RABBIT SEMINAL PLASMA PROTEOME: THE IMPORTANCE OF THE GENETIC ORIGIN

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Abstract

The present study was conducted to characterise rabbit seminal plasma proteins (SP proteins) focusing on the influence of the genetic origin and seasonality. In addition, β-NGF protein quantity in SP was determined. Semen samples were recovered from January to December 2014 using 6 males belonging to genotype A and six from genotype R. For each genotype, one pooled sample at the beginning, middle and end of each season was selected to develop the experiment. A total of 24 pools (3 for each season and genetic line) were analysed. SP proteins of the two experimental groups were recovered and subjected to in-solution digestion nano LC-MS/MS and bioinformatics analysis. The resulting library included 402 identified proteins validated with ≥ 95% Confidence (unused Score ≥ 1.3). These data are available via ProteomeXchange with identifier PXD006308. Only 6 proteins were specifically implicated in reproductive processes according to Gene Ontology annotation. Twenty-three proteins were differentially expressed between genotypes, 11 over-expressed in genotype A and 12 in genotype R. Regarding the effect of season on rabbit SP

proteome, results showed that there is no clear pattern of protein variation throughout the year. Similar β-NGF relative quantity was observed between seasons and genotypes. In conclusion, this study generates the largest library of SP proteins reported to date in rabbits and provide evidence that genotype is related to a specific abundance of SP proteins.

Keywords: rabbit, seminal plasma, proteome, genotype, season, LC-MS/MS.

1. Introduction

The control of rabbit reproduction has experienced great changes in the last decade, mainly as a consequence of the development of new techniques such as commercially applicable artificial insemination (AI) (Safaa et al., 2008). The use of AI in intensive meat rabbit production is currently a common practice (Piles et al., 2013), like in the vast majority of livestock (Hansen, 2014), and it utilisation has contributed to improve the knowledge of rabbit spermatozoa and bucks' management (Boiti et al., 2005; Castellini et al., 2008; Lavara et al., 2005; Pascual et al., 2016; Safaa et al., 2008; Theau-Clément et al., 2015, 2016; Viudes-de-Castro et al., 2014). Rabbit ejaculates present some peculiarities that should be taken into account, for instance, they present occasionally gel plug or gelatinous mass and contain several vesicles that have been related to modulate different sperm functions such as motility, capacitation and acrosome reaction (Castellini et al., 2006, 2012, 2013; Collodel et al., 2012). In addition, rabbit belongs to the few species in which ovulation is induced by copulation (Fisher et al., 2012), like cats, camelids, koala, voles and sumatran rhinos (McGraw et al., 2015). In these species, a specific protein named β−NGF has been studied in seminal plasma because of its potential role in inducing ovulation in camelids (Adams and Ratto, 2013; Berland et al., 2016; Druart et al., 2013; Kershaw-Young et al., 2012; Li et al., 2010; Silva et al., 2011). Nevertheless, in rabbits, the intramuscular administration of seminal

plasma did not provoke ovulation (Silva et al., 2011), but plays a role in promoting the formation and development of the testis and the differentiation, maturation, and movement of the spermatozoa (Li et al., 2010).

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

Seminal plasma contributes to the safe environment for sperm maturation, sperm viability and fertilization in mammals (Muiño-Blanco et al., 2008; Rodríguez-Martínez et al., 2011; Manjunath et al., 2007; Bromfield, 2016). Moreover, seminal plasma is a promising source for the study of potential reproductive biomarkers, because it is a complex mixture of secretions from testis, epididymis and male accessory sex glands (González-Cadavid et al., 2014). Sperm maturation is acquired during the transit of the spermatozoa through the epididymis, where its plasma membrane undergoes intense changes in protein composition and in localization of their components (Dacheux et al. 2003). The protein composition of mammalian seminal plasma varies among species, and has important effects on sperm function (Rodríguez-Martínez et al., 2011). Even though seminal plasma contains hundreds of proteins, their functions are not completely understood. In rabbits, seminal plasma has a positive effect in maintaining sperm motility and viability during *in vitro* storage (Castellini et al., 2000).

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

2. Materials and methods

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

2.1. Localization and animals

The experiment was carried out with 24 males from two Spanish commercial rabbit genetic lines (genotypes A and R) from January to December 2014. All bucks were of proven

fertility and subjected to a weekly pattern of ejaculate collection. Line A is based on New Zealand White rabbits selected since 1980 by a family index for litter size at weaning over 45 generations (Fig. 1 right). Line R comes from the fusion of two lines, one founded in 1976 with Californian rabbits reared by Valencian farmers and another founded in 1981 with rabbits belonging to specialised paternal lines (Fig. 1 left). The selection method was individual selection on post-weaning daily gain, with weaning taking place at 28 days and the end of the fattening at 63 days. All animals were housed at the Animal Technology and Research Centre (CITA-IVIA, Segorbe, Castellón, Spain) experimental farm in flat deck indoor cages (75×50×30 cm), with free access to water and commercial pelleted diets (minimum of 15 g of crude protein per kg of dry matter (DM), 15 g of crude fibre per kg of DM, and 10.2 MJ of digestible energy per kg of DM). The photoperiod was set to provide 16 h of light and 8 h of dark, and the room temperature was regulated to keep temperatures between 14°C and 28°C.

2.2. Semen collection and preparation of seminal plasma samples

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

For each genotype, one pooled sample at the beginning, middle and end of each season was selected to develop the experiment (Fig. 2). A total of 24 pools (3 for each season and genetic line) were analysed. Total protein concentration was quantified in duplicate by the bicinchoninic acid method (BCA) using BSA as standard protein (Smith et al., 1985) and seminal samples were adjusted to 5 µg/µL in saline solution.

2.3. In-solution digestion

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

2.4. Nano LC-MS/MS analysis

Two µL of each sample were loaded onto a trap column (nano LC Column, 3µm particles size C18-CL, 350 µm diameter x 0.5mm long; Eksigent Technologies) and desalted with 0.1% TFA at 3µL/min during 5 min. The peptides were then loaded onto an analytical column (LC Column, 3 µm particles size C18-CL, 75µm diameter x 12cm long, Nikkyo)

equilibrated in 5% acetonitrile (ACN) 0.1% formic acid (FA). Peptide elution was carried out with a linear gradient of 5% to 35% of solvent B in A for 120 min. (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 300 nL/min. Peptides were analysed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSCIEX).

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

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

2.5. Protein identification

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

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

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

2.6. Label-free protein quantification using Chromatographic Areas

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

2.7. Bioinformatics analysis

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

2.8. Statistical analysis

The quantitative data obtained by PeakView® were analysed by Marker View® v1.3 (AB Sciex). First, areas were normalized by total areas summa. A t-test was used to

identify the differentially expressed proteins between genotypes. Proteins were considered differentially expressed if the adjusted p-value < 0.05. Mean quantity of proteins were calculated and the fold-changes between the two groups were estimated. No multiple corrections were performed. The standard deviation was pooled out by calculating a separate t value for each peak. Group comparison is performed by calculating the square of t according to the following equation:

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

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

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

3. Results

3.1. Rabbit seminal plasma proteome

The Proteomics System Performance Evaluation Pipeline (PSPEP) Software was used to perform a false discovery rate analysis on Paragon™ algorithm results. The complete spectral library included 88,385 spectral corresponding to 4,600 peptides and 402 proteins validated with ≥ 95% Confidence (unused Score ≥ 1.3) when using at least 2 peptides for identification (Table S1). These 402 proteins were quantified based on their chromatographic or peak areas (Table S2).

The complete rabbit seminal plasma proteome was classified under different categories based on their molecular function, biological process and cellular components

(PANTHER analysis). The results are shown in Figure 3. For molecular function (Fig. 3a), a total of 251 hits were found. The catalytic activity was the predominant function (50%), followed by binding (27%) and structural molecule activity (13%). Regarding biological process (Fig. 3b), a total of 471 hits were found. The metabolic (28%) and the cellular process (26%) were the most abundant categories, but it is worth mentioning that 6 hits (1%) were classified in the category of reproduction specifically to gamete generation and fertilization functions. Finally, a total of 195 hits were found for cellular component category (Fig. 3c). Cell part (52%), organelle (27%), macromolecular complex (9%) and extracellular region (7%) were the most abundant cellular components of the studied proteins.

3.2. Effect of genetic origin on seminal plasma proteome

The results of the seminal plasma proteome comparison between both genetic lines (A and R) are shown in Fig. 4. PLS-DA analysis showed a clear effect of the genetic origin. Proteins with a vip score > 1.5 (high influence in the response variable) were selected and a heat map was generated (Fig. 5). The hierarchical clustering of seminal plasma proteins separated the twenty-four seminal samples into two different main clusters, differentiating between genotypes. Given that the proteome between both genotypes presented high variability, a t-test was done. Results showed a total of 23 differentially expressed proteins ($p < 0.05$) between genotypes (Table S3). Of the differentially expressed proteins, 11 proteins were over-expressed in genetic line A and 12 proteins over-expressed in line R (Table 1).

3.3. Effect of season on seminal plasma proteome

The results of the comparison between seasons are shown in Fig. 6. PLS-DA analysis showed unclear separation between seasons, existing overlaps between winter, spring, summer and autumn samples. Seminal samples from autumn seem to be the most differential

ones compared with the others; however, half of them overlap with other seasons samples. After applying vip score function to the previous PLS-DA results and only selecting the proteins with a vip score > 1.5 (high influence in the response variable), a heat map was generated (Fig. 7). Predictably, in the heat map, the hierarchical clustering of seminal plasma proteins separated the 24 seminal samples into four different main clusters, but these clusters did not match the four seasons but a mixture of them.

3.4. β-NGF relative quantification in rabbit seminal plasma

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

4. Discussion

To the best of our knowledge this study generates the largest library of seminal plasma (SP) proteins reported to date in rabbits. Moreover, one of the most important contributions of this study is the significant relationship found between genetic origin and SP proteins in rabbit. In previous studies, rabbit seminal plasma proteome has already been proved different between rabbit genotype A and R (Casares-Crespo et al., 2016a; Safaa et al., 2008; Viudes-de-Castro et al., 2004). In these previous studies a traditional 1-D polyacrylamide gel was done, identifying only major proteins visible after Coomassie Colloidal Blue staining and obtaining the relative quantity of these protein bands through scanning and analysing the gel with a 1D software. The fact that in the present study a more accurate technique such as LC-MS/MS was used in order to identified the differentially expressed proteins, could explain the

276 differences found between previous (Casares-Crespo et al., 2016a; Safaa et al., 2008; Viudes-
277 de-Castro et al., 2004) and current results. Indeed, the exceptional sensitivity and resolving
278 power of today's mass spectrometers allow for the detection of proteins and peptides at low
279 femtomole quantities (Wither et al., 2016). That is why, in the current study, with the
280 application of LC-MS/MS, we identified and quantified 402 rabbit SP proteins, compared to
281 the seven rabbit seminal plasma proteins identified previously (Casares-Crespo et al., 2016a).
282 Bioinformatics analysis of rabbit SP proteome revealed that 50% of identified proteins
283 were related to catalytic activity and the second dominant group of proteins were assigned to
284 a binding function (27%). These proportions agree with previous proteomic studies of human,
285 ram, carp and boar seminal plasma (Dietrich et al., 2014; Pérez-Patiño et al., 2016; Pilch and
286 Mann, 2006; Souza et al., 2012). Inside the category of catalytic activity, the aminopeptidase
287 B protein is included. This enzyme has an important role in rabbit AI because it is responsible
288 for degrading the GnRH analogue when it is added to the seminal dose to induce doe
289 ovulation. In previous works, we have demonstrated that aminopeptidase activity in rabbit
290 seminal plasma reduces the bioavailability of the GnRH analogue (Viudes-de-Castro et al.,
291 2014) and new extenders with aminopeptidase inhibitors are being developed (Casares-
292 Crespo et al., 2016b, 2017). Regarding biological process, the metabolic (28%) was the most
293 abundant category in rabbit SP, which coincides with human, carp and boar SP proteins
294 (Dietrich et al., 2014; Pérez-Patiño et al., 2016; Souza et al., 2012). It is also noticeable that
295 only 6 of the 402 proteins identified in rabbit SP are to date recorded in GO as being directly
296 associated with reproductive processes. Similar results were found in boar SP, where only 20
297 of the 374 proteins identified were annotated as related to reproduction (Pérez-Patiño et al.,
298 2016).
299 To date, recent research on seminal plasma of major domestic mammalian species
300 (Aquino-Cortez et al., 2017; Druart et al., 2013; Pérez-Patiño et al., 2016; Pini et al., 2016;

Souza et al., 2012), human (Pilch and Mann, 2006) and fish (Dietrich et al., 2014; Gombar et al., 2017; Nynca et al., 2017), including semen quality (Sarsaifi et al., 2015), fertilizing markers (Kwon et al., 2015) and freezability (Dietrich et al., 2017; Vilagran et al., 2015) have been reported. Nevertheless, it is unknown at present if there is a variation in seminal plasma protein composition among genotypes within the same species. Our results clearly indicate that SP proteins abundance in rabbit seems to be related to a specific genotype, which in several previous studies have demonstrate differences in sperm quality, fertility and prolificacy (Safaa et al., 2008; Vicente et al., 2000). As stated, we identified a higher abundance of 11 proteins in genotype A seminal plasma, while another 12 proteins were more abundant in genotype R seminal plasma.

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

spermatozoa and the enhanced fertility and prolificacy previously described in genotype A (Lavara et al., 2005; Safaa et al., 2008).

Other over-expressed proteins in line A such as plastin 1 and ubiquitin carboxyl-terminal hydrolase have been found related to spermatogenesis in other species (Kwon et al., 2004; Li et al., 2015). Plastins are a family of actin binding proteins known to cross-link actin microfilaments in mammalian cells, creating actin microfilament bundles necessary to confer cell polarity and cell shape (Li et al., 2016). There are three types of plastins: plastin 1, 2, and 3. All three are expressed in Sertoli cells and plastin 1 and 2 in testes germ cells (Li et al., 2016). Plastin protein has been found in boar seminal plasma exosomes (Piehl et al., 2013) and in rat testis (Li et al., 2015). Plastin 1 deficient mice were fertile and displayed a normal reproductive rate (Grimm-Gunter et al., 2009), what suggests an additional role of plastin far from the fertility process. On the other hand, ubiquitin carboxyl-terminal hydrolase isozyme 3 may function in the meiotic differentiation of spermatocytes into spermatids (Kwon et al., 2004).

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

recovery and performance of thawed semen from genotype A compared to R (Mocé et al., 2003).

The rest of the over-expressed proteins in line A were enzymes such as carbonic anhydrase 2, which has been found to have a role in the regulation of bicarbonate concentration in horse seminal plasma and accordingly regulate seminal plasma pH (Asari et al., 1996), aspartate aminotransferase (AST) which is an important regulator of glutamate (http://www.uniprot.org/uniprot/P33097) and peptidyl-prolyl cis-trans isomerise which keeps in an inactive conformation of the TGF-beta type I serine/threonine kinase receptor, preventing TGF-beta receptor activation in absence of ligand (http://www.uniprot.org/uniprot/P26883).

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

Regarding the effect of the season on the rabbit seminal plasma proteome, a previous study showed a season effect on the abundance of three proteins (FAM115E-like, haemoglobin subunit zetalike and nerve growth factor) (Casares-Crespo et al., 2016a), but again, these relative quantity protein differences were obtained with a less resolute proteomic technique. In the current work, results showed that there are slight protein differences between seasons but it does not exist a clear pattern of protein variation between genotypes. This lack of variation could be explained by the controlled environmental conditions used in our study where animals were kept under 16 h light/8 h dark and maintained between 14ºC and 28ºC using cooling and heating systems over the year.

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

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

5. Conclusions

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

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Figure Legends

Figure 1. Picture of rabbit genotypes R (left) and A (right).

Figure 2. Experimental design scheme.

Figure 3. Pie charts showing the distribution of rabbit seminal plasma proteins based on their a) molecular function, b) biological process and c) cellular component, using UniProt database in combination with PANTHER.

Figure 4. Partial Least Squares Discriminant Analysis (PLS-DA) showing the classification of seminal samples from genotypes A and R, based on relative protein amount.

Figure 5. Heat map representing levels of differentially expressed seminal plasma proteins between genetic origins A and R and hierarchical clustering, showing two main clusters comprising genotype A and R.

Figure 6. Partial Least Squares Discriminant Analysis (PLS-DA) showing the classification of seminal samples belonging to the four seasons, based on relative protein amount.

Figure 7. Heat map representing levels of differentially expressed seminal plasma proteins between seasons and hierarchical clustering, showing four main clusters comprising a mixture of samples from different seasons.

Supporting Information

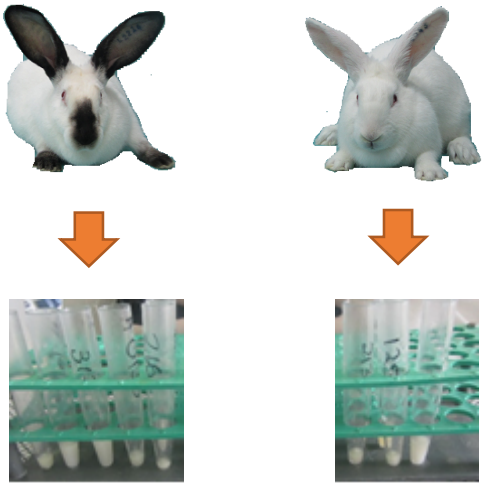
Supporting Information Table 1 contains the complete list of the 402 proteins identified in rabbit seminal plasma with a cut off of two unique peptides and validated with ≥ 95% Confidence (unused Score ≥ 1.3).

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

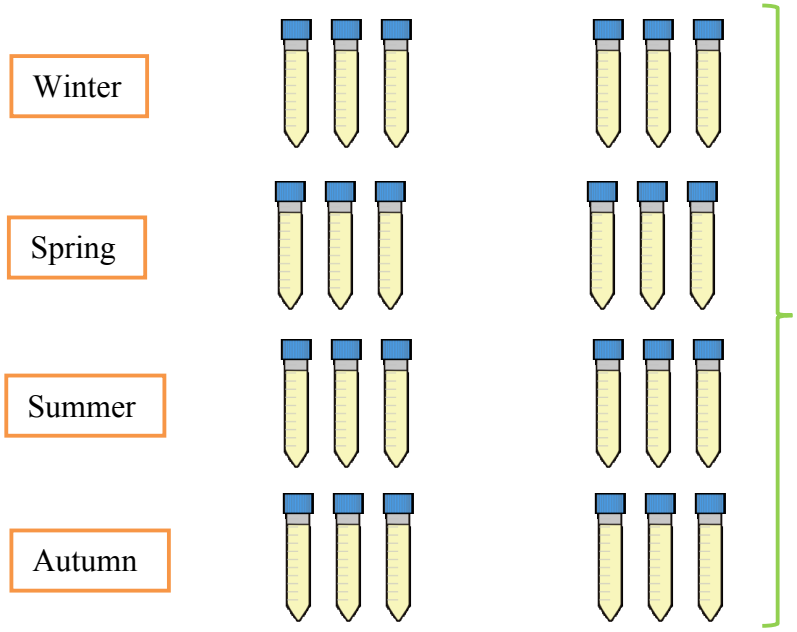
Supporting Information Table 3 shows the results of the protein quantity T-test comparison between genotypes, including mean protein quantity, t-value, p-value, fold change and log (fold change) of the 402 quantified proteins.

Figure 1

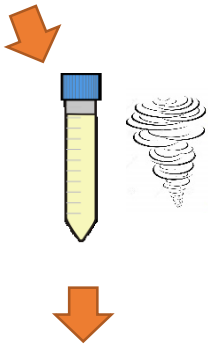
Figure 2



Semen samples were recovered from January to December 2014.



For each genotype, one pooled sample at the beginning, middle and end of each season was selected to develop the experiment. The selected samples were collected the exact same day in both genotypes.

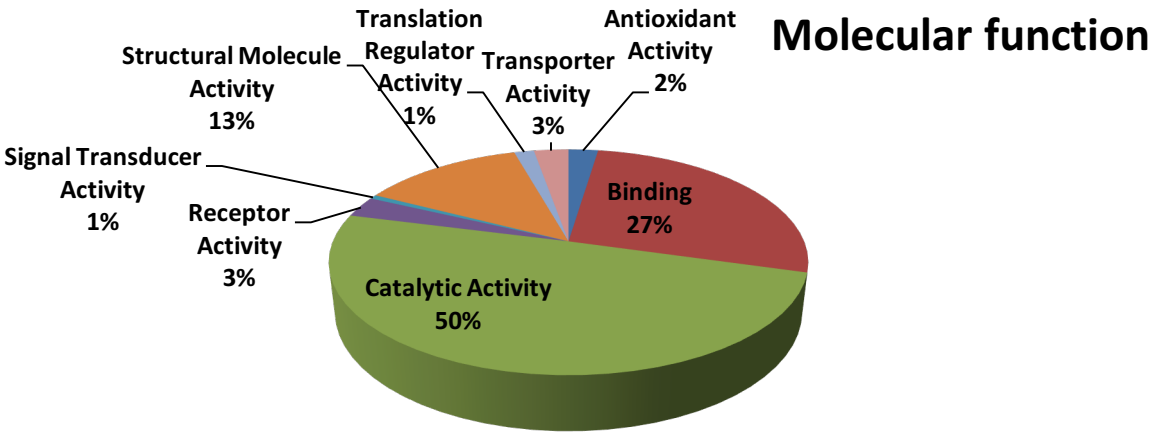


Seminal plasma from selected samples was recovered by centrifugation.

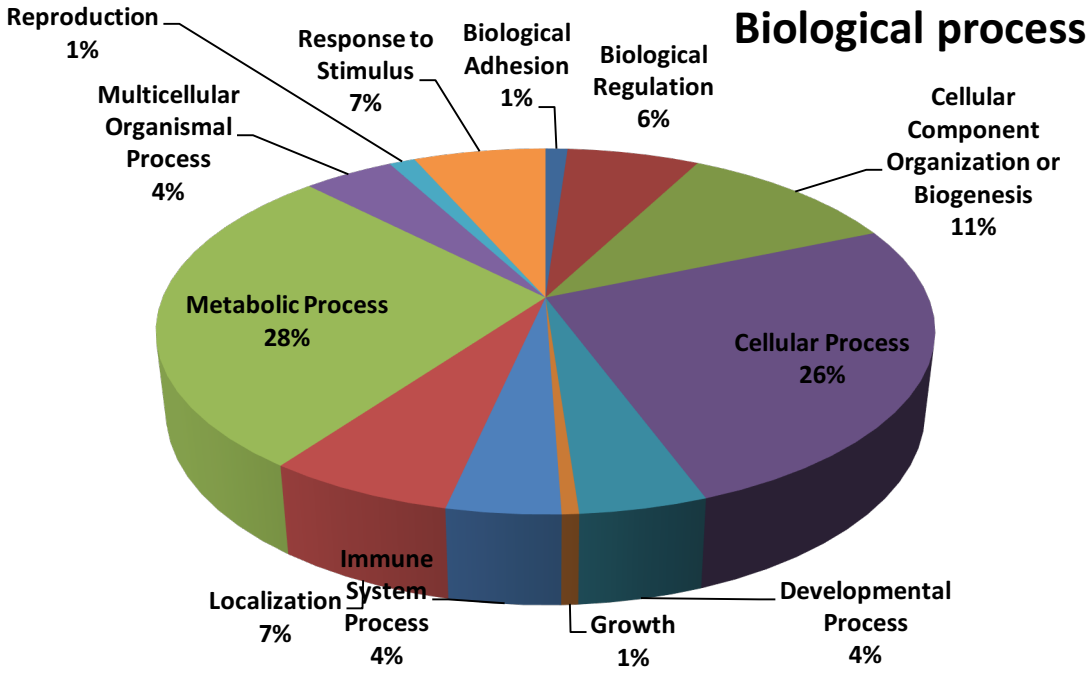
Three biological replicates were used for each season and genotype and subjected to nano LC/MS-MS. In total, 24 samples were analysed.

Figure 3

a)


Molecular function
Translation Regulator Activity 1%
Antioxidant Activity 2%
Structural Molecule Activity 13%
Transporter Activity 3%
Signal Transducer Activity 1%
Binding 27%
Receptor Activity 3%
Catalytic Activity 50%

b)


Biological process
Reproduction 1%
Response to Stimulus 7%
Biological Adhesion 1%
Biological Regulation 6%
Multicellular Organismal Process 4%
Cellular Component Organization or Biogenesis 11%
Metabolic Process 28%
Cellular Process 26%
Immune System Process 4%
Localization 7%
Growth 1%
Developmental Process 4%

c)

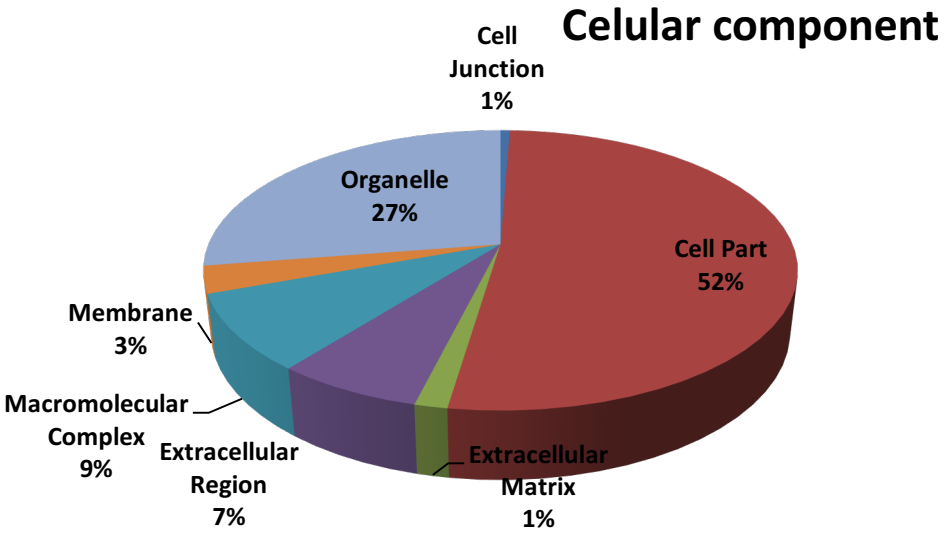

Celular component
Cell Junction 1%
Organelle 27%
Cell Part 52%
Membrane 3%
Macromolecular Complex 9%
Extracellular Region 7%
Extracellular Matrix 1%

Figure 4

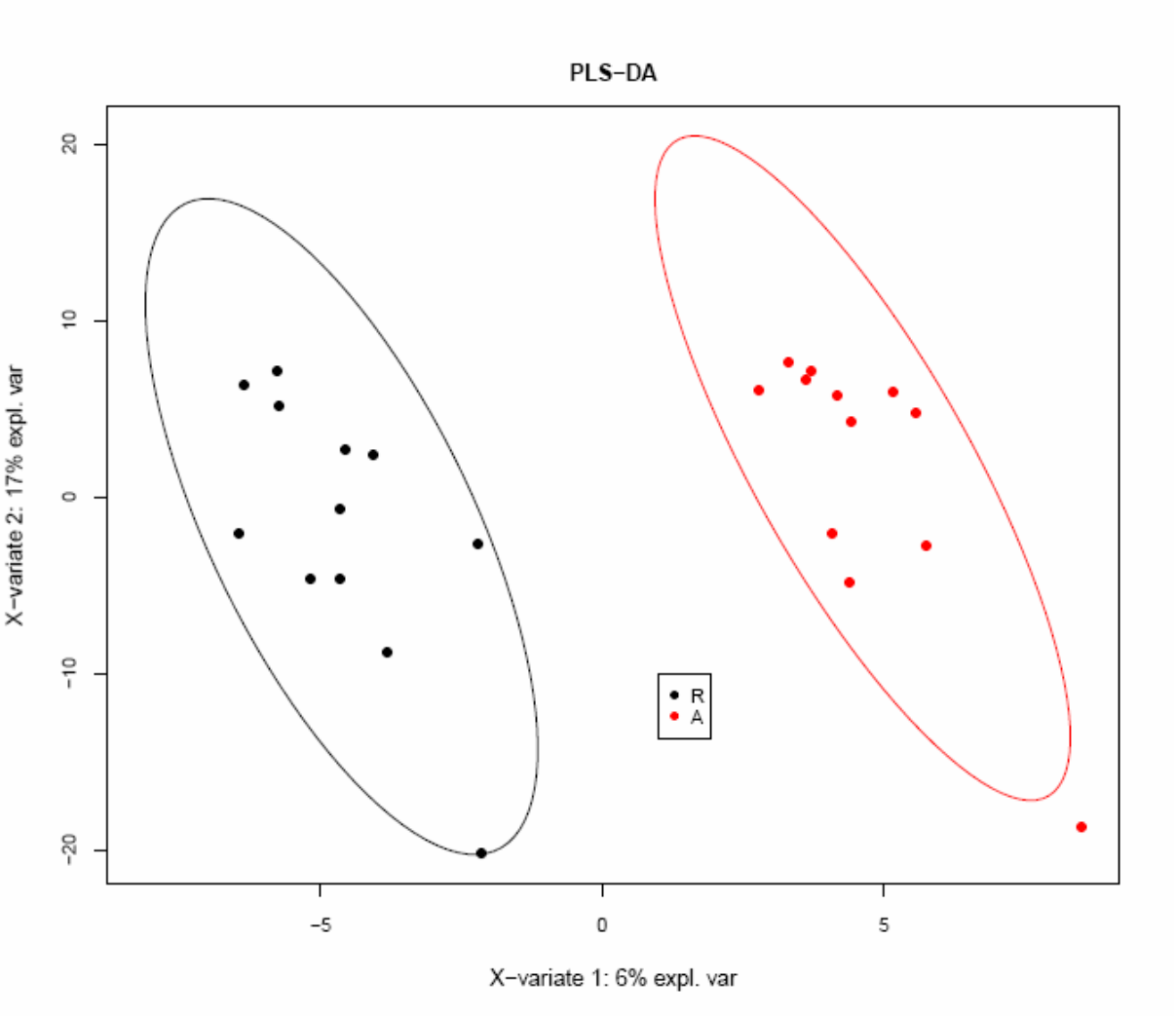

PLS-DA
X-variate 2: 17% expl. var
X-variate 1: 6% expl. var
R
A
20
10
0
-10
-20
-5
0
5

Figure 5

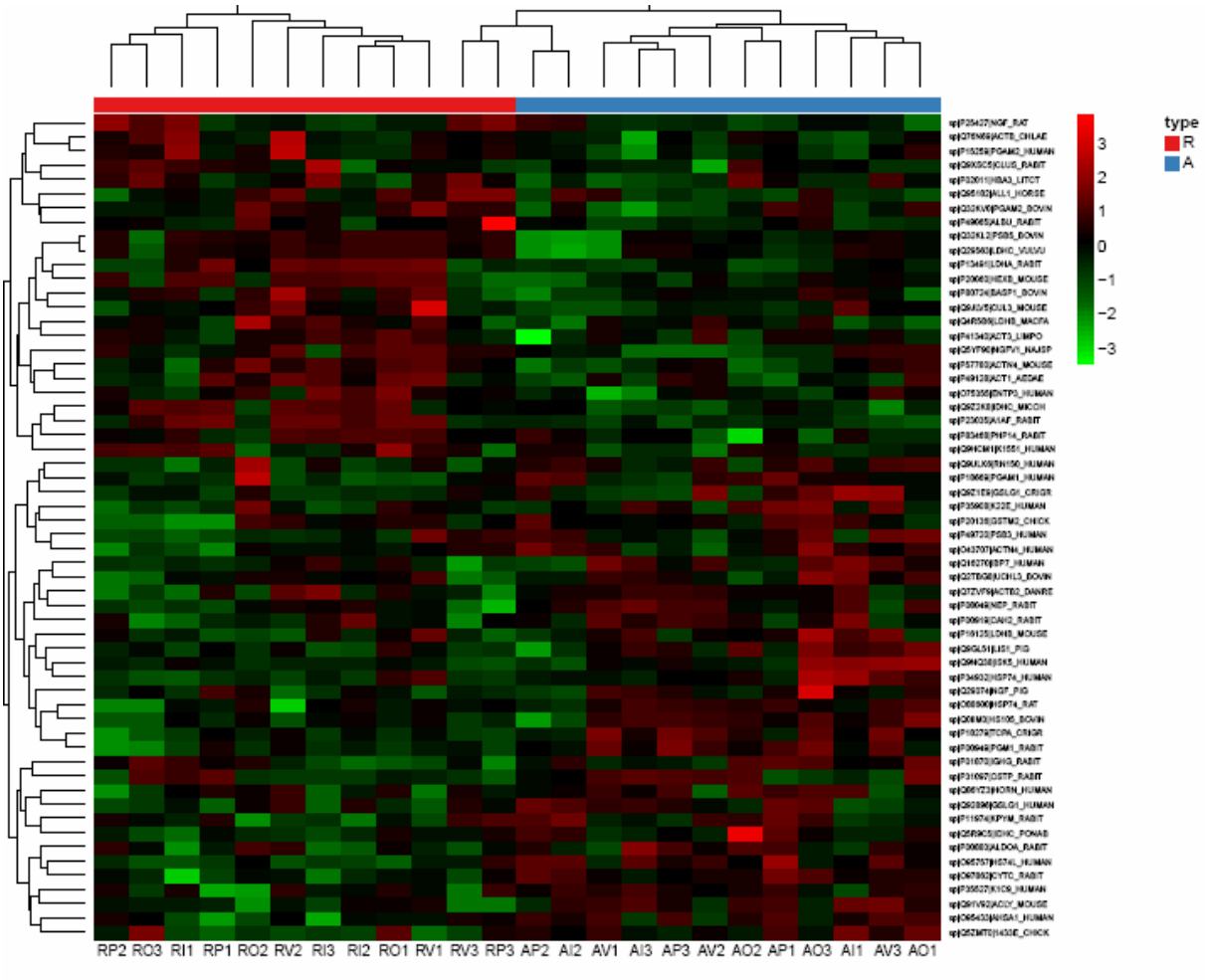
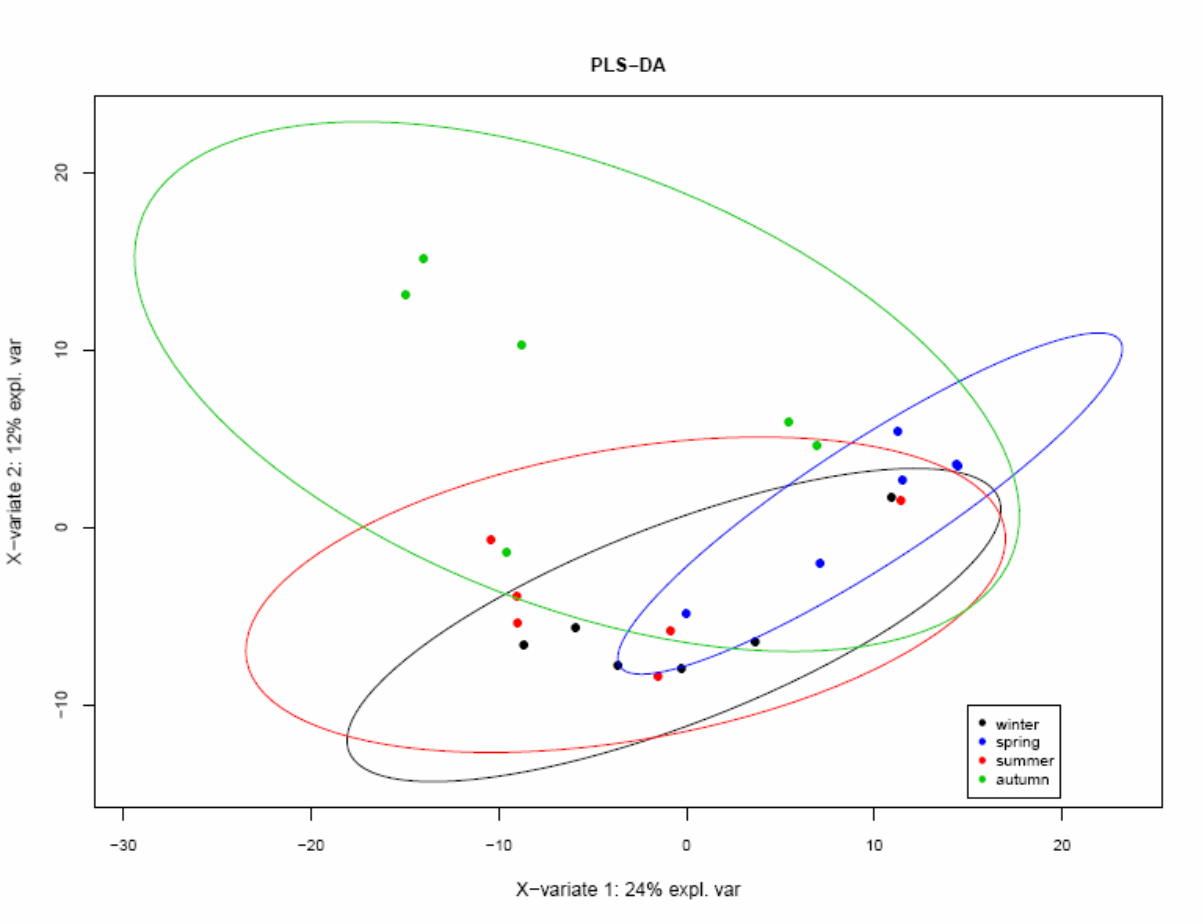
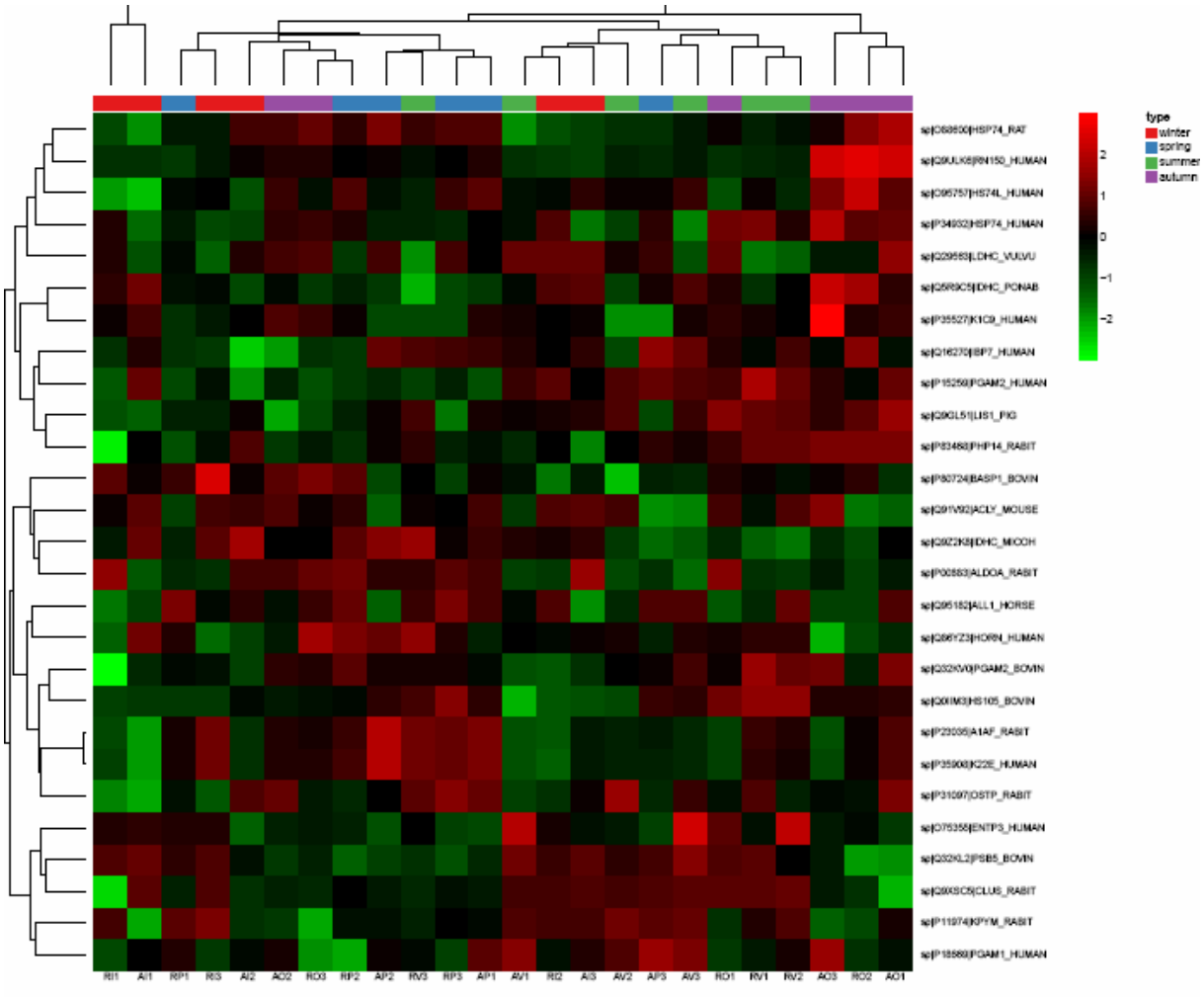

type
R
A
3
2
1
0
−1
−2
−3
RP2 RO3 RI1 RP1 RO2 RV2 RI3 RI2 RO1 RV1 RV3 RP3 AP2 AI2 AV1 AI3 AP3 AV2 AO2 AP1 AO3 AI1 AV3 AO1

Figure 6


PLS-DA
X-variate 2: 12% expl. var
X-variate 1: 24% expl. var
winter
spring
summer
autumn
20
10
0
-10
-30
-20
-10
0
10
20

869 **Figure 7**

870



871 **Table 1.** List of differentially expressed proteins in rabbit seminal plasma between genotypes

872 A and R.

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

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

873

874

875 **Highlights:**

876 -First in-depth characterization of rabbit seminal plasma proteome.

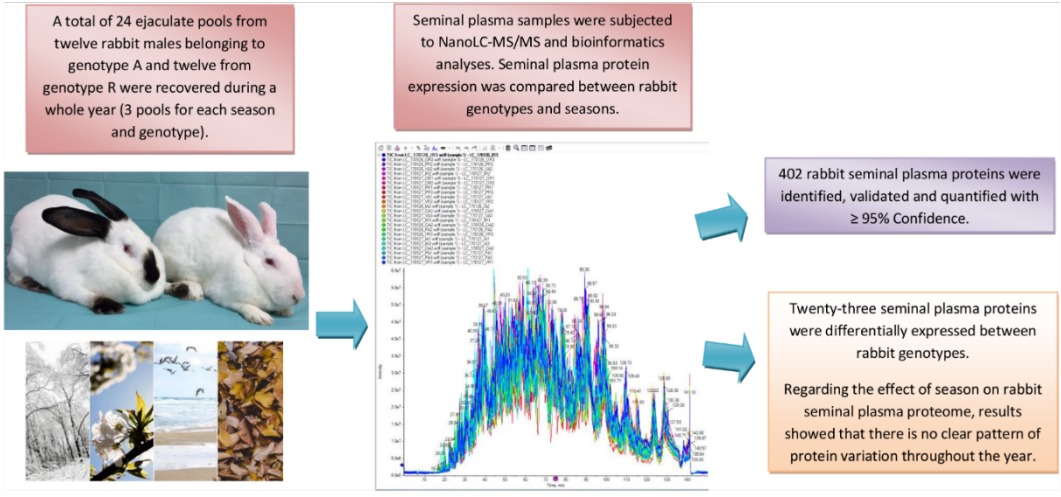
877 -402 proteins were identified and quantified in rabbit seminal plasma.

878 -Genotype is related to specific proteins abundance in seminal plasma.

879 -A publicly accessible database of the rabbit seminal plasma proteome was created.

880

881 **Graphical Abstract**



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