDX1	Peroxiredoxin-1	20463,159	48178,975	-0.372	0.01975	
ACTC1	Actin, alphacardiac muscle 1	17064660,120	19815038,34	-0.065	0.02015	Cell cycle, endocytosis, exocytosis, intracellular protein transport
RAB14	Ras-relatedprotein Rab-14	11035,514	7647,710	0.159	0.02028	
ME1	NADP-dependent malic enzyme	63596,915	24116,024	0.421	0.02191	Generation of precursor metabolites and energy, carbohydrate metabolic process, cellular amino acid catabolic process

G1T1S4	Uncharacterized protein	7527,446	24318,855	-0.509	0.02279	
CCT2	T-complex protein 1 subunit beta	139552,440	70079,970	0.299	0.02652	Protein folding, protein complex assembly
MYO1C	Unconventional myosin-Ic	9273,656	27610,368	-0.474	0.02678	Mitosis, cellular component movement and organization, cytokinesis, intracellular protein transport, intracellular signal transduction
CSE1L	Exportin-2	43538,784	31223,895	0.144	0.02712	Cellular process, nuclear transport, protein localization and targeting
CCT6B	T-complex protein 1 subunit zeta-2	46030,918	30228,978	0.183	0.02933	Protein folding, protein complex assembly
ARSA	ATPase ASNA1	235936,189	118217,646	0.300	0.02996	Ion transport, reponse to toxic substance
TCP1	T-complex protein 1 subunit alpha	280703,715	159641,857	0.245	0.03036	Protein folding, protein complex assembly
PSMB5	Proteasome subunit beta type-5	38988,095	20945,469	0.270	0.03086	
MPST	3-mercapto pyruvate	17474,356	7178,149	0.386	0.03302	Anion transport, immune system

	sulfurtransferase					process, response to toxic substance
TXNRD1	Thioredoxin reductase 1, cytoplasmic	15003,186	4118,362	0.561	0.03578	Ferredoxin metabolic process, nitrogen compound metabolic process, respiratory electron transport chain
AP1G1	AP-1 complex subunit gamma-1	4014,558	9804,403	-0.388	0.03596	Intracellular protein transport, receptor-mediated endocytosis
SULT1C4	Sulfotransferase 1C4	207417,507	46024,524	0.654	0.03816	Sulfur compound metabolic process
GSTP1	Glutathione S-transferase P	6118,846	24472,019	-0.602	0.03941	Muscle contraction
ACTB	Actin, cytoplasmic 1	16089029,870	17666884,880	-0.041	0.04093	Cell cyle, endocitosis, exocytosis, intracelular protein transport
RSPH1	Radial spoke head 1 homolog	37959,409	25088,770	0.180	0.04116	
ZAN	Zonadhesin	851340,621	657973,081	0.112	0.0424	Cell adhesion, cellular process, fertilization
LY6K	Lymphocyte antigen 6K	17476,014	10019,657	0.242	0.04276	
GPI	Glucose-6-phosphate isomerase	357966,171	251913,213	0.153	0.04322	Gluconeogenesis, glycolysis
CUL3	Cullin-3	43018,851	27406,064	0.196	0.04441	Catabolic process, cellular

						process, cellular protein modification process, proteolysis
NT5C3A	Cytosolic 5'-nucleotidase 3A	23230,678	44228,290	-0.280	0.04754	
YWHAE	14-3-3 protein epsilon	187532,157	243472,884	-0.113	0.04769	
SERPINE2	Glia-derived nexin	5889,179	1960,672	0.478	0.04867	Regulation of biological process
KLHL10	Kelch-like protein 10	14523,219	9150,116	0.201	0.04907	Cellular process, celullar protein modification process

DISCUSSION

To the best of our knowledge this is the first study in which rabbit sperm proteins are characterised. As a consequence of the lack of knowledge of rabbit proteomics in comparison with other mammalian species, among the 487 identified proteins 325 were catalogued as uncharacterized proteins. In UniProtKB database the 98% of the protein sequences have been derived from cDNA or genomic sequencing, thus most of the available protein sequences are reliant on the quantity and quality of DNA or RNA-derived information for that species (Bayram et al., 2016). As a result, studies of the reproductive proteome to date have been limited to model species such as human, mice and fruit fly (Baker et al., 2008a; Wasbrough et al., 2010; Amaral et al., 2014) supported by extensive, high quality genomic information or with dedicated genome projects such as honeybee (Poland et al., 2011) among which rabbit species is not included. In addition, Bayram et al. (2016) studied the species origin of database matches using mammalian proteome database search in UniProtKB and the dominant species were few: human, mouse, rat, sheep and cattle, which account for 87.5% of the sequences entries. Therefore, the fact that rabbit is a non-model species and lacks a fully annotated genome explains the high number of uncharacterised proteins found in rabbit spermatozoa in the present work. To solve this drawback, all protein accession numbers were translated into gene names in order to analyse the results.

Bioinformatics analysis of rabbit spermatozoa proteome revealed that 49% of identified proteins were related to catalytic activity and the second dominant group of proteins were assigned to a binding function (26%). These proportions agree with previous proteomic studies of honeybee and carp spermatozoa (Zareie et al., 2013; Dietrich et al., 2016). Regarding biological process, the cellular process (29%) was the most abundant category in

rabbit spermatozoa followed by metabolic process (25%), which coincides with honeybee sperm proteins (Zareie et al., 2013). It is also noticeable that only seven of the 487 proteins identified in rabbit spermatozoa are to date recorded in GO as being directly associated with reproductive processes such as fertilization and gamete generation. These proteins related with reproductive processes are the following: acrosin-binding protein (ACRBP), zonadhesin (ZAN), sperm equatorial segment protein 1 (SPESP1), serine protease inhibitor Kazal type 8 (SPINK8), dual specificity testis-specific protein kinase 2 (TESK2), dynein heavy chain 9 (DNAL1) and four and a half LIM domains protein 1 (FHL1). It is surprising that so few spermatozoa proteins are found in the category of reproduction and that many of the proteins studied in this work have not been assigned a specific role in GO. In previous studies in boar and in rabbit seminal plasma, authors encountered the same situation (Pérez-Patiño et al., 2016; Casares-Crespo et al., 2018). Despite of this, we are sure that the identified sperm proteins are directly or indirectly related to reproduction processes. Finally, GO analysis revealed that the majority of rabbit spermatozoa proteins were extracted from cell parts, organelles and membranes.

Among the twenty-five more abundant proteins in genotype A, we found proteins related to different biological functions such as: protein folding, glycolysis, protein transport, metabolism, ion transport and fertilization.

CCT8, CCT3, CCT2 and CCT6B are chaperones that contain the TCP1 complex and whose function is to assist the folding of other proteins such as tubulin and actin upon ATP hydrolysis [http://www.uniprot.org/uniprot/G1SHZ8]. In addition, arylsulfatase A protein (ARSA) has been localized in the acrosomal region of human spermatozoa and it is known to increase its surface expression significantly during capacitation (Redgrove et al., 2012). These proteins have been related to sperm zona pellucida interaction role in mouse and

human spermatozoa by several authors (Dun et al., 2011; Redgrove et al., 2011, 2012). On the other hand, zonadhesin protein' (ZAN) role is to mediate species-specific zona pellucida adhesion (Tardif et al., 2010) and has been localized in the anterior acrosome of rabbit spermatozoa (Lea et al., 2001). In a previous work (Casares-Crespo et al., 2018), zonadhesin, was also found more abundant in seminal plasma of rabbit A genotype compared to genotype R. Furthermore, other proteins over-expressed in genotype A are: cullin 3 (CUL3), which has been recently found to have an important role in the human sperm flagellum (Jumeau et al., 2017), phosphoglycerate kinase 2 (PGK2), that has shown to be essential for sperm motility and male infertility in mice (Danshina et al., 2010) and SERPINE2, which may play a role as a decapacitation factor (Lu et al., 2011). All of these findings, especially the increased amount of these proteins observed in genotype A in comparison with genotype R could explain in part the enhanced fertility and prolificacy previously described in genotype A (Lavara et al., 2005; Safaa et al., 2008).

On the other hand, among the fifteen more abundant proteins in genotype R, we found proteins related to different biological functions such as: antioxidant activity, binding, catalytic activity, transporter activity and structural molecule activity.

For instance, the protein HSPB1 belongs to a superfamily of mammalian small stress proteins and its function has been suggested to be related to the cytoskeleton (Fontaine et al., 2003). Just like ACTB protein, which is an actin involved in cell motility and cytokinesis (Zeng et al., 2014). On the other hand, peroxiredoxins such as PRDX1 are highly sensible to oxidative stress proteins which are involved in the antioxidant protection of the mammalian spermatozoa (Liu and O'Flaherty, 2017). Finally, BASP1 protein, which has been located in rat mature spermatozoa, may be participating in biochemical processes through the activation of calcium (Mosevitsky et al., 2012).

In summary, the present study characterises for the first time rabbit sperm proteins and generates a publicly accessible database of rabbit sperm proteome, which still remains poorly understood. In addition, our data provide evidence that genotype has a huge impact on protein abundance in rabbit sperm. The present work together with the previous study of rabbit seminal plasma lead to the complete characterization of rabbit semen. Further studies are needed in order to elucidate the reproductive role of these identified proteins and the different evolution of these genotypes that gives rise to the intraspecies variation found.

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IX.GENERAL DISCUSSION

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1. DEVELOPMENT OF NEW ARTIFICIAL INSEMINATION EXTENDERS SUPPLEMENTED WITH GnRH ANALOGUES

The use of artificial insemination (AI) in rabbit farms has become a common practice in European countries, being currently used in more than 80% of the Spanish and EU rabbit farms (Quintela et al., 2009). AI offers the same benefits for rabbit breeding as in other species in the control of genetic diversity, rapid upgrading of stock, establishment of pregnancies in females which refuse to mate, and avoidance of the spread of diseases (Morrell, 1995). In addition, using AI, an ejaculate from one male can be used to inseminate a large number of females and conception rates after AI with fresh semen are equivalent to or even better than those obtained by natural mating (Daniel and Renard, 2010; Kitajima, 2009).

The rabbit (*Oryctolagus cuniculus*) is considered a reflexively ovulating species in which ovulation is induced by sensory stimulation associated with mating. In this species, a short mating bout including ejaculation induces genital somatosensory cues that contribute to the activation on GnRH neurons and the consequential generation of a preovulatory LH surge from the pituitary gland (Rebollar et al., 2012). Therefore, when using AI, the administration of a GnRH analogue is mandatory to induce doe ovulation due to the lack of nervous stimuli evoked by the male. This treatment requires an intramuscular injection, which can result in stress to the animal and additional work for the farm operators (Dal Bosco et al., 2011). Moreover, in most rabbit farms, GnRH injection is usually performed by the farmer, with a certain risk of misuse, and

increasing the time needed for the artificial insemination of each doe (Quintela et al., 2009). Recent studies have investigated the possibility of ovulation induction in rabbits by vaginal absorption after supplementation of the seminal dose with different GnRH synthetic analogues (Gogol, 2016a, 2016b; Dal Bosco et al., 2014; Quintela et al., 2004, 2009; Ondruška et al., 2008; Vicente et al., 2008, 2011; Viudes-de-Castro et al., 2007, 2014). There are clear breeding advantages of intravaginal administration of GnRH analogue (noninvasive route, less treatment distress, labor for the farmers, and operating time), but unfortunately, to achieve fertility results similar to those with GnRH intramuscular injection, the intravaginally hormone concentration should be much higher than the amount administered intramuscularly (Viudes-de-Castro et al., 2014). The absorption of GnRH by vaginal mucosa is influenced by several factors. The main barrier is mucosal permeation, but another factor that limits the bioavailability of GnRH analogue is the proteolytic activity found in the seminal plasma as well as in the female vagina. For this reason, in order to obtain fertility rates comparable with those obtained with the usual intramuscular injection, the intravaginal GnRH dose has to be at least 15-fold higher (Quintela et al., 2009), becoming a potential health risk for farmers. Various approaches to improve protein delivery by vaginal route include: use of enzyme inhibitors, absorption enhancers, mucoadhesive polymers and/or novel carrier systems such as nanoparticles.

With this background, the first three chapters of this thesis aimed to develop new AI extenders supplemented with different substances in order to increase the bioavailability of the GnRH analogue without affecting ovulation induction or the reproductive performance.

In the first chapter, our hypothesis was that if we used protease inhibitors in AI extenders, part of the enzyme activity that degrades the GnRH analogue would be inhibited and therefore, the bioavailability of the hormone would be higher. Therefore, the objective was to evaluate the effect of the inclusion of protease inhibitors in semen extender on *in vitro* rabbit semen quality parameters and on reproductive performance after AI. The results showed that rabbit semen extender supplementation with a protease inhibitors cocktail affected neither semen quality (motility, viability, acrosome integrity) nor fertility rate, but affected the prolificacy rate by decreasing the total number of kits born per litter (9.3 *vs*. 8.2, positive control and AMIS group, respectively).

Several studies have tested the effect of different protease inhibitors on mammal sperm *in vitro* quality. For instance, leupeptin had no effect in rabbit sperm motility (de Lamirande et al., 1986). In pig, spermatozoa incubated in medium with AEBSF were less motile after 6 h of incubation, yet progressive motility, VAP, VSL, acrosome status and mitochondrial potential remained unaltered (Beek et al., 2015). These results are consistent with ours, as seminal quality parameters were similar between control and protease inhibitors group after 2 h of incubation.

The fact that the inclusion of protease inhibitors in semen extender affected the prolificacy rate, having this group one kit less per delivery, could be explained because proteases play an important role during mammalian fertilization. Seminal plasma composition is designed to assure the successful fertilization of the oocyte and is characterized by a high abundance of proteins which play important roles in sperm survival and are involved in various events such as epididymal sperm maturation, sperm capacitation, sperm membrane stabilization, modulation of the uterine immune

response, sperm transport in the female genital tract, gamete interaction and fusion and even pregnancy establishment (Okabe et al., 1993; Topfer-Petersen et al., 1998; Gwathmey et al., 2006; Rodríguez-Martínez et al., 2011; Laflamme et al., 2013). The abundance of proteases and protease inhibitors in seminal plasma show the importance of this system in this body fluid (Pilch et al., 2006). In human, for example, the 60% of the seminal plasma proteome has enzymatic activity. Therefore, the inhibition of a wide variety of proteases with the cocktail used, could have negatively affected this process. In addition, the part of the fecundation process affected by protease inhibitors seems to be species-specific. For instance, in guinea pig, the incubation of sperm with leupeptin inhibited the completion of the acrosomal reaction, but bestatin had no effect (Flaherty et al., 1993). In bovine sperm, the use of trypsin and chymotrypsin inhibitors, but not metalloproteases, were effective in inhibiting the acrosomal reaction process and in addition, sperm incubation with these proteases did not reveal statistically differences in the sperm zona pellucida binding capacity in relation to control sperm (Deppe et al., 2008). In pig, the effect of AEBSF on *in vitro* fertilization and polyspermy rates were a decreased by at least 50% (Beek et al., 2015). In mouse and human, the use of specific serine proteases inhibitors decreases the fertilization rate (Fraser, 1982; Llanos et al., 1993). Finally, studies in sea urchin provide evidence for the involvement of metalloproteases in membrane fusion during the acrosome reaction (Farach et al., 1987).

Therefore, considering the results of our first study, we conclude that as a consequence of the addition of a wide variety of protease inhibitors in the rabbit semen extender, prolificacy rate was negatively affected. For this reason, we conducted a second experiment in which we only inhibited the aminopeptidase

activity (APN), because in a previous work by Viudes-de-Castro et al. (2014), authors observed that the bioavailability of buserelin acetate when added to the seminal dose appeared to be determined by the activity of the existing seminal plasma aminopeptidases. We supplemented AI extenders with bestatin and EDTA as aminopeptidase inhibitors (AMIs) and we evaluated their effect on *in vitro* rabbit semen traits (motility, acrosome status and viability) and on *in vivo* reproductive performance (fertility and prolificacy) after AI. Moreover, a secondary objective of this second work was to study the effect of AMIs on β-nerve growth factor (β-NGF) protein's protection from enzyme degradation. β-NGF is a highly conserved protein of about 14 kDa, capable of inducing ovulation when administered intramuscularly to other reflex ovulators species such as alpaca, llama and camel females (Kershaw-Young et al., 2012; Silva et al., 2015; Berland et al., 2016; Adams et al., 2016; El Allali et al., 2017). β-NGF has been identified in rabbit seminal plasma (Casares-Crespo et al., 2016a, 2018b) and its mRNA in adult rabbit's prostate, seminal gland and testis (García-García et al., 2017). However, in rabbit species, β-NGF protein or seminal plasma intramuscular administration to rabbit does does not provoke ovulation (Silva et al., 2011; García-García et al., 2017). Although the function of the β-NGF protein in rabbit reproduction remains unknown, we thought that it was interesting to find out if AMIs were able to protect it while protecting GnRH analogue.

The results of this second study showed that the addition of bestatin and EDTA in the rabbit semen extender had neither effect on semen quality nor on the fertilizing capacity of spermatozoa. In agreement with our results, the addition of bestatin to guinea pig sperm had no effect on membrane fusion (Flaherty et al., 1993) and the incubation of bovine sperm with EDTA did not affect the acrosome reaction (Deppe et

al., 2008). Thus, the fecundation process damaged in the first work by protease inhibition (Casares-Crespo et al., 2016b) seemed not to be affected by bestatin and EDTA, showing a similar prolificacy rate between groups with or without AMIs (10.12 *vs.* 10.15, respectively). On the other hand, the presence of AMIs improves the bioavailability of β-NGF in semen up to 12 h of storage. As a consequence, the possible ovulation/fertilization modulator role of β-NGF was assured over this time by the presence of bestatin and EDTA in the extender, being able to protect β-NGF from enzyme degradation.

Given that it was demonstrated that AI extender supplementation with aminopeptidase inhibitors (AMIs) such as bestatin and EDTA did not affect rabbit seminal quality nor reproductive performance (Casares-Crespo et al., 2018a), but inhibited part of the seminal plasma aminopeptidase activity, the third study aimed to evaluate the *in vivo* effect of a 20% reduction of hormone concentration in extenders supplemented with AMIs. Besides, we have managed to develop chitosan (CS)-dextran sulfate (DS) nanoparticles containing buserelin acetate (Fernández-Serrano et al., 2017). In this previous work, we achieved a hormone entrapment efficiency of 40-50% and showed that these nanoparticles did not affect rabbit seminal quality parameters and, in addition, significantly increased the acrosome integrity of spermatozoa. Therefore, in this third chapter we tested the effect of a 20% reduction of hormone concentration in extenders supplemented with AMIs and with the GnRH analogue free or entrapped in CS-DS nanoparticles on rabbit reproductive performance.

According to our results, when the buserelin acetate was non encapsulated, although the extenders were supplemented with bestatin and EDTA, the utilization of 4 µg hormone/doe significantly reduced fertility rate compared to group with 5 µg

hormone/doe (0.70 *vs.* 0.85, respectively). This fact shows that even though part of the enzymatic activity of seminal plasma is inhibited, the bioavailability of GnRH is not enough to allow a 20% reduction in the concentration of hormone in the extender without compromising fertility. Maybe it is possible that we are working with a limiting hormone concentration (5 μg/doe) and even a small hormone reduction could affect fertility. In this sense, there is only another work in which a GnRH analogue concentration lower than 5 μg/doe has been used in rabbit ovulation induction, and the results were the same as ours, with fertility rate significantly lower and similar prolificacy rate (2.5 μg/doe GnRH-Lecirelinum in seminal dose) (Ondruška et al., 2008). On the other hand, when buserelin acetate was encapsulated in CS-DS nanoparticles, no differences in fertility and prolificacy were observed between 4 μg hormone/doe or 5 μg hormone/doe, showing similar values than C5 group (0.85, 0.82 and 0.85, respectively). Thus, with the use of nanoparticles, the GnRH analogue seems to be protected against degradation and a 20% hormone reduction does not affect fertility. In resemblance with our results, Trapani et al. (2010) employed CS based nanoparticles in oral administration of a small peptide (glutathione), and they achieved to protect the drug from the enzymatic gastric degradation and induce permeabilization of the intestinal epithelia. In addition, Han et al. (1995), in an *in vitro* study in rabbit, observed that the permeability of the vaginal membrane to GnRH increased twice when EDTA was used, suggesting that enzyme inhibition effect of EDTA resulted in substantial enhancement of vaginal absorption. Therefore, the enzyme inhibitor role of bestatin and EDTA besides the absorption enhancement effect of EDTA and the protection role of chitosan and dextran sulfate nanoparticles and their mucoadhesive function, all together, could explain the fertility rate improvement of Q4 group

compared to C4 group. In conclusion, the CS-DS nanoparticles prepared by coacervation process as carrier for buserelin acetate overcome some of the limitations associated with the vaginal application of the hormone in rabbit artificial insemination and allow to reduce the concentration of hormone used in an extender supplemented with bestatin and EDTA without affecting the fertility and prolificacy of rabbit females. Therefore, nano encapsulation seems to be a promising system to protect the GnRH analogue in order to decrease the hormone concentration in rabbit artificial insemination extenders.

2. PROTEOMIC CHARACTERIZATION OF RABBIT SEMEN

The use proteomics in order to identify and quantify the semen proteins of commercially relevant species has provided a lot of new information lately. In species such as boar, stallion, bull, ram, rooster, turkey, salmon, carp and trout, seminal plasma and/or sperm proteins have been studied with this method (Dietrich et al., 2014a, 2014b; Gombar et al., 2017; Marzoni et al., 2013; Novak et al., 2010; Nynca et al., 2014a, 2014b; Pérez-Patiño et al., 2016; Pini et al., 2016; Rego et al., 2016; Slowinska et al., 2017). Although rabbit is an important mammalian species worldwide, being at the same time of commercial interest and a research model animal and European rabbit meat production corresponds to a 30% share of world production (Petracci et al., 2009), a limited number of studies have performed an analysis of rabbit seminal plasma or sperm proteins (Arruda-Alencar et al., 2012; Davis and Davis, 1983; de Lamirande et al., 1983; Lavon, 1972; Minelli et al., 2001; Okabe et al., 1993; Thomas et al., 1986; Viudes-de-Castro et al., 2004). Because rabbit seminal plasma and sperm proteome remained unsolved, we decided to study them, becoming the second

objective of the present thesis. In chapter four, five and six, we looked into rabbit semen proteins from two different methodology approaches (1D polyacrylamide gel *versus* LC-MS/MS). Moreover, we compared the protein abundance between rabbit genetic breeds A and R and between seasons.

In the fourth and fifth chapters, seminal plasma of rabbits from genotype A (maternal line) and R (paternal line) was collected during a natural year and was subjected to polyacrylamide gel electrophoresis (SDS-PAGE) or to nano LC-MS/MS, respectively. In the fourth chapter, a traditional 1D polyacrylamide gel was done; being able to identify only the major proteins visible after Coomassie Colloidal Blue staining and obtaining the differentially expressed bands by the relative quantity of the protein bands through scanning and analysing the gel with 1D software. When using this approach, seven protein bands were significantly different between genetic lines and among these, three protein bands were significantly different between seasons. The differentially expressed proteins were: FAM115E-like (220, 113 and 59 kDa), ectonucleoside triphosphate diphosphohydrolase 3 isoform X2 (72 kDa), annexin A5 (32 kDa), lipocalin allergen Ory c 4 precursor (19 kDa), and hemoglobin subunit zeta-like (13 kDa) between genetic lines and FAM115E-like (113 kDa), hemoglobin subunit zeta-like (13 kDa) and β-Nerve growth factor (12 kDa) between seasons. However, with the same samples and through nano LC-MS/MS technique, we were able to identify and quantify 402 proteins and twenty-three proteins were significantly different between genotypes. Therefore, the fact that in the fifth study a more accurate technique such as LC-MS/MS was used in order to identified the differentially expressed proteins, could explain the differences found between previous (Casares-Crespo et al., 2016a; Safaa et al., 2008; Viudes-de-Castro et al., 2004) and current

results. Indeed, the exceptional sensitivity and resolving power of today's mass spectrometers allow for the detection of proteins and peptides at low femtomole quantities (Wither et al., 2016).

Bioinformatics analysis of rabbit SP proteome revealed that 50% of identified proteins were related to catalytic activity and the second dominant group of proteins were assigned to a binding function (27%). These proportions agree with previous proteomic studies of human, ram, carp and boar seminal plasma (Dietrich et al., 2014; Pérez-Patiño et al., 2016; Pilch and Mann, 2006; Souza et al., 2012). Inside the category of catalytic activity, the aminopeptidase B protein is included. This enzyme has an important role in rabbit AI because it is responsible for degrading the GnRH analogue when it is added to the seminal dose to induce doe ovulation. In previous works, we have demonstrated that aminopeptidase activity in rabbit seminal plasma reduces the bioavailability of the GnRH analogue (Viudes-de-Castro et al., 2014) and new extenders with aminopeptidase inhibitors are being developed (Casares-Crespo et al., 2016b, 2017). Regarding biological process, the metabolic (28%) was the most abundant category in rabbit SP, which coincides with human, carp and boar SP proteins (Dietrich et al., 2014; Pérez-Patiño et al., 2016; Souza et al., 2012). It is also noticeable that only 6 of the 402 proteins identified in rabbit SP are to date recorded in GO as being directly associated with reproductive processes. Similar results were found in boar SP, where only 20 of the 374 proteins identified were annotated as related to reproduction (Pérez-Patiño et al., 2016).

Additionally, our results clearly indicate that SP proteins abundance in rabbit seems to be related to a specific genotype, which in several previous studies have demonstrate differences in sperm quality, fertility and prolificacy (Safaa et al., 2008; Vicente et al.,

2000). As stated, we identified a higher abundance of 11 proteins in genotype A seminal plasma, while another 12 proteins were more abundant in genotype R seminal plasma.

Among the over-expressed proteins in genotype A, we find uteroglobin and zonadhesin. Uteroglobin has been identified in rabbit seminal plasma and in rabbit uterus secretions (Kirchner and Schroer, 1976; Müller, 1983) and zonadhesin in spermatozoa (Lea et al., 2001), but their role remains unknown to date. While uteroglobin, also present in the prostate, may be responsible for suppressing sperm antigenicity in the rabbit (Mukherjee et al., 1983), zonadhesin is located exclusively in the anterior acrosome and may be one of the proteins that anchors the acrosomal shroud to the zona pellucida (http://www.uniprot.org/uniprot/P57999), thereby allowing the spermatozoa to continue penetration and fertilization to proceed spermatozoa (Lea et al., 2001). In addition, we also observed a greater amount of ectonucleoside triphosphate diphosphohydrolase 3 protein, which agrees with the results of chapter four and has been related with acrosome alteration when it concentration decreased (Taha et al., 2011). All of these findings, especially the increased amount of these proteins observed in genotype A in comparison with genotype R could explain in part the better acrosome integrity of spermatozoa and the enhanced fertility and prolificacy previously described in genotype A (Lavara et al., 2005; Safaa et al., 2008).

Other over-expressed proteins in line A such as plastin 1 and ubiquitin carboxyl-terminal hydrolase have been found related to spermatogenesis in other species (Kwon et al., 2004; Li et al., 2015). Plastins are a family of actin binding proteins known to cross-link actin microfilaments in mammalian cells, creating actin microfilament

bundles necessary to confer cell polarity and cell shape (Li et al., 2016). There are three types of plastins: plastin 1, 2, and 3. All three are expressed in Sertoli cells and plastin 1 and 2 in testes germ cells (Li et al., 2016). Plastin protein has been found in boar seminal plasma exosomes (Piehl et al., 2013) and in rat testis (Li et al., 2015). Plastin 1 deficient mice were fertile and displayed a normal reproductive rate (Grimm-Gunter et al., 2009), what suggests an additional role of plastin far from the fertility process. On the other hand, ubiquitin carboxyl-terminal hydrolase isozyme 3 may function in the meiotic differentiation of spermatocytes into spermatids (Kwon et al., 2004).

Seminal plasma contains antioxidants that are free radical scavengers that protect sperm cells against oxidative stress (Bousnane et al., 2017). For instance, catalase serves to protect cells from the toxics effects of hydrogen peroxide (http://www.uniprot.org/uniprot/Q64405). In bulls, the levels of catalase in seminal plasma have been found higher in high-fertile males than in subfertile bulls (Kumar et al., 2016). In addition, the supplementation of post-thawed rooster semen with 100 µg/mL of catalase has beneficial effects on semen quality (Amini et al., 2015). In line with this, the protein named elongation factor 4 is required for accurate and efficient protein synthesis under certain stress conditions (http://www.uniprot.org/uniprot/Q5KWZ3). Therefore, the over-expression of catalase and elongation factor 4 proteins in seminal plasma of genotype A supports the better recovery and performance of thawed semen from genotype A compared to R (Mocé et al., 2003).

The rest of the over-expressed proteins in line A were enzymes such as carbonic anhydrase 2, which has been found to have a role in the regulation of bicarbonate concentration in horse seminal plasma and accordingly regulate seminal plasma pH

(Asari et al., 1996), aspartate aminotransferase (AST) which is an important regulator of glutamate (http://www.uniprot.org/uniprot/P33097) and peptidyl-prolyl cis-trans isomerise which keeps in an inactive conformation of the TGF-beta type I serine/threonine kinase receptor, preventing TGF-beta receptor activation in absence of ligand (http://www.uniprot.org/uniprot/P26883).

On the other hand, genotype R presents higher abundance in several proteins related with reproductive function such as insulin-like growth factor-binding protein 7 which is important for correct spermatogenesis (Belardin et al., 2016) and polyubiquitin C which is involved in sperm-zona pellucida interactions and antipolyspermy defense in pig (Yi et al., 2007). Besides, genotype R seminal plasma has more quantity of Heat shock 70 kDa 1-like protein. Heat shock proteins (70 and 90 kDa) are chaperones implicated in a wide variety of cellular processes, including protection of the proteome from stress, folding and transport of newly synthesized polypeptides, activation of proteolysis of misfolded proteins and the formation and dissociation of protein complexes (http://www.uniprot.org/uniprot/P0CB32). In several species like porcine, ovine and bovine, heat shock 70 kDa protein 8 was found to prolong the survival of spermatozoa at body temperature *in vitro* (Elliot et al., 2009; Lloyd et al., 2009). The greater abundance of this heat shock protein in line R could explain the better performance of line R spermatozoon when they are stored *in vitro* during several days (unpublished work, ICTA, 2016). Based on the foregoing, our results provide evidence that genotype has a clear effect on seminal plasma protein abundance.

Regarding the effect of the season on the rabbit seminal plasma proteome, in chapter four we showed a season effect on the abundance of three proteins (FAM115E-like, haemoglobin subunit zetalike and nerve growth factor), but again, these relative

quantity protein differences were obtained with a less resolute proteomic technique. In the current work, results showed that there are slight protein differences between seasons but it does not exist a clear pattern of protein variation throughout the year for both genotypes. This lack of variation could be explained by the controlled environmental conditions used in our study where animals were kept under 16 h light/8 h dark and maintained between 14ºC and 28ºC using cooling and heating systems over the year.

Finally, we determined the variation of β-NGF in rabbit seminal plasma. β-NGF quantity in other reflexively ovulating species such as llama represents 30% of the total seminal plasma protein content (20 mg/ejaculate) (Berland et al., 2016), whereas in rabbit seminal plasma it only represents about 1.4%, independently of the genotype. Kershaw-Young et al. (2012) observed that intramuscular administration of llama seminal plasma (equivalent to <1/4 of an ejaculate) resulted in high rate ovulation induction of females (94% compared to 0% when saline was administered). Interestingly, in other works, the intramuscular administration of rabbit seminal plasma induced ovulation in llamas, but not in rabbits (Silva et al., 2011). The low proportion of β-NGF protein in rabbit seminal plasma and the fact that this protein is also present in a relatively low proportion in the seminal plasma of the majority of spontaneous ovulators (Druart et al., 2013), could lead us to think that β-NGF may have different function. In cows, Stewart et al. (2018) have shown that β-NGF from bull seminal plasma enhances corpus luteum formation and conceptus development. On the other hand, Maranesi et al. (2015) hypothetized that the role of β-NGF protein in rabbit seminal plasma may be related to the modulation of the ovulation/fertilization events. Moreover, β-NGF concentration in rabbit seminal plasma decreased in winter

compared to the other seasons, which agrees with the results in chapter four. This may be related to the natural reluctance of rabbits to breed in the early winter and it accords with Zhang et al. (2015) findings in wild ground squirrels, which showed that the production of NGF in testes was decreased during the non-breeding season and increased in the breeding season.

Finally, in chapter six we characterised rabbit sperm proteome by nano LC-MS/MS focusing on the influence of the genetic origin. Bioinformatics analysis of rabbit spermatozoa proteome revealed that 49% of identified proteins were related to catalytic activity and the second dominant group of proteins were assigned to a binding function (26%). These proportions agree with previous proteomic studies of honeybee and carp spermatozoa (Zareie et al., 2013; Dietrich et al., 2016). Regarding biological process, the cellular process (29%) was the most abundant category in rabbit spermatozoa followed by metabolic process (25%), which coincides with honeybee sperm proteins (Zareie et al., 2013). It is also noticeable that only seven of the 487 proteins identified in rabbit spermatozoa are to date recorded in GO as being directly associated with reproductive processes such as fertilization and gamete generation. These proteins related with reproductive processes are the following: acrosin-binding protein (ACRBP), zonadhesin (ZAN), sperm equatorial segment protein 1 (SPESP1), serine protease inhibitor Kazal type 8 (SPINK8), dual specificity testis-specific protein kinase 2 (TESK2), dynein heavy chain 9 (DNAL1) and four and a half LIM domains protein 1 (FHL1). It is surprising that so few spermatozoa proteins are found in the category of reproduction and that many of the proteins studied in this work have not been assigned a specific role in GO. In previous studies in boar and in rabbit seminal plasma, authors encountered the same situation (Pérez-Patiño et al., 2016; Casares-

Crespo et al., 2018b). Despite of this, we are sure that the identified sperm proteins are directly or indirectly related to reproduction processes. Finally, GO analysis revealed that the majority of rabbit spermatozoa proteins were extracted from cell parts, organelles and membranes.

Among the twenty-five more abundant proteins in genotype A, we found proteins related to different biological functions such as: protein folding, glycolysis, protein transport, metabolism, ion transport and fertilization.

CCT8, CCT3, CCT2 and CCT6B are chaperones that contain the TCP1 complex and whose function is to assist the folding of other proteins such as tubulin and actin upon ATP hydrolysis [http://www.uniprot.org/uniprot/G1SHZ8]. In addition, arylsulfatase A protein (ARSA) has been localized in the acrosomal region of human spermatozoa and it is known to increase its surface expression significantly during capacitation (Redgrove et al., 2012). These proteins have been related to sperm zona pellucida interaction role in mouse and human spermatozoa by several authors (Dun et al., 2011; Redgrove et al., 2011, 2012). On the other hand, zonadhesin protein' (ZAN) role is to mediate species-specific zona pellucida adhesion (Tardif et al., 2010) and has been localized in the anterior acrosome of rabbit spermatozoa (Lea et al., 2001). In the previous chapter (Casares-Crespo et al., 2018b), zonadhesin, was also found more abundant in seminal plasma of rabbit A genotype compared to genotype R. Furthermore, other proteins over-expressed in genotype A are: cullin 3 (CUL3), which has been recently found to have an important role in the human sperm flagellum (Jumeau et al., 2017), phosphoglycerate kinase 2 (PGK2), that has shown to be essential for sperm motility and male infertility in mice (Danshina et al., 2010) and SERPINE2, which may play a role as a decapacitation factor (Lu et al., 2011). All of

these findings, especially the increased amount of these proteins observed in genotype A in comparison with genotype R could explain in part the enhanced fertility and prolificacy previously described in genotype A (Lavara et al., 2005; Safaa et al., 2008).

On the other hand, among the fifteen more abundant proteins in genotype R, we found proteins related to different biological functions such as: antioxidant activity, binding, catalytic activity, transporter activity and structural molecule activity.

For instance, the protein HSPB1 belongs to a superfamily of mammalian small stress proteins and its function has been suggested to be related to the cytoskeleton (Fontaine et al., 2003). Just like ACTB protein, which is an actin involved in cell motility and cytokinesis (Zeng et al., 2014). On the other hand, peroxiredoxins such as PRDX1 are highly sensible to oxidative stress proteins which are involved in the antioxidant protection of the mammalian spermatozoa (Liu and O'Flaherty, 2017). Finally, BASP1 protein, which has been located in rat mature spermatozoa, may be participating in biochemical processes through the activation of calcium (Mosevitsky et al., 2012).

In summary, this last chapter characterises for the first time rabbit sperm proteins and generates a publicly accessible database of rabbit sperm proteome, which still remains poorly understood. In addition, our data provide evidence that genotype has a huge impact on protein abundance in rabbit sperm. The last three chapters of this thesis lead to the complete characterization of rabbit semen. Further studies are needed in order to elucidate the reproductive role of these identified proteins and the different evolution of these genotypes that gives rise to the intraspecies variation found.

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X.CONCLUSIONS

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-The inhibition of rabbit seminal plasma peptidase activity by a protease inhibitor cocktail affects reproductive performance decreasing prolificacy.

-The use of AMIs (bestatin and EDTA) in order to inhibit part of the aminopeptidase activity is not enough to allow us to reduce the GnRH concentration in the insemination extender without affecting fertility.

-The encapsulation of the GnRH analogue by chitosan-dextran sulfate nanoparticles in the insemination extender, makes possible to diminish the hormone quantity in a 20% (from 5 to 4 µg/doe), without altering rabbit reproductive performance.

-Rabbit seminal plasma and sperm proteins are mainly related to catalytic and binding activity with only six and seven of them known to be involved to date in reproduction processes, respectively.

-β-NGF protein was identified and quantified for the first time in rabbit seminal plasma. This protein only represents about 1.4% of the total protein amount in rabbit seminal plasma and its quantity is independent of the genotype.

-Genotype is the main reason for the protein abundance differences observed between rabbit genetic lines A and R. There are slight protein differences between seasons but it does not exist a clear pattern of protein variation along the year in both genotypes.

-The first publicly accessible database of the rabbit seminal plasma and sperm proteome was created.

XI.SUPPLEMENTARY TABLES

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CHAPTER V. RABBIT SEMINAL PLASMA: THE IMPORTANCE OF THE GENETIC ORIGIN.

Table S5.1. contains the complete list of the 402 proteins identified in rabbit seminal plasma with a cut off of two unique peptides and validated with ≥ 95% Confidence (unused Score ≥ 1.3).

Table S5.2. contains the complete list of the chromatographic areas of the 402 proteins identified in the two rabbit genotypes and the four seasons (3 replicates per sample).

Table S5.3. shows the results of the protein quantity T-test comparison between genotypes, including mean protein quantity, t-value, p-value, fold change and log (fold change) of the 402 quantified proteins.

CHAPTER VI. CHARACTERIZATION OF RABBIT (*ORYCTOLAGUS CUNICULUS*) SPERM PROTEOME.

Table S6.1. contains the complete list of the 487 proteins identified in rabbit spermatozoa with a cut off of two unique peptides and validated with ≥ 95% Confidence (unused Score ≥ 1.3).

Table S6.2. contains the complete list of the chromatographic areas of the 487 proteins identified in the two rabbit genotypes (3 replicates per sample).

Table S6.3. shows the results of the protein quantity T-test comparison between genotypes, including mean protein quantity, t-value, p-value, fold change and log (fold change) of the 487 quantified proteins.