UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Embryo cryopreservation and transfer to rederive a paternal rabbit line after 18 generations. Evaluation of growth and reproductive traits.

Ph.D. Thesis by

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By

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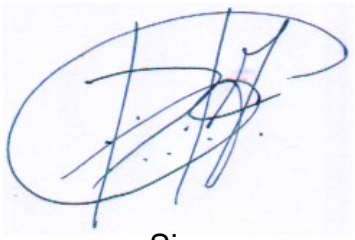

Sig.

Thesis Director

Prof. José Salvador Vicente Antón

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A mi papá y mamá:

José Daniel y María Estela

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ABSTRACT

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Selection for post-weaning average daily gain in rabbits has managed to increase growth rate in a paternal line such as the R from the Polytechnic University of Valencia. Nevertheless, the impact of response obtained in growth rate on reproductive performance has not yet been properly evaluated. This line shows low reproductive rates that are more evident generation after generation, not knowing if it has been due to selection. To evaluate the effects of the selection process, growth traits and reproductive performance from the offspring of the current generation (R36) was compared under the same environment with a control population rederived from the embryos stored of a previous generation (R18). Moreover, to reduce or avoid the effect of the cryopreservation process (vitrification and transfer) on phenotypic traits that can mask the evaluation of the selection program, embryos of the current generation (R36) were cryopreserved and transferred to obtain a third population (R37V). On these three populations and over two generations, both reproductive performances (components of litter size, fertility and prolificacy) and growth characteristics (foetal and postnatal) has been evaluated.

In chapter 1, R37V was rederived from embryos of 28 donors females and 15 different sire families of 36th generation, and offspring of 36th generation born by artificial insemination at the same time of the year originated the control population (generation 37, R37). Offspring were bred in non-overlapping generations over two generations. Growth trait parameters were evaluated for all generations. Reproductive traits and litter size components were assessed for the 38th generation. Differences in postnatal growth traits (end fattening weight and average daily gain) were observed in the three generations assessed. Although foetal growth, litter size components and reproductive traits did not show significant differences. In conclusion, cryopreservation and embryo transfer processes cause changes in growth traits of reconstituted populations that influence the following generations, without changes in reproductive traits in a paternal line of rabbits.

In the following chapters, to reduce or avoid the effects of the rederivation process, genetic drift and environmental factors on phenotypic traits, only the rederived populations were used to compare reproductive and growth performances.

In chapter 2, taking into account that males from a paternal line selected for growth traits were used to produce semen doses at insemination centres and farms in a breeding scheme for rabbit meat production, we studied whether a programme of selection by daily gain in fattening period changed the seminal traits, plasma and sperm proteome and the fertility of semen when used in artificial insemination. Thirty-nine males obtained by re-derivation from vitrified embryos with a difference of 18 generations (R21 and R39). Sperm production parameters, morphological traits, sperm motility parameters and viability were evaluated from ejaculates. Seminal plasma and sperm proteome of three pool ejaculates from 10 mature males of each group were analysed and semen doses were used to inseminate 311 females. Only the percentage of abnormal sperm showed significant differences, with R21 presenting fewer abnormal sperm than R39. The discriminant analysis (DA-PLS) showed a clear effect of the generation for plasma and sperm proteome. In seminal plasma, 643 proteins were reported and 64 proteins were differentially expressed, of which 56 were overexpressed in R39 (87.5%). Sperm proteome reported 1360 proteins with 132 differentially abundant proteins. Of the total, 89 proteins were overexpressed in R39 (67.4%). From the 64 and 132 differentially abundant proteins of plasma and sperm, 19 and 26 had a FC>1.5, 12 and 13 of them belonging to the Oryctolagus cuniculus taxonomy, respectively. Despite observing differences in important proteins related to capacitation, sperm motility or immunoprotection and consequently to the fertilization process (TMPRSS2, Serpin family, Fam71f1, ATPase H+ transporting accessory protein 2, carbonic anhydrase 2, UDP-glucose glycoprotein glucosyltransferase 2), no differences in fertility and prolificacy were detected when commercial seminal doses were used for insemination from both male groups. However, overabundance of KIAA1324 protein can be related to the increase in abnormal sperm after selection by growth rate.

In chapter 3, we evaluated the effect of a long-term selection for post-weaning average daily weight gain (ADG) over 37 generations. After two generations of both rederived populations (R21 vs. R39 generations), all evaluated traits showed some progress as a result of the selection, the response being 0.113 g/day by generation. This response does not seem to affect the estimated Gompertz growth curve parameters in terms of the day and the weight at the inflexion point or the adult weight. Moreover, a sexual dimorphism favouring females was observed in this paternal line. Results demonstrated that the selection programme had improved ADG without variations in adult body weight but, after 37 generations of selection, this trait seems exhausted. Given the reduction in the cumulative reproductive performance and as a consequence in the selection pressure, or possibly/perhaps due to an unexpected effect, rederivation could be the cause of this weak selection response observed from generation 18 onwards.

In chapter 4, we compared female reproductive traits between rabbit populations separated for 18 generations under growth rate selection pressure. To eliminate confounding maternal and embryo handling effects, foetal growth and litter size traits were measured in the second generation after rederivation (R20 and R38 generations). Our study suggests that selection for growth rate has no adverse effect on litter size components. Thus, in the R38 generation we observed a significant increase in embryo implantation (7.2±0.71 vs 5.1±0.79) and litter size (7.1±0.29 vs 6.5±0.32). Besides, the foetal sac area at day 12 of gestation (2.44±0.070 vs 2.07±0.071 mm2, for R38 vs R20, respectively), and foetal placenta area (136.7±6.14 vs 116.0±6.31 mm2, for R38 vs R20, respectively) and crown-rump length of the foetus (38.0±0.68 vs 35.8±0.68 mm, for R38 vs R20, respectively) at day 19 of gestation were higher in the R38 generation. Altogether, these results show that selection for growth rate does not adversely affect components of litter size, foetal growth and reproductive performance. However, the extent to which foundation criteria play a role in the high prenatal and perinatal mortality rate remains unclear in paternal lines.

In conclusion, our study provides further evidence of the effects of cryopreservation on growth traits persisting two generations after rederivation, observations that reinforce the findings in parallel studies of the group on omics and phenotypic variations, such as

in the liver. Therefore, for genetics studies it would be recommendable that both populations should be cryopreserved and transferred to surrogate mothers. Moreover, the paternal line showed signs of genetic progress exhaustion due to low reproductive performance and high postnatal mortality. Selection by daily weight gain between weaning and the end of the fattening period influenced changes in foetal growth and ejaculate proteome, but did not affect the reproductive performance of females or the fertility and prolificacy of seminal doses of males in the last 18 generations of selection.

Embryo cryopreservation and transfer to rederive a paternal rabbit line after 18 generations. Evaluation of growth and reproductive traits.

RESUMEN

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La selección por ganancia media diaria post-destete en conejos ha conseguido aumentar la velocidad de crecimiento en una línea paterna como la R de la Universidad Politécnica de Valencia. Sin embargo, el impacto de la respuesta obtenida en la velocidad de crecimiento sobre el rendimiento reproductivo aún no ha sido debidamente evaluado. Esta línea muestra bajas tasas reproductivas que son más evidentes generación tras generación, desconociendo si se ha debido a la selección. Para evaluar los efectos del proceso de selección, se compararon los rasgos de crecimiento y el rendimiento reproductivo de la descendencia de la generación actual (R36) bajo el mismo ambiente con una población control rederivada de los embriones almacenados de una generación anterior (R18). Además, para reducir o evitar el efecto del proceso de criopreservación (vitrificación y transferencia) sobre los rasgos fenotípicos que pueden enmascarar la evaluación del programa de selección, se criopreservaron y transfirieron embriones de la generación actual (R36) para obtener una tercera población (R37V). Sobre estas tres poblaciones y a lo largo de dos generaciones, se han evaluado tanto los rendimientos reproductivos (componentes del tamaño de la camada, fertilidad y prolificidad) como las características de crecimiento (fetal y postnatal).

En el capítulo 1, la población R37V se rederivó a partir de embriones de 28 hembras donantes y 15 familias de machos diferentes de la generación 36, y la descendencia de la generación 36 nacida por inseminación artificial en la misma época del año originó la población de control (generación 37, R37). Las crías se reproducían en generaciones no solapadas a lo largo de dos generaciones. Se evaluaron los parámetros de crecimiento durante el engorde y los reproductivos de todas las generaciones. En la generación 38 de ambas poblaciones se estudió el desarrollo prenatal y los componentes del tamaño de la camada. Se observaron diferencias en los rasgos de crecimiento postnatal (peso final de engorde y ganancia media diaria) en las tres generaciones evaluadas. Sin embargo, el crecimiento fetal, los componentes del tamaño de la camada y los rasgos reproductivos no mostraron diferencias significativas. En conclusión, los procesos de criopreservación y transferencia de embriones causan cambios en los caracteres de

crecimiento de las poblaciones reconstituidas que influyen en las siguientes generaciones, sin que se produzcan cambios en los caracteres reproductivos en esta línea paterna de conejos.

En los siguientes capítulos, para reducir o evitar los efectos del proceso de rederivación, la deriva genética y los factores ambientales sobre los rasgos fenotípicos, se utilizaron únicamente las poblaciones rederivadas para comparar los rendimientos reproductivos y de crecimiento. En el capítulo 2, teniendo en cuenta que los machos de una línea paterna seleccionados por caracteres de crecimiento se utilizaron para producir dosis de semen en los centros de inseminación y en las granjas de producción de carne de conejo, se evaluó si un programa de selección por ganancia diaria en periodo de engorde modificaba los caracteres seminales, el proteoma plasma seminal y el de los espermatozoides y, la fertilidad del semen cuando se utilizaba en inseminación artificial. Se utilizaron 39 machos obtenidos a partir de embriones vitrificados con una diferencia de 18 generaciones (R21 y R39). Se evaluaron los parámetros de producción espermática, los rasgos morfológicos, los parámetros de motilidad espermática y la viabilidad de los eyaculados. Se analizó el proteoma del plasma seminal y de los espermatozoides de tres mezclas de eyaculados de 10 machos maduros de cada grupo y se prepararon dosis de semen a partir de éstos para inseminar a 311 hembras. Sólo el porcentaje de espermatozoides anormales mostró diferencias significativas, presentando los machos R21 menos espermatozoides anormales que los R39. El análisis discriminante (DA-PLS) mostró un claro efecto de la generación para el proteoma plasmático y espermático. En el plasma seminal, se reportaron 643 proteínas y 64 proteínas fueron diferencialmente expresadas, de las cuales 56 fueron sobreexpresadas en R39 (87,5%). Del proteoma de los espermatozoides se obtuvieron 1.360 proteínas, de las cuales 132 eran diferencialmente abundantes. Del total, 89 proteínas estaban sobreexpresadas en R39 (67,4%). De las 64 y 132 proteínas diferencialmente abundantes del plasma y del esperma, 19 y 26 tenían una FC>1,5, 12 y 13 de ellas pertenecientes a la taxonomía de *Oryctolagus cuniculus*, respectivamente. A pesar de que se observaron diferencias en importantes proteínas relacionadas con la capacitación, la motilidad o la inmunoprotección de los espermatozoides y, en consecuencia, con el proceso de fecundación (TMPRSS2, familia Serpin, Fam71f1,

proteína accesoria transportadora de H+ ATPasa 2, anhidrasa carbónica 2, UDP-glucosa glicoproteína glucosiltransferasa 2), no se detectaron diferencias en la fertilidad y la prolificidad cuando se utilizaron dosis seminales comerciales para la inseminación de ambos grupos de machos. Sin embargo, la sobreabundancia de la proteína KIAA1324 puede estar relacionada con el aumento de espermatozoides anormales tras la selección por tasa de crecimiento.

En el capítulo 3, analizamos el efecto de la selección por ganancia media diaria de peso (GMD) post-destete en las últimas 18 generaciones. Después de 2 generaciones tras la re-derivación, todos los caracteres evaluados mostraron cierto progreso como resultado de la selección, siendo la respuesta a la selección de 0,113 g/día por generación. Esta respuesta no parece afectar a los parámetros estimados de la curva de crecimiento de Gompertz en cuanto al día y el peso en el punto de inflexión o el peso adulto. Además, se observó en esta línea paterna un dimorfismo sexual favorable a las hembras. Los resultados demostraron que el programa de selección ha mejorado la GMD sin variar el peso corporal adulto, pero, después de 37 generaciones de selección, este carácter parece agotado. Esto podría ser consecuencia de un empeoramiento de la eficiencia reproductiva de la línea como consecuencia de la presión de selección, o tal vez debido a un efecto inesperado en el que la rederivación podría ser la causa la débil respuesta observada.

En el capítulo 4, comparamos los parámetros reproductivos de las conejas entre las poblaciones de conejos separadas por 18 generaciones. Para reducir o eliminar los efectos de la manipulación embrionaria y maternos derivados del proceso de vitrificación y transferencia, los caracteres de desarrollo prenatl y los componenetes del tamaño de camada se midieron en la segunda generación después de la rederivación (generaciones R20 y R38). Nuestro estudio sugiere que la selección de la tasa de crecimiento no tiene efectos adversos sobre los componentes del tamaño de la camada. Así, en la generación R38 observamos un aumento significativo de la implantación de embriones (7,2±0,71 frente a 5,1±0,79) y del tamaño de la camada (7,1±0,29 frente a 6,5±0,32). Además, el área del saco fetal en el día 12 de gestación (2,44±0,070 frente a 2,07±0,071 mm2, para R38 frente a R20, respectivamente), y el área de la placenta fetal

(136,7±6,14 frente a 116,0±6,31 mm2, para R38 frente a R20, respectivamente) y la longitud cráneo-rabadilla del feto (38,0±0,68 frente a 35,8±0,68 mm, para R38 frente a R20, respectivamente) en el día 19 de gestación fueron mayores en la generación R38. En conjunto, estos resultados muestran que la selección por la tasa de crecimiento no afecta negativamente a los componentes del tamaño de la camada, el crecimiento fetal y el rendimiento reproductivo. Sin embargo, sigue sin estar claro hasta qué punto los criterios de fundación desempeñan un papel en la elevada tasa de mortalidad prenatal y perinatal en las líneas paternas.

En conclusión, nuestro estudio demuestra que los efectos de la criopreservación sobre los caracteres de crecimiento persisten dos generaciones después de la rederivación, observaciones que refuerzan los hallazgos en estudios paralelos del grupo sobre las variaciones ómicas y fenotípicas a nivel hepático. Por lo tanto, sería recomendable que en la evaluación del progreso genético cuando se utilicen poblaciones control re-derivadas a partir de embriones crioconservados, ambas poblaciones sean criopreservadas y transferidas. Además, la línea paterna mostró signos de agotamiento del progreso genético probablemente debido al bajo rendimiento reproductivo y a la elevada mortalidad postnatal. La selección por ganancia diaria de peso entre el destete y el final del periodo de engorde influyó en los cambios del crecimiento fetal y del proteoma del eyaculado, pero no afectó al rendimiento reproductivo de las hembras ni a la fertilidad y prolificidad de las dosis seminales de los machos en las últimas 18 generaciones de selección.

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RESUM

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La selecció de guany mitjà de pes diari post-deslletament en conills ha aconseguit augmentar la taxa de creixement en una línia paterna com la R de la Universitat Politècnica de València. No obstant això, l'impacte de la resposta obtinguda en la velocitat de creixement sobre el rendiment reproductiu encara no s'ha avaluat adequadament. Aquesta línia mostra unes taxes reproductives baixes que són més evidents generació rere generació, sense saber si ha estat deguda a la selecció. Per avaluar els efectes del procés de selecció, es van comparar els trets de creixement i el rendiment reproductiu de la descendència de la generació actual (R36) sota el mateix entorn amb una població control derivada dels embrions emmagatzemats d'una generació anterior (R18). A més, per reduir o evitar l'efecte del procés de criopreservació (vitrificació i transferència) sobre els trets fenotípics que poden emmascarar l'avaluació del programa de selecció, embrions de la generació actual (R36) es van criopreservar i transferir per obtenir una tercera població (R37V). En aquestes tres poblacions i al llarg de dues generacions, s'han avaluat tant el rendiment reproductiu (components de la mida de la camada, la fertilitat i la prolificació) com les característiques de creixement (fetal i postnatal).

Al capítol 1, es van transferir embrions vitrificats de 28 femelles donants i 15 famílies de pares diferents de la 36a generació (R37V), i la descendència de la 36a generació nascuda per inseminació artificial a la mateixa època de l'any va originar la població control (generació 37, R37). La descendència es va criar en generacions no superposades durant dues generacions. Es van avaluar els paràmetres de creixement per a totes les generacions i els trets reproductius i els components de la mida de la ventrada es van avaluar a la següent generació (R38V vs R38). Es van observar diferències en els trets de creixement postnatal (pes de l'engreix final i guany mitjà de pes diari) a les tres generacions avaluades. Tot i que el creixement fetal, els components de la mida de la ventrada i els trets reproductius no van mostrar diferències significatives. En conclusió, els processos de criopreservació i transferència d'embrions provoquen canvis en els

trets de creixement de les poblacions reconstituïdes que influeixen en les generacions següents, sense canvis en els trets reproductius en una línia paterna de conills.

En els capítols següents, per reduir o evitar els efectes del procés de rederivació, la deriva genètica i els factors ambientals sobre els trets fenotípics, només es van utilitzar les poblacions rederivades per comparar els rendiments reproductius i de creixement.

En el capítol 2, tenint en compte que els mascles d'una línia paterna seleccionada pels trets de creixement s'utilitzaven per produir dosis de semen en centres d'inseminació i granges en un esquema de cria per a la producció de carn de conill, es va estudiar si un programa de selecció per guany mitja de pes diari en període d'engreix. pot canviar els trets seminals, el proteoma del plasma i dels espermatozoides i la fertilitat del semen quan s'utilitza en la inseminació artificial. Es van utilitzar trenta-nou mascles obtinguts per re-derivació d'embrions vitrificats amb una diferència de 18 generacions (R21 i R39). Es van avaluar els paràmetres de producció d'esperma, els trets morfològics, els paràmetres de motilitat i la viabilitat dels espermatozoides a partir dels ejaculats. Es va analitzar el plasma seminal i el proteoma d'esperma de tres mescles heteroespèrmiques de 10 mascles madurs de cada grup i es van utilitzar dosis de semen per inseminar 311 femelles. Només el percentatge d'espermatozoides anormals va mostrar diferències significatives, amb R21 presentant menys espermatozoides anormals que R39. L'anàlisi discriminant (DA-PLS) va mostrar un efecte clar de la generació del proteoma del plasma i dels espermatozoides. En el plasma seminal, es van informar 643 proteïnes i 64 proteïnes es van expressar de manera diferent, de les quals 56 es van sobre-expressar a R39 (87,5%). El proteoma de l'esperma va informar de 1360 proteïnes amb 132 proteïnes diferencialment abundants. Del total, 89 proteïnes estaven sobre-expressades en R39 (67,4%). De les 64 i 132 proteïnes diferencialment abundants de plasma i espermatozoides, 19 i 26 tenien un FC>1,5, 12 i 13 d'elles pertanyen a la taxonomia d'Oryctolagus cuniculus, respectivament. Tot i observar diferències en proteïnes importants relacionades amb la capacitat, la motilitat o la immunoprotecció dels espermatozoides i conseqüentment amb el procés de fecundació (TMPRSS2, família Serpin, Fam71f1, ATPasa H+ transportant proteïna accessòria 2, anhidrasa carbònica 2,

UDP-glucosa glicoproteïna glucosiltransferases en 2), La fertilitat i la prolificitat es van detectar quan es van utilitzar dosis seminals comercials per a la inseminació dels dos grups de mascles. Tanmateix, la sobreabundància de proteïna KIAA1324 pot estar relacionada amb l'augment d'espermatozoides anormals després de la selecció per velocitat de creixement.

Al capítol 3, vam avaluar l'efecte d'una selecció a llarg termini per a l'augment de pes mitjà diari (ADG) després del deslletament durant 37 generacions. Després de dues generacions d'ambdues poblacions rederivades (generacions R21 vs. R39), tots els trets avaluats van mostrar algun progrés com a resultat de la selecció, la resposta va ser de 0,113 g/dia per generació. Aquesta resposta no sembla afectar els paràmetres estimats de la corba de creixement de Gompertz pel que fa al dia, al pes al punt d'inflexió o al pes de l'adult. A més, es va observar un dimorfisme sexual a favor de les femelles en aquesta línia paterna. Els resultats van demostrar que el programa de selecció havia millorat l'ADG sense variacions en el pes corporal adult, però, després de 37 generacions de selecció, aquest tret sembla esgotat. Donada la reducció del rendiment reproductiu acumulat i com a conseqüència de la pressió de selecció, o possiblement/potser per un efecte inesperat, la rederivació podria ser la causa d'aquesta resposta de selecció feble observada a partir de la generació 18.

Al capítol 4, vam comparar els trets reproductius entre poblacions de conilles separades durant 18 generacions. Per reduir o eliminar els efectes materna i embrionària sobre el desenvolupament fetal i els components de la mida de la ventrada es van mesurar els trets a la segona generació després de la rederivació (generacions R20 i R38). El nostre estudi suggereix que la selecció per a la velocitat de creixement no té cap efecte advers sobre els components de la mida de la ventrada. Així, a la generació R38 vam observar un augment significatiu dels embrions que implanten (7,2±0,71 vs 5,1±0,79) i la mida de la camada (7,1±0,29 vs 6,5±0,32). A més, l´area del sac fetal el dia 12 de gestació (2,44±0,070 vs 2,07±0,071 mm2, per R38 vs R20, respectivament) i l´area de la placenta fetal (136,7±6,14 vs 116,0±6,31 mm2, per R38 vs R20, respectivament) i la longitud de la corona i la gropa del fetus (38, 0 ± 0, 68 vs 35, 8 ± 0, 68 mm, per a R38 vs R20, respectivament) el dia 19 de gestació eren més grans a la generació R38. En conjunt,

aquests resultats mostren que la selecció de la velocitat de creixement no afecta negativament els components de la mida de la ventrada, el creixement fetal i el rendiment reproductiu. No obstant això, no està clar fins a quin punt els criteris de la fundació tenen un paper en l'elevada taxa de mortalitat prenatal i perinatal en les línies paternes.

En conclusió, el nostre estudi proporciona més proves dels efectes de la criopreservació sobre els trets de creixement que persisteixen dues generacions després de la rederivació, observacions que reforcen les troballes en estudis paral·lels del grup sobre òmiques i variacions fenotípiques, com en el fetge. Per tant, per als estudis de genètica genètica es recomanaria l'ús de dues poblacions criopreservades i transferides a mares subrogades. A més, la línia paterna va mostrar signes d'esgotament del progrés genètic a causa del baix rendiment reproductiu i l'alta mortalitat postnatal. La selecció mitjançant l'augment de pes diari entre el deslletament i el final del període d'engreix va influir en els canvis en el creixement fetal i en el proteoma ejaculat, però no va afectar el rendiment reproductiu de les femelles ni la fertilitat i la prolificitat de les dosis seminals dels mascles en les últimes 18 generacions de selecció.

Embryo cryopreservation and transfer to rederive a paternal rabbit line after 18 generations. Evaluation of growth and reproductive traits.

ABBREVIATIONS

ABBREVIATIONS

A

ABN Percentage of abnormal forms
ADG Average daily weight gain
ALH Amplitude of Lateral Head displacement
ART Artificial Reproduction Technology

B

BCF Beat Cross Frequency
BP Biological Process
BW Body weight

C

CC Cellular Component
CONC Spermatic Concentration
CRL Crown-rump length

D

DAMs Differentially Accumulated Metabolites
DE Digestible energy
DM Dry matter
DMSO Dimethyl Sulfoxide
DPBS Dulbecco's Phosphate-Buffered Saline

E

EC Embryo Cryopreservation
EDTA Ethylenediaminetetraacetic acid
EFW End of the fattening weigth
EG Ethylene Glycol
ELR Embryonic loss rate
ET Embryo Transfer

F

F1 F1 Generation
F2 F2 Generation
F3 F3 Generation
FA Fatty Acid
FC Fold Change
FDR False Discovery Rate
FLR Foetal loss rate
FS Foetal sac
FT Fresh-Transferred

G

GLM General Linear Model

GO Gene Ontology
GSH Glutathione

H

HOST Hypo-osmotic swelling test
HM Heat Map
HPLC High Performance Liquid Chromatography
HRMS High Resolution Mass Spectrometry

I

IE Implanted embryos rate

K

KEGG Kyoto Encyclopaedia of Genes and Genomes
KI Kindling interval

L

LIN Linearity Coefficient (VSL/VCL × 100)
LS Litter size
LSM Least square mean

M

MD Multiparous non-lactating does
MF Molecular Function
MLD Multiparous lactating does
MOT Percentage of sperm motility
mRNA Messenger Ribonucleic Acid
MS Mass Spectrometry

N

NAR Percentage of normal apical ridge
NC Naturally-Conceived

O

OR Ovulation rate

P

P450 Cytochrome P450
PCA Principal Component Analysis
PD Primiparous does
PLD Primiparous lactating does
PROG Percentage of progressive motility

R

R Rabbit paternal line
R19V (G19V) Rederived animals from generation 19
R37V (G37V) Rederived animals from generation 37

R20 (G20V) First filial generation from R19V generation
R21 (G21V) Second filial generation from R19V generation
R38 (G38V) First filial generation from R37V generation
R39 (G38V) Second filial generation from R37V generation
ROS Reactive Oxygen Species

S

SE Standard error
Spz Spermatozoa
STR Straightness Coefficient
SWATH Sequential Window Acquisition of all Theoretical Fragment Ion Spectra

T

TFA Trifluoroacetic Acid
TSE Total Sperm Per Ejaculate

U

UPV Universidad Politecnica de Valencia

V

VAP Average Path Velocity
VCL Curvilinear Velocity
VET Vitrified Embryo Transfer Procedure
VIAB Percentage of viable sperm
VOL Ejaculate volumen
VSL Straight-Line Velocity
VT Vitrified-Transferred
VTc Vitrified-Transferred in Cryotop
VTs Vitrified-Transferred in Straw

W

WOB Wobble Coefficient (VSL/VAP × 100)
WW Weaning weight

Z

Zn Zinc

Embryo cryopreservation and transfer to rederive a paternal rabbit line after 18 generations. Evaluation of growth and reproductive traits.

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GENERAL INTRODUCTION

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1. GENERAL INTRODUCTION

1.1. GENERALITIES.

Rabbit production decreased from 1,222,384 tonnes in 2010 to 883,936 in 2019, with Asian rabbit meat producers accounting for roughly 70.9% of world rabbit meat production. In the same decade in Europe, rabbit meat production fell from 297,673 tonnes (24.4% of world production) to 170,751 tonnes of meat (19.3% of world production, FAOSTAT, 2021). Today, Spain is still one of the largest producers of European rabbit meat, along with France and Italy (*Ministerio de Agricultura Pesca y Alimentación*, 2018 and 2019).

In recent years in Spain, there has been a significant reduction in the number of local producers and increased professionalisation, linked to declining consumption per capita and export distribution circuits. The result of all this has been larger and more productive farms. On the other hand, hunting production farms and breeding farms for pet rabbits seem to thrive (Ministerio de Agricultura Pesca y Alimentación, 2018). Similar trends are occurring across Europe; rabbit meat consumption is progressively decreasing due to price, improper presentation, lack of processed products and socio-economic factors (Trocino *et al.*, 2019). For this reason, the industry is currently considering strategies to re-launch rabbit meat consumption, such as integration of rabbit value chains, use of alternative and economically sustainable production systems taking into account emerging animal welfare standards, development of new processed rabbit meat products or improved marketing and communication strategies (Cullere and Dalle Zotte, 2018).

In this context, sustainable rabbit production must be based on the healthy and respectful breeding of genetic lines that provide high productivity levels, taking into account the quality of the product and with little or zero environmental impact. Rabbit breeding of robust, long-lived, reproductively efficient animals with excellent feed conversion rates must be one of the ways to achieve this.

1.2. GENETIC IMPROVEMENT OF MEAT RABBIT

Several genetic improvement programmes for maternal and paternal lines involved in three-way crosses schemes have been carried out in Spain since 1976 (Gómez *et al.*,1999; Baselga, 2002). Maternal lines (Line A, V, H and Prat) were developed to increase litter size at weaning and paternal lines (Caldes and R line) were for weight gain from weaning to slaughter (average daily gain) (Gómez *et al.*, 1999). The three-way crossbreeding scheme used in rabbit meat production crosses two maternal lines to take advantage of the positive heterosis effects in reproductive traits and to dissipate the inbreeding accumulated within lines, and a paternal line for terminal crosses contributing to growth complementarity and faster growth at slaughter (Gómez *et al.*, 1999; Baselga, 2005).

Development of paternal lines began in Spain in 1976 from California rabbits reared by Valencian farmers and selected for individual growth during the fattening period (B line, Estany *et al.*, 1992; Baselga, 2002). Later, other paternal lines were founded in the Polytechnic University of Valencia (R line, 1981) or IRTA (Caldes line, 1983). The current paternal line R resulted from fusing two paternal lines (B and R). Both paternal lines were chosen by individual selection for daily gain between weaning and slaughter (Estany *et al.*, 1992; Baselga, 2002 and 2005). This selection criterion improves feed conversion (a significant trait in meat rabbit production) because of its negative and critical genetic correlation (Feki *et al.*, 1996; Baselga, 2002).

Selection for residual feed intake has been another criterion used in paternal lines to improve feed efficiency (Larzul and De Rochambeau, 2005). The lack of use of this selection criterion is due to its difficulty and cost measurement. Another criterion associated with paternal lines could be meat quality, but rabbit meat quality is not currently paid for in the markets (Blasco *et al.*, 2018).

An extensive analysis for direct and indirect selection for growth, carcass and meat quality traits in rabbits concluded that in selection programmes in paternal lines, the average daily gain is preferred for selection during the post-weaning period rather than

individual weight, as it is less affected by common litter effects and for its moderate to high heritabilities (0.13 to 0.48). Moreover, it is more efficient to improve feed conversion ratio than feed efficiency (Khalil and Al-Saef, 2008) and the range of estimated heritability for slaughter weight is even greater 0.03 to 0.72 (Khalil and Al-Saef, 2008; Antonini and Cordiviola, 2010).

1.2.1. Effects of growth rate selection on weight and meat quality

In meat production for different domestic species, there are many factors to consider. For example, in swine production, the selection strategy focuses on negative halothane lines (MHS test) due to decreased variation in carcass and meat characteristics and minimising their faults (Krieter and Tholen, 2001). In European beef cattle breeds, the trend is for genomic selection for meat quality improvement (carcass traits, marbling, tenderness) in combination with different feeding systems according to market requirements (Brito *et al.*, 2014). In Nellore (India), zebu cattle selection has made it possible to increase muscle growth and back fat deposition, but it has not yet improved intramuscular fat deposition and meat tenderness (Malheiros *et al.*, 2020). In comparison, sheep meat production systems are much more specific, due to consumer preferences for meat-type linked to the characteristic of the production system (Prache *et al.*, 2021). However, genetic selection programmes have been applied to increase muscle, carcass weight and yield and decrease fat accretion. One ovine breed widely used for improved meat quality traits (meat yield, carcass percentage, meat colour stability and sensory characteristics) is the Merino (Jacob and Pethick, 2014).

Until very recently, rabbit genetic selection aimed to improve quantitative aspects of production, such as growth rate and muscle development, with little interest in meat quality aspects. The rabbit meat industry currently does not pay for meat quality in the market; furthermore, it has been observed that selection by growth rate does not seriously affect meat quality (Blasco *et al.*, 2018).

The productive performance of the R line has been constantly evaluated since 1981. Estany *et al.* (1992) analysed the responses in the two founder lines (B and R), observing

an increment of 2.7% and 2.2% from the initial weight at 63 days of age per year, respectively, and showing that selection for individual weight gain was effective but was less than expected. Gómez *et al.* (1999) reported 47 to 48 g/day post-weaning gain by generation selection in this paternal line. A first approach to estimate the selection response for post-weaning growth rate was obtained by Piles *et al.* (2000), comparing rederived rabbits from frozen embryos, belonging to generations (generation 3 and 4) with a selected one (generation 10), observed a higher growth rate for the selected one (49.8 ± 0.75 g/d vs 45.9 ± 0.76 g/d). This difference was mainly by increasing weight at slaughter age (2350 ± 34 vs 2180 ± 40). In the same populations, Blasco *et al.* (2003), using a Gompertz model, estimated that adult weight was affected by selection for growth rate, the females being heavier than males. It was also noted that adult weight gain could lead to more difficulties in the reproductive management of this line (Blasco *et al.*, 2003).

Some studies were performed in the R line to evaluate the effect of the selection programme by growing rate in traits involved in carcass composition and meat quality by reconstitution of a population from cryopreserved embryos. Piles *et al.* (2000) assessed this effect between two populations separated by seven generations (generation 3 vs 10), concluding that there were no adverse effects of selection for increased growth rate on carcass composition and meat quality (Piles *et al.*, 2000). Similar studies, comparing relative growth, carcass and meat quality at 9 and 13 weeks of age between a selected (generation 18) and a control population (generation 7) in this paternal line were carried out by Hernandez *et al.* (2004); Ramirez *et al.* (2004) and Pascual *et al.* (2008), concluding that selection for growth rate affected water holding capacity of meat and texture properties but did not affect relative growth or carcass quality. Similar results have been shown in other paternal lines, where selection by average daily gain did not affect quality meat and carcass traits, although if selection were performed by thigh or loin quality, the outcome might be different (Larzul and Gondret, 2005).

1.2.2. Effects of growth rate selection on reproductive performance

1.2.2.1 Semen quality traits

Genetic programmes to improve growth rate in rabbits have not usually considered the reproductive performance of males in their selection aims. In paternal lines of rabbits selected for growth rate, genetic parameters for seminal traits were not included in the genetic programme, despite their significant role in farm productivity, considering that one male could affect the fertility and prolificacy of about a hundred females when artificial insemination is performed (Viudes-de-Castro and Vicente, 1997). An extensive review of seminal variation factors was described by Alvariño (2000), stating that breed, age, seasonality and feeding programme affected semen quality traits in rabbits. The highest volume, motility, concentration and percentage of live sperm was observed in crossbreed males due to the heterosis effect (El-Tarabany *et al.*, 2015). Thus, in crossbred sire lines, a moderate advantage in viability and the percentage of the normal apical ridge were improved, but a negative heterotic effect on fertility was observed (Garcia-Tomás *et al.*, 2006).

Genetic parameters of seminal traits have been reported in some mammal livestock species. Moderate or high heritability has been observed for seminal parameters such as motility, viability, teratozoospermia and concentration; it could be interesting to include these criteria in paternal lines (Smital *et al.*, 2005; Gottschalk *et al.*, 2016, Berry *et al.*, 2019). Gonzalez-Pena *et al.* (2016) compared the financial benefits of the advanced selection strategy incorporating semen traits (ejaculate volume, sperm concentration, percentage of motile sperm and morphologically abnormal cells) to those of the traditional selection strategy in a swine production system, suggesting the integration of the advanced selection strategy, as greater financial benefits were obtained.

Rabbit selection for daily gain (DG) would not affect sperm production adversely (Lavara *et al.*, 2011). Including sperm quality traits in the selection programme could be effective due to the moderate heritability estimates and genetic correlations between DG and

sperm traits (Lavara *et al.*, 2012). Similar findings were observed when divergent lines were selected for 63-d body weight, low and high body weight, concluding that growth selection does not seem to greatly affect sperm quality (Brun *et al.*, 2006).

In recent decades, with the development of new technologies, studies to determine, quantify and analyse sperm and seminal plasma proteomes have been performed in many species and humans. These studies provide information to define infertility factors and to maintain or select sperm fertility, optimising either the identification of good freezer donors, the systems for semen preservation and in vitro fecundation or to develop new sex control technology (Gaviraghi *et al.*, 2010; González-Cadavid *et al.*, 2014; Li *et al.*, 2016; Pérez-Patiño *et al.*, 2018). For example, in pigs, a panel of differentially expressed seminal plasma proteins were found in boars showing fertility differences in terms of fertility and prolificacy, some of them playing an essential role in the reproductive process, making their use as biomarkers in semen possible (Pérez-Patiño *et al.*, 2018). Nevertheless, very few studies have evaluated the effect of genetic selection for growth in the seminal proteome. In domestic chicken, research to evaluate whether long-term selection had an impact on seminal fluid found proteome differences, observing an enrichment of proteins and peptides related to immune-modulation (gallinacin-9, Ig λ-chain C, TGF-β2 and CXCL10) presumably due to the selection for production traits during chicken breeding (Atikuzzaman *et al.*, 2017).

In rabbits, differences in seminal protein profile between maternal and paternal lines have been reported (Casares-Crespo *et al.*, 2016; 2018 and 2019). More than 400 sperm proteins were identified in these studies, and about forty proteins were differentially expressed between a maternal (line A) and a paternal line (line R), 25 overexpressed in the maternal line and 15 in the paternal line. From the differentially expressed protein, uteroglobin with an immune-modulation role and zonadhesin and ectonucleoside triphosphate diphosphohydrolase 3 protein link to acrosome stabilisation and fertility was overexpressed in the maternal line with greater fertility than the paternal line with poor sperm quality and fertility (Casares-Crespo *et al.*, 2018 and 2019).

1.2.2.2. Effects on prenatal development and reproductive performance

The prenatal period offers a great opportunity to study mammalian growth, as external influences are better regulated than in another period of life (MacDowell *et al.*, 1927). Many factors can have a significant impact on embryonic development. The effects could be direct when including genetic imprinting, mitochondrial DNA transmission, maternal immune activation, hormonal influences, interactions between the foetal and the maternal intrauterine environment, or maternal behaviour that is not the result of an interaction of the mother with her offspring. Indirect, when the effect is postnatal and affects the offspring by establishing mother-pup interactions (Fligny *et al.*, 2009). In rabbits, the maternal effects have been demonstrated in litter size, birth weight and the production of suckling and growing rabbits, and these effects could even affect subsequent adult performance (Szendrő and Maertens, 2001).

Genetic differences in prenatal losses have been intensely studied in polytocous species (reviewed by Brien 1986; Blasco *et al.*, 1993 and Holt *et al.*, 2004). A thorough review of the literature by Bunger *et al.* (2005) concluded that while some studies suggest a small negative relationship between growth and reproduction in livestock species, the estimates of the parameters were often insufficiently accurate to be conclusive about such a relationship. In this way, estimates of genetic correlations in rabbits are inconsistent, and both positive and negative estimates have been reported, possibly depending on the line and its selection criteria. Maternal lines selected by reproductive traits (ovulation rate or prolificacy) could affect foetal weight, lead to lower weight at birth, and subsequently affect weaning and commercial weight. Nevertheless, genetic correlation reported between litter size and growth traits has been low or null, with few or non-negative consequences on growth rate in commercial maternal lines (Peiró *et al.*, 2019, Garcia and Argente, 2020, Peiró *et al.*, 2021). These results have not been corroborated in paternal lines selected for growth rate, where no studies have been carried out.

Paternal line R showed problems in ovulation, with more implantational, foetal, gestational and perinatal losses than maternal line A, resulting in a smaller litter size at

birth (Gómez *et al.*, 1999; Vicente *et al.*, 2012, Naturil-Alfonso *et al.*, 2016). This negative performance could be due to impaired levels of 17β-estradiol, progesterone, LH and IGF-I observed in paternal line R in comparison with maternal line A. Moreover, maternal and paternal lines differ greatly in their genetic backgrounds, as different priority is given to the ability to acquire resources and their allocation patterns (Arnau-Bonachera *et al.*, 2018).

In rabbits, the increase in uterine capacity increases the uterine length and decreases weights of the foetus and foetal placenta (Argente *et al.*, 2003), and a divergent selection for uterine capacity shows that foetal weight at term depends on the recipient genotype and foetal placental weight on the donor genotype (Mocé *et al.*, 2004). Vicente *et al.* (2013) and Naturil-Alfonso *et al.* (2015), comparing paternal and maternal genotypes in a reciprocal embryo transfer assay, observed that embryos and the recipient does from the paternal line (R) affected embryo survival negatively at implantation, and foetal survival rate and the foetal and placenta weights positively at the end of gestation.

1.2.2.3. Effect on health and robustness

Selection for reproductive longevity allowed offspring to have greater maturity and immunological status at weaning, giving them a better ability to resist post-weaning digestive disorders than lines selected exclusively for productive criteria, such as growth rate to fattening or litter size at birth (Garcia-Quirós *et al.*, 2014), reaching a greater acquisition capacity by assigning their resources to maintain prolificacy without increasing excessive mobilisation of body reserves, achieving a longer productive life than maternal lines selected for prolificacy (Theilgaard *et al.*, 2009; Savietto *et al.*, 2015).

In Spain, it has been determined that the major causes for the culling of females and males in production farms are infertility and other reproductive disorders, with higher mortality and culling risks during the first three births (Rosell and De La Fuente, 2016; Rosell and De La Fuente, 2009). Despite a higher body weight and body condition score

in paternal lines than in maternal lines (De La Fuente and Rosell, 2012), R paternal line does seem to have a minor capacity to assign their resources to maintain gestation and suckling rabbits, and as a result, high nest mortality and infertility problems can be associated with this line.

1.3. CONTROL POPULATIONS TO EVALUATE SELECTION EFFECTS

Response to selection for a trait is assessed in several ways: estimating breeding values, divergent selection, random mating of control population and a control population cryopreserved generations ago (Baselga, 2005; Khalil & Al-Saef, 2008; Antonini and Cordiviola, 2010). Cryopreservation techniques have been demonstrated to be a successful alternative to preserve and rederive populations many years later (Marco-Jiménez *et al.*, 2018). Khalil & Al-Saef (2008) reviewed the advantages and disadvantages of diverse methodologies to estimate the response to selection, stating that a major advantage of using cryopreserved populations is avoiding genetic drift and maintaining a control population without selection. Populations rederived by cryopreservation and transfer methods to compare the effects of selection do not seem to affect the health of animals born and reproductive behaviour or seminal quality, despite changes at embryo level in transcriptome and metabolome, in prenatal growth or postnatal liver weight and function (Lavara *et al.*, 2014; Garcia-Dominguez *et al.*, 2020c). Similar results have been reported in sheep, where lambing rate, growth performance and viability of offspring were not affected, and embryos could be stable for up to 7.5 years (Yao *et al.*, 2012). In humans, the use of IVF and ICSI reported alterations in the cardiovascular system and metabolic dysregulations that could result in a high risk of cardiometabolic disease (Heber and Ptak, 2021). A concern of this technique is the short- and long-term effects on genotype and phenotype of rederived animals. The short-term effect is related to changes in embryo early gene expression, implantation, transcriptomic and proteomic foetal placenta and foetal survival (Saenz-de-Juano *et al.*, 2012, 2014 and 2015) and their effects in postnatal life and their offspring by paternal or maternal inheritance (Lavara *et al.*, 2014 and 2015; Garcia-Dominguez *et al.*, 2018, 2020a, b and c; Marco-Jiménez *et al.*, 2020).

Rederived rabbits have been used to evaluate selection response since 2000 in our research team [JV1]. The first assessment between two populations separated by 6 generations (15 versus 21), using cryopreserved populations, was performed on a maternal line selected for litter size at weaning (Line V), finding that the direct response to selection between both generations was 0.51 ± 0.25 rabbits weaned/litter (0. 085 per generation) (Garcia and Baselga, 2002a), and the genetic correlation with growth traits (weaning weight –WW-, slaughtered weight-SW- and average daily gain-ADG) was close to zero. However, the genetic trends were positive and significant but small for WW and ADG, resulting in no correlated response to selection for litter size at weaning on growth traits, feed intake and feed efficiency to a constant litter size (Garcia and Baselga 2002b).

Piles and Blasco (2003) evaluated the selection response in the first years of R paternal line, observing similar results when comparing the estimated response using an animal model where environmental and genetic effects are separated or using a rederived population obtained after embryo freezing and transfer as the control population. Later, other studies were performed using rederived rabbit populations, for example to study the interaction between feeding programmes and selection by litter size, and non-relevant effects were observed (Quevedo *et al.*, 2006). A control population rederived from vitrified embryos was also used to estimate the direct and correlated response to selection for ovulation rate, demonstrating an increase of ovulation rate (+2) in 10 generations, but without a correlated response to litter size at birth (Laborda et al., 2012). Likewise, to evaluate the correlated response between ovulation rate and growth traits, determining a very low correlation between both (from 0.03 to 0.11), suggesting that female effects (including maternal and common litter effect) are not modified after selection (Peiró *et al.*, 2021).

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OBJECTIVES

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2. OBJECTIVES

General objective:

Estimate the effect of the average daily gain selection programme on reproductive and growths traits in the last 18 generations of selection of a rabbit paternal line, using rederivation by embryo vitrification and transfer to establish the control population.

Specific objectives:

1. Evaluate the impact of embryo vitrification and transfer procedures on the growth and reproductive traits of a paternal rabbit line selected for average DG from weaning (28 days old) to fattening (63d-old).

2. Evaluate whether a selection programme by DG in fattening period affects ejaculate traits, seminal plasma and sperm proteome and semen fertility.

3. Evaluate the effect of long-term selection for average DG (37 generations) on commercial growth traits and Gompertz parameters, using two populations rederived from vitrified embryos with 18 generational intervals.

4. Evaluate whether the selection programme for DG in fattening period has changed foetal growth, prenatal survival and reproductive performance.

CHAPTER I

EFFECTS OF REDERIVATION BY EMBRYO VITRIFICATION ON PERFORMANCE IN A RABBIT PATERNAL LINE

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ABSTRACT

Embryo cryopreservation is a valuable tool for maintaining genetic variability and preserving breeds and lines, allowing to assess the response to selection and enabling genetic diffusion. This study aimed to evaluate 7 the impact of rederivation by embryo vitrification and transfer procedures on the growth and reproductive traits in a paternal rabbit line selected for average daily gain from weaning (28 days old) to fattening (63 days old). The rederived population were breed over two generations at the same time as a control population of this paternal line and, growth trait parameters (weights at weaning, end of the fattening period and average daily gain) and reproductive performance (kindling rate, litter size at birth and at weaning) were compared to three filial generations. Moreover, fetal growth and litter size components were assessed for the second generation by ultrasonography and laparoscopy. Differences in postnatal growth traits (end of fattening weight and average daily gain) were observed in the three generations assessed. However, fetal growth, litter size components and reproductive traits did not show significant differences. In conclusion, cryopreservation and embryo transfer processes cause changes in growth traits of reconstituted populations that influence the following generations, without changes in reproductive traits in a paternal line of rabbits.

Keywords: Embryo vitrification, fetal growth, litter size, growth line, rabbit.

3.1. INTRODUCTION

Estimation of response to selection in rabbit breeding programs has been extensively described, and one alternative is the comparison between two different generations by rederivation of a control population cryopreserved generations ago (Khalil and Al-Saef, 2008), which could be stored for many years, achieving reasonable pregnancy rates and survival at birth (Lavara et al., 2011; Yao et al., 2012; Marco-Jiménez et al., 2018;). The implementation of reproductive biotechnologies such as cryopreservation and embryo transfer in rabbits has been used in our research group for approximately three decades (Vicente and García-Ximénez, 1993; García-Dominguez et al., 2019). Vitrifying has proven to be one of the most cost-effective and optimal freezing technologies for rabbit embryos, achieving high survival rates at birth and high parturition rates (Kasai et al., 1992; Vicente and García-Ximénez, 1994), allowing the creation of an embryo bank (Vicente et al., 2003).

Rederivation of a control population has been used to estimate the response to selection in both maternal (García and Baselga, 2002a; García and Baselga, 2002b) and paternal lines (Piles and Blasco, 2003, Juarez et al., 2020a and b; Peiró et al., 2021). In some cases, without taking into account that rederivation process have phenotypic effects on some growth-related traits during the prenatal period, such as placenta, foetus and newborn weights (Mocé et al., 2010; Saenz-de-Juano et al., 2014), and in the postnatal period in organ weight, such as the liver (Lavara et al., 2015, García-Dominguez et al., 2020a and b). Moreover, Lavara et al. (2014) observed effects on reproductive traits such as litter size in derived female reproduction (F1 females) and transgenerational effects on female F1 offspring (F2 females) from a maternal rabbit line.

These phenotypic changes suggested that embryo cryopreservation is not neutral. Embryo cryopreservation itself presents specific issues, embryos are exposed to osmotic, chemical and temperature stress, to which they have to respond. Consequently, developmental alterations emerge in the embryo, fetus and postnatal life of vitrified-transferred embryos. Recent studies have demonstrated that vitrified-

transferred embryos could respond to stressful procedures by altering gene expression or even incorporating lasting epigenetic marks with effects on the epigenome, transcriptome, proteome, and metabolome of adult rabbits (Marco-Jiménez et al. 2020; García-Dominguez et al., 2020a, b and c; and 2021). These last studies provided evidence that embryo cryopreservation induced an adaptive response resulting in phenotypic plasticity without negative consequences on health. It has been observed that this adaptive response was still present in later generations through changes in organs such as the liver and heart or bodyweight; moreover, liver epigenetic and metabolome variations have also been found (García-Dominguez et al., 2020a, b and c; and 2021).

In mice, vitrification and slow freezing of embryos has been shown to increase the expression of genes associated with fat mass but did not alter physiological development, movement coordination function or brain development, even though mice become heavier at eight weeks of age (Wang et al., 2020). This could be associated with significant dysfunction of glucose metabolism in the liver due to increased insulin resistance reported in male mice (Qin et al., 2021). Even in humans, the freezing process might cause singletons to be born with a greater risk of high birth weight and larger for gestational age than singletons after fresh embryo transfer and naturally conceived (Spijkers et al., 2017).

This study aimed to evaluate the impact of embryo vitrification and transfer procedures on the growth and reproductive traits of a paternal rabbit line selected for average daily gain from weaning (28 days old) to fattening (63 days old).

3.2. MATERIALS AND METHODS

Before the experimental phase began, all animal experimentation protocols used were reviewed and approved by the Ethics and Animal Welfare Committee of the Universitat Politècnica de València (code number 2015/VSC/PEA/00061). Likewise, all experiments followed the guidelines and regulations set forth in Directive 2010/63/EU/ EEC. Facilities and cages used were adequate for experimentation on this species (code: ES462500001091).

3.2.1. Animals and experimental design.

A paternal rabbit line (R) selected at the Universitat Politècnica de Valencia was used. This line was founded in 1989 from two closed paternal lines (Estany et al., 1992). Since then, the line has been selected for individual weight daily gain from 28 days (weaning) to 63 days old (end of fattening). The rederivation procedure of the population was described by Marco-Jiménez et al., 2018) from embryos of 28 donors belonging to 15 different sire families of 36th generation. Sire families were established to avoid matings between relatives sharing a grandparent. Control population was the offspring of 36th generation born by artificial insemination at the same time as the rederived population. Offspring from both populations were bred over two generations. The offspring of vitrified embryos were named R37V and inseminated conceived named R37 and the following filial generations (R38V and R39V and R38 and R39). Table 3.1 and Figure 3.1 show number of parents used to generate the offspring and trait measured in each generation.

Table 3.1. Total number of parents used to generate the offspring analysed.

Population	Filial Generation	Generation	Females	Males	Total
37th	F1	R37	77	16	93
	F2	R38	49	9	58
	F3	R39	78	27	105
		Total	204	52	256
37th Vitrified	F1	R37V	11	10	21
	F2	R38V	26	13	39
	F3	R39V	78	18	96
		Total	115	41	156

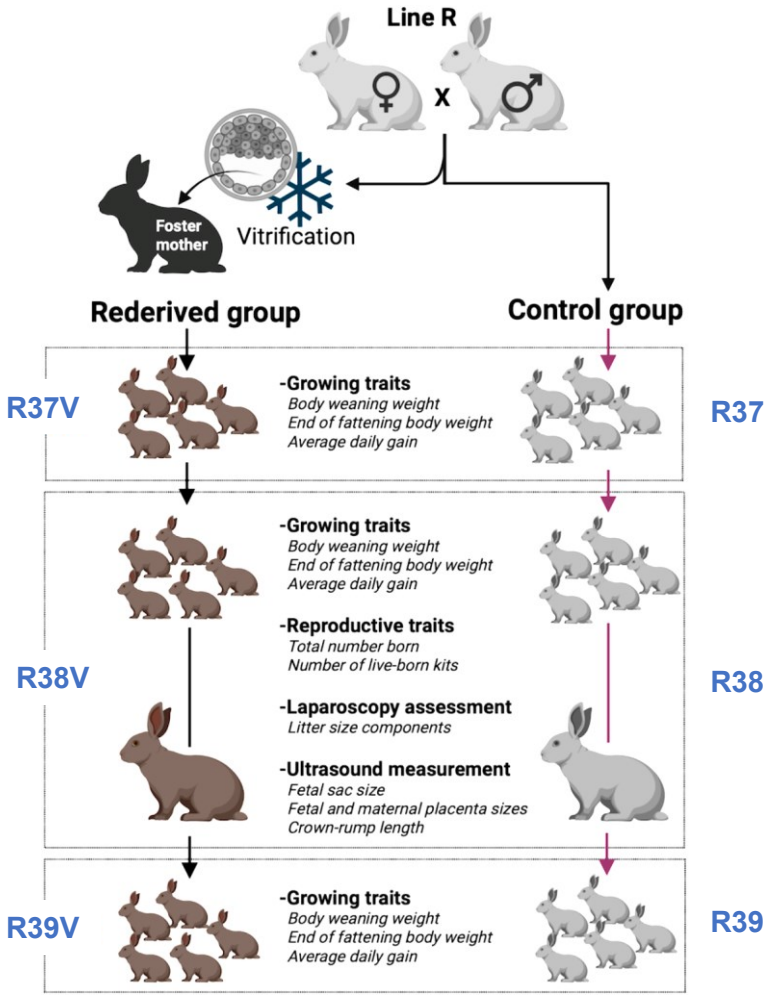

Figure 3.1. Graphical abstract.

The animals were housed at the Universitat Politècnica de València experimental farm in flat deck indoor cages (75 x 50 x 30 cm), with free access to water and commercial pelleted diets (Cunilap, NANTA S.A., Spain, minimum of 15 g of crude protein per kg of dry matter (DM), 15 g of crude fiber per kg of DM and 10.2 MJ of digestible energy (DE) per kg of DM). The photoperiod was 16 h of light and 8 h of dark, with a regulated room temperature between 14 °C and 28 °C.

3.2.2. Growing traits analysis

Individual weaning weight (WW, 28 days old), individual weight at the end of the fattening period (EFW, 63 days old) and average daily weight gain (weight gained from day 28 to 63 divided by 33, ADG) during the fattening period were recorded for all generations.

3.2.3. Female reproductive management

Reproductive management of both populations (Rederived and Control) and their offspring avoided mating among relatives with common grandparents, achieving non-overlapping generations. The first reproductive cycle took place at ~5 months of age, and after kindling, the new insemination was tried 10-12 days later. Briefly, two ejaculates per male were collected in each replica using an artificial vagina. Only ejaculates with total motility higher than 70% and less than 30% of abnormal sperm were used. After semen evaluation, optimal ejaculates from each male were extended to 40 million/mL. All females were synchronized by intramuscular injection with 15UI eCG (Cuniser, HIPRA S.A., Spain) 48h before being inseminated with 0.5 mL of extended semen using a curved plastic pipette. Females were induced to ovulate by intramuscular injection of 1 μg of buserelin acetate (Suprefact, Hoechst Marion Roussel, S.A., Spain) at insemination time. Pregnancy was checked at 14 days from insemination and non-pregnant does were inseminated again at 21 days after the previous insemination. In addition, it was noted whether rabbits underwent a lactation-gestation overlap and the dams were classified into off in four groups, according to reproductive status: offspring from primiparous does without overlapping (PD), offspring from primiparous lactating does (females that were pregnant while suckling their first litter, PLD), multiparous lactating does (females with more than one parturition that were pregnant while suckling their litter, MLD) and multiparous non-lactating does (females from more than one parturition that were pregnant after lactation, MD). Total litter size, liveborn and litter size at weaning were recorded for each female.

3.2.4. Evaluation of litter size components.

A total of 139 laparoscopies were carried out on females from fourth and fifth parity (80 does from R38 and 59 does from R38V). Females were sedated according to the procedure described by Juarez et al. (2021). In brief, the females were sedated with an intramuscular injection of 5 mg xylazine/kg (Rompun, Bayer AG, Leverkusen, Germany) and 3 mg/kg morphine chloride. Five minutes later, 35 mg/kg Ketamine hydrochloride (Imalgene, Merial, S.A., France) was administered intravenously. After laparoscopy,

does were treated with antibiotics (200,000 IU procaine penicillin and 250 mg streptomycin, Duphapen Strep, Pfizer, S.L., Spain), 0.03 mg/kg of buprenorphine hydrochloride every 12 hours and 0.2 mg/kg of meloxicam every 24 hours for 3 days. The number of corpora lutea, the number of implanted embryos at 12 days (IE) and litter size at birth (LS) were recorded per female. The following variables were calculated using the above data. Ovulation rate (OR), defined as the number of corpora lutea, embryonic loss rate (ELR), estimated as (OR-IE)/OR, and fetal loss rate (FLR), estimated as (IE-LS)/IE.

3.2.5. Foetal growth. Ultrasound measurement

Twenty-nine pregnant does from laparoscopized females (13 from R38 and 16 from R38V) were examined on day 12, 19 and 26 of gestation using a portable color Doppler ultrasound device (Esaote, Spain) with a 7.5 MHz linear probe (4–12 MHz range). Does were sedated according to the procedure described above and placed in a polystyrene cage where they were prevented from moving. The ultrasound examination was performed according to the procedure described by Juarez et al. (2021). After localization of different fetal sacs, 4–6 whole fetal sac were examined per doe. The identifiable structures (fetal sac, fetus, and fetal and maternal placenta) were measured from frozen frame pictures on the monitor, using the Esaote 16 ultrasound software.

3.2.6. Statistical analyses

Growth traits

A first analysis on growth traits was carried out to assess the effects of cryopreservation procedures on the reconstituted population:

$$Y_{ijklm} = \mu + P_i + R_j + MY_k + PR_{ij} + CO_l + Cov\ X_m + e_{ijklm}\ (1)$$

where Y_{ijklm} was the trait to analyze, μ was the general mean, P_i was the fixed effect of rederivation (control population vs rederived), R_j was the fixed effect of reproductive status of the doe used in the WW analysis (PD, PLD, MLD and MD), MY_k was the fixed

effect of month-year in which the fattening period ended (39 levels), PR_{ij} was the effect of interaction between rederived population and reproductive status of the mothers used for WW analysis, CO_l was the random effect of common litter, $Cov\ X_m$ was the covariate of the number born alive (BA) used for the WW trait or the covariate of WW used for weight at the end of the fattening period (EFW) and ADG traits and e_{ijklm} was the error term of the model.

A second analysis of growth traits to evaluate the effect of rederivation in each filial generation (Control [R37, R38 and R39] vs Rederived [R37V, R38V and R39V]) was performed using the mixed linear model described above:

$$Y_{ijklm} = \mu + P_i + R_j + MY_k + PR_{ij} + CO_l + Cov\ X_m + e_{ijklm}\ (2)$$

where P_i was the fixed effect of filial generation (R37 and R37V or R38 and R38V or R39 and R39V). In the WW analysis of the first filial generation, the fixed effect month-year (MY_k) had 33 levels, for the filial generation 2 had 31 levels and for filial generation 3, had 32 levels.

Reproductive traits

The general linear model was used to assess reproductive performance, including as fixed effects the rederived generation group (P_i, R38 and R38V), reproductive status of does (R_j, nulliparous, primiparous lactating, multiparous lactating and non-lactating does), month-year in which insemination was done (MY_k, 9 levels) and the interaction between generation group and reproductive status of the mothers (PR_{ij}).

$$Y_{ijklm} = \mu + P_i + R_j + MY_k + PR_{ij} + e_{ijklm}\ (3)$$

Litter size components (ovulation rate, implanted embryos, litter size, liveborn and rates of the embryo and foetal losses) were analysed by a generalised linear model, including as fixed effects the rederived generation group (P_i, R38 and R38V) and lactating or non-lactating status (L_j) and their interactions (PL_{ij}).

$$Y_{ijklm} = \mu + P_i + L_j + PL_{ij} + e_{ijklm} \quad (4)$$

To analysis foetal sac area, crown-rump length of foetus, foetal and maternal placenta areas at days 12, 19 and 26 of gestation, and the weight of liveborn kits, a mixed linear mode was used:

$$Y_{ijklm} = \mu + P_i + R_j + PR_{ij} + CO_k + Cov\ X_l + e_{ijkl} \quad (5)$$

, where Y_{ijkl} was the trait to analyse, μ was the general media, P_i was the fixed effect of the rederived generation group (R38 and R38V), R_j was the fixed effect of reproductive status of the doe used to analysis of weaning weight (lactating and non-lactating doe); PR_{ij} was the effect of interaction between rederived population and reproductive status of the mothers, CO_k was the random effect of common litter, $Cov\ X_l$ was the covariate of the number of implanted embryos and e_{ijklm} was the error term of the model. Values were considered statistically different at $P < 0.05$. Results were reported as least square means with standard error of the mean (SEM). All analyses were performed with SPSS 26.0 software package (SPSS Inc., Chicago, Illinois, USA, 2012).

3.3. RESULTS

Data from 2326 (3614 liveborn) animals that finished fattening from the three filial generations were obtained from 530 parturitions (106, 155 and 269 in the first, second and third generations respectively).

In general, the mean weight at weaning (WW) was 720 g, the average body weight at the end of fattening (EFW) was 2.32 kg (at 63 days old) and the average daily gain (ADG) was 48.40 g/day.

The descriptive values for the growth parameters analyzed and the live birth covariate are shown in the supplementary table 3.5.

3.3.1. Growing traits. Rederived effect

Cryopreservation and embryo transfer procedure significantly affected EFW and ADG, but not for WW (Table 3.2). However, reproductive status of does affected WW, showing that young and adult mothers without lactation-gestation overlap (ND and MD, respectively) had the lowest WW (0.67 ± 0.01 and 0.68 ± 0.01 vs. 0.78 ± 0.02 and 0.78 ± 0.01 kg to ND, MD, PLD and MLD, respectively, $p < 0.05$, data not shown in tables). MY fixed and common litter random effects were significant for all analyses.

Table 3.2. Effect of rederivation by embryo cryopreservation on weaning weight (WW, kg), weight at end of fattening (EFW, Kg) and average daily gain (ADG, g/day) from a paternal rabbit line.

Population	n	WW (kg)	EFW (kg)	ADG (g/day)
R37 (R37+R38+R39)	1117	0.73 ± 0.010	2.34 ± 0.010[a]	49.24 ± 0.298[a]
R37V (R37V+R38V+R39V)	1209	0.73 ± 0.011	2.26 ± 0.011[b]	46.61 ± 0.339[b]
Covariate effect		0.015 ± 0.001***	0.11 ± 0.001***	9.77 ± 1.21***

Significance of estimated effects and covariates (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3.3.2. Evolution of growth traits by filial generation

Differences in WW were greater for the R37 than R37V, but in subsequent generations were greater for offspring of the rederived population (R38V and R39V) than control (R38 and R39). Otherwise, for EFW and ADG, in the first generation the rederived population (R37V) had higher values, but in the following generations, it was greater to the control population (R38 and R39) in both traits (Figure 3.2).

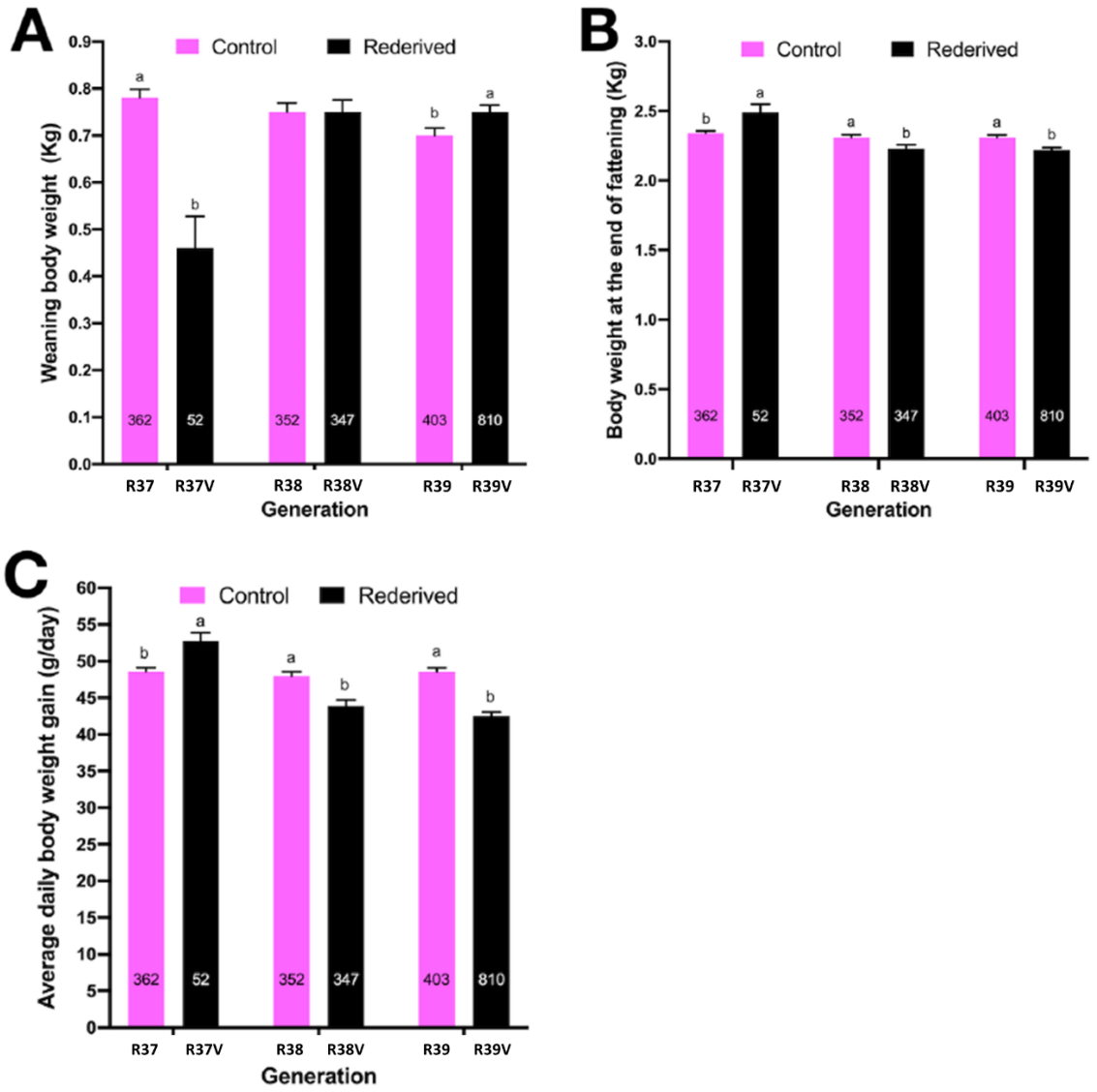

Figure 3.2. Effect of rederivation by embryo cryopreservation on: A, weaning weight (WW, kg); B, weight at end of fattening (EFW, Kg) and C, average daily gain (ADG, g/day) from filial generations (F1-R37 and R37V, F2-F38 and F38V and F3-R39 and R39V). Number of rabbits for each population and filial generation is noted into columns. [a, b] Values in each treat and filial generation with different superscripts are statistically different ($P < 0.05$)

3.3.3. Reproductive Traits

3.3.3.1. Reproductive performance of F2 (R38 and R38V) females

No differences in litter size at birth and at weaning, perinatal and lactation mortalities and kindling interval were found between the populations assessed (R38 and R38V,

Table 3.3). Likewise, no significant differences in the reproductive status of does were registered, except for the kindling interval, registering a higher KI in multiparous non-lactating does (MD), almost twice the interval (Table 3.3).

Similarly, no effects of interaction between population and reproductive status of doe were reported for all reproductive parameters evaluated.

3.3.3.2. Litter size components of F2 (R38 and R38V) females

In all the litter size components assessed, no significant differences were found between populations (R38 and R38V) or between lactation status at insemination, and the interaction between generation and lactation status of the doe was not significantly different (Table 3.4).

3.3.3.3 Foetal growth during gestation in F2 (R38 and R38V) females.

No differences were observed in the size of foetal sac, maternal placenta, foetal placenta and crown-rump length of the fetus in any day of gestation assessed between both populations (Figure 3.3). Differences in fetal sac size were observed relative to the lactational status of the doe only at 12 days of gestation, being larger in non-lactating does (2.51 ± 0.087 vs. 2.27 ± 0.073 in lactating does). The bodyweight of the liveborn kits were no different between populations and was not affected by reproductive status (66.5 ± 2.43, data not shown in tables).

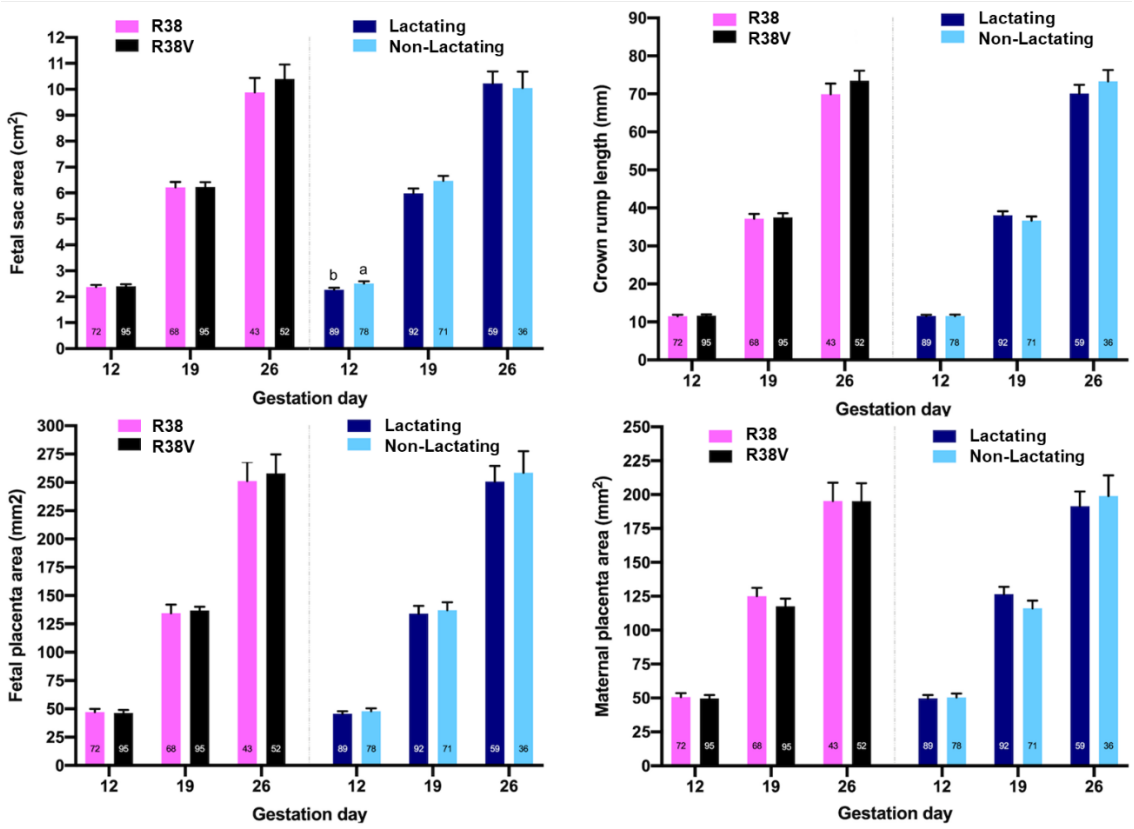

Figure 3.3. Effect of rederivation by embryo cryopreservation on fetal growth at 12, 19 and 26 days of gestation from a paternal rabbit line. Number of fetal traits measured is noted into columns. A, Fetal sac area (cm^2); B, crown-rump length of fetuses (mm); C, maternal placenta area (mm^2) and fetal placenta (mm^2). ^{a,b} Values in each trait and age with different superscripts are statistically different ($P < 0.05$).

Table 3.3. Effect of rederivation by embryo cryopreservation on total litter size, liveborn, litter size at weaning, perinatal and lactation mortality rates from a paternal rabbit line.

Type		n	Litter size			Mortality rate		KI
			Total	Liveborn	At weaning	Perinatal	Lactation	
Generation	**R38**	98	6.38 ± 0.366	5.50 ± 0.402	4.00 ± 0.367	15.56 ± 3.278	36.50 ± 4.325	56.41 ± 1.619
	R38V	172	7.21 ± 0.453	5.86 ± 0.497	3.55 ± 0.456	21.36 ± 4.059	46.55 ± 5.419	58.35 ± 2.007
Lactation status	**Nulliparous**	115	6.29 ± 0.423	4.84 ± 0.464	2.97 ± 0.434	25.33 ± 3.790	51.39 ± 5.265	
	Primiparous Lactating	50	7.20 ± 0.617	6.34 ± 0.677	4.08 ± 0.618	12.09 ± 5.531	35.35 ± 7.364	46.33 ± 2.409[a]
	Multiparous lactating	103	6.81 ± 0.468	5.79 ± 0.514	4.16 ± 0.481	17.43 ± 4.196	38.85 ± 5.693	42.08 ± 1.878[a]
	Non-lactating	75	6.88 ± 0.490	5.74 ± 0.538	3.87 ± 0.495	19.00 ± 4.393	40.51 ± 5.849	83.73 ± 1.877[b]

n: Number of births. Data are expressed as least squared mean ± standard error of means. [a,b] Values with different superscripts in column differ significantly ($p < 0.05$).
KI: Kindling Interval

Table 3.4. Effect of rederivation by embryo cryopreservation on litter size components from a paternal rabbit line.

Type		Ovulation rate[1]	Implanted embryo		Loss rate		Litter size
			Implanted embryo	Implanted embryo[2]	[1]Embryonic	Foetal	
Generation	**R38**	12.1 ± 0.32	6.87 ± 0.566	9.43 ± 0.457	24.01 ± 3.458	41.84 ± 4,836	6.02 ± 0.539
	(n)	80	80	59	59	59	55
	R38V	12.7 ± 0.40	6.79 ± 0.700	8.83 ± 0.563	32.71 ± 4.096	31.43 ± 5.762	6.70 ± 0.623
	(n)	59	59	45	45	44	44
Lactation status	**Non-lactating**	13.0 ± 0.39	7.08 ± 0.687	8.89 ± 0.547	31.62 ± 3.981	40.53 ± 5.568	6.07 ± 0.610
	(n)	64	64	51	51	51	48
	Lactating	11.8 ± 0.33	6.58 ± 0.581	8.89 ± 0.494	25.10 ± 3.590	32.74 ± 5.059	6.65 ± 0.554
	(n)	75	75	53	53	52	51

Data are expressed as least squared mean ± standard error of means. n: number of laparoscopies.

[1] Determined as the number of *corpora lutea.*

[2] Implanted embryos in pregnant does.

[a,b] Values in the same column and factor with different superscripts are statistically different (P < 0.05).

3.4. DISCUSSION

3.4.1. Growth traits

The selection objective of paternal line R is the average daily gain at fattening (Estany et al., 1992). ADG has been estimated to increase from 0.45 - 1.23 g/d per generation (Baselga, 2005), having a moderate heritability (0.17) ideal for improvement. These estimates could be refined using an unselected, divergently selected, or a population rederived by cryopreservation as control. The latter offers a more economical tool and avoids the genetic drift of the population. However, recently there are more and more studies showing genetic and phenotypic effects in rederivative populations that could question their use as control populations. Our study shows effects on the end of fattening weight (EFW) and average daily gain (ADG), which indicates some effect derived from the cryopreservation and transfer process performed. Both populations were coetaneous, received the same feed and were placed at the same facilities, avoiding the environmental effect on traits under evaluation. Differences in each trait become more evident when each generation is compared, showing that growth traits are affected in all generations. Direct or short-term effects of embryo manipulation (production, cryopreservation and transfer) have been reported in previous studies (Lavara et al., 2015; Saenz-de-Juano et al., 2014; Saenz-de-Juano et al., 2015; Vicente et al., 2013) and by other authors in other animal species (Cuello et al., 2021; Leme et al., 2020; López-Damián et al., 2020), and even humans (Barsky et al., 2016; Beyer and Griesinger, 2016; Chen et al., 2020; Heber and Ptak, 2021), which may be involving epigenetic changes at the embryonic level (Chatterjee et al., 2017) that could cause post-implantation losses, changes in fetal and maternal placental tissues, and higher birth weight in rabbits (Saenz-de-Juano et al., 2014; Saenz-de-Juano et al., 2015). Long-term (transgenerational) effects have also been reported in maternal lines (selected for litter size, García-Dominguez et al, 2020d; Lavara et al., 2014) and recently in paternal lines (selected for growth rate, García-Dominguez et al., 2020c), where growth traits are affected more than reproductive traits.

Differences observed from the first generation (F1, R37 and R37V) were significant in the three growth traits assessed (WW, EFW, ADG). A higher EFW achieved by the Rederived population has been previously reported associated with disturbances in liver and adrenal gland size and lipid and fatty acid metabolism (Marco-Jiménez et al., 2020, García-Dominguez et al., 2020b). Direct effects could be due to the stress to which the embryos are subjected during recovery, vitrification, warming and transfer, in addition to the maternal effects derived from the host rabbits. Thus, in F1 WW was higher for R37 population, while for EFW and ADG it was higher for R37V population. This differentiated and lower growth before weaning could be related to the metabolism of Zn and Fe, which has been reduced in children born from ART (Xia et al., 2019), and which could influence the lower weight achieved at weaning by R37V population, but apparently, these differences are reversed at the end of fattening where this population has highest results. These disturbances should be taken into account when selecting animals from populations rederived by vitrification to be used as a control group, even in subsequent generations.

It was demonstrated that cryopreservation and embryo transfer processes in lines selected for daily weight gain cause changes in the reconstituted population in weaning weight that stabilize in the following generations, but the changes in pubertal and adult weight, and liver and heart weight, are maintained, as well as in some seminal characteristics and changes in the transcriptome and metabolome profile of the liver of adult rabbits (García-Dominguez, et al., 2020e). These findings would indicate that the change caused in a first generation influences the following generations in the characteristics studied (WW, EFW and ADG), causing higher WW, lower EFW and therefore lower ADG in the descendants of the rederived population.

3.4.2. Reproductive traits.

Reproductive traits, such as litter size and live births, seem not to be influenced by the effects of cryopreservation either by vitrification or slow freezing, but by the seasons and the physiological state of the dam (Cifre et al., 1999; Cifre et al., 1996). However, Lavara et al. (2014) showed that cryopreservation and embryo transfer increased litter

size, live births, and postnatal survival in cryopreservation-born females (F1) and their female offspring (F2), whereas in our study, no differences are observed in any of the reproductive traits examined in F2 females; differences are only reported based on the lactation status of does, being higher in non-lactating does. These discordant results could be due to the genotype of the populations studied.

Similarly, foetal sac size was smaller in lactating rabbits than non-lactating rabbits (2.27 ± 0.073 vs 2.51 ± 0. 087, respectively, Figure 3.3.) at 12 days of gestation, but as gestation progresses these differences disappear. As in primiparous rabbits, lactation could be detrimental for receptivity, conception and ovulation rate, embryo and foetal survival and foetal growth (Fortun-Lamothe and Prunier, 1999), possibly due to the nutritional deficit that occurs during lactation, which induces a competition between the mammary glands and the pregnant uterus for the supply of nutrients (Fortun-Lamothe *et al.*, 1999).

The effects of vitrification and embryo transfer processes may not affect the reproductive characteristics evaluated in this line because they showed lower reproductive performance than maternal lines (Baselga, 2002; García and Baselga, 2002b; Vicente *et al.*, 2003 and 2013, Naturil-Alfonso *et al.*, 2015).

Embryo cryopreservation in rabbits has been used to estimate genetic gain by rederivation of frozen populations from previous generations (García and Baselga, 2002b; García and Baselga, 2002a; Pascual, *et al.*, 2008), either in maternal lines selected for their reproductive traits or in paternal lines selected for their growth traits. It has also been found to be a valuable technique to re-establish populations cryopreserved fifteen years ago (Marco-Jiménez *et al.*, 2018). Likewise, the transgenerational effect on some reproductive traits has been noted (Lavara *et al.*, 2014), as has the effect on birth weight, transcriptomic and metabolomic changes and on liver size of offspring of individuals born by these reproductive techniques (García-Dominguez, *et al.*, 2020e). However, our work shows for the first time the impact of these biotechnologies on the traits under selection in paternal lines without detriment or changes in their reproductive traits two generations later, evidencing possible transgenerational epigenetic changes, suggesting that when estimating genetic gain using former

generations cryopreserved years ago, this should be done with a current population subjected to cryopreservation and embryo transfer processes.

3.5. CONCLUSIONS

Embryo vitrification and embryo transfer processes cause changes in growth traits of reconstituted populations that influence the following generations, without changes in female reproductive traits in a paternal line of rabbits. This finding should be taken into account when these populations are used as controls to estimate genetic changes in these characters. A possible solution would be a double control, rederiving the contemporary population with the same procedure.

Supplementary tables

Table 3.5. Number of young rabbits weighted from weaning to end of fattening period and average and standard deviation to birth alive (BA), weaning weight (WW), weight at end fattening (EFW) and average daily weight gain (ADG).

Trait	Generation						
	R37	**R37V**	**R38**	**R38V**	**R39**	**R39V**	**Total**
BA	6.7 ± 2.30	6.6 ± 3.05	7.4 ± 2.76	7.7 ± 2.94	7.8 ± 2.71	8.4 ± 2.88	7.8 ± 2.83
WW (Kg)	0.75 ± 0.18	0.64 ± 0.14	0.74 ± 0.16	0.72 ± 0.17	0.71 ± 0.18	0.71 ± 0.17	0.72 ± 0.17
EFW (Kg)	2.39 ± 0.32	2.17 ± 0.25	2.40 ± 0.32	2.33 ± 0.31	2.32 ± 0.33	2.25 ± 0.31	2.32 ± 0.32
ADG(g/day)	49.64 ± 6.64	46.30 ± 5.58	50.26 ± 7.25	46.88 ± 6.81	48.88 ± 7.20	43.62 ± 6.37	48.40 ± 6.91
Animals	414	52	352	347	403	810	2326

R37V: Rederived from vitrified and embryo transfer, R38V and R39V filial generations

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CHAPTER II

EVALUATION BY RE-DERIVATION OF A PATERNAL LINE AFTER 18 GENERATIONS ON SEMINAL TRAITS, PROTEOME AND FERTILITY

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ABSTRACT

Males from a paternal line selected for growth traits were used to produce semen doses at insemination centres and farms in a breeding scheme for rabbit meat production. The aim of this study was to assess whether a program of selection by daily gain in fattening period changed the seminal traits, plasma and sperm proteome and the fertility of semen when used in artificial insemination. Thirty-nine males from a paternal line were obtained by re-derivation from vitrified embryos with a difference of 18 generations (G21V and G39V). Sperm production parameters, morphological traits, sperm motility parameters and viability were evaluated from ejaculates. Seminal plasma and sperm proteome of three pool ejaculates from 10 mature males of each group were analysed and semen doses were used to inseminate 311 females. Only the percentage of abnormal sperm showed significant differences, with G21V presenting fewer abnormal sperm than G39V (10.5 ± 2.63 vs23.8 ± 1.98). The discriminant analysis (DA-PLS) showed a clear effect of the generation for plasma and sperm proteome. In seminal plasma, 643 proteins were reported and 64 proteins were differentially expressed, of which56 were overexpressed in G39V (87.5%). Sperm proteome reported 1360 proteins with 132 differentially abundant proteins. Of the total, 89 proteins were overexpressed in G39V (67.4%). From the 64 and 132 differentially abundant proteins of plasma and sperm, 19 and 26 had a FC>1.5, 12 and 13 of them belonging to the *Oryctolagus cuniculus* taxonomy, respectively. Despite observing differences in important proteins related to capacitation, sperm motility or immunoprotection and consequently to the fertilization process (TMPRSS2, Serpin family, Fam71f1, ATPase H+ transporting accessory protein 2, carbonic anhydrase 2, UDP-glucose glycoprotein glucosyltransferase 2), no differences in fertility and prolificacy were detected when commercial seminal doses were used for insemination from both male groups. However, overabundance of KIAA1324 protein can be related to the increase in abnormal sperm after selection by growth rate.

Keywords: Sperm, Proteome, Growth rate, Selection, Rabbit

4.1. INTRODUCTION

Breeding schemes for meat production in rabbits involved a three-way cross of specialized lines in which paternal line males inseminate maternal crossbred females. Paternal line or terminal sires are selected for growth traits (Rochambeau et al., 1989; Estany et al., 1992; Lukefahr et al., 1996; Larzul et al., 2005), as the males are used for the production of seminal doses at insemination centres and farms. Therefore, males from growth lines must produce semen in sufficient quantity and quality to meet the demand for insemination. Nevertheless, several studies have shown that selection for growth has effects on reproductive performance in both females and males (Bunger et al.,2005). In rabbits, negative effects have been observed in ovulation induction, prenatal survival and genetic correlation to fertility (Vicente et al., 2012; Piles et al., 2012) and ejaculate traits such as mass motility, volume, abnormal sperm rate or head sperm morphometry (Brun et al., 2006; Lavara et al., 2012and 2013).

Many factors influence the production and quality of rabbit semen, such as collection frequency (Nizza et al., 2003), environment (season or photoperiod, Marai et al., 2002, Pascual et al., 2004, Roca et al.,2005; Theau-Clément et al., 2015, Sabés-Alsina et al., 2015), nutrition (Pascual et al., 2004and 2016) and genetic line (Vicente et al., 2000;Brun et al., 2002and 2006;García-Tomás et al., 2006a; Piles et al.,2013). Genetic parameters for ejaculate traits show a moderate repeatability and low to moderate heritability in most of them (García-Tomás et al., 2006b; Lavara et al., 2011, Tusell et al., 2012; Brun et al.,2016). Tusell et al. (2012) found a moderate heritability for concentration, volume and sperm production in a rabbit line selected by daily gain and, a low or uncorrelated genetic response between daily gain and these ejaculate traits, as consequence non detrimental effect is expected on sperm production. In this sense, Lavara et al. (2012 and 2013) observed no effects on sperm production but showed moderate negative correlations between daily weight gain and normal acrosome status, sperm motility and the morphometry of sperm heads, suggesting that genes that favor daily weight gain slightly decrease normal acro-some status and increase abnormal sperm forms.

The production of semen doses requires the estimation of different parameters of seminal quality, among which motility and morphology are the most widely used. It is accepted that conventional seminal parameters provide a low correlation with male fertility. Due to these limitations, efforts must be made to understand and identify sperm biomarkers at molecular level in seminal plasma and sperm. In this sense, some works have tried to better understand the role of seminal plasma. Castellini et al. (2000) observed that seminal plasma enhanced both the resistance of rabbit spermatozoa to in vitro storage and their motility characteristics. Seminal plasma contains, in the others components, several proteinases involved in physiological events, ranging from immunosuppressive activity to the enhancement of sperm cell motility or fertility. Viudes de Castro et al. (2014and 2015) reported differences between genetic lines and showed that high levels of aminopeptidase activity of rabbit seminal plasma was related with ab-normal sperm rates and lower percentages of normal apical ridge, however, no effects on fertility was observed. In mouse, deficient aminopeptidase activity was associated with infertility, lack copulatory behavior and impaired spermatogenesis (Osada et al., 2001). Several authors have observed that some seminal parameters significantly influenced kindling rate in rabbit, such as acrosome integrity and chromatin structure (Courtens et al., 1994), mass motility and total motile sperm per dose (Brun et al., 2002and Hagen et al., 2002) and the percentage of abnormal sperm (Lavara et al., 2005). Most of the previous studies have been focused on the effects of selection on the seminal and sperm parameters, but little attention has been paid to the protein seminal plasma or sperm composition and whether these changes could affect the fertility of seminal doses obtained from the paternal males. In this context, the study of the proteome is of great interest, as plasma and sperm proteins play a key role in the maintenance of sperm morphology, motility patterns, acrosome formation and reaction, capacitation and fertilization. Recently, Casares-Crespo et al. (2018and 2019) analysed the effect of the genetic origin of two rabbit lines (maternal and paternal) and season on the seminal and sperm proteome. They identified 402 and 487 proteins in seminal plasma and spermatozoa respectively, providing evidence that genotype had a huge impact on protein abundance in rabbit ejaculates. Whether these different proteome patterns justify cryotolerance and fertility differences observed previously in these lines has yet to be resolved (Mocé et al., 2003). Finally, Bezerra et al. (2019)

identified137 different seminal plasma proteins and identified potential associations between the major seminal plasma proteome and some semen traits in rabbits. Among other findings, they noted that sperm motility had a positive association with beta-nerve growth factor and cysteine-rich secretory protein 1-like and a negative one with galectin-1, that intact sperm membrane was related to seminal plasma protein FAM115complex and tropomyosin or that morphologically normal sperm was positively linked to carcinoembryonic antigen-related cell adhesion molecule 6-like and down regulated by seminal plasma isocitrate dehydrogenase. The aim of this study was to evaluate whether a selection program by daily gain in fattening period affects ejaculate traits, seminal plasma and sperm proteome and semen fertility.

4.2. MATERIALS AND METHODS

All chemicals, unless otherwise stated, were reagent-grade and purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain). All the experimental procedures used in this study were performed in accordance with the principles of animal care published by Spanish Royal Decree 53/2013 (BOE 2013) and the Directive 2010/63/EU EEC for animal experiments and reviewed and approved by the Ethics and Animal Welfare Committee of the Universitat Politècnica deValència (Research code, 2015/VSC/PEA/00061).

4.2.1. Animals

A total of 39 males from genetic line R from the Universitat Politècnica de València were used. Line R is a paternal line selected over 36 generations for daily gain from 28 to 63 days of age (Estany et al., 1992). Selection is based on phenotypic values of daily gain and is conducted in non-overlapping generations. Environmental conditions were maintained using a control system for light (16:8 light/dark photoperiod), with free access to water and commercial pelleted diets (minimum of 15 g of crude protein per kg of dry matter, 15 g of crude fiber per kg of DM, and 10.2 MJ of digestible energy per kg of dry matter). A total of 311 commercial crossbreed females were used to perform the fertility and prolificacy study. Females were kept in similar environmental conditions.

4.2.2. Experimental design

Two populations of R line males were used for this experiment. Both were obtained from embryos vitrified in 2015. The 18th generation was re-derived from vitrified embryos stored in 2000 (G19V) and a sample of embryos from the current generation (36th) was vitrified and transferred at the same time to establish the G37V population. The reconstitution of respective generations was successfully done with 8 families from G19V and 9 from G37V of different male origin (Fig. 4.1).

After two generations without selection, to avoid possible effects of cryopreservation and transfer procedures on growth and reproductive performance, males from different generations (G21V and G39V) were trained and evaluated.

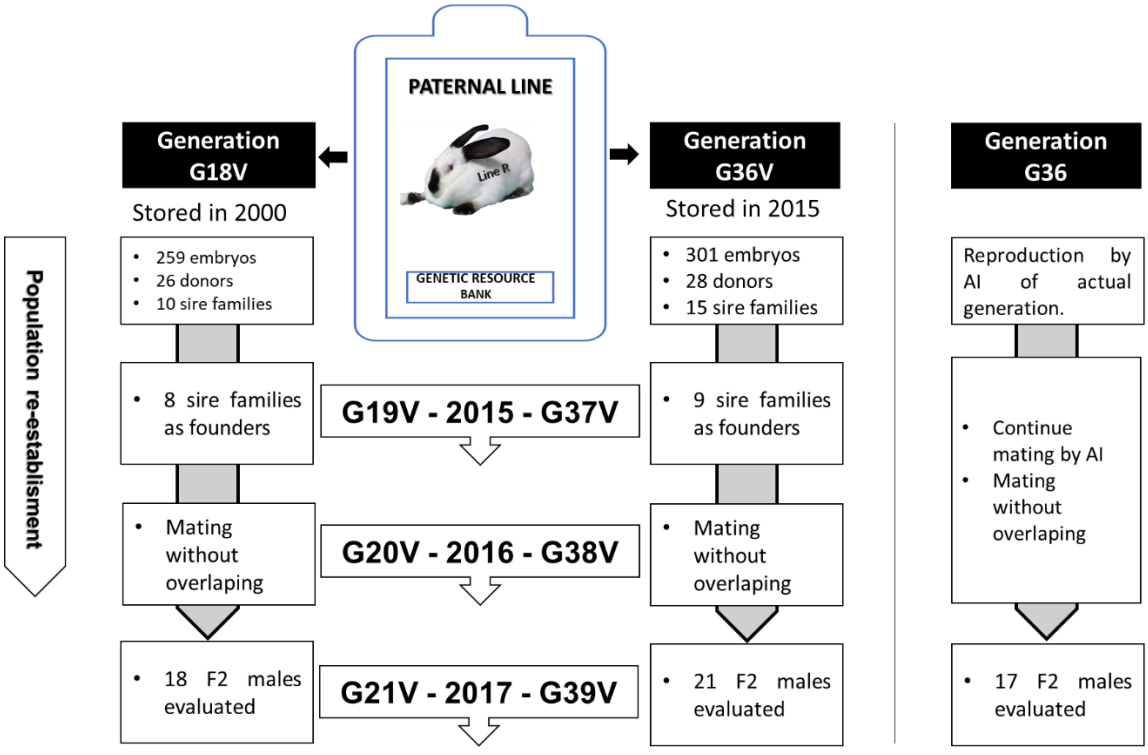


Fig. 4.1. Flowchart of the experiment performed to obtain the evaluated generations of a rabbit line selected by growth rate.

4.2.3. Semen collection

At 5 months of age, males started a four-weeks training period with artificial vaginas; one ejaculate was collected per male weekly. Semen collection was performed using a receptive doe and the response of the males to the collection procedure was recorded during the training period until the beginning of the experimental period. At the 6th month of age males were subjected to experimental evaluation. Collections were performed on the same day for 10 weeks; one ejaculate was collected per male and per week to assess the seminal parameters. Only ejaculates exhibiting a white color were used in the experiment; if the first ejaculate was not available (with urine, sediment or cell debris), second one was collected 20 min later. Gel was removed if present. Males were weighted weekly during experimental period.

4.2.4. Evaluation of ejaculates

4.2.4.1. Ejaculate quantity and sperm production

The ejaculate volume was measured in a graduated tube. To determine sperm concentration, aliquots of each ejaculate were diluted1:50 with 0.25% of glutaraldehyde solution in Dulbecco's phosphate buffered saline, using a Thoma-Zeiss counting cell chamber (Marienfield, Germany). Total sperm per ejaculate (TSE) was calculated using volume and concentration from each ejaculate.

4.2.4.2. Ejaculate quality

Sperm morphological traits. To measure acrosome integrity percentage (normal apical ridge) and percentage of abnormal forms (abnormal head and tails), a sample of spermatozoa from each ejaculate was fixed with a solution of glutaraldehyde (0.25% in Dulbecco’s phosphate buffered saline) and the samples were examined under a phase contrast optical microscope at x400 magnification.

***Sperm motility parameters*.** Ejaculate samples were diluted in a Tris-citrate-glucose extender (TCG:250mMtris-hydroxymethylaminomethane, 83 mM citric acid, 50 mM

glucose, pH6.8 ± 7.0, 300 mOsm/kg−1) to obtain a concentration of 30 × 106sperm/mL. An aliquot from each sample was then adjusted to 7.5 × 106sperm/mL with TCG extender supplemented with 2 g/L BSA, then 10µlwere placed in a Makler counting chamber pre-warmed at 37°C on a thermal plate and evaluated in an Integrated Semen Analysis System v.1.0.17 (ISAS; Projectes i ServeisR++DS.L.). The system was set to record images at 30 frames/s. Motility was assessed at 37°C at 200Xusing a negative phase contrast microscope. For each sample, six microscopic fields were analysed and a minimum of 400 sperm evaluated. The curvilinear velocity (VCL, the average velocity measured over the actual point to point track followed by the cell), straight-line velocity (VSL, the average velocity measured in a straight line from the beginning to the end of the track), average path velocity (VAP, the average velocity of the smoothed cell path), linearity index (LIN; the average value of the ratio VSL/VCL), straightness (STR, the ratio between VSL and VAP), wobble (WOB = (VAP/VCL) x 100, a measure of the oscillation of the actual trajectory about its spatial average path), amplitude of lateral head displacement (ALH, the mean width of the head oscillation as the sperm cells swim) and beat cross frequency (BCF, the frequency of sperm head crossing the average path in either direction) were evaluated. All captures were saved and analysed later. Before field analysis, we proceeded to identify each sperm trajectory to eliminate debris (false captures) and reduce the risk of confusing trajectories.

Viability and HOST analysis*.* The percentage of viable sperm was determined using a dual fluorescent staining with SYBR-14/PI according to Viudes de Castro et al. (2014). A minimum of 100 sperm cells were counted per ejaculate, and only the percentages of live sperm were considered in the results (SYBR-14-positive and PI-negative). All dilutions were performed at 22 °C.

A hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane (Jeyendran et al., 1984). Semen was diluted 1:20 in a HOST solution of 75 mOsm at 25–30 °C for15 min. A minimum of 100 sperm cells were evaluated and HOST was calculated as the percentage of spermatozoa with swollen coiled tails/total spermatozoa.

4.2.5. Plasma and sperm protein extraction samples

Ejaculates from 20 mature males (10 for each experimental group “G21V and G39V”) were collected and pooled. Six pooled ejaculates (three for each group of males) were obtained in three different weeks and used for insemination. Before preparing the sperm doses, a sample of 500 µl from ejaculate pools was centrifuged at 7400 x g for 10 min at22 °C. The supernatants (seminal plasma) were collected, supplemented with a 1% v/v protease inhibitor cocktail (P2714, Sigma) and stored at−80°C until use. The resulting pellets were washed twice by centrifugation at 900 x g for 10 min in PBS. Sperm proteins were extracted according to the Casares-Crespo et al. (2019) protocol. Briefly, sperm pellets were resuspended in 1% SDS (w/v) in TCG (Tris-citrate-glucose supplemented with a 1% v/v protease inhibitor cocktail, P2714) 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 15,000 g at 4°C. Protein lysates were stored at−80°C until use.

4.2.6. Proteomic relative quantification analysis: SWATH (DIA) MSMS analysis

The proteomic analyses were performed in SCSIE of the Universitat de València (PRB3-ISCIII ProteoRed Proteomics Platform).

Initial protein concentration from seminal plasma was measured by Nanodrop (Thermo Scientific) using diluted (1 to 10) samples in ultrapure water and the concentration of sperm protein sample by Machery Nagel quantitation kit (Ref. 740967.50), following the manufacturer’s protocol. A pool of seminal plasma and another with sperm samples were prepared with 50 µg of protein and resolved in 1D PAGE gel.

4.2.6.1. Spectral libraries building

In gel protein digestion: 5 gel slides in each gel were digested with sequencing grade trypsin (Promega) as described by Shevchenko et al. (1996). Gel slides were digested using 200 and400 ng of trypsin and digestion was set to 37 °C (on seminal and sperm

slides, respectively). The trypsin digestion was stopped with 10% trifluoroacetic acid (TFA) and the supernatant (SN) was removed, then the library gel slides were dehydrated with pure acetonitrile (ACN). The new peptide solutions were combined with the corresponding SN. The peptide mixtures were dried in a speed vacuum (ISS 110 SpeedVac System, Thermo Savant, Thermo Scientific, Langenselbold, Germany) and resuspended in 2% ACN; 0.1% TFA. The volumes were adjusted according to the intensity of the staining.

LCMSMS data dependent acquisition (DDA) analysis: 5 µl of the digested fragments were loaded into a trap column (NanoLC Column,3µ C18-CL, 75µ x 15 cm; Eksigent) and desalted with 0.1% TFA at 3 µl/min for 5 min. The peptides were loaded into an analytical column (LC Column, 3µ C18-CL, 75µ x 12 cm, Nikkyo Technos, Tokyo, Japan) equilibrated in 5% ACN 0.1% formic acid (FA). Peptide elution was carried out with a linear gradient of 5 to 35% of solvent B for 60 min (A:0.1% FA in water; B: 0.1% FA in ACN) at a flow rate of 300nL/min. Peptides were analysed in a nanoESI qTOF mass spectrometer (5600TripleTOF, ABSCIEX).

The tripleTOF was operated in information-dependent acquisition mode, in which a 250-ms TOF MS scan from 350 to 1250 m/z, was performed, followed by 150-ms product ion scans from 350 to 1500 m/z on the 25 most intense 2–5 charged ions. The rolling collision energies equations were set for all ions as for 2+ ions, according to the following equations: |CE|=(slope)x(m/z)+(intercept).

4.2.6.2. ProteinPilot v5.0. search engine (Sciex)

ProteinPilot default parameters were used to generate a peak list directly from 5600 TripleTof wiff files. The Paragon algorithm (Shilov et al., 2007) of ProteinPilot was used to search the UniprotMammalia database (03.2018) with the following parameters: trypsin specificity, cys-alkylation, without taxonomy restriction, and the search effort set to through and False Discovery Rate (FDR) correction for proteins. The protein grouping was done by Pro-group algorithm. Here, the formation of protein groups is guided

entirely by observed peptides only, which originate from the experimentally ac-quired spectra. Because of this, the grouping can be considered to be guided by use of spectra.

4.2.6.3. Swath analysis of samples

Protein digestion of seminal plasma samples: 25 µg of every sample were reduced by 2 mM dithiothreitol (DTT; Vf=25µ L) for 20 min at60°C. The thiol groups were alkylated by 5.5 mM Iodoacetamide (IAM, Vf=30 µL) for 30 min at room temperature in the dark. The excess of IAM was quenched with 10 mM DTT (Vf=60 µL) at 37°C for 1 h For protein digestion, 500 ng of trypsin were added (Vf=65 µL) and digestion was left overnight. All the reagents were prepared in 50 mM Ammonium bicarbonate solution. The protein digestion was stopped with 5 µL of 10% Trifluoro-Acetic acid (TFA) in water. The final mixture volume was 70 µL. Samples were concentrated by rotatory evaporator to 25 µL. The individual SWATH injections were randomized in blocs.

Protein digestion sperm samples: the protein gel mixtures were digested as described by Shevchenko et al. (1996), using 500 ng of trypsin for each sample and digestion was set to 37 °C. The trypsin digestion was stopped with 10% TFA and the SN was removed, then the library get slides were dehydrated with pure ACN. The new peptide solutions were combined with the corresponding SN. The peptide mixtures were dried in a speed vacuum and resuspended in 2% ACN; 0.1% TFA. The volume was adjusted to a final concentration of 0.5 µg/µL.

SWATH LCMSMS analysis: 5 µl of every sample were chromato-graphically resolved as in 2.6.1 but with a 120-min gradient. The tripleTOF was operated in Swath mode, in which a 0.050-s TOF MS scan from 350 to 1250 m/z was performed, followed by 0.080-s product ion scans from 350 to 1250 m/z on the 32 defined windows (3.05 s/cycle). The Swath windows used were: 15 Da window widths from 450 to1000 Da, 37 windows.

Protein quantification: the wiff files obtained from the Swath experiment were analysed by Peak View 2.1. The processing settings used for the peptide selection were: a maximum number of peptides per protein of 20, a number of transitions or fragment

ions per peptide of 6, more than 95% to peptide confidence threshold and less than 1% to FDR. After peptide detection, peptides were aligned among different samples using high confidence detected peptides from the library. Peptides with the correlated retention time were extracted using the cited processing set with 10 min Extracted Ion Chromatogram extraction. A total of 6 samples were analysed and 643 seminal plasma and1362 sperm proteins were quantified.

The proteomics data and result files from the analysis have been deposited with the ProteomeXchange Consortium (Vizcaíno et al.,2014) via the PRIDE partner repository (data identifier PXD015510 and PXD015516 and, PXD015511 and PXD015517 for sperm and seminal plasma data, respectively).
Bioinformatics analysis of identified plasma and sperm proteins was performed using the comprehensive bioinformatics tool for functional annotation UniProt KB database (www.uniprot.org) in combination with David Functional Annotation Tool (version 6.8; October 2016).

4.2.7. Fertility parameters. artificial insemination

A total of 311 crossbreed does were inseminated in three replicates,159 inseminated with seminal doses of G21V group and 152 with seminal doses of G39V group. Ten males per experimental group were used.

4.2.7.1. Semen collection and evaluation

Two ejaculates per male were collected in each replica using an artificial vagina. The percentage of motile, abnormal and normal apical ridge and sperm production were evaluated as described above. Only white ejaculates were used.

4.2.7.2. Semen extension

After semen evaluation, ejaculates form each group were pooled and extended with TCG to 40 million/mL. The semen was diluted at room temperature (20°−25°C).

4.2.7.3. Insemination procedure

All females used in this experiment were multiparous crossbred does and were synchronized with 12UI eCG injected intramuscularly 60 h before they were inseminated. Insemination was carried out 10–12thday post-partum and females were induced to ovulate using a synthetic analogue of GnRH (1µg of buserelin acetate, Hoechst) injected intramuscularly. Twenty million total sperm/female were inseminated (0.5 ml of semen/doe), using a plastic curved pipette. Females were randomly assigned. Kindling rate (number of does giving birth/number of inseminated does) and prolificacy (number of total kits born) were the reproductive performances considered.

4.2.8. Statistical analyses

To analyze the effect of generation on semen characteristics, a mixed linear model was used. The generation (G) and batch (B) were taken as fixed effects, the male weight (W) as a covariable and litter of origin (CO) and the male (M) as random effects.
The mixed model used for the semen traits was:

$$Y_{ijklm} = \mu + G_i + B_j + W_k + CO_l + M_m + \epsilon_{ijklm}$$

where Y_{ijklm} is a record of the semen trait measured in the each male, µ is the overall mean for each trait, G_j is the fixed effect of generation with two levels (G21V and G39V), B_i is the fixed effect of the batch in which the ejaculate was collected with 10 levels, as covariable W_k is the weight of the male at the evaluation, CO_l is the random effect of the litter in which the male was born, M_m is the male and ε_{ijklm} is the residual.

A generalized linear model including male group (G21V and G39V) as fixed effect was performed to compare litter size. For kindling rate, a probit link with binomial error distribution was used in the analysis, assigning 1 to pregnant and delivery does and 0 to non-pregnant and non-delivery does.

A p-value less than 0.05 was considered to indicate a statistically significant difference. The data are shown as least square mean ±standard error mean. Statistical analyses were carried out using a commercially available software program (SPSS 21.0 software package; SPSS Inc., Chicago, Illinois, USA, 2002).

For plasma and sperm protein analysis, Multiexperiment Viewer (MeV software, Saeed et al., 2003) was used for statistical normalization following the software instructions. A t-test was used to identify the differentially expressed plasma and sperm proteins among the six ejaculate pools. Analysis was done only on proteins identified in all sperm samples. Proteins were considered differentially expressed with an adjusted p-value < 0.05 and those with a fold change (FC)≥1.5after log2 transformation were highlighted. Inferno software was used to perform DA-PLS among samples and ClustVis software was used for the Heat Map clustering of differentially expressed proteins (DEPs). Functional annotation of DEPs, enrichment analysis of their associated gene ontology terms (GO terms) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis were computed using the Bioinformatic software: David Functional Annotation Tool (version 6.8; October 2016), considering a p-value < 0.05.

4.3. RESULTS

4.3.1. Ejaculates and sperm traits

A different percentage of males between experimental groups responded to artificial vagina stimulus (P<0.05, data not shown in tables). The percentage of non-responding males was greater in G21Vgroup (7/18, 38.9% versus 2/21, 9.5% G39V).

Of males responding to artificial vagina stimulus, 105 and 213ejaculates were obtained from the G21V and G39V groups, respectively, of which 13 (12.4%) and 23 (10.8%) were discarded due to the presence of urine, debris or feces in the G21V and G39V groups, respectively. Only the percentage of abnormal sperm showed significant differences, being lower in G21V (10.5 ± 2.63 versus 23.8 ± 1.98). The remaining seminal parameters were similar between groups (Table 4.1).

Table 4.1. Seminal traits in G21V and G39V.

TRAITS	N° EJACULATES	G21V (LSM ± SE)	G39V (LSM ± SE)
EJACULATE PARAMETERS			
VOL (ml)	424	0.58 ± 0.06	0.48 ± 0.05
CONC (x 106spz/ml)	414	278 ± 65.7	316 ± 47.4
TSE (x 106sperm)	414	155 ± 22.8	144 ± 17.0
SPERM QUALITY PARAMETERS			
MOT (%)	386	45.8 ± 6.47	50.5 ± 4.83
PROG (%)	386	25.3 ± 4.25	25.9 ± 3.13
VIAB (%)	379	72.6 ± 3.25	68.7 ± 2.40
ABN (%)	401	10.7 ± 3.27[a]	23.5 ± 2.27[b]
NAR (%)	401	88.7 ± 2.75	90.0 ± 2.04
HOST (%)	380	68.6 ± 4.55	63.4 ± 3.35
SPERM MOTILITY PARAMETERS			
VCL(µm/s)	372	104 ± 4.0	98.0 ± 2.84
VSL (µm/s)	372	38.3 ± 3.32	37.6 ± 2.31
VAP (µm/s)	372	56.6 ± 3.55	55.7 ± 2.46
LIN (%)	372	38.0 ± 2.67	38.9 ± 1.97
STR (%)	372	67.8 ± 2.15	67.1 ± 1.58
WOB (%)	372	55.0 ± 2.39	56.9 ± 1.77
ALH (µm)	372	3.1 ± 0.18	3.0 ± 0.12
BCF (Hz)	372	12.9 ± 0.74	11.1 ± 0.51

[a, b] Different superscript between rows indicate statistical differences (P<0.05).LSM ± SE: least square mean ± standard error.

VOL: Ejaculate volume; CONC: Spermatic concentration; TSE: Total sperm per ejaculate; MOT: Percentage of sperm motility; PROG: Percentage of progressive motility; VIAB: Percentage of viable sperm; ABN: Percentage of abnormal forms; NAR: percentage of normal apical ridge; HOST: Hypo-osmotic swelling test; VCL: Curvilinear velocity; VSL: straight-line velocity; VAP: average pathvelocity; LIN: linearity index; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat cross-frequency.

4.3.2. Plasma and sperm proteome

Sperm parameters of three ejaculated pools used in the proteome analysis and fertility assay are shown in Table 4.2.

Table 4.2. Seminal parameters of G21V and G39V groups used in proteome analysis and fertility assay.

TRAITS	G21V(LSM ± SE)	G39V (LSM ± SE)
MOT (%)	68.7 ± 6.69	67.3 ± 8.45
PROG (%)	49.0 ± 6.93	41.0 ± 7.93
VIAB (%)	57.3 ± 4.11	62.7 ± 6.90
ABN (%)	16.9 ± 2.34[a]	26.6 ± 1,49[b]
NAR (%)	81.7 ± 4.37	85.6.0 ± 3.58

[a,b] Different superscript between rows indicate statistical differences (P<0.05).LSM ± SE: least square mean ± standard error.
MOT: Percentage of sperm motility; PROG: Percentage of progressive motility; VIAB: Percentage of viable sperm; ABN: Percentage of abnormal forms; NAR: percentage of normal apical ridge.

For both generations, 643 plasma proteins were reported. Three hundred and ninety-seven identified proteins belonged to *Oryctolagus cuniculus* taxonomy. The results of the plasma proteome comparison between both generations (G21V and G39V) are shown in Fig. 4.2a. Discriminant Analysis (DA-PLS) classified the six sperm samples into two different main clusters corresponding to both groups analysed. The analysis showed differences of relative abundance in 64 proteins (Supplementary Table 1). Of the total, 56 proteins were overexpressed in G39V (87.5%). Hierarchical clustering and heat map of differential seminal plasma proteins are shown in Fig. 4.3a, observing two main clusters associated with the experimental groups (G21V and G39V). GO term of molecular function, biological process and cell components are shown in Fig. 4.4a, demonstrating that protein functions related to binding and catalytic activity were mainly affected (37.2 and 43.0%respectively). Biological regulation, metabolic and cellular process presented more than 54% of differential plasma proteins. KEGG pathway analysis showed glutathione metabolism affected in seminal plasma proteome (gamma-glutamylcyclotransferase, glutathione S-transferase mu 2 and glutathione S-transferase mu 3).

Sperm proteome reported 1360 proteins. DA-PLS analysis showed a clear effect of the generation (Fig. 4.2b). Results showed a total of 132 differentially abundant proteins (Supplementary Table 4.2). Of the total, 89 proteins were overexpressed in G39V

(67.4%). Hierarchical clustering of differential sperm proteins and heat map and GO annotation of molecular function, biological process and cells components are shown in Figs. 4.3b and 4.4b, respectively. Fig. 4.3b shows a hierarchical clustering with two main clusters associated with experimental groups (G21V andG39V) and Fig. 4.4b reveals that proteins related to binding and catalytic activity were mainly affected (36.8 and 37.4% respectively). Biological regulation, metabolic and cellular process presented more than 53% of differential plasma proteins (Fig. 4.4b). KEGG pathway analysis showed non-specific routes such pancreatic secretion (ATPase Na+/K+ transporting subunit beta 3 and ATPase plasma membrane Ca2+ transporting 4), Renin-angiotensin system (ATPase H+ transporting accessory protein 2 angiotensin I converting enzyme) and Proximal tubule bicarbonate reclamation (ATPase Na+/K+ transporting subunit beta 3 and carbonic anhydrase 2).

4.3.3. Fertility parameters

According to the characteristics of the ejaculates from the two experimental groups, seminal doses differed only in the abnormal sperm percentage (Table 4.2). Kindling rate, total litter size and live born were similar for both generational groups. Sixty-eight percent of inseminated does became pregnant and gave birth (kindling rate), the total litter size was 11.8 and live litter size was 10.7 (Table 4.3).

Table 4.3. Reproductive performance of inseminated does (least square mean ± standard error least).

Male group	N° Does	Kindling rate	Total litter size	Alive born
G21V	159	0.73 ± 0.036	11.3 ± 0.35	10.5 ± 0.37
G39V	152	0.69 ± 0.038	12.3 ± 0.37	10.9 ± 0.40
Total	311	0.71 ± 0.026	11.8 ± 0.25	10.7 ± 0.27

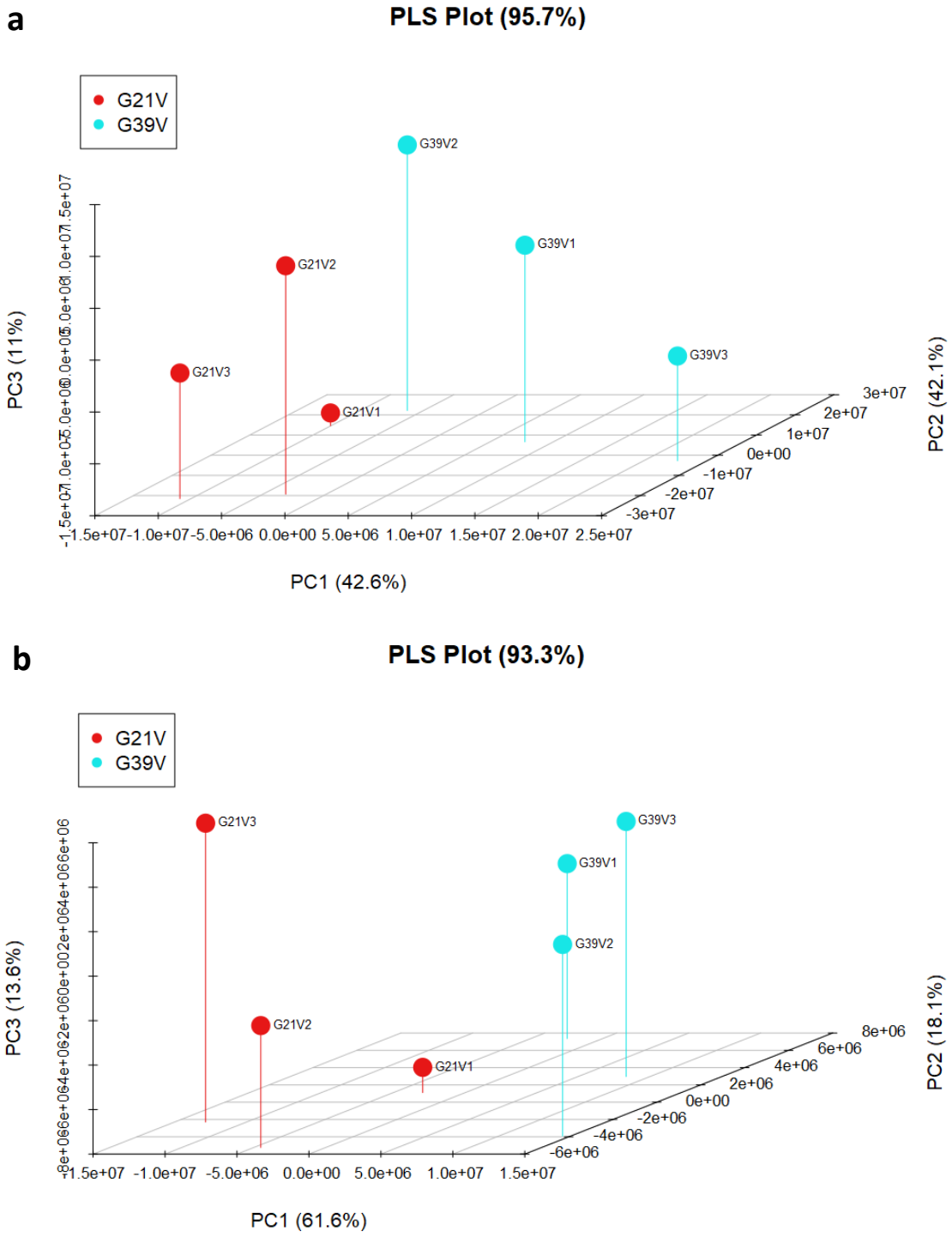
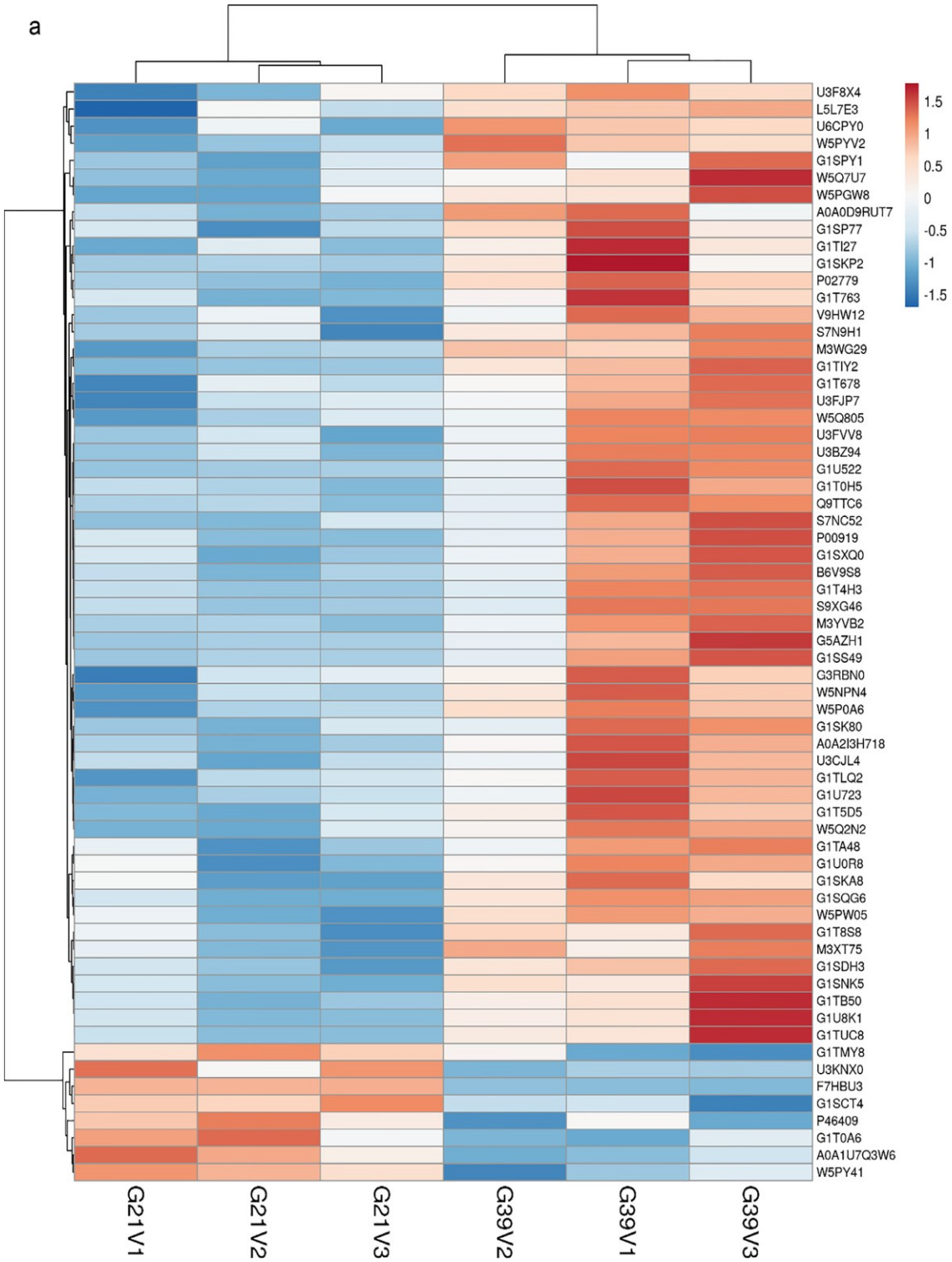


Fig. 4.2. a. Partial Least Squares Discriminant Analysis (PLS-DA) showing the classification of seminal plasma samples belonging to G21V and G39V. b. Partial Least Squares Discriminant Analysis (PLS-DA) showing the classification of sperm samples belonging to G21V and G39V.


a
U3F8X4
L5L7E3
U6CPY0
W5PYV2
G1SPY1
W5Q7U7
W5PGW8
A0A0D9RUT7
G1SP77
G1TI27
G1SKP2
P02779
G1T763
V9HW12
S7N9H1
M3WG29
G1TIY2
G1T678
U3FJP7
W5Q805
U3FVV8
U3BZ94
G1U522
G1T0H5
Q9TTC6
S7NC52
P00919
G1SXQ0
B6V9S8
G1T4H3
S9XG46
M3YVB2
G5AZH1
G1SS49
G3RBN0
W5NPN4
W5P0A6
G1SK80
A0A2I3H718
U3CJL4
G1TLQ2
G1U723
G1T5D5
W5Q2N2
G1TA48
G1U0R8
G1SKA8
G1SQG6
W5PW05
G1T8S8
M3XT75
G1SDH3
G1SNK5
G1TB50
G1U8K1
G1TUC8
G1TMY8
U3KNX0
F7HBU3
G1SCT4
P46409
G1T0A6
A0A1U7Q3W6
W5PY41
G21V1
G21V2
G21V3
G39V2
G39V1
G39V3
1.5
1
0.5
0
-0.5
-1
-1.5

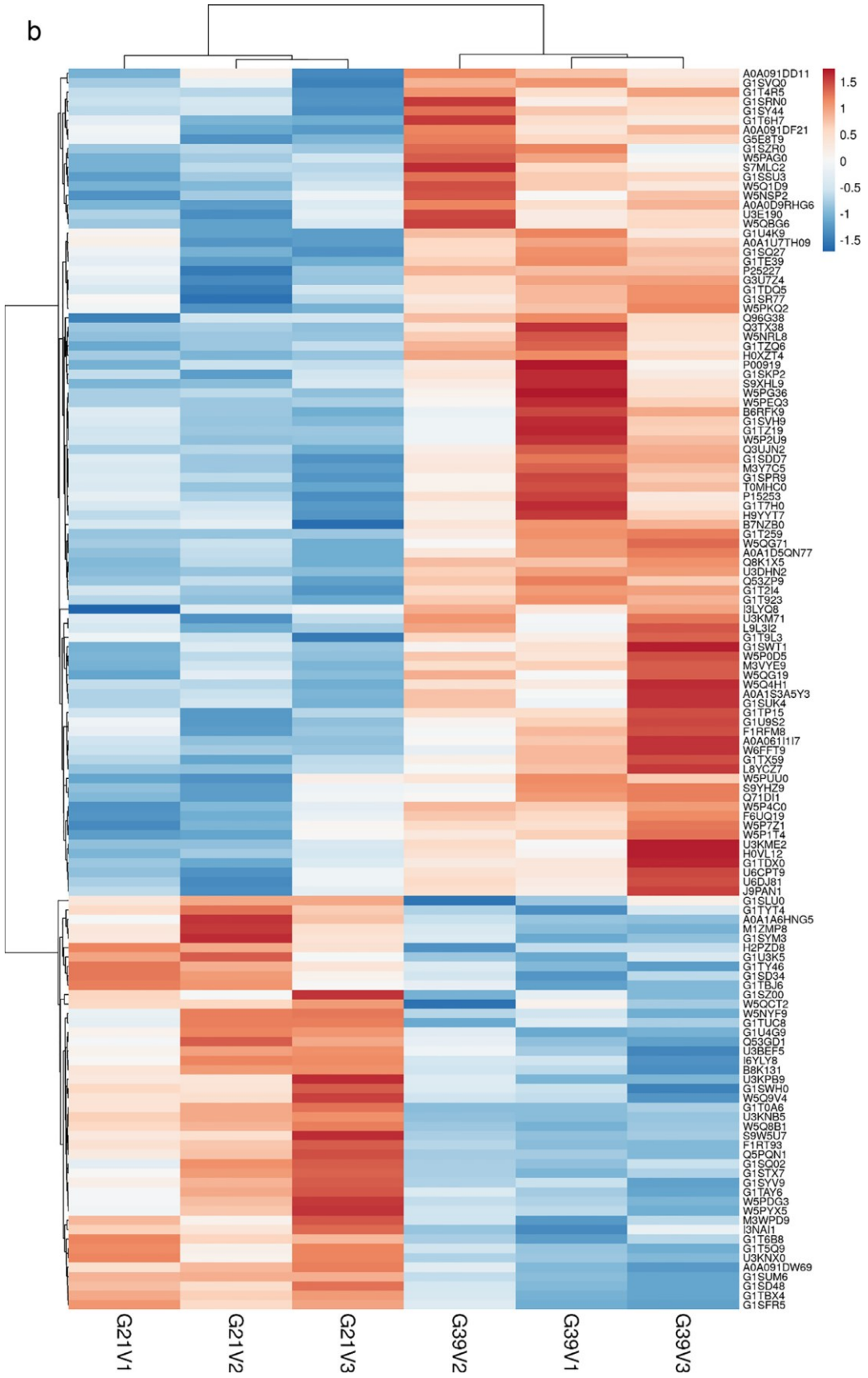
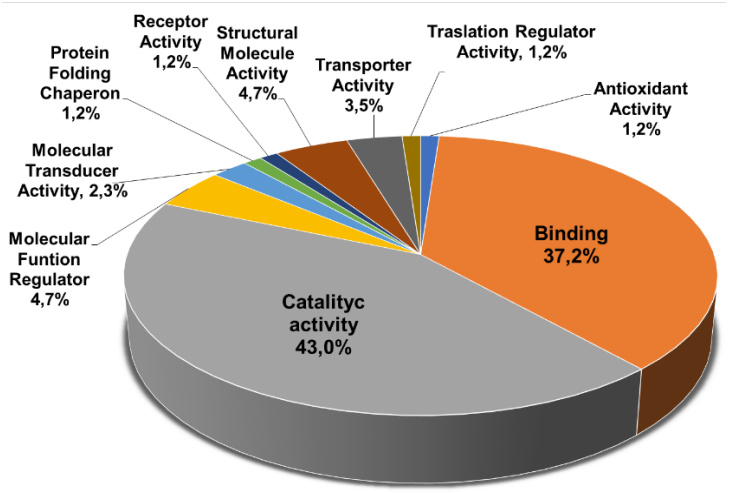


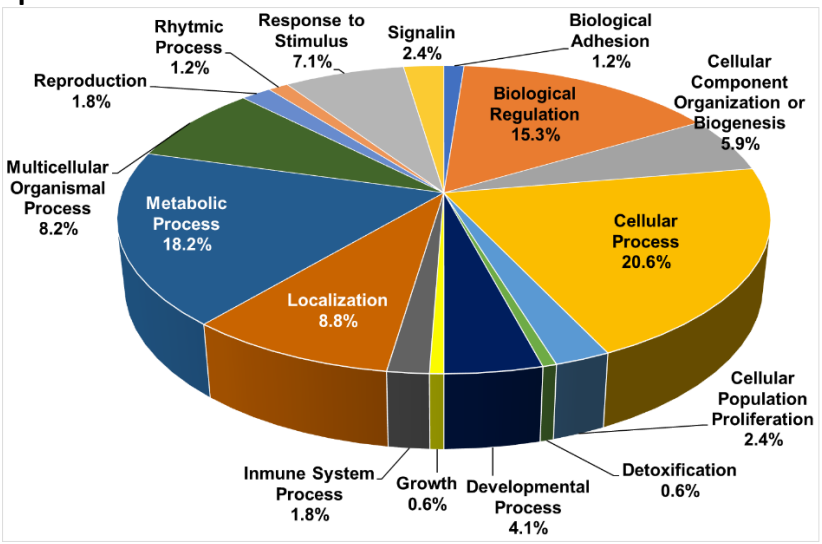
Fig. 4.3. a. Heat map representing levels of differentially expressed seminal plasma proteins between male groups (G21V and G39V). b. Heat map representing levels of differentially expressed sperm proteins between male groups (G21V and G39V).

A

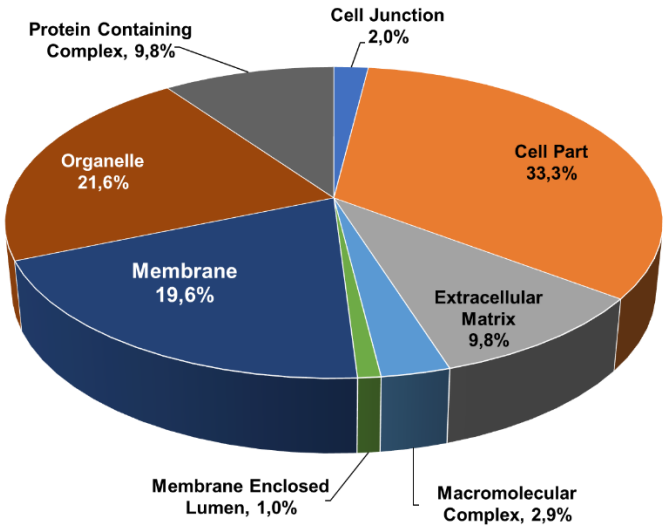
a) Molecular function



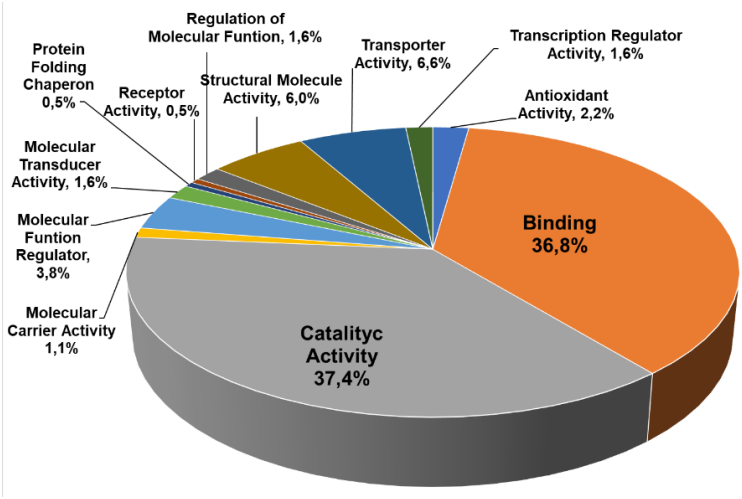
b) Biological process



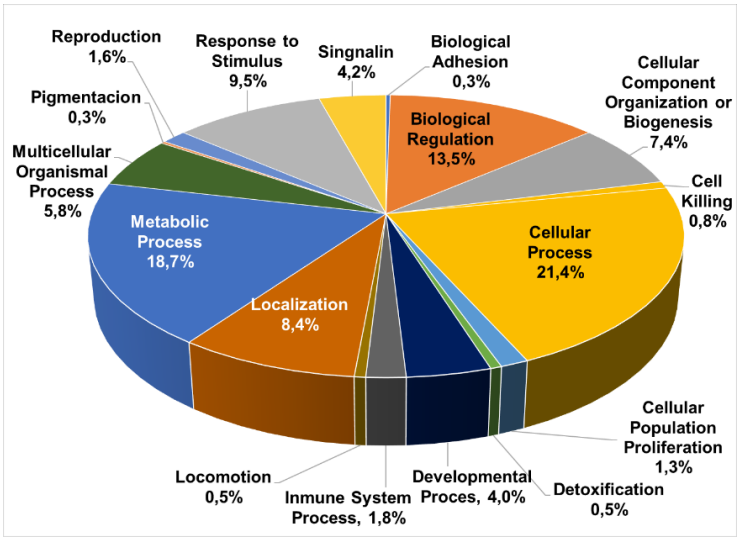
c) Cellular component



a) Molecular function



b) Biological process



c) Cellular component

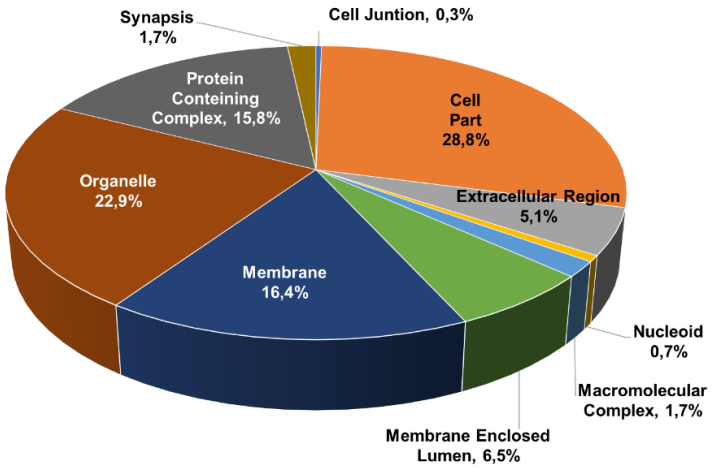


Fig. 4.4. a. Distribution of molecular function, biological process and cell components of differentially expressed seminal plasma proteins between male groups (G21Vand G39V). b. Distribution of molecular function, biological process and cell components of differentially expressed sperm proteins between male groups (G21V andG39V).

Of the 64 and 133 differentially abundant proteins of plasma and sperm, 19 and 26 had a FC>1.5, 12 and 13 of them belonging to the *Oryctolagus cuniculus* taxonomy, respectively (Table 4.4a and b). Moreover, of the total of 197 differentially abundant proteins, 10 were present in both plasma and sperm proteome and 7 of them were less abundant in G21V, highlighting proteins such as Carbonic anhydrase 2, Glutathione S-transferase or Izumo family member 4. Two of them, Chromosome 16 open reading frame 89 and uncharacterized protein(U3KNX0), were overabundant.

Table 4.4a Highlighted differentially seminal plasma proteins between male groups (G21Vand G39V) with a fold change (FC) ≥ 1.5 after log_2 transformation.

Peak name	Protein name	Fold Change	p-value
G1TIY2_RABIT	Uncharacterized protein	−3.96	0.004
G1SKP2_RABIT	Importin 5	−2.91	0.046
G1SQG6_RABIT	Serpin family A member 5	−2.35	0.004
P02779_RABIT	Uteroglobin	−2.25	0.003
G1T4H3_RABIT	Protein disulfide isomerase family A member 6	−2.15	0.049
G1SPY1_RABIT	1,4-alpha-glucan branching enzyme 1	−2.03	0.027
G1U8K1_RABIT	Serpin domain-containing protein	−1.70	0.029
P00919_RABIT	Carbonic anhydrase 2	−1.63	0.044
G1SNK5_RABIT	Uncharacterized protein	−1.55	0.019
G1TMY8_RABIT	Transmembrane serine protease 2	1.52	0.035
G1T0A6_RABIT	Chromosome 16 open reading frame89	2.21	0.031
U3KNX0_RABIT	Uncharacterized protein	2.41	0.015

Table 4.4b. Highlighted differentially sperm proteins between male groups (G21V andG39V) with a fold change (FC) ≥ 1.5 after log_2 transformation.

Peak name	Protein name	Fold Change	p-value
G1T259_RABIT	Family with sequence similarity 71member C	−3.30	0.005
G1T923_RABIT	ATPase H+ transporting accessory protein 2	−2.30	0.000
P00919_RABIT	Carbonic anhydrase 2	−2.12	0.047
G1U4K9_RABIT	Uncharacterized protein	−1.96	0.038
G1TE39_RABIT	UDP-glucose glycoprotein glucosyltransferase 2	−1.59	0.007
G1SVH9_RABIT	KIAA1324	−1.54	0.049
G1SUM6_RABIT	Uncharacterized protein	1.65	0.000
B8K131_RABIT	Zeta globin (Predicted)	1.67	0.008
G1TBJ6_RABIT	Pro-epidermal growth factor	1.88	0.030
U3KPB9_RABIT	Uncharacterized protein	1.97	0.029
U3KNX0_RABIT	Uncharacterized protein	3.26	0.009
G1T0A6_RABIT	Chromosome 16 open reading frame89	3.62	0.002
U3KNB5_RABIT	Lipocln_cytosolic_FA-bd_dom domain-containing protein	4.47	0.000

4.4. DISCUSSION

In accordance with Piles et al. (2013), selection for average daily gain does not seem to be genetically correlated with the majority of seminal traits and male fertility. Unexpectedly, the percentage of discarded animals (unable to adapt to the artificial vagina) is higher for males of the younger G21V group than for those of G39V (38.9% vs9.5%). Brun et al. (2006) did not observe differences in sexual behavior at semen collection between divergent lines selected by growth rate. Male libido measured as successful collection rate seems to be lowly heritable and more strongly affected by management practices rather than genetic selection (Tusell et al., 2012). In this study, only abnormal sperm percentage showed significant differences as a consequence of the selection for daily gain in fattening period, being 12% higher after 18 generations of selection. This result coincides with the estimated heritability of 0.19 and a positive

genetic correlation of 0.25 calculated for this trait in this paternal line by Lavara et al. (2012). Moreover, the increased percentage of abnormal sperm in this study corroborates earlier findings. Thus, Vicente et al. (2004) and Lavara et al. (2005) obtained similar percentages of abnormal sperm using males belonging to the 18th generation, while Safaa et al. (2008) and Lavara et al. (2012) after 6–7 generations (24–25th) observed increased percentages (17 to 20%) more closely to those of the current generation. No differences were observed in other sperm parameters after 18 generations, perhaps because parameters such as concentration, volume, and sperm production have a positive but low heritability, while motility parameters showed low heritability and an uncorrelated response to selection (Lavara et al., 2011; Tusell et al., 2012).

Abnormal sperm might be associated with worsening of the spermatogenesis process and linked to the poor reproductive performance of does. High failures in ovulation frequency and gestational losses related with deficient LH, 17β-estradiol and progesterone production and alterations in the insulin growth factor system were observed in females from this paternal line (Llobat et al., 2012; Vicente et al., 2012; Naturil-Alfonso et al., 2016).

Seminal plasma and sperm proteome showed that the generational step increased the abundance of most of the differentially expressed proteins (87.5% and 66.9%, respectively). Moreover, highlighting proteins differentially expressed with FC>1.5 and focusing on Oryctolagus cuniculus taxonomy, fourteen critical proteins related with the sperm functions were affected by selection for growth rate (9 and 5 from plasma and sperm proteome, respectively). Sperm leave the testes morphologically defined but lacking motility, as well as crucial proteins involved in oocyte binding and fertilization. Key proteins related to motility or later to interaction with the egg surface are added to the sperm membrane in the epididymis and, moreover, at the time of ejaculation, seminal plasma proteins from accessory glands coat the sperm surface and stabilize the membrane, inhibiting fertilization ability (Gervasi and Visconti, 2017). The G21V group showed overabundant proteins, such as transmembrane serine protease 2 (TMPRSS2), chromosome 16 open reading frame 89 and uncharacterized protein (U3KNX0). The first

of them has been found in human seminal prostasomes (Kim et al., 2006; Antalis et al., 2011), and rabbit seminal plasma is also rich in seminal vesicles produced and secreted by the prostate to prevent sperm capacitation (Davis et al., 1983; Castellini et al., 2006 and 2012). The function of the second is unknown, but a predicted functional partner is Ropporin-1A, a pKA-dependent signaling protein involved in sperm motility and prominent in capacitated spermatozoa (Rahman et al., 2017), and the third has high homology with WGA16, a prostate-derived seminal plasma glycoprotein that is deposited on the sperm surface at the moment of ejaculation to prevent premature capacitation (Garénaux et al., 2015; Pérez-Patiño et al., 2018). In contrast, another 6 seminal sperm proteins involved in immunoprotection (G1TIY2 and uteroglobin), capacitation (serpin family, uteroglobin, importin 5, carbonic anhydrase II) and membrane fusion (protein disulfide isomerase family A member 6) and consequently in fertilization process were less abundant. It has been suggested that G1TIY2, an IgG that was detected in lumen of epididymis, accessory glands and spermatozoa in rabbit (Weininger et al., 1982), would play a role of immunoprotection in fertilization (Yan et al., 2016). Uteroglobin is related to suppression of sperm antigenicity and capacitation-inhibiting activity (Luconi et al., 2000). Serpin family proteins are inhibitors of several serine proteases which would reinforce the effect of TMPRSS2 overexpression (Law et al., 2006). Importins, originally characterized for their central role in protein transport through the nuclear pores, contribute to the formation of subcellular domains in sperm during the maturation as acrosome (Loveland et al., 2015). Protein disulfide isomerase family A member 6 are involved in the activation of membrane fusion and required fertilization process (Ellerman et al., 2006). Carbonic anhydrase II regulates HCO_3^- homeostasis in sperm and the composition in genital tract fluids, affecting sperm motility and capacitation for what is required for normal fertilization (Liao et al., 2009; Wandernoth et al., 2015).

Sperm proteome showed several remarkable proteins whose abundance has been modified and they are mainly related with sperm maturation, morphology and motility. Among the overabundant ones, in addition to chromosome 16 open reading frame 89 and U3KNX0-RABIT in G21V already found in seminal plasma proteome, we observed a protein from the lipocalin family strongly expressed (U3KNB5_RABIT) and involved in

sperm maturation. This protein family is a carrier of small hydrophobic ligands (fatty acids, steroids, thyroid hormones, retinoids, etc.), and several members were reported to be associated with poor semen parameters such as decreased sperm count, percentage of motility, and percentage of normal morphology when their level is diminished (Gerena et al., 1998; Leone et al., 2002; Samanta et al., 2018). Finally, in this overabundant group, Zeta globin was correlated with the percentage of cells with both membrane and acrosome damaged in rabbit sperm (Arruda-Alencar et al., 2012). Among the least abundant proteins in G21V male group are carbonic anhydrase, already observed in the plasma proteome Fam71f1 (Family with sequence similarity 71 member C). This protein family has been identified in sperm nucleus and tail (Kwon et al., 2017; Ma et al., 2017) and has predicted functional partners related with spermatogenesis and motility. ATPase H+ transporting accessory protein 2 is an ATP-dependent proton pump that acidifies intracellular compartments and is negatively correlated with asthenozoospermia, in a similar way to carbonic anhydrase (Peralta-Arias et al., 2015; Lestari et al., 2017). This last protein was identified with ATPase plasma membrane Ca2+ transporting 4 and carbonic anhydrase in the KEGG pathways renin-angiotensin system and proximal tubule bicarbonate reclamation, respectively. These ATPases and carbonic anhydrase play a main role in hyperactivity, capacitation and acrosome reaction by regulating intracellular pH, membrane potential and intracellular calcium release (Freitas et al., 2017; Thundathil et al., 2018). However, no effect has been observed between the groups of males on total motility or on the speed and trajectory parameters in the present study. UDP-glucose glycoprotein glucosyltransferases (UGGT 1 and 2) are central components of the endoplasmic reticulum glycoprotein-folding quality control system. UGGT expression is increased through stress and has been observed to be predominantly up-regulated in the semen of infertile men (Cadavid et al., 2014).

Finally, overabundance of KIAA1324 protein has been observed in teratozoospermic human (Choucair, 2018), and, accordingly, this protein was less abundant in semen samples of G21V and overabundant in the G39V male group and could contribute together with the lipocalin family protein to high levels of abnormal spermatozoa observed in males from this latter male group. Despite the changes produced by growth rate in abnormal sperm or ejaculate proteome, no differences were observed in

pregnancy and prolificacy rates when seminal doses of both experimental groups were used to inseminated crossbred females. It seems that the 14 proteins related with capacitation and fertilization function affected by selection for growth rate had no impact on the results of insemination. The commercial seminal dose used (20 million spermatozoa/ml) would compensate for the potential deleterious effects of these differences. Viudes de Castro and Vicente (1997) showed that commercial seminal doses of about 4 sperm millions are enough to obtain normal pregnancy and prolificacy rates. How the changes produced by growth selection in the ejaculate proteome can alter the fertilizing capacity of the semen at a level that is appreciable by the rabbit farmers and insemination centres is difficult to assess in a species in which the seminal doses are heterospermic and the amount of sperm per dose is at least 5 times higher than necessary to guarantee the fertility and prolificacy of rabbits. A more restrictive assay with individual males and low sperm doses might define the importance of modifications introduced by genetic selection. In conclusion, our study reveals how the effect of selection schemes for daily average gain in a paternal line (R line) increases over ab-normal sperm and alters seminal plasma and sperm proteome. Some proteomic changes may be related to the increasing abnormal sperm rate observed, but no effects on fertility and prolificacy were observed after insemination with commercial semen doses.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.livsci.2019.103894.

4.5. REFERENCES

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CHAPTER III

REDERIVATION BY CRYOPRESERVATION OF A PATERNAL LINE OF RABBITS SUGGESTS EXHAUSTION OF SELECTION FOR POST-WEANING DAILY WEIGHT GAIN AFTER 37 GENERATIONS

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ABSTRACT

Rabbit selection programmes have mainly been evaluated using unselected or divergently selected populations, or populations rederived from cryopreserved embryos after a reduced number of generations. Nevertheless, unselected and divergent populations do not avoid genetic drift, while rederived animals seem to influence phenotypic traits such as birth and adult weights or prolificacy. The study aimed to evaluate the effect of a long-term selection for post-weaning average daily weight gain (ADG) over 37 generations with two rederived populations. Specifically, two coetaneous populations were derived from vitrified embryos with 18 generational intervals (R19 andR37), reducing or avoiding genetic drift and environmental and cryopreservation effects. After two generations of both rederived populations (R21 vs. R39 generations), all evaluated traits showed some progress as a result of the selection, the response being 0.113 g/day by generation. This response does not seem to affect the estimated Gompertz growth curve parameters in terms of the day, the weight at the inflexion point or the adult weight. Moreover, a sexual dimorphism favouring females was observed in this paternal line. Results demonstrated that the selection programme had improved ADG without variations in adult body weight but, after 37 generations of selection, this trait seems exhausted. Given the reduction in the cumulative reproductive performance and as a consequence in the selection pressure, or possibly/perhaps due to an unexpected effect, rederivation could be the cause of this weak selection response observed from generation 18 onwards.

Keywords: Selection programme; embryo vitrification; Gompertz growth curve; biobanking; reproductive performance

5.1. INTRODUCTION

Traits related to prolificacy for maternal lines, and feed conversion rate and carcass and meat quality for paternal lines, are commonly used in selection programmes in rabbits (Estany et al., 1989; Lukefahr et al., 1996; Baselga 2004; Nagy et al., 2006; Khalil and Al-Saef, 2008; Martinez et al., 2016; Blasco et al., 2018; de Rochambeau et al., 1989). Some of the mare difficult or expensive to measure (for instance, feed conversion rate trait), so correlated traits such as growth rate have been successfully used in selection (Lukefahr et al., 1996; Blasco et al., 2018; Mgheni and Christensen 1985; Torres et al., 1992; Estany et al., 1992; Moura et al., 1997; Blasco et al., 2003; Piles et al., 2004). Accurately, post-weaning daily weight gain has been estimated from 0.45 to 1.73 g/day (18 to 68 g) per generation in rabbit (Lukefahr et al., 1996; de Rochambeau et al., 1989; Estany et al., 1992; Moura et al., 1997; Drouilhet et al., 2013; Garreau et al., 2000; Larzul et al., 2005; Piles and Blasco, 2003). Body weight at slaughter time ranged in heritability from 0.12 to 0.67 as a consequence of environmental variability, the improvement of facilities, nutrition and feed systems, making it difficult to assess during the selection programme (Blasco et al., 2018). The success of rabbit selection programmes has been evaluated using a control population, in some cases from rederived cryopreserved embryos or by divergent selection (Blasco et al., 2003; Larzul et al., 2005; Piles and Blasco, 2003; Gondret et al., 2002; García and Baselga, 2002a; Piles et al., 2017). Nevertheless, unselected control or divergent populations do not avoid genetic drift, while rederivation of animals by cryopreservation seems to affect phenotypic traits such as birth and adult weights or prolificacy, not only in animals born after transfer, but also their offspring (Lavara et al., 2014; Lavara et al., 2015). Whether the phenotypic changes are only intragenerational, as a consequence of the embryonic stress response to cryopreservation and transfer, or transgenerational, as a consequence of heritable changes introduced at the epigenome level, is yet to be assessed (García-Dominguez et al 2020a and b). However, cryopreservation offers the great advantage of being able to measure the genetic progress through generations, making evaluation possible in the same environment, facilities and feed diets of individuals separated by many generations (Marco-Jiménez et al., 2018). A control population obtained by cryopreservation has rarely been used in selection experiments. In rabbits, it has been

used to evaluate both the response to selection for litter size in maternal lines and for average daily weight gain in paternal lines (Piles and Blasco, 2003; García and Baselga, 2002b. García and Baselga (2002b) observed that the estimated responses obtained when using a rederived population of cryopreserved embryos or a mixed model that took into account the kinship matrix (genetic relationship) were different, suggesting that this latter model is less appropriate. Piles and Blasco (2003) demonstrated that the selection for average daily gain between the 3rd–4th and 10th generations was successful and the response obtained was similar, using a rederived control population or a model with genetic relationships (0.62 to 0.65 g/day by generation). The latter study was performed with the paternal line used in this work. This study aimed to evaluate the effect of a long-term selection for average daily gain (37 generations) on commercial growth traits and Gompertz parameters, using two population rederived from vitrified embryos with18 generational intervals to reduce or avoid genetic drift, environmental and cryopreservation effects.

5.2. MATERIALS AND METHODS

All the experimental procedures used in this study were performed following Directive 2010/63/EU EEC for animal experiments and reviewed and approved by the Ethical Committee for Experimentation with Animals of the Universitat Politeècnica de València, Spain (research code: 2015/VSC/PEA/00061).

5.2.1. Animals.

A rabbit paternal line (R) selected at the Universitat Politècnica de València was used. This line was founded in 1989 from two closed paternal lines selected according to individual weight gain from weaning to end of fattening period (77 days old) during 12 and 9 generations (Estany et al., 1992). Since then, the line has been selected for individual daily weight gain from 28 days (weaning) to 63 days of age (end of fattening phase). Animals from two different generations of selection were used. R19V population was rederived from 256 embryos of 25 donors belonging to ten different sire families of 18th generation vitrified in 2000. R37V population was rederived from 301 embryos of

28 donors belonging to 15 different sire families of 36th generation and they were vitrified in 2015. Both animal groups were transferred at the same time in 2015 (see details in Marco-Jiménez et al., 2018). Offspring were bred in non-overlapping generations during 2 generations (R20–R21 and R38–R39 from R19V and R37V, respectively). Figure 5.1 and Table 5.1 show the experimental design and the parents used to generate the offspring analysed in this study.

The animals were housed at the Universitat Politècnica de València experimental farm in flat deck indoor cages (75 x 50 x 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 16 h of light and 8 h of dark, with a regulated roomtemperature between 14◦C and 28◦C.

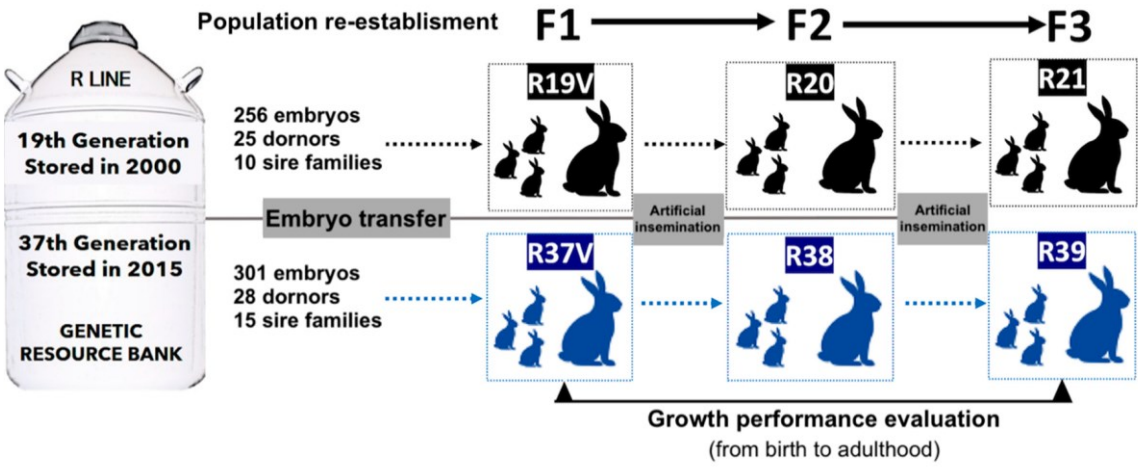

Figure 5.1. Experimental design: Two experimental progenies were developed from vitrified embryos stored in 2000 (19th generation of selection) and 2015 (37th generation of selection). All rabbits were identified and weighted at weaning and end of the fattening period to calculate the average daily gain. A sample of males and females were weighted weekly from birth to 20 weeks age in F1 and F3 generation to estimate Gompertz curve parameters.

5.2.2 Embryo vitrification and transfer to population rederivation

Five-hundred-and-fifty-seven embryos were used from donors belonging to R18 and R36 generations. Vitrification and transfer were described elsewhere (Marco-Jiménez et al., 2018; Vicente et al., 1999; García-Dominguez et al., 2019). Briefly, the vitrification was

carried out in two steps at room temperature (approximately 20–22◦C). In the first step, embryos from each donor doe were placed for 2 min in an equilibrium solution consisting of 12.5% dimethylsulfoxide (DMSO) and 12.5% of ethylene glycol (EG) in Dulbecco's phosphate-buffered serum (DPBS)supplemented with 0.1% (w/v) of bovine serum albumin (BSA). In the second step, embryos were suspended for 1 min in the vitrification solution containing 20% DMSO and 20% EG in DPBS supplemented with 0.1% of BSA. Then, embryos suspended in vitrification medium were loaded into 0.125 mL plastic straws (ministraws, L'Aigle, France) and plunged directly into liquid nitrogen. After storage in liquid nitrogen, embryos were warmed and vitrification solution was removed, loading the embryos into a solution containing DPBS and 0.33 M sucrose for 5 min, followed by one bath in a solution of DPBS for another 5 min before transfer. Immediately after warming, the embryos were evaluated morphologically, and only embryos without damage in mucin coat or pellucid zone were transferred by laparoscopy into the oviduct of synchronized recipient females from a maternal line (Line A, García-Domínguez et al., 2019) following the procedure described by Besenfelder and Brem (1993) and García-Domínguez et al. (2019). The re-establishing of both populations to generate the 19th (R19V) and37th (R37V) generation was described by Marco-Jiménez et al. (2018).

5.2.3. Reproduction management in rederived and filial generations

Rederived rabbits were conducted in non-overlapping generations and the generation interval was approximately 9–10 months. The first reproductive cycle took place at ~5 months of age, and after kindling the new insemination was tried 10–12 days later. Insemination between relatives sharing a grandparent was avoided. Briefly, two ejaculates per male were collected in each replica using an artificial vagina. Ejaculates were diluted 1:5 with Tris-Citrate-Glucose extender and both motility and abnormal spermatozoa were assessed under phase optic at 200×. Only ejaculates with total motility higher than 70% and less than 30% of abnormal sperm were used. After semen evaluation, optimal ejaculates from each male were pooled and extended to 40 million/mL. All females were synchronized with 15UI eCG injected intramuscularly 48h before being inseminated with 0.5 mL of extended semen using a plastic curved pipette.

Females were induced to ovulate by intramuscular injection of 1µgof buserelin acetate at insemination time. Pregnancy was checked at 14 days from insemination and non-pregnant does were inseminated again at 21 days after the previous insemination. In addition, it was noted whether rabbits underwent a lactation–gestation overlap, classifying the reproductive status of their dams in four levels: offspring from primiparous does without overlapping (PD), offspring from primiparous lactating does (females that were pregnant while suckling their first litter, PLD), multiparous lactating does (females with more than one birth that were pregnant while suckling their litter, MLD) and multiparous non-lactating does (females from more than one parturition that were pregnant after lactation, MD).

5.2.4. Growing traits analysis

The offspring born of vitrified embryos were named R19V and R37V, the first filial generation (R20and R38) and the second filial generation (R21 and R39). Individual weaning weight (WW, 30 days old), individual weight at end of the fattening period (EFW, 63 days old) and average daily weight gain (weight gained from day 30 to 63 divided by 33, ADG) during the fattening period were noted for all generations. To determine differences in growth curve, 76 and 113 rabbits from the rederived population (18 and 21 females and 16 and 21 males from R19V and R37V, respectively) and second filial generation (29 and 39 females and 18 and 27 males from R21 and R39, respectively) were identified at birth with chip and weighed weekly from born to 20 weeks old.

5.2.5. Statistical analyses

A first analysis for growth traits was performed, attending to the generation interval of rederived population,

$$Y_{ijklm} = \mu + P_i + R_j + MY_k + PR_{ij} + CO_l + Cov\ X_m + e_{ijklm} \qquad (1)$$

where Y_{ijklm} was the trait to analyse, μ was the general mean, P_i was the fixed effect of selection generation of rederived population R19 (R19V, R20, R21) and R37 (R37V, R38, R39), R_j was the fixed effect of reproductive status of the doe used in the WW analysis (PD, PLD, MLD and MD), MY_k was the fixed effect of month-year in which the fattening period ended (39 levels), PR_{ij} was the effect of interaction between rederived population and reproductive status of the mothers used for WW analysis, CO_l was the random effect of common litter, Cov X_m was the covariate of the number born alive (BA) used for the WW trait or the covariate of WW used for weight at the end of the fattening period (EFW) and ADG traits and e_{ijklm} was the error term of the model.

A second analysis of growth traits to evaluate each filial generation (F1-R19V vs. R37V, F2-R20 vs. R38- and F3-R21 vs. R39) was performed using the mixed linear model described above:

$$Y_{ijklm} = \mu + P_i + R_j + MY_k + PR_{ij} + CO_l + Cov\ X_m + e_{ijklm} \quad (2)$$

where P_i was the fixed effect of filial generation (R19V and R37V or R20 and R38 or R21 and R38). In the WW analysis of the first filial generation, the effect of reproduction status of the mothers (R_j) and the interaction (PR_{ij}) were not included, all hosted females were multiparous non-lactating (MD) and the fixed effect month-year had 4 levels. In the analysis of filial generation 2, the fixed effect month-year (MY_k) had 19 levels. For filial generation 3, MY_k fixed effect had 25 levels.

Finally, Gompertz curve parameters were estimated for each rabbit from R19V and R37V, and R21and R39 for each rabbit:

$$X_{jm} = a_m \exp\left[-b_m \exp(-k_m t)\right] + e_{jm} \quad (3)$$

where X_{jm} is the body weight (BW) of m animal at t age (days); am, bm and km are the Gompertz growth curve parameters of m animal; and e_{jm} is the residual. As to the meaning of parameters, a can be interpreted as the mature BW maintained independently of short-term fluctuations, b is a timescale parameter related to the initial BW and k is a parameter related to the rate of maturing. The inflexion time (tI) is

the moment when growth rate is maximum and is determined by $t_I = (\log e^b)/k$ and inflexion weight (w_I) were fixed at 0.37 of adult weight (a) by Gompertz curves. A mixed linear model was fitted for the analysis of Gompertz growth curve parameters as follows:

$$Y_{ijkl} = \mu + P_i + S_j + PS_{ij} + CO_k + CovB_l + e_{ijkl} \quad (4)$$

where Y_{ijkl} was the Gompertz parameter, P_i is the fixed-effect population (R19V and R37V or R21V and R39V), S_j the fixed effect of the sex, PS_{ij} was the effect of interaction between population and sex, CO_k is the random effect of common litter, $CovB_l$ was the weight at birth as a covariate and e_{ijkl} was the error term of the model.

5.3. RESULTS

5.3.1. Descriptive growing Traits

A total of 2025 (2991 liveborn) animals from the three filial generations that finished fattening were obtained from 437 parturitions (27, 134 and 276 parturitions in the first, second and third generations respectively, Table 5.1).

Table 5.1. The total number of parents used to generate the offspring analysed.

Rederived Population	Filial Generation	Generation	Females	Males	Total
19th	F1	R19V	14	11	25
	F2	R20	22	7	29
	F3	R21	50	16	66
		Total	86	34	120
37th	F1	R37V	11	10	21
	F2	R38	26	13	39
	F3	R39	78	18	96
		Total	122	41	163

V: Rederived from vitrified embryos.

Average body weight at the end of fattening was 2.240 kg (EFW, 63 days old) and ADG was43.95 g/day (Table 5.2).

Table 5.2. Number of young rabbits weighted from weaning to end fattening period and average and standard deviation to birth alive (BA), weaning weight (WW), weight at end fattening (EFW) and average daily weight gain (ADG).

Trait	Generation						
	R19V	**R37V**	**R20**	**R38**	**R21**	**R39**	**Total**
BA	3.4 ± 1.40	5.8 ± 3.30	6.7 ± 2.75	6.6 ± 2.95	6.8 ± 2.41	7.4 ± 3.16	6.8 ± 2.94
WW(Kg)	0.75 ± 0.236	0.64 ± 0.139	0.72 ± 0.162	0.72 ± 0.171	0.69 ± 0.165	0.71 ± 0.173	0.71 ± 0.171
EFW(Kg)	2.20 ± 0.302	2.17 ± 0.249	2.24 ± 0.279	2.33 ± 0.309	2.17 ± 0.323	2.25 ± 0.314	2.24 ± 0.313
ADG(g/day)	43.8 ± 5.72	46.3 ± 5.57	44.2 ± 5.98	46.9 ± 6.81	42.0 ± 7.08	43.6 ± 6.37	44.0 ± 6.72
Animals	42	52	285	347	489	810	2025

V: Rederived from vitrified embryos.

Mortality by rederived generations (R19V-R20-R21 vs. R37V-R38-R39) was 17.5% and 20.5% in the lactation period and 15.2% and 16.6% in the fattening period, respectively. For each filial generation, the mortality at the end of the lactation and fattening period reached levels of 3.3% and 19.6% for F1,21.3 and 10.2 for F2 and 19.3% and 18.4% for F3, respectively. Data not shown in tables.

5.3.2. Growing traits by generation interval of rederived populations. Selection effect

Selection had a significant effect on studied traits and the estimated effects on WW, EFW and ADG were 0.031±0.014 kg, 0.058±0.013 kg and 1.55±0.392 g/day (R37-R19, $p<0.05$, Table 5.3). Moreover, reproductive status of does affected the WW, observing that the young mothers without lactation-gestation overlap (ND) had the lowest WW (0.632±0.016 vs. 0.734±0.019, 0.746±0.015and 0.718±0.016 kg to PD, PLD, MLD and MD, respectively, $p<0.05$, data not shown in tables). No interaction between selection generation of rederived population and reproductive status was found.MY fixed and common litter random effects were significant for all analyses (data not shown in tables). As expected, the warm months of July, August and September from each year had adverse effects on the traits studied.

Table 5.3. Effect of selection for growth rate on weaning weight (WW, kg), weight at end of fattening period (EFW, kg) and average daily weight gain (ADG, g/day) from two rederived population after 18 generations.

Rederived Population	n	WW (kg)	EFW (kg)	ADG (g/day)
19th (R19V+R20+R21)	816	0.692±0.014^{b}	2.219±0.013^{b}	43.85±0.375^{b}
37th (R37V+R38+R38)	1209	0.723±0.012^{a}	2.277±0.011^{a}	45.41±0.324^{a}
Covariate effect		−0.028±0.0024***	1.35±0.029***	10.58±0.847***

n: number of young rabbits. V: Rederived from vitrified embryos. Data are expressed as least squared mean ± standard error of means. ^{a,b} Values with different superscripts in column differ significantly ($p<0.05$). Significance of estimated value of covariates (***$p<0.001$).

5.3.3. Evolution of growth traits by filial generation.

Weaning weight (WW) was not different in the first two filial generations (F1 and F2), and it was different and favourable to R39 at third filial generation (0.053±0.022 and 0.066±0.019, respectively, p<0.05). On the contrary, the estimated effects on weight at the end of fattening (EFW) and ADG were always significant and favourable in each comparison to the last generations (Table 5.4).

Table 5.4. Differences of least-square means of weaning weight (WW, Kg), weight at end of fattening period (EFW, kg) and average daily weight gain (ADG, g/day) from filial generations. V: Rederived from vitrified embryos.

Trait	Generation		
	F1 (R37V-R19V)	F2 (R38-R20)	F3 (R39-R21)
WW (Kg)	0.043 ± 0.066	−0.040 ± 0.025	0.066 ± 0.019**
EFW (Kg)	0.135 ± 0.056*	0.062 ± 0.020**	0.056 ± 0.018**
ADG (g/day)	4.270 ± 1.578*	1.658 ± 0.606**	1.468 ± 0.544**
Covariate effect			
BA(WW)	−0.028 ± 0.0122*	−0.030 ± 0.0038***	−0.027 ± 0.0030***
WW(EFW)	1.20 ± 0.120***	1.33 ± 0.046***	1.36 ± 0.039***
WW(DG)	5.60 ± 3.581	10.4 ± 1.35***	11.0 ± 1.13 ***

Significance of estimated effects and covariates (*p<0.05, **p<0.01, ***p<0.001).

Reproductive status of does in F2 and F3 affected the WW. It was observed that the young mothers without lactation–gestation overlap had the lowest WW. No interaction between selection generation of rederived population and reproductive status was found. MY fixed were significant for all analyses, while common litter effect was not significant for EFW and ADG in F1 (R37V-R19V).

5.3.4. Estimated growth curves: F1 rederived populations (R19V and R37V)

No differences in Gompertz parameters were found for R19V and R37V population and sex (Table 5.5).

Table 5.5. Gompertz curve parameters from R19V and R37V generations.

Group	Gompertz Parameters				
	a	b	k	t (days)	W (g)
Rederived Population					
R19V	4906 ± 133	4.62 ± 0.269	0.026 ± 0.001	60.6 ± 2.33	1815 ± 49
R37V	4905 ± 128	5.08 ± 0.288	0.026 ± 0.001	62.5 ± 2.46	1815 ± 47
Sex					
Female (F)	4939 ± 113	4.75 ± 0.211	0.025 ± 0.001	62.1 ± 1.84	1828 ± 42
Male (M)	4872 ± 114	4.93 ± 0.210	0.026 ± 0.001	61.0 ± 1.84	1803 ± 42
Rederived Population x Sex					
R19V x F	4887 ± 170	4.64 ± 0.297	0.030 ± 0.002	60.6 ± 2.63	1808 ± 63
R19V x M	4925 ± 168	4.57 ± 0.292	0.030 ± 0.002	60.6 ± 2.58	1822 ± 62
R37V x F	4992 ± 159	4.85 ± 0.306	0.030 ± 0.002	63.6 ± 2.66	1847 ± 59
R37V x M	4818 ± 155	5.29 ± 0.303	0.030 ± 0.002	61.4 ± 2.63	1783 ± 57

V: Rederived from vitrified embryos. [a,b]: Different superscript between columns indicate statistical differences ($p<0.05$). a: mature body weight; b: a timescale parameter related to the initial body weight; k: growth velocity; t: Inflexion age; w: weight at inflexion. LSM ± SEM: least square mean ± standard error of means.

Estimated growth patterns showed that both population and sex reached a maximum growth rate between 59 and 61 days old and with a weight about 1800 g. In addition, the estimated adult weights were between 4800 and 5000 g (Figure 5.2).

5.3.5. Estimated Growth Curves: F3 Rederived Population (R21 and R39)

Gompertz parameters for R21 and R39 populations suggest no relevant differences between both populations (Table 5.6, Figure 5.3A). However, Gompertz parameters showed sexually dimorphic growth. Thus, males reached the inflexion point sooner (~3 days) and had a lower adult weight (Table 5.6, Figure 5.3B). Moreover, an interaction

between population and sex was observed, suggesting a significant dimorphic pattern in R21 population as a consequence of a minor growth rate of R21females (Figure 5.4).

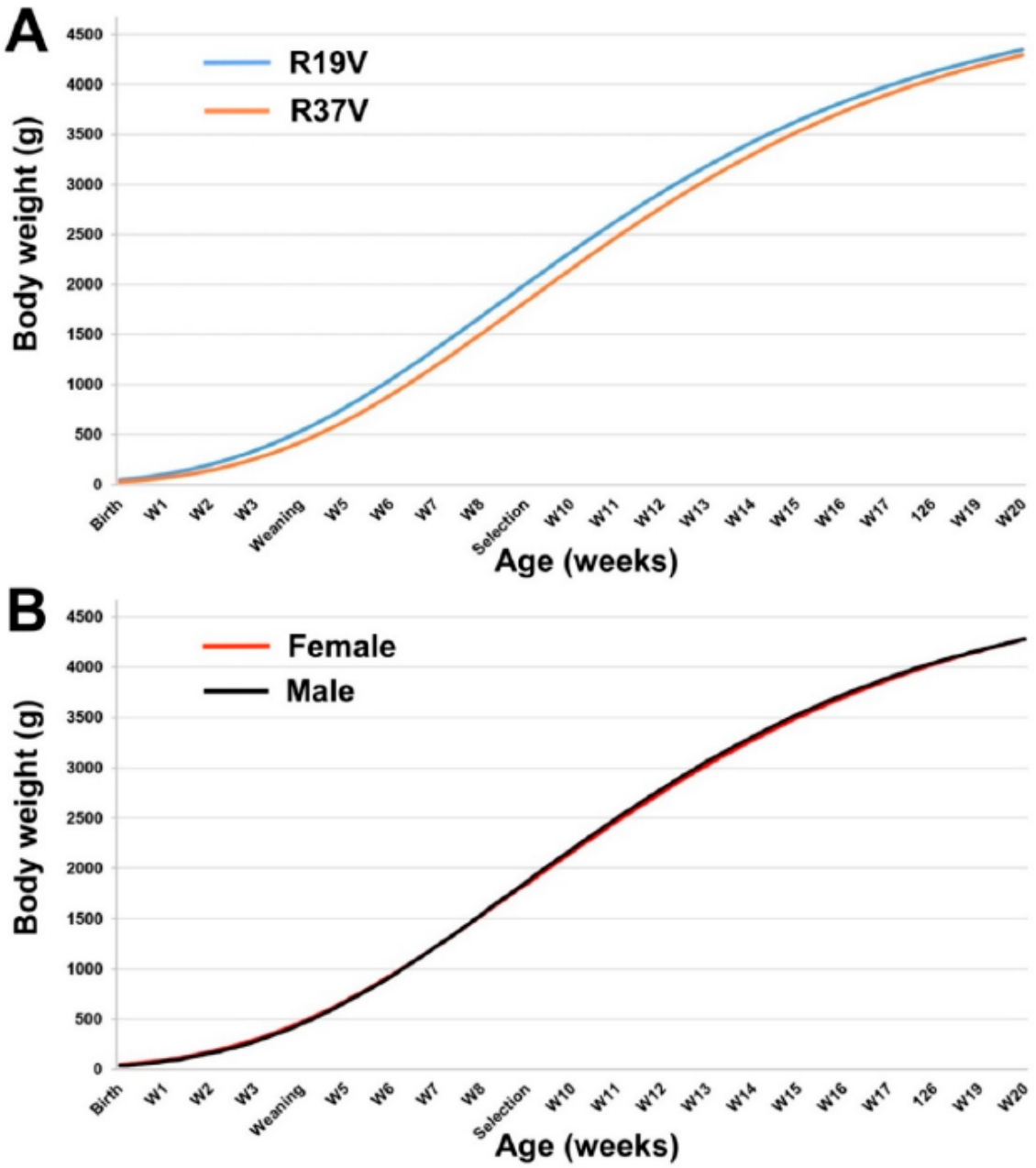

Figure 5.2. Growth curves from birth to 20-week-old between F1 rederived populations from R19V and R37V generations. V: Rederived from vitrified embryos. Growth curves were fitted using the Gompertz equation for (A) R19V and R37V generations and (B) sex.

Table 5.6. Gompertz curve parameters from R21 and R39 generations.

Group	Gompertz Parameters				
	a	**b**	**k**	**t (days)**	**W (g)**
Population					
R21	5518 ± 154	4.77 ± 0.095[a]	0.026 ± 0.001	60.1 ± 1.22	2042 ± 56.9
R39	5400 ± 135	4.49 ± 0.083[b]	0.027 ± 0.001	56.9 ± 1.07	1998 ± 50.1
Sex					
Female (F)	5815 ± 113[a]	4.63 ± 0.070	0.026 ± 0.000a	60.1 ± 0.89[a]	2151 ± 41.7[a]
Male (M)	5102 ± 132b	4.64 ± 0.082	0.027 ± 0.001b	56.9 ± 1.05[b]	1888 ± 48.8[b]
Rederived Population _ Sex					
R21 x F	5925 ± 172	4.68 ± 0.107	0.025 ± 0.001[a]	62.7 ± 1.37	2192 ± 63.6
R21 x M	5110 ± 195	4.86 ± 0.122	0.028 ± 0.001[b]	57.4 ± 1.56	1891 ± 72.1
R39 x F	5704 ± 153	4.57 ± 0.095	0.027 ± 0.001[b]	57.5 ± 1.22	2111 ± 56.5
R39 x M	5095 ± 171	4.42 ± 0.106	0.027 ± 0.001[b]	56.4 ± 1.37	1885 ± 63.3

[a,b] Different superscript between columns indicate statistical differences ($p<0.05$). a: mature body weight; b: a timescale parameter related to the initial body weight; k: growth velocity; t: inflexion age; w: weight at inflexion. LSM ± SEM: least square mean ± standard error of means.

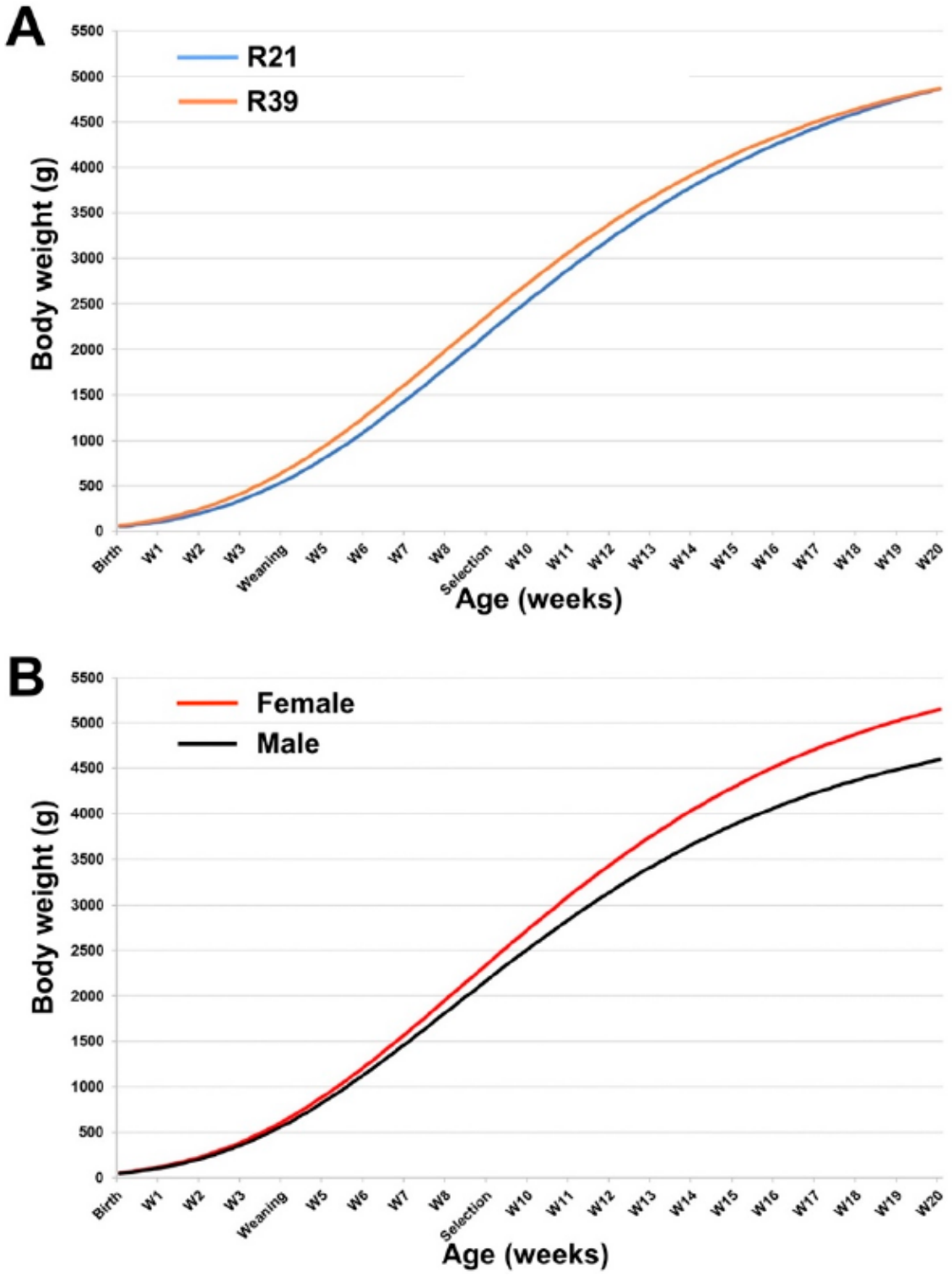

Figure 5.3. Growth curves from birth to 20-week-old between F3 filial generations from R21 and R39 generations. Growth curves were fitted using the Gompertz equation for (A) R21 and R39 generations and (B) sex.

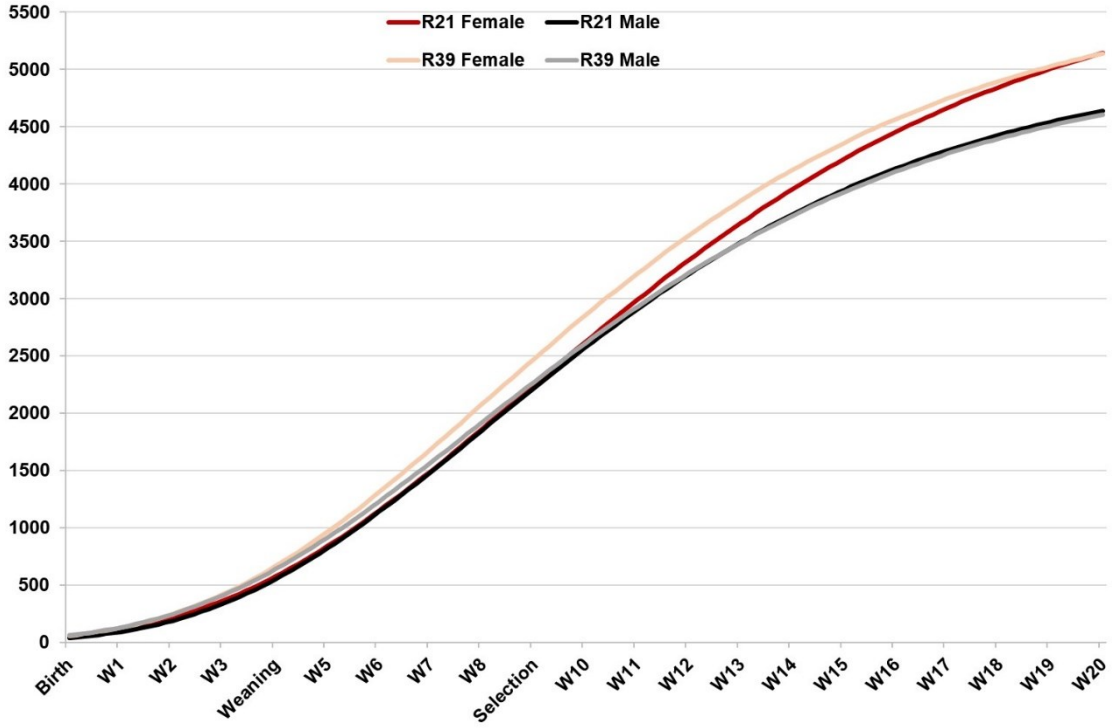

Figure 5.4. Gompertz growth curve from birth to 20-week-old for interaction between populations and sex of animals.

5.4. DISCUSSION

Rabbit meat programmes, like other animal breeding programmes, have based selection on the improvement of target criteria in controlled environments (e.g., litter size, BW daily gain, meat quality or feed efficiency (Estany et al., 1989; Baselga, 2004; Khalil and Al-Saef, 2008; Martínez-Álvaro et al., 2016; de Rochambeau et al., 1989; Estany et al., 1992; Piles et al., 2004; Drouilhet et al., 2013; Piles and Blasco, 2003; García And Baselga, 2002a; Larzul and De Rochambeau, 2005; Garreau et al., 2015; Garreau et al., 2016; Martínez-Álvaro, et al., 2018). Individual selection by ADG has been a common criterion for selecting rabbits in paternal lines, as it is effortless to record and has a moderate heritability (0.15–0.18), and favourable genetic correlation with the conversion index (Moura et al., 1997; Piles et al., 2004). Related to this, response to selection for ADG has been estimated by comparison with a control population (Lukefahr et al., 1996; De Rochambeau et al., 1989; Larzul et al., 2005), or by divergent selection (Moura et al., 1997). The response obtained by generation ranged between 0.56 g/day to 6 g/day (Moura et al., 1997; Piles and Blasco, 2003). Nevertheless, Magheni and Christensen

(1985) demonstrated an asymmetrical response to divergent selection, the response being greatest in the downward rather than in the upward lines, and therefore higher realised heritabilities were recorded in the downward than in upward lines. It is worth mentioning that in all studies, the response was lower than expected, attending to heritability estimates (Blasco et al 2018). In our study, the average daily gain was compared between two rederived populations separated by 18 generations (approximately 15 years). This experimental approach reduces the influence of environmental effects, management, nutrition and even the rederivation procedure, as both populations were cryopreserved, transferred in the same host maternal line and reproductively conducted in the same way under the same environmental and feeding conditions. In this study, the response to the selection for ADG between the 19th and 37th generations was lowest compared to the results obtained in the same line between the 3–4th versus 11th generation (Piles and Blasco, 2003). These findings suggest that the response for ADG has been reduced across generations. Despite further research being required, we hypothesize that in part it would be as a consequence of an unfavourable effect on reproductive performance (Vicente et al., 2012; Naturli-Alfonso et al., 2016) and high mortality rates both during lactation (26% in Lavara et al. (2002) and 17.5 and 20.5% in the present study to all R19 and R37 rederived generations, respectively) and the fattening period (15.2% and 16.6% to all R19 y R37 rederived generations, respectively), reducing the applied selection pressure and variability. On the other hand, rederivation from cryopreserved embryos could be a non-effective way to generate a control population (García-Dominguez et al., 2020a and b). All this evidence contributed to the response to selection being lower in the last 18 generations from a line with a total of 37 generations (0.09 g/day, Table 5.3). To the best of our knowledge, this is the first study carried out in rabbit after more than 37 generations of selection for ADG, if we take into account the lines from which it was founded in 1989. On the contrary, other studies have reported better results for genetic progression achieved after a few generations of selection (Lukefahr et al., 1996; Estany et al., 1992; Larzul et al., 2005; Piles and Blasco, 2003; Blasco and Gómez, 1993).

In recent years, different studies have shown that assisted reproduction technologies and specifically embryo cryopreservation are not neutral (Garcia-Dominguez et al.,

2020a and b). Embryos are subjected to extreme environments in either recovery, cryopreservation and transfer that alter the gene expression, prenatal and postnatal development, and even modify reproductive performance (for example, focused on rabbit (Lavara et al., 2014 and 2015; Garcia-Dominguez et al., a and b; Cifre et al., 1999; Vicente et al., 2013; Saenz-De-Juano et al., 2014 and 2015)). These effects not only modify the phenotype of those born from cryopreserved embryos, but their effects might also be affected and transferred to subsequent generations through heritable and non-heritable epigenetic changes (Garcia-Dominguez et al., a and b). First, in the first generation, newborns could be affected both by the vitrification transfer procedure and by maternal effects when developing in a different uterine and lactation environment, due to the mother in this experiment and the resulting litter size (Marco-Jiménez et al., 2018; McLaren, 1981; Cowley et al., 1989; Atchley et al., 1991; Naturil-Alfonso et al., 2015). The second generation can be affected by the conditions in which parents developed. Finally, in the third generation, the heritable epigenetic effects generated by the rederived technique could emerge and become present. Lavara et al. (2015) demonstrated that vitrification and transfer procedures involved in a rederivation programme for rabbit embryos have long-term consequences on rabbit growth patterns and might affect some growth-related traits in rabbits. Therefore, if the genetic artefacts introduced by the rederivation process were similar in both generations, evaluating their phenotypic differences from the third generation post-rederivation should be the most appropriate method with a minor cumulative genetic drift variance (Smith, 1977). It has been observed that rederived populations showed differences in WW, end fattening weight and ADG as a consequence of selection. However, these differences were not constant after rederivation. Thus, the growth pattern was similar between the rabbits born from vitrified embryos (rederived populations, R19V and R37V) and the subsequent filial generations (R20 and R38). At weaning and end of fattening, the body weights were not different between generations. Only the first filial generation had a relevant difference for ADG (4.27 g/day), with a response of 0.237 g/day by generation. In the third filial generation (R21 andR39), all traits showed some progress as a result of the selection and the response was 0.113 g/day by generation. These deviations can be appreciated in the growth curve pattern, although they did not reach a significant difference either for the day or the weight at which the inflexion point is reached or the

estimated adult weight. It should be noted that the inflexion point was slightly above the rabbit marketing age in Spain (56–58 days old) and near the selection age (63 days old). Blasco et al. (2003), comparing a cryopreserved rederived population from 3rd and 4th generations from this paternal line with a 10th generation, concluded that estimated adult BW was increased by 7% after six generations, while other parameters of the Gompertz curve were scarcely affected by selection. In this context, the reduced sample size of rederived population advises caution regarding the conclusions of this study. The parameters and the resulting growth curve revealed a significant female sexual dimorphism pattern at the inflection point and estimated adult BW. Favourable female dimorphism was already being reported by several authors (Ralls, 1976; Pascual et. al., 2008; De la Fuente and Rosell, 2012) in different breeds or synthetic lines of this species. Rall (1976) described that females were larger than males by a proportion of 1.3:1, while De La Fuente and Rosell (2012) reduce this ratio to 1.03. In this line, Blasco and Gómez (1993) found no effect of sex in Gompertz parameters after 12 generations of ADG selection. Our data showed that sexual dimorphism was affected by ADG, going from a ratio of 1.0:1 to 1.14:1 in 18 generations. An undesirable consequence of selection for ADG could be the increment in adult BW, as it augments the costs of maintaining a parent population. Blasco et al. (2003) found that Gompertz estimated adult BW increased with selection, whereas the parameters related to the slope of the curve practically did not change. In this circumstance, these latter authors predicted that male lines will become giant lines and the reproduction management will be more difficult, unless artificial insemination is used. An interesting finding of the study on long-term selection in these populations separated by 18generations was that the selection for ADG did not change the estimated adult weight, although it seems to have reduced the adult female body weight (221 g, approximately−4% between R21 andR39 females). It is necessary to emphasize that from the parameters obtained in the populations directly rederived by vitrified embryo transfer (R19V and R37V), it was not possible to observe differences. This is probably due to the associated procedure and maternal effects derived from the transfer (smaller litter size and mother of the maternal line). Recent studies that demonstrated the incorporation of inheritable epigenetic marks (García-Dominguez et al., 2020b) and the lack of knowledge as to whether or not they selectively affect certain genetic loci, could question the model used. Therefore,

although rederived populations from cryopreserved embryos have some advantages, by optimising experimental facilities and reducing genetic drift this rederivation procedure could also contribute to distorting the control population (Garcia-Dominguez et al., 2020a and b). Recently, García-Domínguez et al. (2020c) compared naturally conceived animals with progeny generated after embryo cryopreservation, observing transgenerational effects in differentially expressed transcripts and metabolites in hepatic tissue that could be associated with a lower adult weight of the rederived population. Another effect to take into account would be the differential storage time, although these studies were performed to analyse the effect on post-thaw embryo survival and pregnancy outcomes, but not on the liveborn development (Riggs et al., 2010; Sanchez-Osorio et al., 2010; Fogarty et al., 2000; Mozdarani et al., 2007; Lavara et al., 2011; Fang et al., 2014). No studies have been performed to evaluate if the pattern of omics and phenotypic alteration was influenced by storage time. As expected, the effect of the rabbit's reproductive status (number of parturitions and overlap or not with lactation) on the WW was significant and unfavourable in the rabbits at first parity. Although the effect is restricted in young mothers without lactation and gestation overlap, our results were similar to those reported in earlier generations of R line (Gómez et al., 1999), and in line with other paternal and maternal lines between primiparous and multiparous does (Pascual et al., 1998; Fortun-Lamothe et al., 1999; Rebollar et al., 2009). This effect was related to the capacity of the doe to produce milk, which depends on the maturity of the female and the number of suckling kits (Rommers et al., 1999; Fortun-Lamothe and Prunier, 1999; Fortun-Lamothe, 2006; Maertens, 2006). In this regard, we observed that young females that became pregnant again after 10–12 days post-partum achieved a litter size weight at weaning similar to that of multiparous rabbits. Likewise, they showed a better health and physiological status than young mothers that were not pregnant after being inseminated.

5.5. CONCLUSION

In conclusion, the present study describes the selection programme to improve post-weaning daily weight gain without increasing adult body weight in R line, but after 37 generation of selection this trait seems exhausted. A low accumulative reproductive

performance or an unexpected rederived effect on growth traits might be the cause of this little selection response. A refoundation of line and selection for other criteria such as feed efficiency and maternal traits will be necessary.

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CHAPTER IV

EVALUATION OF FOETAL GROWTH, LITTER SIZE AND REPRODUCTIVE PERFORMANCE IN RABBIT AFTER 18 GENERATIONS OF SELECTION FOR GROWTH RATE USING CRYOPRESERVED EMBRYOS

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ABSTRACT

In livestock, adverse effects on reproductive performance, health traits and robustness have been demonstrated in animals selected for high production and efficiency. Using embryo cryopreservation and rederivation, we compared phenotypic traits between rabbit populations separated for 18 generations under growth rate selection pressure (R18 vs R36). To do so, embryos from the ancestral population (R18) and the most recent population (R36) were vitrified in 2000 and 2015, respectively, and rederived and grown together in a randomized controlled environment in 2015. To eliminate confounding maternal and embryo handling effects, traits were measured in the second generation after rederivation (R20 and R38 generations). Our study suggests that se-lection for growth rate has no adverse effect on litter size components. Thus, in the R38 generation we observed a significant increase in embryo implantation (7.2 ±0.71 vs 5.1 ±0.79) and litter size (7.1 ±0.29 vs 6.5 ±0.32). Besides, the foetal sac area at day 12 of gestation (2.44 ±0.070 vs 2.07±0.071 mm2, for R38 vs R20, respectively), and foetal placenta area (136.7 ±6.14 vs 116.0 ±6.31 mm2, for R38 vs R20, respectively) and crown-rump length of the foetus (38.0 ±0.68 vs 35.8 ±0.68 mm, for R38 vs R20, respectively) at day 19 of gestation were higher in the R38 generation. Altogether, these results show that selection for growth rate does not adversely affect components of litter size, foetal growth and reproductive performance. However, the extent to which foundation criteria play a role in the high prenatal and perinatal mortality rate remains unclear in paternal lines.

Keywords: Foetal growth, Reproductive performance, Paternal line, Rederived population, Rabbit

6.1. INTRODUCTION

Rabbit lines specialized for growth rate or feed efficiency and litter size are a common selection objective, as in other animal farms, due to their economic interest (Blasco et al., 2018). Nevertheless, the relationship between growth and maternal traits and their correlated responses, unclear or not, is always positive. Although maternal effects have long been acknowledged as potentially important factors in artificial selection, their magnitude of response remains unclear. In mammals, the role of maternal effects is especially complex due to the fact that progeny experience two distinct maternal environments (prenatal uterine and postnatal nursing) influenced by numerous factors, such as the number of foetuses or litter size, parity, age, breed, heat stress and nutrition (Vuguin, 2007; Wolf et al., 2011). The success of selection for high prolificacy in polytocous species is related with negative consequences in survival, foetal growth and birth weight in rabbit (Vicente et al., 1995; Argente et al., 2003 and 2008) and pigs (Damgaard et al., 2003; Foxcroft et al., 2006; Wolf et al., 2008). Argente et al. (2003) found a reduction in placental and foetal development in rabbits with each additional implanted embryo at 25 days of gestation. Moreover, higher intrauterine crowding has been correlated with higher foetal mortality at 18 days of gestation (Argente et al., 2008). In pigs, it has been shown that selection for increased litter size entails the possibility of various degrees of intrauterine growth retar-dation associated with impaired foetal and placental growth, which can result in lower birth weights (Town et al., 2004). Thus, postnatal variation in growth performance variation may be pre-programmed during foetal development in the uterus. Furthermore, it is likely that these pre-programmed limitations in growth performance will only finally express themselves in the late grower and not at the early finisher stage of production (Du et al., 2010). Some studies suggest that long-term selection for growth rate results in physiological and reproductive changes (Rauw et al., 1998). For example, mice selected for increased early body weight gains showed a decreased response to superovulation and oestrous synchronization, and when they were used as recipients, they produced pups that were significantly larger with respect to body weight and tail length compared with litters gestated in females non-selected or selected divergently (Ernst et al., 2000). These authors suggested that retarded pre-pubescent reproductive development results in

reproductive uterine horns more efficient for sustaining pregnancy, foetal development and growth. Thus, cattle breeds with postnatal different growth impetus and muscularity show differences in foetal development, especially in muscle tissue deposition and development (Mao et al., 2008). In rabbit paternal line, the reproductive performance traits are not a selection factor, as reproductive traits like parity and litter size had a negligible effect on growth rate (Piles and Blasco, 2003), but is unclear whether a selection programme for growth rate affects reproductive traits in rabbits. Low or null correlations between litter size at birth and postnatal growth rate have been observed in rabbit (García and Baselga, 2002; Moć e and Santacreu, 2010; Drouilhet et al., 2013; Mínguez et al., 2016; Peiró et al., 2019) and an uncertain pattern was found in pigs (Damgaard et al., 2003 Wolf et al., 2008; Zhang et al., 2016). Meanwhile, a positive genetic correlation was estimated between postnatal growth rate and ovulation rate in pig and rabbit (Bidanel et al., 1996; Ruiz--Flores and Johnson, 2001; Drouilhet et al., 2013; Peiŕ o et al., 2019). In rabbit, reproductive differences have been described between maternal and paternal lines. Females from the paternal line showed widespread failures, such as altered LH and steroidogenic patterns, low response to ovulation frequency and high losses in implantation during foetal development and birth (Vicente et al., 2012; Naturil-Alfonso et al., 2015 and 2016). Paternal line males did not present normal sexual behaviour, observing low libido, lower sperm production (Pascual et al., 2004; Rosell and De La Fuente, 2009) and changes in seminal and sperm proteome (Lavara et al., 2011; Casares-Crespo et al., 2018; Juárez et al., 2020). The aim of this study was to evaluate whether the selection programme for daily gain in fattening period has changed foetal growth and prenatal survival, using two rederived populations separated for 18 generations. To this end, embryos from the population R18 and the most recent population (R36) were vitrified, rederived and grown together in a randomized controlled environment. To rule out confounding maternal and embryo handling effects, prenatal growth traits and litter size components were measured in the second generation after rederivation (R20 and R38).

6.2. MATERIALS AND METHODS

The animal study protocol was reviewed and approved (code number 2015/VSC/PEA/00,061) by Ethical Committee of the Universitat Politècnica de València before initiating the study. All experiments were performed following guidelines and regulations outlined in Directive 2010/63/EU EEC. Animal experiments were conducted at an accredited animal care facility (code: ES462500001091).

6.2.1. Animals

A rabbit paternal line (R) selected at the Universitat Politècnica de València was used. This line was founded in 1989 from two closed paternal lines selected according to individual weight gain from weaning to end of fattening period (77 days old) during 12 and 9 generations (Estany et al., 1992). Since then, the line has been selected for individual weight daily gain from 28 days (weaning) to 63 days of age (end of fattening). For the present study, two populations (R19V and R37V) separated for 18 generations of selection were used. R19V population was rederived from 256 embryos of 25 donors belonging to 10 different sire families of 18th generation and vitrified in 2000. R37V population was rederived from 301 embryos from 28 donors belonging to 15 different sire families of 36th generation, which were vitrified in 2015. Both populations were rederived at the same time in 2015 (see details in Marco-Jiménez et al., 2018). Offspring were bred in non-overlapping generations; 101 females from generations 20 and 38 were used in this experiment (named R20 and R38, respectively). Environmental conditions were maintained using a control system for light (16:8 light/dark photoperiod), ventilation and temperature (18–25 ◦C) and relative humidity: 60 to 75% by a forced ventilation system. Rabbits does were fed ad libitum throughout the gestation and lactation period with a commercial pelleted diet (2900 kcal of digestible energy / kg, 3.5% crude fat, 15.5% crude fibre and 17% crude protein dry matter). Non-pregnant rabbit does were fed with 140 g/animal/day until a positive pregnancy diagnosis.

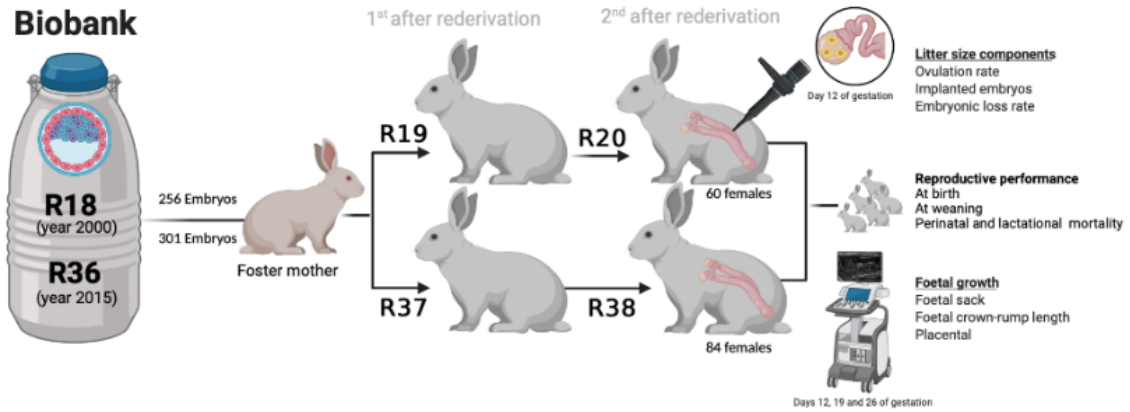

Fig. 6.1. Graphical abstract of the experimental work.

6.2.2. Reproduction management

One hundred and forty-two females were inseminated by males from the corresponding generation (60 from R20 and 82 from R38). To control inbreeding, males and females sharing a grandparent were avoided. Receptivity of does was improved with 12–15 UI of eCG via intramuscular 48 h before insemination. First insemination was performed at 20 weeks of age and then 10–12 days after parturition. Fourteen days post-insemination, pregnancy was diagnosed by abdominal palpation and, if they were non-pregnant, females were inseminated again 7 days later. Young rabbits were weaned at 28 days of age. Insemination was performed after evaluation of ejaculate with 0.5 ml and 20 to 40 million sperm per seminal dose. Only ejaculates with more than 70% of motility rate and less than 20% of abnormal sperm were used. Ejaculates were diluted with tris-citric-glucose diluent to adjust the concentration (Viudes-de-Castro and Vicente, 1997). Immediately after insemination, ovulation was induced by an intramuscular injection of 1 µg of Buserelin Acetate (Suprefact, Hoechst Marion Roussel, S.A., Madrid, Spain). Reproductive status of does at insemination time (nulliparous, primiparous lactating, multiparous lactating and non-lactating does), total litter size, liveborn and litter size at weaning were recorded for each female.

6.2.3. Laparoscopy and evaluated litter size components

A total of 85 does were used and 103 laparoscopies were carried out on females from fourth and fifth parity (38 does from R20 and 47 does from R38). In brief, the females were sedated with intramuscular injection of 5 mg xylazine/kg (Rompun, Bayer AG, Leverkusen, Germany) and 3 mg/kg morphine chloride (). Five minutes later, 35 mg/kg Ketamine hydrochloride (Imalgene®, Merial, S.A., Lyon, France) was administered intravenously. After laparoscopy, does were treated with antibiotics (200,000 IU procaine penicillin and 250 mg streptomycin, Duphapen® Strep, Pfizer, S.L.), 0.03 mg/kg of buprenorphine hydrochloride every 12 h and 0.2 mg/kg of meloxicam every 24-h for 3 days. The number of corpora lutea, the number of implanted embryos at 12 days (IE) and litter size at birth (LS) were recorded per female. The following variables were calculated using the above data. Ovulation rate (OR), defined as the number of corpora lutea, was recorded to determine ovulation rate (OR), embryonic loss rate (ELR), estimated as (OR-IE)/ OR, and foetal loss rate (FLR), estimated as (LS-EI)/LS.

6.2.4. Foetal growth. ultrasound measurement

Thirty-one pregnant does from laparoscopised females (15 from R20 and 16 from R38) were examined on day 12, 19 and 26 of gestation using a portable colour Doppler ultrasound device (Esaote, Spain) with a 7.5 MHz linear probe (4–12 MHz range). Does were sedated according to the procedure described above and placed in a polystyrene cage where they were prevented from moving. The ultrasound examination was performed from right to left with the probe in sagittal orientation and, after localization of different foetal sacs, 4–6 whole foetal sac examinations per doe were performed. The identifiable structures (foetal sac, foetus and foetal and maternal placenta) were measured from frozen frame pictures on the monitor, using the Esaote 16 ultrasound software. Measurements on different days of gestation are illustrated in Fig. 1. Briefly, foetal sac (FS, A, C and E) measurements were taken when the largest surface area appeared on the screen. For whole foetus measurements, crown-rump length (CRL) was determined as the maximum distance from crown to tail base, with the foetus on a sagittal plane (Fig. B, D and F). Placental size was difficult to assess, but placental

measurements were determined when the maximal placental surface with the two-lobed foetal (L1FP and L2FP) and maternal placenta (MP) were visible on screen (Fig. 1A, 2A, 3A).

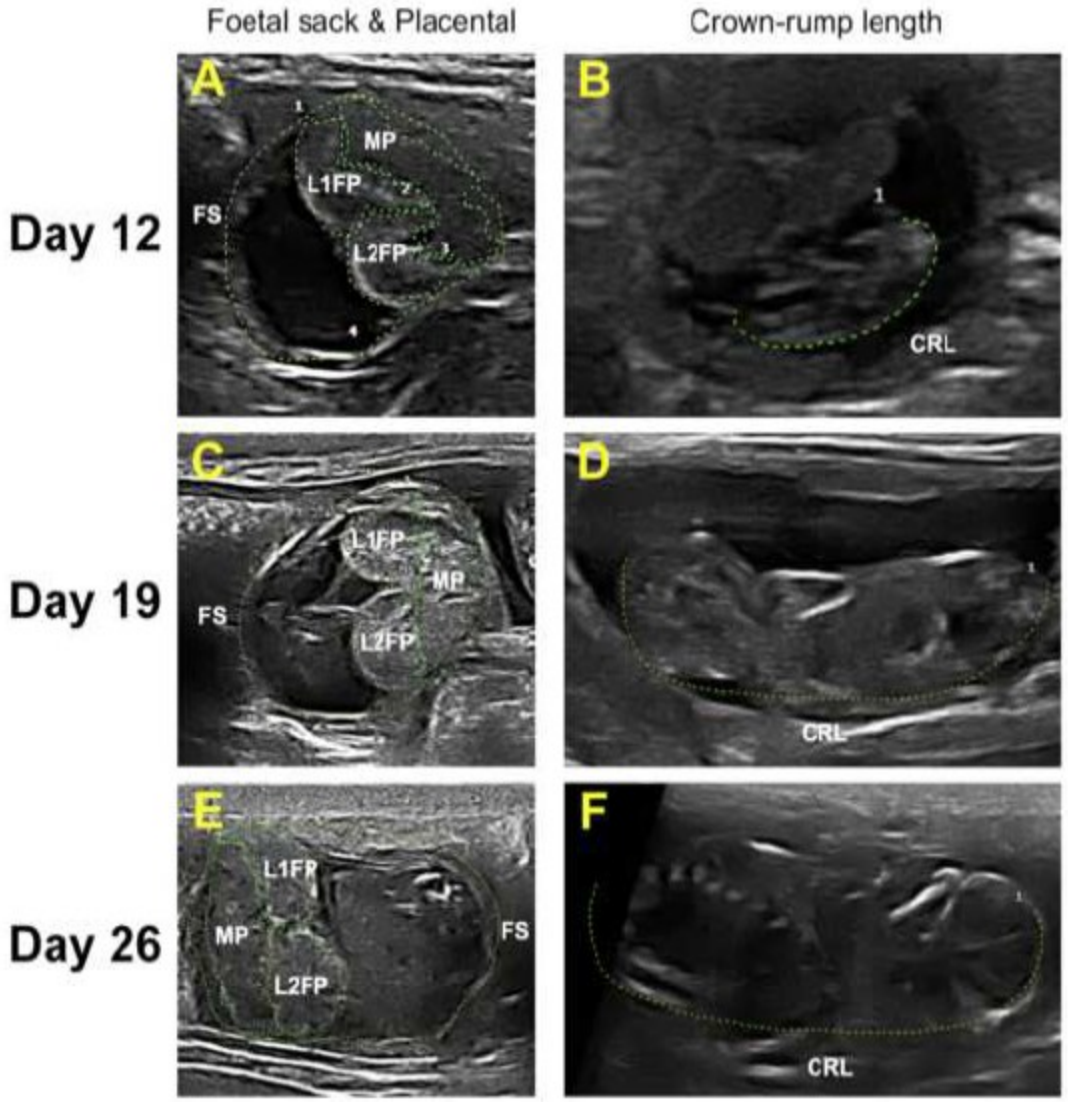

Fig. 6.2. Ultrasonography measurements of the foetal sac (FS), crown-rump length (CRL) of foetus and the placental measurements of the two-lobed foetal (L1FP and L2FP) and maternal (MP) at 12, 19 and 26 day of gestation.

6.2.5. Statistical analyses

Reproductive performance was analysed with a linear general model including as fixed effects rederived generation group (R20 and R38), reproductive status of does (nulliparous, primiparous lactating, multiparous lactating and non-lactating does),

month-year in which insemination was done (18 levels) and the interaction between generation group and reproductive status of the mothers. Delivery rates were analysed using a probit link with binomial error distribution, included in the generalized model described above. Litter size components (ovulation rate, implanted embryos, litter size, liveborn and rates of embryo and foetal losses) were analysed by a generalized linear model including as fixed effects rederived generation group (R20 and R38) and lactating or non-lactating status and their interactions. To analysis foetal sac area, crown-rump length of foetus, foetal and maternal placenta areas at days 12, 19 and 26 of gestation and, weight of liveborn kits, a mixed linear mode was used:

$$Y_{ijkl} = \mu + P_i + R_j + PR_{ij} + CO_k + CovX_l + \epsilon_{ijkl}$$

where Y_{ijkl} was the trait to analyse, μ was the general media, Pi was the fixed effect of the rederived generation group (R20 and R38), R_j was the fixed effect of reproductive status of the doe used to analysis of weaning weight (lactating and non-lactating doe); PR_{ij} was the effect of interaction between rederived population and reproductive status of the mothers, CO_k was the random effect of common litter, Cov X_l was the covariate of number of implanted embryos and ε_{ijkl} was the error term of the model. Values were considered statistically different at $P < 0.05$. Results were reported as least square means with standard error of the mean (SEM). All analyses were performed with SPSS 26.0 software package (SPSS Inc., Chicago, Illinois, USA, 2012).

6.3. RESULTS

6.3.1. Reproductive performance between rederived populations

Of the different reproductive parameters evaluated, only litter size at parturition was significantly different between the two rederived populations, with the litter size being larger in the most recent generation R38 (6.5 ± 0.32 vs 7.1 ± 0.29, Table 6.1). However, the high mortality in both groups around delivery and during the lactation period (more than 20% and 40%, respectively, Table 1) make this difference irrelevant. In addition, the delivery rate was also similar in both groups (Table 6.1). Considering the

reproductive status of the doe at the time of insemination, nulliparous females had the highest delivery rates, the lowest litter size and the highest mortality rate during lactation (Table 6.1). Furthermore, it is necessary to highlight that the females with 1 or more deliveries that were not pregnant presented problems to gestate again, obtaining the lowest delivery rate (Table 6.1). Non-significant effects of year-month and interactions between generational group and week of reproductive status were observed for all evaluated traits.

6.3.2. Litter size components between rederived groups

Litter size components evaluated such as ovulation rate, foetal and embryo losses were not different between the rederived populations, despite the 18 generations of selection that separate them (Table 6.2). However, a significant increase in the number of implanted embryos was observed in R38 vs R20 population if the number of implanted embryos included those females that did not implant any. Both rederived populations showed high rates of loss at implantation and from implantation to parturition, but not significantly different (Table 6.2). Total implantation failure was 38.6% (17) in R20, while this occurred at 23.7% (14) in the R38 population. Non-significant interactions between generational group and lactation status were observed for evaluated traits.

6.3.3. Foetal growth during gestation

Early differences in foetal sac growth, foetal placenta and Crown-rump length of foetuses were observed at day 12 and 19 of gestation, respectively, between both populations (Table 6.3). At the onset of gestation, the lactation status affected the foetal sac size at 12 and 19 days and the foetal placenta size at 19 days. On day 26 of gestation, neither the generation nor the lactation status affected any trait. The bodyweight of the liveborn kits was significantly different between generations, but was not influenced by the lactating status.

Table 6.1. Effect of selection for growth rate on total litter size, liveborn, litter size at weaning, perinatal and lactation mortality rates and delivery rate from two rederived population separated by 18 generations.

Type		n	Litter size		Mortality rate (%)			Delivery rate
			Total	Liveborn	At weaning	Perinatal	Lactation	(%)
Generation	R20	179	6.5 ± 0.32^{b}	5.1 ± 0.36	3.1 ± 0.34	23.2 ± 3.33	46.1 ± 4.11	0.57 ± 0.029
	R38	278	7.1 ± 0.29^{a}	5.8 ± 0.33	3.7 ± 0.31	21.9 ± 3.03	42.5 ± 3.68	0.59 ± 0.024
Lactation status	Nulliparous	142	5.9 ± 0.37^{b}	4.1 ± 0.35^{b}	2.3 ± 0.39^{b}	29.8 ± 3.86	56.7 ± 4.80^{b}	0.76 ± 0.032^{a}
	Primiparous Lactating	75	7.1 ± 0.46^{a}	5.7 ± 0.52^{a}	3.2 ± 0.49^{ab}	23.3 ± 4.76	46.4 ± 5.91^{ab}	0.59 ± 0.044^{b}
	Multiparous lactating	143	7.1 ± 0.36^{a}	6.1 ± 0.41^{a}	4.2 ± 0.38^{a}	17.3 ± 3.72	33.8 ± 4.60^{a}	0.58 ± 0.032^{b}
	Non-lactating	97	7.1 ± 0.37^{a}	5.9 ± 0.42^{a}	3.9 ± 0.39^{a}	19.9 ± 3.86	40.3 ± 4.72^{a}	0.37 ± 0.031^{ca}

n: Number of inseminations. Data are expressed as least squared mean ±standard error of means. ^{a,b} Values with different superscripts in column differ significantly ($p < 0.05$).

Table 6.2. Effect of selection and lactation status on litter size components from two rederived population separated by 18 generations.

Type		Ovulation rate[1]	Implanted embryo	[2]Implanted embryos	Loss rate (%)		Litter size
					[1]Embryonic	Foetal	
Generation	R20	12.1 ± 0.45	5.1 ± 0.79[b]	8.1 ± 0.73	37.0 ± 5.19	42.8 ± 7.31	5.0 ± 0.80
	(n)	(44)	(44)	(27)	(27)	(27)	(26)
	R38	12.7 ± 0.39	7.2 ± 0.71[a]	9.4 ± 0.60	28.5 ± 4.26	33.4 ± 6.00	6.7 ± 0.65
	(n)	(59)	(59)	(45)	(45)	(45)	(44)
Lactation status	Non-lactating	12.8 ± 0.47	6.5 ± 0.86	8.3 ± 0.71	36.7 ± 5.11	40.0 ± 7.19	5.7 ± 0.79
	(n)	(36)	(36)	(28)	(28)	(28)	(27)
	Lactating	12.0 ± 0.37	5.8 ± 0.64	9.1 ± 0.61	28.9 ± 4.36	36.2 ± 6.14	6.1 ± 0.66
	(n)	(67)	(67)	(44)	(44)	(44)	(43)

Data are expressed as least squared mean ±standard error of means. n: number of laparoscopies.
[1] It was determined as the number of corpora lutea.
[2] Implanted embryos in pregnant does.
[a,b] Values in the same column and factor with different superscripts are statistically different (P <0.05).

The highest birth weight was obtained in the offspring of R38 population (57.2 ±3.47 vs 69.3 ±3.89 for R20 and R38 from 48 to 42 liveborn kits, respectively. Data not shown in tables).

6.4. DISCUSSION AND CONCLUSSIONS

To the best of our knowledge, this is the first in-depth study to evaluate the effect of selection for growth on reproductive parameters or traits using populations rederived (re-established) from cryopreserved embryos. Although rarely used in selection experiments, cryopreserved populations offer the advantages of optimizing the experimental facilities and reducing genetic drift (García and Baselga, 2002; Piles and Blasco, 2003), and it is a successful strategy to re-establish a population to continue the breeding programme (Marco-Jiménez et al., 2018). Our study suggests that selection for growth rate has no adverse effect on litter size, foetal growth and reproductive performance, and we even observed a slight improvement in the embryo implantation and litter size and bodyweight at birth after 18 generations of selection. Livestock animals selected for high production efficiency (litter size, growth, feed efficiency or carcass composition and meat quality) have impaired reproductive performance (Rauw et al., 1998; Bunger et al., 2005) or health traits and robustness (Rauw et al., 1998; Prunier et al., 2010; Phocas et al., 2014; Rauw and Gomez-Raya, 2015). These adverse effects of selection are often difficult to reveal as a consequence of not being registered or because the feeding or environmental conditions are being modified. For example, in swine a long-term selection for a combined breeding goal (growth, feed efficiency, body composition and litter size) has been accompanied by an improvement in litter size and weight, but unfavourable effects of selection for several traits such as an increase in stillbirths and in postnatal mortality, reduced longevity and productive life, a reduced milk production and robustness (Silalahi et al., 2016 and 2017). It is known that rabbit selection for growth traits has negative genetic correlations on ejaculate traits such as mass motility, volume, abnormal sperm rate or head sperm morphometry (Brun et al., 2006; Lavara et al., 2012 and Lavara et al., 2013) and the female contribution to fertility has been found (Piles and Tussel, 2012).

Table 6.3. Effect of selection and lactation status on foetal sac and placentae area and foetal size at 12, 19 and 26 days of gestation from two rederived population separated by 18 generations.

	Group	Day of gestation	n	Foetal sac area (cm2)	Maternal placenta area (mm2)	Foetal placenta area (mm2)	Crown-rump length of foetus (mm)
Generation	R21	12	82	2.07 ±0.071[b]	44.9 ±2.46	42.0 ±2.29	11.2 ±0.35
	R39		95	2.44 ±0.070[a]	50.7 ±2.44	46.7 ±2.28	11.5 ±0.34
	R21	19	77	5.81 ±0.178	111.8 ±6.30	116.0 ±6.31[b]	35.8 ±0.68[b]
	R39		94	6.27 ±0.176	118.2 ±6.13	136.7 ±6.14[a]	38.0 ±0.68[a]
	R21	26	66	10.01 ±0.530	197.8 ±12.21	247.6 ±15.67	73.1 ±1.85
	R39		62	10.24 ±0.560	193.1 ±13.57	244.5 ±17.59	72.0 ±1.92
Lactation status	Non- lactating	12	70	2.36 ±0.072[a]	48.4 ±2.49	46.8 ±2.32	11.3 ±0.35
	Lactating		107	2.15 ±0.058[b]	47.2 ±2.02	42.0 ±1.88	11.4 ±0.29
	Non- lactating	19	69	6.49 ±0.181[a]	116.2 ±6.23	139.0 ±6.27[a]	36.9 ±0.71
	Lactating		104	5.59 ±0.147[b]	113.8 ±5.13	113.7 ±5.14[b]	36.9 ±0.57
	Non- lactating	26	54	10.15 ±0.695	211.7 ±17.01	233.2 ±22.04	71.6 ±2.42
	Lactating		74	10.10 ±0.602	179.1 ±13.90	258.9 ±17.91	73.6 ±2.08

[a,b] Values in the same column and factor with different superscripts are statistically different (P <0.05).

Moreover, several studies showed an impaired reproductive performance of paternal line R when it was compared with maternal lines, with high embryonic, foetal, perinatal losses and during the lactation period (Vicente et al., 2012 and Vicente et al., 2013; Naturil-Alfonso et al., 2016). Nevertheless, the present study showed that selection for growth rate does not adversely affect litter size and reproductive performance. It is worth mentioning that, after 18 generations of the selection process, females increased in implanted embryos, ending in improved litter size. Moreover, similar prenatal losses were observed between both generations, in line with our previous results in which implantation and gestational losses were around 20–30% and 50%, respectively (Vicente et al., 2012). In maternal or crossed rabbit lines, embryonic and foetal loss rates are 10 and 20%, respectively, and up to 15% for perinatal losses (Santacreu et al., 1992; Fortun et al., 1993; García and Baselga, 2002; Santacreu et al., 2005; Moć e et al., 2005; Vicente et al., 2012; Ragab et al., 2014). Some of the causes of the high implantation failures and foetal losses of this paternal line could be linked to high levels of IGF-1 and leptin, lower oestrogen and progesterone levels and lower mRNA expression levels of insulin-like growth factor II receptor (IGF-IIR) at endometrial tissue found in the females (Vicente et al., 2012; Llobat et al., 2012; Naturil-Alfonso et al., 2016). Additionally, perinatal and lactation mortality rates were similar and high between both generations. Perinatal and lactation losses found in both can be associated with impaired maternal behaviour and were already reported for this line by Lavara et al. (2002). Moreover, this could be related to the abnormal levels of oestradiol and progesterone during gestation observed in this line (Vicente et al., 2013) and with a low litter size. This endocrine disruption might trigger a cascade of events that would affect the construction of the nest, the pheromonal cues, nursing behaviour and, finally, milk production. González-Mariscal et al. (2016) reviewed maternal behaviour and sibling interactions in rabbits, describing the role of changing concentrations of oestradiol, progesterone and prolactin throughout gestation to prime the mother's brain to respond to the newborn and as regulators of nest-building, and how the duration and periodicity of nursing will depend on the stimulation of the teats by the kits (suckling young). This should be evaluated in a subsequent study in order to improve repro-ductive efficiency, amongst other things, carry out adoptions at birth to prevent the number of young rabbits from being less than 6 so that an adequate nursing behaviour develops. Regarding negative outcomes of non-

lactating does, it is probable that in spite of feed control, these females tended to accumulate fat and had difficulty mobilizing during gestation or lactation. R line does seem to prioritize maintaining their heavier body size rather than litter development, a difference from other lines (Arnau-Bonachera et al., 2018a and Arnau-Bonachera et al., 2018b), which may be further aggravated if does do not become pregnant and continue to gain weight (Rommers et al., 1999) and consequently, negatively affects their long-term reproductive function (Castellini et al., 2006). This study also enabled us to explore whether selection for growth rate affects placentae and foetal growth. Our findings revealed that the foetal sac and foetal placenta area at day 12 of gestation and foetal placenta area at day 19 of gestation was higher in the R38 generation. Nevertheless, no differences between generations were found at day 26 of gestation, indicating that the possible effects of both selection and the gestation-lactation overlap were compensated at the end of gestation but not in the weight of liveborn kits. This could be because during the last week of gestation the fastest increase in bodyweight takes place (Vicente et al., 1995 and Vicente et al., 2013; Argente et al., 2003). So, we have observed evidence that growth selection influenced foetal structures and final weight of foetuses. The changes in foetal structures take place at a critical period between day 12 and 19 of gestation, in which organogenesis is defined, which could be important in the final growth of gestation and during postnatal life (Vuguin, 2007; Sartori et al., 2020). During foetal development, extrauterine signalling provides a link between environmental changes and physiology of the foetus as the impetus to prepare the organism for the postnatal environment, guided mainly by epigenetic changes (Gluckman et al., 2005; Sarkies, 2020). If these adaptive responses are directed to a nutritionally deficient post-natal environment, they could potentially affect muscle, adipose and reproductive tissue development (Ford et al., 2007; Ford and Long, 2012). Skeletal muscle or reproductive tissue have a lower priority in nutrient partitioning compared to the brain and heart in response to challenges to the foetus during development and are particularly vulnerable to nutrient deficiency (Caton et al., 2019; Crouse et al., 2019). Females from the paternal rabbit line used in this study, regardless of the repercussions due to the selection process, shows some negative phenotypic characteristics at endocrine level and a different nutritional partition that could be triggered during prenatal development and in the first steps of postnatal development (suckling). So, a more in-depth study of

these stages is necessary. In conclusion, selection for growth rate does not adversely affect components of litter size, foetal growth and reproductive performance in rabbit does. Nevertheless, this study reinforces some significant reproductive problems, such as high prenatal and perinatal mortality in this paternal line, that were already present in generation 18. Therefore, further studies must elucidate how the founders and not the selection process could play a fundamental role in the adverse reproduction outcomes.

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GENERAL DISCUSSION

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7. GENERAL DISCUSSION

One of the methodologies for assessing genetic progress consists of rederiving populations cryopreserved generations ago. Likewise, the great advantage of using cryopreserved populations from previous generations is avoiding genetic drift and extra facilities for populations without selection (Khalil and Al-Saef, 2008). Typically, the effect of these embryonic technologies has been neglected by estimating that they are not detrimental. Nevertheless, it is known that the manipulation of embryos, from collection, freezing, thawing and even transfer, has specific effects that may be evident generations later. Thus, considering the effects described at the omic and phenotypic level in previous studies carried out in this species after cryopreserving the embryos, the thesis aimed to apply and evaluate the impact of these technologies in two populations from a paternal line obtained from vitrified embryos 18 generations apart.

The use of rederived populations by embryo cryopreservation and transfer to establish a new line or to estimate genetic progress and genetic correlations between traits of interest have been used previously in rabbits (Cifre *et al.*, 1999; Piles *et al.*, 2000; García and Baselga, 2002a and b; Piles and Blasco, 2003; Blasco *et al.*, 2003; Laborda *et al.*, 2012; Lavara *et al.*, 2014; Peiró *et al.*, 2021). In all these studies, an attempt was made to avoid the possible effects arising from the application of assisted reproductive techniques (ART) and the maternal effect related to the host mother, reproducing those born from ART and studying the characteristics of interest in the offspring.

However, the use of embryo cryopreservation techniques, either by slow freezing or vitrification, not only has effects on the viability and foetus development, which reduces litter size at birth and affects postnatal weight (Mocé *et al.*, 2010; Saenz-de-Juano *et al.*, 2012; Saenz-de-Juano *et al.*, 2014a; Vicente *et al.*, 2013; García-Dominguez *et al.*, 2019). Moreover, it has been demonstrated that the cryopreservation programme (embryo vitrification or freezing and transfer) affects the epigenomic, transcriptomic, proteomic and metabolomic levels in embryos. This will not only have consequences in the short term in foetus development and long term in postnatal growth, but also in the following

generations (transgenerational effect) (Saenz-de-Juano *et al.*, 2012; Vicente *et al.*, 2013; Saenz-de-Juano *et al.*, 2014b, 2015; Lavara *et al.*, 2015 and García-Dominguez *et al.*, 2020 b,c,d,e and f and, 2021). The changes derive from the adaptability of the embryos to unfavourable environments that take place during the process (osmotic and thermal stress and toxicity of cryoprotectants, among others). A recent study shows that the stress caused by embryo recovery, scoring and transfer already induces changes at the metabolomic level in rabbit embryos, suggesting a synergistic effect to cause further alterations (García-Dominguez *et al.*, 2020b).

Therefore, using the offspring of the animals born from cryopreserved embryos may not be enough to avoid the alterations introduced with phenotypic repercussions. Moreover, when these technologies are applied to establish control populations to evaluate the genetic progress in a line selected for growth traits, as in most studies carried out in rabbits or other species, it is observed that the primary genes affected are related to the response to stress, development and metabolic pathways (Uechi *et al.*, 1997; Dhali *et al.*, 2007; Martino *et al.*, 2013; Zhu *et al.*, 2020 and Qin *et al.*, 2021 in mice, Stinshoff *et al.*, 2011; Maldonado *et al.*, 2015 and Gutierrez-Castillo *et al.*, 2021 in bovine; Cuello *et al.*, 2021 in pigs).

In rabbits, genes involved in the implantation process and embryo and foetus development have been found, some at day 6 upregulated such as complement C1q tumour necrosis factor-related protein 1 (C1QTNF1), insulin-like growth factor 1 (IGF1), uteroglobulin (SCGB1A1), epithelial membrane protein (EMP1), annexin A3 (ANXA3), EGF-like (EGFLAM) and tumour necrosis factor-inducible gene 6 protein (TNFAIP6) (Saenz-de-Juano *et al.*, 2012, 2014a). Others, such as alpha-fetoprotein (AFP), serotransferrin (TRF), serum albumin precursor (ALB), phosphoglycerate mutase 1 (PGAM1), alpha (HBA) and beta (HBB) subunit of haemoglobin and transthyretin (TTR) at day 14 and ALB, PGAM1 and isocitrate dehydrogenase 1 (IDH1) were also identified overexpressed at day 24 (Saenz-de-Juano *et al.*, 2014b, 2015). These changes seem to be part of a phenotypic adaptation process of embryos from foetal life to prepubertal age (Marco-Jiménez *et al.*, 2020). It is necessary to point out that changes in lipid metabolism at the transcriptome, proteome and metabolome levels have been

observed both in embryos and in the liver of born rabbits (García-Dominguez *et al.*, 2020c and d).

Our results show that cryopreservation effects are present in growth traits such as weaning weight (WW), end of fattening weight (EFW) and average daily gain (ADG) without affecting any of the reproductive traits in females. Similar trends are observed in the next two generations (F2 and F3) for growth traits, which are the selection objectives for a paternal line. In previous studies by our research team, to estimate the effect of selection, the offspring from the cryopreserved rederived population was compared to the current generation, trying to avoid the adverse effects of embryo manipulation (Piles and Blasco, 2003; García and Baselga 2002a and b; Laborda *et al.*, 2012; Santacreu *et al.*, 2005; Peiró *et al.*, 2021). However, these effects were noted for the first time in litter size in their offspring, being higher in the rederived does than in the current generation of selection from a maternal line (Lavara *et al.*, 2015).

In the paternal rabbit line used in this study, changes have also been reported in rabbits born from vitrified embryos, reporting higher birth weights, lower growth rates and adulthood weights than rabbits natural conceived (García-Dominguez *et al.*, 2020d). In addition, negative effects on liver and heart size were found as well as in expressed proteins related to oxidative phosphorylation, downregulated zinc and lipid metabolism, which is a possible cause of the altered growth pattern. Nevertheless, no alterations were observed in prolificacy and health status from these rabbits groups (García-Dominguez *et al.*, 2020c). Similar studies in a maternal line demonstrated effects in adult weight, oxidative and lipid metabolism and milk composition (García-Dominguez *et al.*, 2020e, Marco-Jiménez *et al.*, 2020) and intragenerational transmission by the germinal paternal route (García-Dominguez *et al.*, 2020f). Moreover, these effects have been observed two generations later, evidencing the introduction of hereditable epigenetic changes (García-Dominguez *et al.*, 2020c).

The effect of cryopreservation on growth traits in a paternal line in rabbits was demonstrated in the first chapter by comparing two populations of the current generation; one non-cryopreserved as control and the other cryopreserved. As

mentioned above, it is recommendable to evaluate the offspring of cryopreserved populations rather than the populations themselves to avoid the adverse effects observed (Cifre *et al.*, 1999). Estimates of the response to selection using cryopreserved populations from previous generations have already been evaluated in either maternal (García and Baselga, 2002a, 2002b; Santacreu *et al.*, 2005; Laborda *et al.*, 2012; Peiró *et al.*, 2021;) or paternal lines (Piles and Blasco, 2003; Sánchez *et al.*, 2005; Pascual *et al.*, 2008), but estimates for growth traits either using cryopreserved population or divergent selection were lower than expected based on previous heritability estimates (Blasco *et al.*, 2018). Based on our results, we have observed that the rederived current population showed a lower average daily weight gain and a lower weight at the end of fattening, which would cause an overestimation of parameters when these growth characters are compared between the ongoing generation and the population rederived from several previous generations. Some of the studies previously done on this paternal line may have overestimated the genetic progress achieved. On the contrary, it is necessary to highlight that when the rederived population is obtained by cryopreservation, a bias could be committed by having only the genotypes that survive the procedure, in addition to the absence of an interaction between the rederivation technique and generation.

In chapter two, comparing the cryopreserved generations and their offspring after 18 generations, ADG and SW's growth traits were higher for the older generations, but weaning weights were only higher in the 3rd generation (F3). The response to selection was not as expected (0.09 g/day, per generation), being lower than that reported in another similar work (0.56 g/day) at the beginning of the selection programme and with generations less distant (generation 3-4 vs generation 11) (Piles and Blasco, 2003). This difference could be due to tangible effects of the cryopreservation process and intragenerational effects on the offspring, added to the fact that the selection for this trait has been exhausted after 37 generations of selection, partly due to the low reproductive indexes and the increase in adult weight (Blasco *et al.*, 2003), especially in females, according to our results.

The reproductive traits evaluated in males were the ejaculate, sperm quality and motility parameters, with the percentage of abnormalities (ABN) as the only parameter where significant differences were observed between both generations (G21 vs G39), having increased after 18 generations (10.7% to 23.5%, respectively) but without affecting prolificacy and pregnancy rates. Heritabilities for semen quality parameters were estimated, varying from 0.19 for ABN to 0.07 for total sperm per ejaculate (TSE), and there is no evidence that selection for ADG impaired sperm production. Similar results were observed in other lines selected for growth rate, concluding that seminal traits are not negatively affected by selection for growth traits (Tusell *et al.*, 2012; Brun *et al.*, 2016). Nevertheless, seminal parameters should be considered in the breeding programme, as these males will be used as donors in the insemination centres (Lavara *et al.*, 2011, 2012), in addition to the fact that these ABN were already observed in previous studies in generation 18 and 25 in the same line (Vicente *et al.*, 2004; Lavara *et al.*, 2005; Safaa *et al.*, 2008).

The selection process also seems to have affected the expression of proteins in both seminal plasma and sperm, as 64 and 132 differentially expressed proteins were identified in seminal plasma and sperm, respectively. Nevertheless, it did not affect fertility or prolificacy when ejaculates were used to inseminate crossbred females. Differentially expressed proteins on ejaculate have been reported before by comparing maternal versus paternal line (Casares-Crespo *et al.*, 2018, 2019), where two differentially expressed proteins, uteroglobin and carbonic anhydrase 2 showed a similar tendency to be overexpressed in R line compared to A line (Casares-Crespo *et al.*, 2018), as well as in the selected population (39th Generation) of our study compared to the control population (21st Generation). Moreover, it has also been found that the most abundant proteins in seminal plasma are haemoglobin subunit zeta-like, annexins, lipocalin, FAM115 protein and albumin, molecules related to prevention of damage caused by lipid peroxidation and oxidative stress, membrane functionality, lipid transport to the sperm membrane and temperature regulation (Martins *et al.*, 2019), which were not reported differentially expressed in either plasma or spermatozoa in our study. This is the first time that it has been documented that selection for growth trait

modifies the sperm proteome, although from the productive point of view, no relevant effect is observed.

Genetic selection allowed increased production in livestock species, but undesirable effects have been observed, such as decreased resistance to some diseases and reproductive failure (Rauw *et al.*, 1998). In our study, it is shown that selection by growth rate does not adversely affect litter size and reproductive performance. Moreover, we observed that cryopreservation rederivation did not affect the reproductive traits in this paternal line, results that disagree with those obtained by Lavara *et al.* (2014) in a maternal line. In this latter study, the effect of cryopreservation increased litter size, a variation observed even in offspring, suggesting a transgenerational effect (Lavara *et al.*, 2014).

Very few studies have addressed intragenerational or transgenerational effects of embryo cryopreservation or the application of reproductive technologies. In mice, blastocysts obtained by in vitro culture had no effect on fertility across the generations tested, but the progeny of the cultured blastocysts had lower body weights at the time of weaning compared to in vivo blastocysts in the F0 and F1 generations, but not in the F2 generation (Mahsoudi *et al.*, 2007). Calle *et al.* (2012) reported that males obtained by in vitro culture have reduced fertility and transmit organomegaly and Rexhaj *et al.* (2013) observed that ART-generated mice and their offspring show endothelial dysfunction and increased stiffness in their vasculature, leading to hypertension and shortened life span, suggesting underlying epigenetic modifications.

In chapter 4, selection by growth rate also seems to affect foetal placental growth and foetal size up to day 19 of gestation, increasing size in the last generation, but as already indicated, almost without modifying litter size or birth weight. However, it is known that the correlation between crown-rump length of the neonate and number of implantations is -0,26 and with litter size is -0.29 in rabbit (Breuer and Claussen, 1977), which explains the smaller litter size of this line with higher birth weights than maternal lines (Arnau-Bonachera *et al.*, 2018b). Previous studies have already observed lower reproductive performance in R line than in maternal lines due to higher failures in

ovulation induction and implantation, with embryonic, foetal and perinatal losses. Substantial losses could be due to lower levels of IGF-IIR expression at day 6 of gestation in the blastocyst and uterine tissue (Llobat et., 2012), with high levels of IGF-1 and lower levels of 17β-estradiol and progesterone (LLobat *et al.*, 2012; Vicente *et al.*, 2012, Naturil-Alfonso *et al.*, 2016). In consequence, these females tend to get fat easily, presenting high levels of leptin that make it even more difficult to obtain adequate levels of LH for correct oocyte maturation and ovulation induction, aggravating their problems (Naturil-Alfonso *et al.*, 2016). This triggers lower fertility and is reflected in the long interval between births of non-lactating multiparous rabbits. Moreover, females from this paternal line prioritise the maintenance of their body condition instead of litter development (Arnau-Bonachera 2018a and b). These reproductive performance rates of the R line after 18 generations were not affected by the selection process, and only a slight non-significant increase in litter size was observed. Nor were they affected by the rederivation process (cryopreservation and embryo transfer) as observed in the first chapter, which leads us to argue that this low reproductive performance could have started with the founder population and due to the low heritability of the reproductive traits (<0.13) they were not affected as notoriously for growth traits under selection.

Finally, I would like to mention that a couple of studies have been carried out to improve the growth and reproductive performance of the paternal line. The first one was to integrate new individuals from two selection nuclei that have had this line for more than a decade. These genotypes were incorporated via embryo transfer (Jorge *et al.*, 2019) and the rabbits bred to adults. Females and males from two new groups (Fabara and San Carles de la Ràpita) were bred and growth traits noted, comparing them with the current generation of the nucleus of the Polytechnic University. The data from this study has not yet been published, but preliminary results indicate both the average daily weight gain in fattening and the reproductive performance of females from the three groups (UPV, Fabara and San Carles de la Ràpita) are low and identical. In a second work, an experiment is being carried out to improve the paternal line's reproductive performance, for which three absorption crosses have been performed on a maternal line. It is currently 5th generation, and the new line's reproductive results seem very similar to those shown in this thesis (the data is pending analysis). This could suggest the

persistence of unfavourable genes in this line contributed by the genome of the paternal line under study.

In conclusion, our study provides further evidence of the effects of cryopreservation on growth traits persisting two generations after rederivation, observations that reinforce the findings in parallel studies of the group on omics and phenotypic variations, such as in the liver. Therefore, for genetics studies it would be recommendable that both populations should be cryopreserved and transferred to surrogate mothers. Moreover, the paternal line showed signs of genetic progress exhaustion due to low reproductive performance and high postnatal mortality. Selection by daily weight gain between weaning and the end of the fattening period influenced changes in foetal growth and ejaculate proteome, but did not affect the reproductive performance of females or the fertility and prolificacy of seminal doses of males in the last 18 generations of selection.

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CONCLUSSIONS

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8. CONCLUSSIONS

The conclusions of the thesis are as follows:

Rederivation by embryo vitrification and transfer affected the growth traits used in selection programmes two generations later, without detriment or changes in their reproductive performance. The transgenerational effect on growth traits shows evidence of epigenetic changes induced by the rederivation procedure.

The response to genetic selection evaluated on two rederived populations separated by 18 generations of selection had the following effect:

- Low response to selection in the current generation, due to low cumulative reproductive performance or an unexpected effect of rederivation on growth traits.

- In males, increased abnormal sperm percentage and altered seminal plasma and sperm proteome. Some proteomic changes may be related to the increasing abnormal sperm rate observed, without effects on fertility and prolificacy after insemination with commercial semen doses.

- Reproductive traits were not adversely affected, but reproductive problems such as prenatal and perinatal mortality remained high after 18 generations of selection. This could indicate an effect of the founder population rather than the selection process.