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 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 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 vs 23.8±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 which 56 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.

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., 2012 and 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., 2004 and 2016) and genetic line (Vicente et al., 2000, Brun et al., 2002 and 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, Tussell et al., 2012; Brun et al., 2016). Tussell 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 favour daily weight gain slightly decrease normal acrosome 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. (2014 and 2015) reported differences between genetic lines and showed that high levels of aminopeptidase activity of rabbit seminal plasma was related with abnormal 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. 2002 and 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. (2018 and 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) identified 137 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 FAM115 complex 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 programme by daily gain in fattening period affects ejaculate traits, seminal plasma and sperm proteome and semen fertility.

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 de València (Research code, 2015/VSC/PEA/00061).

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 fibre 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.

2.2. Experimental design

Two populations of R line males were used for this experiment. Both were obtained from embryos vitrified in 2015. The 18^{th} generation was re-derived from vitrified embryos stored in 2000 (G19V) and a sample of embryos from the current generation (36^{th}) 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 (Figure 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.

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 colour were used in the experiment; if the first ejaculate was not available (with urine, sediment or cell debris), a second one was collected 20 minutes later. Gel was removed if present.

Males were weighted weekly during experimental period.

2.4. Evaluation of ejaculates

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 diluted 1:50 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.

2.4.2. Ejaculate quality

2.4.2.1. 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

153 fixed with a solution of glutaraldehyde (0.25% in Dulbecco's phosphate buffered saline) and
154 the samples were examined under a phase contrast optical microscope at x400 magnification.
155 2.4.2.2. Sperm motility parameters
156 Ejaculate samples were diluted in a Tris-citrate-glucose extender (TCG: 250 mM tris-
157 hydroxymethylaminomethane, 83mM citric acid, 50mM glucose, pH 6.8±7.0, 300 mOsm/kg^{-1})
158 to obtain a concentration of 30x10^6 sperm/mL. An aliquot from each sample was then adjusted
159 to 7.5 x 10^6 sperm/mL with TCG extender supplemented with 2 g/L BSA, then 10 µl were
160 placed in a Makler counting chamber pre-warmed at 37ºC on a thermal plate and evaluated in
161 an Integrated Semen Analysis System v. 1.0.17 (ISAS; Projectes i Serveis R+D S.L.). The
162 system was set to record images at 30 frames/s. Motility was assessed at 37ºC at 200X using a
163 negative phase contrast microscope. For each sample, six microscopic fields were analysed and
164 a minimum of 400 sperm evaluated. The curvilinear velocity (VCL, the average velocity
165 measured over the actual point to point track followed by the cell), straight-line velocity (VSL,
166 the average velocity measured in a straight line from the beginning to the end of the track),
167 average path velocity (VAP, the average velocity of the smoothed cell path), linearity index
168 (LIN; the average value of the ratio VSL/VCL), straightness (STR, the ratio between VSL and
169 VAP), wobble (WOB = (VAP/VCL) x 100, a measure of the oscillation of the actual trajectory
170 about its spatial average path), amplitude of lateral head displacement (ALH, the mean width
171 of the head oscillation as the sperm cells swim) and beat cross-frequency (BCF, the frequency
172 of sperm head crossing the average path in either direction) were evaluated. All captures were
173 saved and analysed later. Before field analysis, we proceeded to identify each sperm trajectory
174 to eliminate debris (false captures) and reduce the risk of confusing trajectories.
175 2.4.2.3. Viability and HOST analysis
176 The percentage of viable sperm was determined using a dual fluorescent staining with
177 SYBR-14/PI according to Viudes de Castro et al. (2014). A minimum of 100 sperm cells were

178 counted per ejaculate, and only the percentages of live sperm were considered in the results
179 (SYBR-14-positive and PI-negative). All dilutions were performed at 22 ºC.

180 A hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of
181 the sperm membrane (Jeyendran et al., 1984). Semen was diluted 1:20 in a HOST solution of
182 75 mOsm at 25-30 ºC for 15 min. A minimum of 100 sperm cells were evaluated and HOST
183 was calculated as the percentage of spermatozoa with swollen coiled tails/total spermatozoa.

184 **2.5. Plasma and Sperm protein extraction samples**

185 Ejaculates from 20 mature males (10 for each experimental group "G21V and G39V")
186 were collected and pooled. Six pooled ejaculates (three for each group of males) were obtained
187 in three different weeks and used for insemination. Before preparing the sperm doses, a sample
188 of 500µl from ejaculate pools was centrifuged at 7,400 x g for 10 min at 22 ºC. The supernatants
189 (seminal plasma) were collected, supplemented with a 1% v/v protease inhibitor cocktail
190 (P2714, Sigma) and stored at -80ºC until use. The resulting pellets were washed twice by
191 centrifugation at 900 x g for 10 min in PBS. Sperm proteins were extracted according to the
192 Casares-Crespo et al. (2019) protocol. Briefly, sperm pellets were resuspended in 1% SDS (w/v)
193 in TCG (Tris-citrate-glucose supplemented with a 1% v/v protease inhibitor cocktail, P2714)
194 and sonicated on ice 6 times for 5 s at 30% amplitude using an Ultrasonic Lab Homogenizer
195 UP 100 H (Hielscher Ultrasonics GmbH). After sonication, the solution was kept in ice for 15
196 min and centrifuged for 10 min at 15,000g at 4ºC. Protein lysates were stored at -80ºC until use.

197 **2.6. Proteomic relative quantification analysis**: **SWATH (DIA) MSMS analysis**

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

200 Initial protein concentration from seminal plasma was measured by Nanodrop (Thermo
201 Scientific) using diluted (1 to 10) samples in ultrapure water and the concentration of sperm
202 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.

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 and 400 ng of trypsin and digestion was set to 37 ºC (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, ThermoScientific, 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: 5µl of the digested fragments were loaded into a trap column (NanoLC Column, 3µ C18-CL, 75µ x 15cm; Eksigent) and desalted with 0.1% TFA at 3µl/min for 5 min. The peptides were loaded into an analytical column (LC Column, 3µ C18-CL, 75µ x 12cm, 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 (5600 TripleTOF, ABSCIEX).

The tripleTOF was operated in information-dependent acquisition mode, in which a 250-ms TOF MS scan from 350–1250 m/z, was performed, followed by 150-ms product ion scans from 350–1500 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).

228 2.6.2. ProteinPilot v5.0. search engine (Sciex).

229 ProteinPilot default parameters were used to generate a peak list directly from 5600
230 TripleTof wiff files. The Paragon algorithm (Shilov et al., 2007) of ProteinPilot was used to
231 search the UniprotMammalia database (03.2018) with the following parameters: trypsin
232 specificity, cys-alkylation, without taxonomy restriction, and the search effort set to through
233 and False Discovery Rate (FDR) correction for proteins. The protein grouping was done by Pro
234 group algorithm. Here, the formation of protein groups is guided entirely by observed peptides
235 only, which originate from the experimentally acquired spectra. Because of this, the grouping
236 can be considered to be guided by use of spectra.

237 2.6.3. Swath analysis of samples

238 Protein digestion of seminal plasma samples: 25 µg of every sample were reduced by 2
239 mM dithiothreitol (DTT; Vf=25µ L) for 20 minutes at 60ºC. The thiol groups were alkylated
240 by 5.5 mM Iodoacetamide (IAM, Vf=30 µL) for 30 minutes at room temperature in the dark.
241 The excess of IAM was quenched with 10 mM DTT (Vf=60 µL) at 37ºC for 1 hour. For protein
242 digestion, 500 ng of trypsin were added (Vf=65 µL) and digestion was left overnight. All the
243 reagents were prepared in 50 mM Ammonium bicarbonate solution. The protein digestion was
244 stopped with 5 µL of 10 % Trifluoro-Acetic acid (TFA) in water. The final mixture volume was
245 70 µL. Samples were concentrated by rotatory evaporator to 25 µL. The individual SWATH
246 injections were randomized in blocs.

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

SWATH LCMSMS analysis: 5µl of every sample were chromatographically resolved as in 2.6.1 but with a 120-minute gradient. The tripleTOF was operated in Swath mode, in which a 0.050-s TOF MS scan from 350–1250 m/z was performed, followed by 0.080-s product ion scans from 350–1250 m/z on the 32 defined windows (3.05 sec/cycle). The Swath windows used were: 15 Da window widths from 450 to 1000 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 and 1362 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).

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.

278 2.7.1. Semen collection and evaluation

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

282 2.7.2. Semen extension

283 After semen evaluation, ejaculates form each group were pooled and extended with
284 TCG to 40 million/mL. The semen was diluted at room temperature (20º-25ºC).

285 2.7.3. Insemination procedure

286 All females used in this experiment were multiparous crossbred does and were
287 synchronized with 12UI eCG injected intramuscularly 60h before they were inseminated.
288 Insemination was carried out 10-12th day post-partum and females were induced to ovulate
289 using a synthetic analogue of GnRH (1 µg of buserelin acetate, Hoechst) injected
290 intramuscularly. Twenty million total sperm/female were inseminated (0.5 ml of semen/doe),
291 using a plastic curved pipette. Females were randomly assigned.

292 Kindling rate (number of does giving birth/number of inseminated does) and prolificacy
293 (number of total kits born) were the reproductive performances considered.

294 **2.8. Statistical analyses**

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

298 The mixed model used for the semen traits was:

299 $$Y_{ijkml} = \mu + G_i + B_j + W_k + CO_l + M_m + \Sigma_{ijkml}$$

300 , where Y_{ijkml} is a record of the semen trait measured in the each male, µ is the overall mean
301 for each trait, G_j is the fixed effect of generation with two levels (G21V and G39V), B_i is the
302 fixed effect of the batch in which the ejaculate was collected with 10 levels, as covariable W_k

303 is the weight of the male at the evaluation, $CO(Mm)_l$ is the random effect of the litter in which
304 the male was born, M is the male and Σ_{ijkml} is the residual.

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

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

313 For plasma and sperm protein analysis, Multiexperiment Viewer (MeV software, Saeed
314 et al., 2003) was used for statistical normalization following the software instructions. A t-test
315 was used to identify the differentially expressed plasma and sperm proteins among the six
316 ejaculate pools. Analysis was done only on proteins identified in all sperm samples. Proteins
317 were considered differentially expressed with an adjusted p-value < 0.05 and those with a fold
318 change (FC) ≥ 1.5 after log2 transformation were highlighted. Inferno software was used to
319 perform DA-PLS among samples and ClustVis software was used for the Heat Map clustering
320 of differentially expressed proteins (DEPs). Functional annotation of DEPs, enrichment
321 analysis of their associated gene ontology terms (GO terms) and the Kyoto Encyclopedia of
322 Genes and Genomes (KEGG) pathways analysis were computed using the Bioinformatic
323 software: David Functional Annotation Tool (version 6.8; October 2016), considering a p-value
324 < 0.05.

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328 **3. Results**

329 **3.1. Ejaculates and sperm traits**

330 A different percentage of males between experimental groups responded to artificial
331 vagina stimulus ($P<0.05$, data not shown in tables). The percentage of non-responding males
332 was greater in G21V group (7/18, 38.9% versus 2/21, 9.5% G39V).

333 Of males responding to artificial vagina stimulus, 105 and 213 ejaculates were obtained
334 from the G21V and G39V groups, respectively, of which 13 (12.4%) and 23 (10.8%) were
335 discarded due to the presence of urine, debris or faeces in the G21V and G39V groups,
336 respectively.

337 Only the percentage of abnormal sperm showed significant differences, being lower in
338 G21V (10.5±2.63 versus 23.8±1.98). The remaining seminal parameters were similar between
339 groups (Table 1).

340 **3.2. Plasma and sperm proteome**

341 Sperm parameters of three ejaculated pools used in the proteome analysis and fertility
342 assay are shown in Table 2.

343 For both generations, 643 plasma proteins were reported. Three hundred and ninety-
344 seven identified proteins belonged to *Oryctolagus cuniculus* taxonomy. The results of the sperm
345 proteome comparison between both generations (G21V and G39V) are shown in Figure 2a.
346 Discriminant Analysis (DA-PLS) classified the six sperm samples into two different main
347 clusters corresponding to both groups analysed. The analysis showed differences of relative
348 abundance in 64 proteins (Supplementary table 1). Of the total, 56 proteins were overexpressed
349 in G39V (87.5%). Hierarchical clustering and heat map of differential seminal plasma proteins
350 are shown in Figure 3a, observing two main clusters associated with the experimental groups
351 (G21V and G39V). GO term of molecular function, biological process and cell components are
352 shown in Figure 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 (Figure 2b). Results showed a total of 132 differentially abundant proteins (Supplementary table 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 Figure 3b and 4b, respectively. Figure 3b shows a hierarchical clustering with two main clusters associated with experimental groups (G21V and G39V) and Figure 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 (Figure 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).

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 3a 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.

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 2). Kindling rate, total litter size and live born were similar for both generational groups. Sixty-eight per cent 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).

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% vs 9.5%). Brun et al. (2006) did not observe differences in sexual behaviour 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 (Tussell 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 18^{th} generation, while Safaa et al. (2008) and Lavara et al. (2012) after 6-7 generations ($24\text{-}25^{th}$) 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,

403 and sperm production have a positive but low heritability, while motility parameters showed
404 low heritability and an uncorrelated response to selection (Lavara et al., 2011; Tusell et al.,
405 2012).

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

411 Seminal plasma and sperm proteome showed that the generational step increased the
412 abundance of most of the differentially expressed proteins (87.5% and 66.9%, respectively).
413 Moreover, highlighting proteins differentially expressed with FC>1.5 and focusing on
414 *Oryctolagus cuniculus* taxonomy, fourteen critical proteins related with the sperm functions
415 were affected by selection for growth rate (9 and 5 from plasma and sperm proteome,
416 respectively). Sperm leave the testes morphologically defined but lacking motility, as well as
417 crucial proteins involved in oocyte binding and fertilization. Key proteins related to motility or
418 later to interaction with the egg surface are added to the sperm membrane in the epididymis
419 and, moreover, at the time of ejaculation, seminal plasma proteins from accessory glands coat
420 the sperm surface and stabilize the membrane, inhibiting fertilization ability (Gervasi and
421 Visconti, 2018). The G21V group showed overabundant proteins, such as transmembrane
422 serine protease 2 (TMPRSS2), chromosome 16 open reading frame 89 and uncharacterized
423 protein (U3KNX0). The first of them has been found in human seminal prostasomes (Kim et
424 al., 2006; Antalis et al., 2011), and rabbit seminal plasma is also rich in seminal vesicles
425 produced and secreted by the prostate to prevent sperm capacitation (Davis et al., 1983,
426 Castellini et al., 2006 and 2012). The function of the second is unknown, but a predicted
427 functional partner is Ropporin-1A, a pKA-dependent signalling 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 HCO3- 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,

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

475 Finally, overabundance of KIAA1324 protein has been observed in teratozoospermic
476 human (Choucair, 2018), and, accordingly, this protein was less abundant in semen samples of
477 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 abnormal 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.

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833 Table 1. Seminal traits in G21V and G39V.

TRAITS	Nº EJACULATES	G21V (LSM±SE)	G39V (LSM±SE)
EJACULATE PARAMETERS			
VOL (ml)	424	0.58±0.06	0.48±0.05
CONC (x 10^6 spz/ml)	414	278±65.7	316±47.4
TSE (x 10^6 sperm)	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

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

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

843

844 Table 2. Seminal parameters of G21V and G39V groups used in proteome analysis and fertility
845 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

846

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

852

853 Table 3a. Highlighted differentially seminal plasma proteins between male groups (G21V and
854 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 frame 89	2.21	0.031
U3KNX0_RABIT	Uncharacterized protein	2.41	0.015

855

856 Table 3b. Highlighted differentially sperm proteins between male groups (G21V and G39V)
857 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 71 member 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 frame 89	3.62	0.002
U3KNB5_RABIT	Lipocln_cytosolic_FA-bd_dom domain-containing protein	4.47	0.000

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Table 4. Reproductive performance of inseminated does (least square mean ± standard error least).

Male group	Nº 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

Figure 1. Flowchart of the experiment performed to obtain the evaluated generations of a rabbit line selected by growth rate.

Figure 2a. Partial Least Squares Discriminant Analysis (PLS-DA) showing the classification of seminal plasma samples belonging to G21V and G39V.

Figure 2b. Partial Least Squares Discriminant Analysis (PLS-DA) showing the classification of sperm samples belonging to G21V and G39V.

Figure 3a. Heat map representing levels of differentially expressed seminal plasma proteins between male groups (G21V and G39V).

Figure 3b. Heat map representing levels of differentially expressed sperm proteins between male groups (G21V and G39V).

Figure 4a. Distribution of molecular function, biological process and cell components of differentially expressed seminal plasma proteins between male groups (G21V and G39V).

Figure 4b. Distribution of molecular function, biological process and cell components of differentially expressed sperm proteins between male groups (G21V and G39V).

Supplementary:

Table 1. List of differentially expressed proteins in rabbit seminal plasma (A) between male groups (G21V and G39V).

Peak name	Protein name	Gene name	Log_2 (Fold Change)	p-value
G1TIY2	Uncharacterized protein		-3.960	0.004
G1SKP2	Importin 5	IPO5	-2.908	0.046
G1SQG6	Serpin family A member 5	SERPINA5	-2.352	0.004
P02779	Uteroglobin	SCGB1A1	-2.246	0.003
G1T4H3	Protein disulfide isomerase family A member 6	PDIA6	-2.150	0.049
W5PGW8	X-prolyl aminopeptidase 1	XPNPEP1	-2.138	0.048
G1SPY1	1,4-alpha-glucan branching enzyme 1	GBE1	-2.027	0.027
W5PW05	Malate dehydrogenase 2	MDH2	-1.774	0.014
W5PYV2	IZUMO family member 4	IZUMO4	-1.700	0.004
G1U8K1	Uncharacterized protein	LOC100346690	-1.699	0.029
A0A0D9RUT7	WD repeat domain 1	WDR1	-1.658	0.023
P00919	Carbonic anhydrase 2	CA2	-1.634	0.044
S9XG46	Tubulin alpha chain (Fragment)	CB1_000302001	-1.599	0.050
G1SNK5	Uncharacterized protein	GGCT	-1.552	0.019
G1T763	Polymeric immunoglobulin receptor	PIGR	-1.474	0.031
G1TI27	Solute carrier family 2, facilitated glucose transporter member 3	SLC2A3	-1.445	0.042
G1TUC8	Actinin alpha 4	ACTN4	-1.415	0.025
G1T8S8	Alpha-mannosidase	LOC100346772	-1.369	0.028
U3BZ94	Tubulin beta chain	TUBB4B	-1.362	0.027
G1T5D5	Dipeptidase	DPEP2	-1.334	0.017
U3FJP7	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	PPP2R1A	-1.317	0.041
L5L7E3	Inositol-3-phosphate synthase 1	PAL_GLEAN10006691	-1.315	0.044
W5Q7U7	Serine/threonine-protein phosphatase	PPP1CC	-1.188	0.049
G1SS49	Haloacid dehalogenase like hydrolase domain-containing 2	HDHD2	-1.177	0.042
M3XT75	Interleukin 4 induced 1	IL4I1	-1.160	0.024
G1SXQ0	Glutathione S-transferase	GSTM3	-1.151	0.037
M3WG29	Thyroglobulin	TG	-1.105	0.002
U3CJL4	Ropporin-1B	ROPN1B	-1.089	0.038
S7NC52	Eukaryotic initiation factor 4A-II	D623_10016117	-1.081	0.049
M3YVB2	Carboxypeptidase	SCPEP1	-1.075	0.027
G3RBN0	Desmoplakin	DSP	-1.043	0.044
G1TA48	EH-domain containing 4	EHD4	-1.038	0.049

G1SP77	Solute carrier family 44 member 5	SLC44A5	-0.997	0.025
B6V9S8	Chaperonin-containing T-complex polypeptide eta subunit		-0.985	0.042
G1SDH3	Prolylcarboxypeptidase	PRCP	-0.969	0.008
G1T0H5	LDL receptor related protein 2	LRP2	-0.936	0.044
G1U0R8	Uncharacterized protein		-0.919	0.046
A0A2I3H718	Capping actin protein of muscle Z-line alpha subunit 1	CAPZA1	-0.911	0.017
G1TLQ2	Uncharacterized protein		-0.884	0.025
U6CPY0	Glutaredoxin-1	GLRX1	-0.880	0.015
G1U522	Protein kinase cAMP-dependent type II regulatory subunit alpha	PRKAR2A	-0.838	0.030
U3FVV8	T-complex protein 1 subunit beta isoform 1	CCT2	-0.834	0.032
W5Q805	Proteasome 26S subunit, ATPase 6	PSMC6	-0.814	0.034
U3F8X4	Clathrin heavy chain	CLTC	-0.814	0.033
W5Q2N2	Proteasome 26S subunit, non-ATPase 12	PSMD12	-0.775	0.017
G1SK80	Zona pellucida binding protein	ZPBP	-0.752	0.046
Q9TTC6	Peptidyl-prolyl cis-trans isomerase A	PPIA	-0.704	0.043
W5NPN4	Heat shock protein family A (Hsp70) member 8	HSPA8	-0.701	0.010
G1U723	3alpha/17beta/20alpha-hydroxysteroid dehydrogenase	PGER5	-0.693	0.034
G1T678	Uncharacterized protein	ACAT2	-0.679	0.043
G1SKA8	Acrosin binding protein	ACRBP	-0.589	0.038
W5P0A6	Platelet activating factor acetyl hydrolase 1b catalytic subunit 2	PAFAH1B2	-0.577	0.004
G5AZH1	5'-nucleotidase	GW7_18824	-0.577	0.043
G1TB50	Syndecan binding protein	SDCBP	-0.551	0.027
S7N9H1	Alpha-aminoadipic semialdehyde dehydrogenase	D623_10026966	-0.482	0.018
V9HW12	Epididymis secretory sperm binding protein Li 2a	HEL-S-2a	-0.461	0.049
P46409	Glutathione S-transferase Mu 1		0.360	0.037
G1SCT4	Uncharacterized protein		0.751	0.007
W5PY41	OTU deubiquitinase, ubiquitin aldehyde binding 2	OTUB2	0.853	0.009
G1TMY8	Uncharacterized protein	TMPRSS2	1.520	0.035
F7HBU3	Chondroadherin	CHAD	1.590	0.000
G1T0A6	Chromosome 16 open reading frame 89	C16orf89	2.208	0.031
U3KNX0	Uncharacterized protein	LOC100350057	2.413	0.015
A0A1U7Q3W6	carbonic anhydrase 2	Ca2	2.750	0.011

Table 2. List of differentially expressed proteins in rabbit sperm between male groups (G21V and G39V).

Peak name	Protein name	Gene name	Log_2 (Fold Change)	p-value
U3DHN2	Choline/ethanolamine kinase	CHKB	-4.292	0.000
G1T259	Family with sequence similarity 71 member C	FAM71C	-3.302	0.005
S9XHL9	Uncharacterized protein	CB1_000231004	-2.932	0.029
A0A1D5QN77	Uncharacterized protein		-2.549	0.004
G1T923	ATPase H+ transporting accessory protein 2	ATP6AP2	-2.301	0.000
W5Q1D9	Mitogen-activated protein kinase 4	MAP2K4	-2.157	0.010
P00919	Carbonic anhydrase 2	CA2	-2.120	0.047
G1U4K9	Uncharacterized protein	LOC100359206	-1.959	0.038
A0A0D9RHG6	IZUMO family member 4	IZUMO4	-1.914	0.006
H0XZT4	Calicin	CCIN	-1.889	0.001
A0A1U7TH09	arginine--tRNA ligase. cytoplasmic	RARS	-1.821	0.034
G1TE39	UDP-glucose glycoprotein glucosyltransferase 2	UGGT2	-1.587	0.007
G1SVH9	KIAA1324	KIAA1324	-1.535	0.049
G1T9L3	Cilia and flagella associated protein 61	CFAP61	-1.363	0.050
W5P2U9	Leucine rich repeat containing 59	LRRC59	-1.311	0.041
B7NZB0	Tryptophan rich basic protein (Predicted)	WRB	-1.272	0.030
Q71DI1	Dermcidin		-1.254	0.041
G1TX59	Serine/threonine-protein phosphatase 2A activator	PTPA	-1.234	0.014
G1SWT1	Phosphodiesterase	PDE10A	-1.161	0.046
G1SSU3	Monoglyceride lipase	MGLL	-1.126	0.003
G1T7H0	Heterogeneous nuclear ribonucleoprotein U	HNRNPU	-1.106	0.044
|L8YCZ7	Oligoribonuclease. mitochondrial	TREES_T100015334	-1,099	0.026
G1SQ27	Clusterin	CLU	-1.098	0.013
G1TZ19	Diablo IAP-binding mitochondrial protein	DIABLO	-1.077	0.049
Q3TX38	Uncharacterized protein	Vdac3	-1.036	0.012
Q53ZP9	Heat shock protein apg-1		-1.027	0.002
I3LYQ8	Radial spoke head 6 homolog A	RSPH6A	-1.019	0.047
A0A1S3A5Y3	26S proteasome non-ATPase regulatory subunit 2	PSMD2	-1.016	0.031
G1SPR9	Ribophorin II	RPN2	-1.015	0.030
L9L3I2	Uncharacterized protein	TREES_T100009695	-0.971	0.028
G1SUK4	Mannose-6-phosphate isomerase	MPI	-0.963	0.041
G1SY44	Dpy-19 like 2	DPY19L2	-0.960	0.009
B6RFK9	Calcium-transporting ATPase		-0.954	0.047
P15253	Calreticulin	CALR	-0.941	0.037
Q3UJN2	RuvB-like helicase	Ruvbl1	-0.938	0.010

W5PG36	Voltage dependent anion channel 2	VDAC2	-0.919	0.034
T0MHC0	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit	CB1_001086058	-0.910	0.021
F6UQ19	ADP-ribosylation factor like GTPase 8B	ARL8B	-0.878	0.012
G1T6H7	Lipase I	LIPI	-0.871	0.029
Q8K1X5	EH-domain containing 1 (Fragment)	Ehd1	-0.860	0.000
G1U9S2	Serum albumin	ALB	-0.844	0.048
H0VL12	Ubiquitin carboxyl-terminal hydrolase	Uchl3	-0.819	0.042
G1SDD7	Polyamine oxidase	PAOX	-0.799	0.014
M3Y7C5	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit DAD1	DAD1	-0.786	0.009
W5Q4H1	Uncharacterized protein	TMED7	-0.774	0.025
G1SKP2	Importin 5	IPO5	-0.739	0.031
S7MLC2	Ribosomal protein L15	D623_10020435	-0.738	0.023
W5P4C0	Uncharacterized protein	RPS13	-0.726	0.007
W5PKQ2	Family with sequence similarity 213 member A	FAM213A	-0.717	0.022
H9YYT7	Calnexin	CANX	-0.714	0.032
G1SRN0	Dynein axonemal heavy chain 7	DNAH7	-0.694	0.031
G1TDX0	Calpain 11	CAPN11	-0.693	0.036
G1SVQ0	Glutathione S-transferase omega 2	GSTO2	-0.689	0.017
F1RFM8	Dynein axonemal heavy chain 10	DNAH10	-0.681	0.045
A0A061I1I7	Clathrin heavy chain 1-like protein	H671_7g18204	-0.668	0.027
U3E190	ADP-ribosylation factor 3	ARF3	-0.668	0.036
G3U7Z4	A-kinase anchoring protein 4	AKAP4	-0.628	0.009
W5PAG0	Lysine--tRNA ligase	KARS	-0.625	0.020
U6DJ81	26S proteasome non-ATPase regulatory subunit 6 (Fragment)	PSMD6	-0.615	0.044
G1T4R5	Glycerophosphodiester phosphodiesterase domain-containing 1	GDPD1	-0.612	0.003
W5P1T4	Serine/threonine-protein phosphatase	PPP4C	-0.610	0.037
W5QG71	Uncharacterized protein		-0.599	0.018
G1TP15	Proteasome 26S subunit. non-ATPase 3	PSMD3	-0.591	0.012
A0A091DF21	4-trimethylaminobutyraldehyde dehydrogenase	H920_09468	-0.584	0.020
W5NSP2	40S ribosomal protein S8	RPS8	-0.581	0.049
W5NRL8	Eukaryotic translation initiation factor 3 subunit A	EIF3A	-0.573	0.004
W6FFT9	Signal peptidase complex catalytic subunit SEC11	SEC11A	-0.570	0.049
S9YHZ9	60S ribosomal protein L7a	CB1_000282013	-0.562	0.046
G1TZQ6	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10. mitochondrial	NDUFA10	-0.549	0.006
G1TDQ5	ATP-dependent 6-phosphofructokinase	PFKM	-0.549	0.010
W5P0D5	Proteasome 26S subunit. non-ATPase 7	PSMD7	-0.548	0.007
G1T2I4	Glutamyl-prolyl-tRNA synthetase	EPRS	-0.547	0.003
J9PAN1	Angiotensin-converting enzyme	LOC100856208	-0.531	0.047

W5PUU0	Uncharacterized protein	RPL31	-0.528	0.048
P25227	Alpha-1-acid glycoprotein	ORM1	-0.525	0.016
G1SZR0	Uncharacterized protein	TEX101	-0.524	0.032
W5QG19	Exportin 1	XPO1	-0.518	0.032
M3VYE9	Uncharacterized protein	USMG5	-0.507	0.005
U6CPT9	Enolase-phosphatase E1	ENOPH	-0.504	0.039
U3KME2	Proteasome 26S subunit. non-ATPase 13	PSMD13	-0.466	0.045
G1SR77	Calcium-transporting ATPase	ATP2B4	-0.441	0.047
Q96G38	Eukaryotic translation initiation factor 3 subunit B (Fragment)	EIF3B	-0.433	0.009
U3KM71	Uncharacterized protein	ATP5L	-0.429	0.038
G5E8T9	Hydroxyacyl glutathione hydrolase	Hagh	-0.420	0.016
W5QBG6	Nuclear pore complex protein Nup93	NUP93	-0.405	0.019
W5PEQ3	Mindbomb E3 ubiquitin-protein ligase 1	MIB1	-0.384	0.026
W5P7Z1	Uncharacterized protein		-0.351	0.038
A0A091DD11	Kinesin light chain 2	H920_10239	-0,258	0.047
I6YLY8	Heat shock cognate 71 kDa protein	HSPA8	0.339	0.026
G1SD34	Sodium/potassium-transporting ATPase subunit beta	ATP1B3	0.410	0.017
G1STX7	Kynurenine aminotransferase 3	KYAT3	0.450	0.016
G1TAY6	Keratin 19	KRT19	0.507	0.032
G1SQ02	Peroxiredoxin 1	PRDX1	0.523	0.045
W5PDG3	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	0.583	0.032
G1SZ00	Cysteine and glycine rich protein 1	CSRP1	0.670	0.048
Q53GD1	Guanine nucleotide-binding protein subunit gamma (Fragment)		0.679	0.041
G1U3K5	Androglobin	ADGB	0.722	0.027
W5NYF9	5'-nucleotidase. cytosolic II	NT5C2	0.723	0.034
G1TY46	Immunoglobulin superfamily containing leucine rich repeat	ISLR	0.745	0.009
S9W5U7	60S ribosomal protein L18	CB1_007371005	0.794	0.017
G1TYT4	Angiotensin-converting enzyme	ACE	0.818	0.009
G1TUC8	Actinin alpha 4	ACTN4	0.844	0.049
G1SYV9	Talin 1	TLN1	0.853	0.012
G1TBX4	Carboxypeptidase	CPVL	0.875	0.002
G1U4G9	Chloride intracellular channel protein	CLIC1	0.896	0.018
M3WPD9	Alpha-methylacyl-CoA racemase	AMACR	0.918	0.019
A0A1A6HNG5	Uncharacterized protein	A6R68_22009	0.928	0.028
W5Q8B1	Glutathione peroxidase	GPX6	0.971	0.001
G1T5Q9	Bactericidal permeability-increasing protein	BPI	0.971	0.011
G1SYM3	Tetraspanin	CD9	1.001	0.030
A0A091DW69	Beta-1.4-galactosyltransferase 1	H920_03195	1.020	0.008
M1ZMP8	Aldehyde oxidase 3		1.026	0.019
G1SLU0	VPS37B. ESCRT-I subunit	VPS37B	1.066	0.050
G1SD48	Glucosylceramidase	GBA	1.130	0.004
W5Q9V4	RAB3D. member RAS oncogene family	RAB3D	1.155	0.020
W5QCT2	COP9 signalosome subunit 8	COPS8	1.238	0.047

G1SFR5	Peptidylglycine alpha-amidating monooxygenase	PAM	1.298	0.005
G1SWH0	Semaphorin 3C	SEMA3C	1.343	0.022
G1T6B8	Lysozyme	LOC100341160	1.470	0.002
U3BEF5	SH3 domain-binding glutamic acid-rich-like protein	SH3BGRL2	1.552	0.036
G1SUM6	Uncharacterized protein	CPE	1.654	0.000
I3NAI1	Uncharacterized protein	Sept9	1.663	0.023
B8K131	Zeta globin (Predicted)	HBZ_1	1.666	0.008
H2PZD8	Transforming growth factor. beta receptor III	TGFBR3	1.682	0.006
G1TBJ6	Pro-epidermal growth factor	EGF	1.878	0.030
W5PYX5	Chromosome 5 open reading frame 49	C5orf49	1.937	0.039
U3KPB9	Uncharacterized protein		1.972	0.029
F1RT93	Chondroadherin	CHAD	2.097	0.004
Q5PQN1	Probable E3 ubiquitin-protein ligase HERC4	Herc4	2.600	0.008
U3KNX0	Uncharacterized protein	LOC100350057	3.263	0.009
G1T0A6	Chromosome 16 open reading frame 89	C16orf89	3.616	0.002
U3KNB5	Lipocln_cytosolic_FA-bd_dom domain-containing protein	LOC103347146	4.467	0.000