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Selection for higher fertility reflects in the seminal fluid proteome of modern domestic chicken

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ABSTRACT

The high egg-laying capacity of the modern domestic chicken (i.e. White Leghorn, WL) has arisen from the low egg-laying ancestor Red Junglefowl (RJF) via continuous trait selection and breeding. To investigate whether this long-term selection impacted the seminal fluid (SF)-proteome, 2DE electrophoresis-based proteomic analyses and immunoassays were conducted to map SF-proteins/cytokines in RJF, WL and a 9th generation Advanced Intercross Line (AIL) of RJF/WL-L13, including individual SF ($n = 4$, from each RJF, WL and AIL groups) and pools of the SF from 15 males of each group, analyzed by 2DE to determine their degree of intra-group (AIL, WL, and RJF) variability using Principal Component Analysis (PCA); respectively an inter-breed comparative analysis of intergroup fold change of specific SF protein spots intensity between breeds. The PCA clearly highlighted a clear intra-group similarity among individual roosters as well as a clear inter-group variability (e.g. between RJF, WL and AIL) validating the use of pools to minimize confounding individual variation. Protein expression varied considerably for processes related to sperm motility, nutrition, transport and survival in the female, including signaling towards immunomodulation. The major conserved SF-proteins were serum albumin and ovotransferrin. Aspartate aminotransferase, annexin A5, arginosuccinate synthase, glutathione S-transferase 2 and L-lactate dehydrogenase-A were RJF-specific. Glyceraldehyde-3-phosphate dehydrogenase appeared specific to the WL-SF while angiotensin-converting enzyme, γ-enolase, coagulation factor IX, fibrinogen α-chain, hemoglobin subunit α-D, lysozyme C, phosphoglycerate kinase, Src-substrate protein p85, tubulins and thioredoxin were AIL-specific. The RJF-SF contained fewer immune system process proteins and lower amounts of the anti-inflammatory/immunomodulatory TGF-β2 compared to WL and AIL, which had low levels- or lacked pro-inflammatory CXCL10 compared to RJF. The seminal fluid proteome differs between ancestor and modern chicken, with a clear enrichment of proteins and peptides related to immune-modulation for sperm survival in the female and fertility.

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1. Introduction

Semen is composed of a complex mixture of organic and inorganic components built by secretions of the male reproductive organs, which the ejaculated spermatozoa bathe in (Mann et al., 1982; Blesbois and Hermier, 1990). In mammals, seminal plasma contains a huge bulk of proteins and peptides (25–60 g/L) per ejaculate, mostly derived from the accessory sex glands (Batruch et al., 2011; Rodríguez-Martínez et al., 2011). In the chicken, due to a lack of accessory sex glands, with the exception of the vascular bodies in the cloaca which resemble the mammalian bulbo-urethral glands, the seminal fluid derives from the testis, the rudimentary epididymis and the ductus deferens (Fujihara, 1992; Etches, 1996) and contains a 10-fold lower protein load than mammals (2.0–2.4 g/dL) (Harris and Sweeney, 1971; Thurston et al., 1982). In mammals, seminal plasma proteins have been ascribed a range of diverse functions, including germicidal effects, promotion of sperm survival, assistance in sperm interactions with different microenvironments in the female genital tract, and the signaling to the immune system of the female

Abbreviations: WL, White Leghorn; RJF, Red Junglefowl; AIL, Advanced intercross line; SF, seminal fluid; TGF-β2, transforming growth factor-beta 2; CXCL10, C-X-C motif chemokine 10.

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so that a state of maternal tolerance to foreign spermatozoa (and embryos and placentae) is established (Rodríguez-Martínez et al., 1998, 2008, 2011; Novak et al., 2010; Kareskoski et al., 2011; Caballero et al., 2012; Druart et al., 2013; Milardi et al., 2013; Rodrigues et al., 2013; Sharma et al., 2013; Bromfield et al., 2014). Whether such roles can also be ascribed to avian seminal fluid (SF) proteins remains to be explored.

Proteomic research in Reproductive Biology, accelerated by methodological developments in mass spectrometry (Calvete et al., 1994; Kelly et al., 2006; Pilch and Mann, 2006; Druart et al., 2013; Rodrigues et al., 2013; Sharma et al., 2013), has increased our knowledge of the composition of seminal plasma in mammals, revealing relevant roles for sperm function (Soggiu et al., 2013; Soler et al., 2016) and immune modulation, including the role of specific cytokines and chemokines (Schjenken and Robertson, 2014; Crawford et al., 2015) with relevance for fertility. Proteomic studies investigating avian sperm and ejaculates are comparatively scarce, yet those that do exist assess the potential correlations with sperm motility (Froman et al., 2011), attempt to identify and classify proteins (Marzoni et al., 2013), or correlate the proteome to the semen phenotype (Labas et al., 2015). Few studies (Marzoni et al., 2013; Labas et al., 2015) describe seminal plasma proteins (e.g. beta-defensin as gallinacins, ovotransferrin, serum albumin and peroxiredoxin-6) and their role in antimicrobial activity and sperm survival (Das et al., 2011). Comparative cytokine/chemokine studies are also restricted to cytokine expression in avian testis (Ocón-Grove et al., 2010; Michailidis et al., 2014) and the oviduct. In the latter, pro-inflammatory cytokines (tumor necrosis factor (TNF) and interleukin (IL)-1β) as well as immunosuppressive cytokines (as transforming growth factor (TGF)-β) and their receptors are expressed in the vagina and utero-vaginal-junction respectively, after seminal deposition (Das et al., 2006, 2008, 2009). The lack of cytokine mappings in the chicken SF, which might be an important factor for sperm function, comparative to what is known in mammals (Barranco et al., 2015), is thus required.

Fertility varies among animals, irrespective of classes. Red Junglefowl (RJF), the wild progenitor of modern domestic chicken breeds, has a low fertility, expressed in terms of fertile oviposition rates per season, while domestic modern laying chicken breeds such as the White Leghorn (WL) display the opposite situation, with high laying/fertility rates. Sperm quality and fertility varies among males, particularly when considering the pressure of selection applied. Interestingly, when a chicken breed is selected for a particular trait — as egg production, a decrease in semen quality has been recorded in the male line (Murugesan et al., 2013). Comparative studies have shown differences in sperm concentration and forward sperm motility between wild RJF and domestic chicken with an advantage for the RJF, without consideration of eventual roles for the seminal fluid (Malik et al., 2013). The composition of the seminal fluid/plasma also varies with varying fertility among males, even if definite links with reproductive outcome are yet to be fully established. Of even greater relevance, semen deposition elicits gene expression shifts in the female genital tract (Fazeli et al., 2004; Atikuzzaman et al., 2015b), calling for the identification of the pertinent signals involved, including components of the SF-proteome that could be related to sperm survival and immunomodulation by peptides as cytokines, an as yet undiscovered chapter in the rooster SF.

The present study aims to map the proteome, including cytokines, of the seminal fluid of the ancestor RJF ($n = 31$ birds), of the highly-selected modern WL ($n = 20$ birds), and of an AIL ($n = 23$ birds) — intercross between wild and domestic chicken (RJF × WL-L13), with clear differences in egg-laying/fertility, evolved during selection. The SF was analyzed for protein profiles as ejaculate pools per breed (15 males/breed) to minimize individual variation or as individual ejaculates (39–79 ejaculates/breed) or pool of ejaculates of a male (10 males/breed) for quantitation of cytokines.

2. Material and methods

2.1. Animals and seminal fluid source

Sexually mature, proven fertile roosters from a RJF-pure line, a pure WL line and from a 9th generation AIL — intercross between RJF and WL-L13 lines have been used (see Johnsson et al., 2012 for details of the cross and breeds used as well as for details on rearing and breeding routines). Food and water were available ad libitum and the chicken were held under controlled temperature and light regimes (12 h light: 12 h darkness cycle, 5 lx) in 1–2 m^2 pens depending on age for their first seven weeks, in compliance with European Community (Directive 2010/63/EU) and Swedish (SJVFS 2012:26) current legislation. Throughout all experiments, animals were handled carefully to avoid any unnecessary stress. The experiments were approved by the 'Regional Committee for Ethical Approval of Animal Experiments' (Linköpings Djurförsöksetiska nämnd) in Linköping, Sweden (permit no. 75–12).

Semen was collected from pre-trained roosters by manual abdominal massage. The success of ejaculation was confirmed by extending 2 μL of semen in Dulbecco's medium (1:10 *v*/*v*) for examination on a Carl Zeiss microscope equipped with a thermal plate (41^{0}C) and positive phase contrast optics (10× objective). Ejaculates mixed with faeces or blood or lacking live sperm were excluded from the study. Semen from 31 RJF (45 ejaculates), 23 RJF/WL-L13 (43 ejaculates) and 20 WL (79 ejaculates) roosters were finally selected for the study. The selected ejaculates were centrifuged at + 5 °C, 21,000 × *g* for 10 min immediately after collection to harvest the extra-cellular SF-supernatant, which was thereafter transferred to liquid nitrogen (LN_2) before storage at —80^{0}C prior to analysis.

2.2. Identification of proteins by 2DE followed by mass spectrometry

Seminal fluid samples collected from four animals per group (breed) were individually analyzed by 2DE to determine their degree of individual variability. As well, pools of SF from 15 males of each group (breed) were built a group/breed sample. The rationale behind this pooling was our interest in unveiling SF proteome differences that may underline a breed population fitness trait, rather than individual differences, and thus to minimize confounding individual intra- and inter-male variation. Each breed pool was analyzed in triplicate by 2DE and only spots consistently found in all three experiments were considered in the subsequent inter-breed comparative analysis.

2.2.1. Sample preparation

Sample preparation: 150 μg of SF from each breed pool (RJF, WL and AIL) was subjected to cleansing using a 2D clean-Up kit (GE Healthcare). The cleansed SF of each breed was suspended in 90 μl of rehydration solution containing 7 M urea, 2 M thiourea, 4% (*w*/*v*) CHAPS, 0.5% (*v*/*v*) IPG buffer, 40 mM DTT plus 1 μL of bromophenol blue. The solution, after centrifugation at 13,000 × *g* for 5 min, was loaded into a prior overnight rehydrated (rehydration solution containing 7 M urea, 2 M thiourea, 2% *w*/*v* CHAPS, 0.5% *v*/*v* IPG buffer, 0.002% bromophenol blue and 40 mM DTT) seven cm long IPG strip (pH range 3–10).

2.2.2. 2DE (two-dimensional gel electrophoresis)

2DE (two-dimensional gel electrophoresis): The isoelectric focusing was carried out following Calvete et al. (2009) at 20^{0}C and 50 μA using Ettan IPGphor 3 (GE Healthcare) with a modification of running schedule: First step and hold, 500 V (125 Vh); Second gradient, 1000 V (500 Vh); and Third gradient, 5000 V (6666 Vh). Prior to perform second dimension sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the IPG strip was equilibrated for 7 min twice in SDS equilibration buffer solution containing 6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol (*v*/*v*), 2% SDS (*w*/*v*) and 0.002% bromophenol blue (*w*/*v*) with a gentle shaking at 15 rotations per min.

The IPG strips were then placed on a SDS polyacrylamide 4% stacking gel (0.75 mm thick) with a SDS polyacrylamide 15% separation gel (1.5 mm thick), according to Laemmli (1970), into a vertical SDS-PAGE gel slab filled with electrode buffer. A 200 kDa molecular weight marker (Mark12™, Invitrogen Corporation) was loaded on to the stacking gel before running the electrophoresis. The electrophoresis was performed at 100 V until the dye reached to the bottom of the separating gels (Calvete et al., 2009).

2.2.3. Protein spot visualization

Protein spot visualization: 2DE gels of individual and pooled SF samples were stained with Coomassie Brilliant Blue G-colloidal at room temperature on a plate shaker at 15 rpm until the spots were visible. The stained gels were then washed with deionized water until the backgrounds became clear. Gels were stored for further analysis immediate after scanning with a high resolution scanner, LabScan (Amersham Pharmacia Biotech, Sweden). The scanned 2D–gel images were analyzed for intragroup (AIL, WL, and RJF) variability using MarkerView™ 1.2.1 Software (AB Sciex, Ontario, Ca), and for intergroup fold change of specific protein spots intensity comparing AIL and WL with RJF, using SameSpots (TotalLab Ltd. Newcastle, UK).

Protein digestion and mass spectrometry: Immediately after scanning, the visible protein spots on 2D gels were cut and subjected to automated reduction with DTT and alkylation with iodoacetamide and digestion with sequencing grade modified porcine trypsin (Promega) using a ProGest™ digestor (Genomic Solutions) following the manufacturer guidelines. The dried digests were re-suspended with 8 μl of 0.1% formic acid, and 6 μl aliquots injected and analyzed by Liquid Chromatography-Electrospray Ionization-Quadropole-Time-of-Flight-Mass Spectrometry (LC-ESI-Q-TOF-MS/MS) using a Synapt G2 instrument (Waters, Manchester, UK) hyphenated to a liquid nano-chromatographic separation system (NanoACQUITY-UPLC, Waters) equipped with a C18 column (100 μm × 100 mm, 1.7 μm particles (BEH130 C18, Waters) operated at a flow rate of 0.6 μL/min. The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). The optimized ultra-performance liquid chromatography (UPLC) elution conditions were 0–1 min 1–12% B; 1–16 min, 12–40% B; 16–18 min, 40–85% B; 18–20 min, 85–1% B; 20–30 min, 1% B. The autosampler was maintained at + 10 °C. All the analyses were performed in data dependent analysis (DDA) mode that automatically triggers the MS/MS experiments. The scan range was from 100 to 2000 *m*/*z*. For positive nanospray mode, the capillary and cone voltage were set at 3 kV and 28 V respectively. The deviation gas was set at 100 L/h, the cone gas at 10 L/h and the source temperature at + 100^{0}C. The mass spectrometer was operated in V-optics mode with 20,000 resolution using dynamic range extension. The data acquisition rate was set to one scan time. All analyses were acquired using the LockSpray to ensure accuracy and reproducibility. Leucine-enkephalin was used as the lockmass at a concentration of 200 ng/mL and a flow rate of 0.5 μL/min. Data were collected in continuum mode and the spray frequency was set at 30 s. The data acquisition and spectra were analyzed by MassLynx4.1 (Waters).

The *Gallus gallus* SF-proteins were identified from their peptide sequences using MASCOT MS/MS Ions Search Engine version 2.5 (Matrix Science, Boston, MA) along with the latest updated version of Swiss-Prot protein database (UniProtKB/Swiss-Prot). Carbamidomethyl cysteine and oxidized methionine residues were selected as fixed and variable modifications, respectively. The peptide tolerance and the MS/MS tolerance were set at 1.2 Da and 0.6 Da, respectively and peptide charges

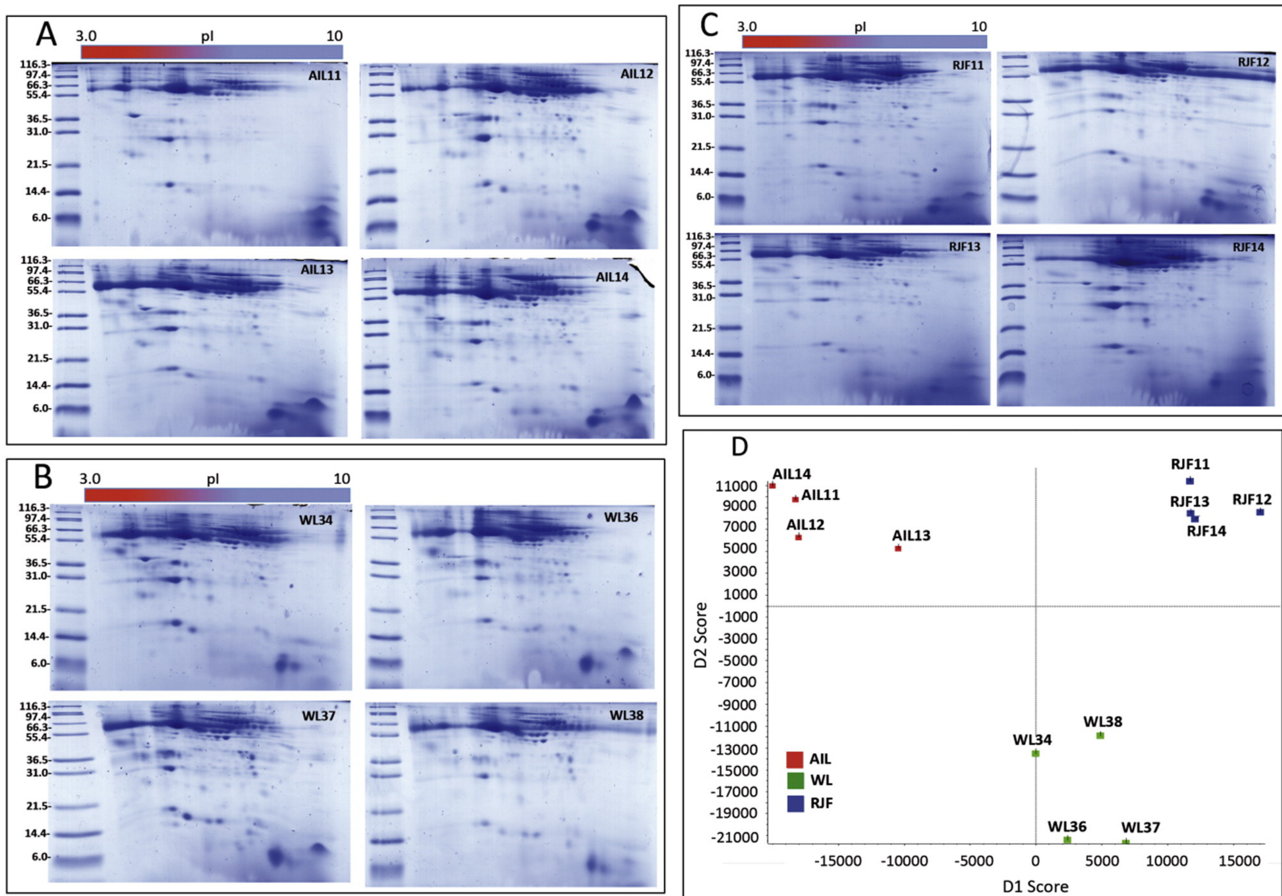


Fig. 1. Coomassie Brilliant Blue stained 2D gels of the seminal fluid of individual males ($n = 4$) from each the Advanced Intercross Line (AIL, panel A), White Leghorn (WL, panel B), and Red Junglefowl (RJF, panel C) groups. Panel D presents a PCA plot showing the overall degree of intra- and intergroup variability. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

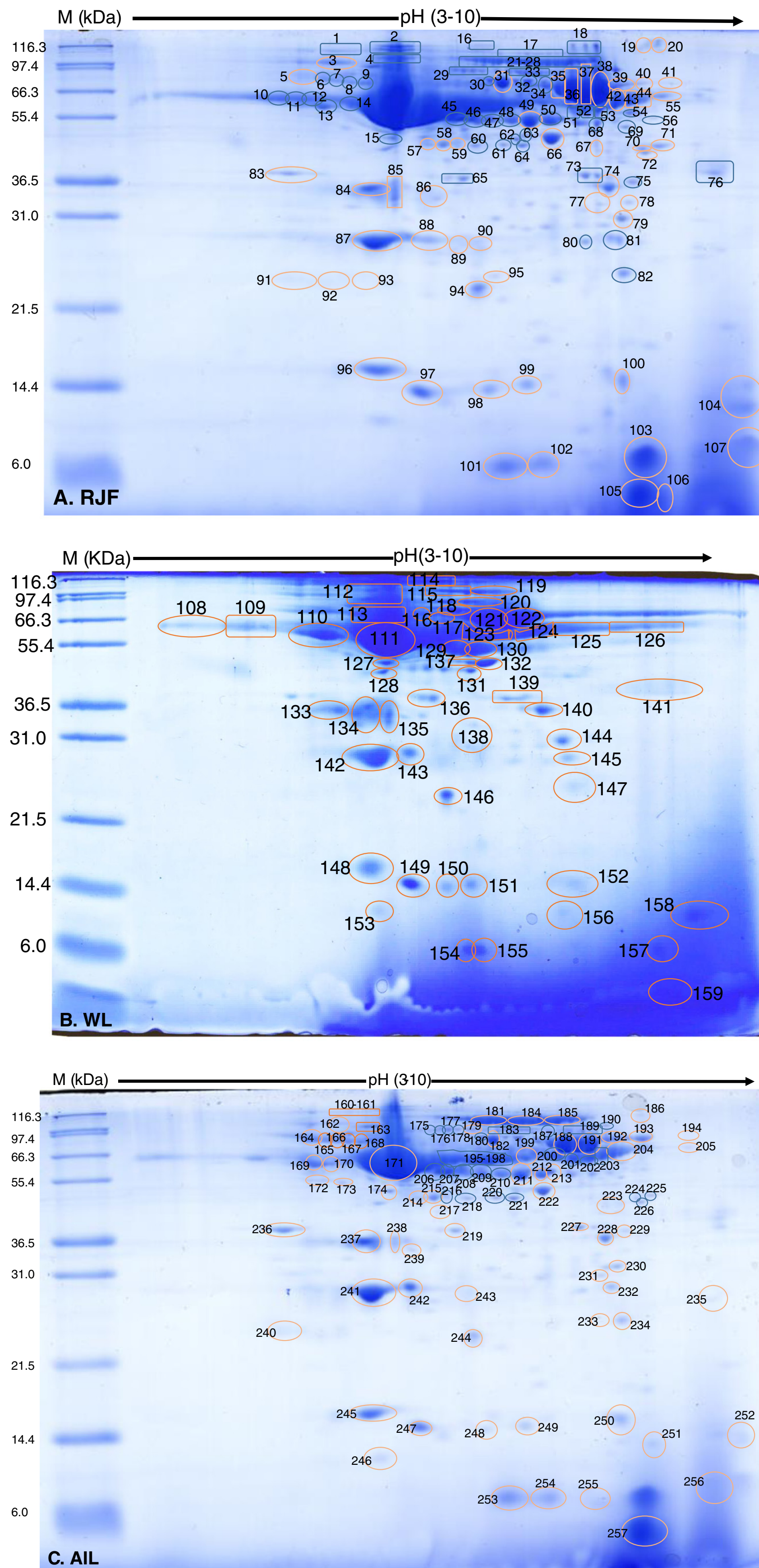


Fig. 2. Coomassie Brilliant Blue stained 2D gels of pooled seminal fluid of Red Junglefowl (A, $n = 15$), White Leghorn (B, $n = 15$) and Advanced Intercross Line (C, $n = 15$) roosters. The gel spots (see the numerical numbers for each spot) were examined by LC-ESI-Q-TOF-MS/MS followed by Mascot ions search using SwissProt high quality, curated protein database. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were set at 2^+, 3^+ and 4^+. Trypsin digestion (allowing two possible missed cleavages) and taxonomy of bony vertebrate were set. The data format and instrument information was set as Micromass (PKL) and ESI-QUAD-TOF, respectively along with decoy. The ion scores have been reported as -10xlog 10 (P) where P is the probability that the observed match is a random even. Individual MS/MS peptide ion scores >39 indicated identity or extensive homology ($P < 0.05$) for the MS/MS ion search have been taken into consideration for protein identification. Identified proteins underwent bioinformatic analysis using the PANTHER classification system (Version 10.0) (Mi et al., 2016).

2.3. Measurement of the concentrations of cytokines and chemokines

Presence and relative concentration of a battery of cytokines and chemokines including IL-6, CXCL8 (IL-8), CCL2 (monocyte chemoattractant protein-1, MCP-1) or the growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF, associated with a pro-inflammatory immune response); the anti-inflammatory IL-10 and TGF-β1, TGF-β2 and TGF-β3; the Th1-associated IFN-γ and CXCL10 (interferon gamma-induced protein, IP-10); the Th2-associated CCL22 (macrophage-derived chemokine, MDC); and the Th17-associated IL-17 and CXCL1 (growth-regulated oncogene, GRO), as well as the inducer of NK cell proliferation IL-15 were first examined in the SF of individual ejaculates of RJF, WL and AIL roosters ($n > 15$ birds per breed, range: 15–79 ejaculates) using a multiplexed microsphere-based flow cytometric assay (Luminex's xMAP®) following the protocol of Barranco et al. (2015). In brief, pre-coated magnetic beads (Cat#HCYTOMAG-60 K-11 for human reactivity, Merck Millipore, Billerica, MA, USA) were used for the determination of cytokines and chemokines except TGF-βs, while a 3-plex kit (Cat#TGFB-64 K-03 for pig, human, mouse, rat, non-human primate, canine, feline reactivity, Merck Millipore) were used for TGF-βs following the methods

Table 1
Comparative identified proteins in chicken seminal fluid detected by 2D SDS-PAGE followed by mass spectrometry. Total protein spots on 2D gels -107 of RJF, 52 of WL and 98 of AIL — were analyzed using LC-ESI-Q-TOF-MS/MS. Percent calculated from total number of spots analyzed in each breed.

Proteins identified in the chicken seminal fluid	Proteins detected in the seminal fluid % (total number of spots identified for a protein)			Spot number(s) on acrylamide gels
	RJF	WL	AIL	
Aspartate aminotransferase, cytoplasmic	1.87 (2)	0 (0)	0 (0)	71, 72
Angiotensin-converting enzyme (Fragment)	0 (0)	0 (0)	1.02 (1)	161
Serum albumin	11.21 (12)	21.15 (11)	13.27 (13)	2, 4, 7, 9, 10, 12, 14, 16, 42, 51, 52, 53, 108, 109, 111, 112, 113, 114, 116, 117, 124, 125, 126, 169, 170, 171, 182, 188, 195, 196, 199, 212, 213, 214, 217, 246
Apolipoprotein A-I	1.87 (2)	5.78 (3)	4.08 (4)	80, 90, 142, 143, 147, 237, 241, 242, 244
Annexin A5	0.93 (1)	0 (0)	0 (0)	86
Argininosuccinate synthase	0.93 (1)	0 (0)	0 (0)	62
Astacin-like metalloendopeptidase	0 (0)	1.92 (1)	1.02 (1)	141, 252
Complement factor B-like protease (Fragment)	0 (0)	1.92 (1)	1.02 (1)	120, 184
Cystatin	0.93 (1)	1.92 (1)	3.06 (3)	100, 152, 248, 250, 251
Alpha-enolase	1.87 (2)	1.92 (1)	1.02 (1)	57, 66, 132, 222
Beta-enolase	0.93 (1)	0 (0)	1.02 (1)	66, 222
Gamma-enolase	0 (0)	0 (0)	1.02 (1)	222
Coagulation factor IX	0 (0)	0 (0)	1.02 (1)	169
Fatty acid-binding protein, brain	1.87 (2)	1.92 (1)	3.06 (3)	97, 98, 149, 247, 248, 249
Fibrinogen alpha chain	0 (0)	0 (0)	1.02 (1)	213
Fibrinogen beta chain (Fragment)	1.87 (2)	0 (0)	4.08 (4)	50, 56, 210, 211, 212, 213
Gelsolin	2.80 (3)	3.85 (2)	2.04 (2)	30, 32, 34, 118, 128, 180, 182
Gallinacin-9	1.87 (2)	1.92 (1)	1.02 (1)	103, 105, 157, 256
Gallinacin-10	4.67 (5)	0 (0)	1.02 (1)	63, 103, 104, 105, 106, 256
Glutathione S-transferase 2	0.93 (1)	0 (0)	0 (0)	79
Glyceraldehyde-3-phosphate dehydrogenase	0 (0)	1.92 (1)	0 (0)	141
Hemoglobin subunit alpha-D	0 (0)	0 (0)	1.02 (1)	251
Ig mu chain C region	1.87 (2)	5.77 (3)	2.04 (2)	6, 31, 114, 116, 117, 182, 188
Ovoinhibitor	2.80 (3)	1.92 (1)	4.08 (4)	45, 46, 47, 129, 206, 207, 208, 209
Trypsin inhibitor ClTI-1	1.87 (2)	3.85 (2)	2.04 (2)	101, 102, 154, 155, 253, 254
Creatine kinase B-type	0.93 (1)	1.92 (1)	2.04 (2)	61, 131, 218, 221
Pyruvate kinase PKM	0.93 (1)	0 (0)	1.02 (1)	54, 204
Ig lambda chain C region	3.74 (4)	1.92 (1)	4.08 (4)	87, 88, 89, 90, 142, 239, 241, 242, 243
L-lactate dehydrogenase A chain	0.93 (1)	0 (0)	0 (0)	75
L-lactate dehydrogenase B chain	0.93 (1)	1.92 (1)	2.04 (2)	74, 140, 228, 229
Lysozyme C	0 (0)	0 (0)	1.02 (1)	252
Malate dehydrogenase, cytoplasmic	0.93 (1)	1.92 (1)	1.02 (1)	73, 139, 227
Protein NEL	0.93 (1)	0 (0)	3.06 (3)	3, 161, 162, 170
Phosphoglycerate mutase 1	0 (0)	0 (0)	1.02 (1)	230
Phosphoglycerate kinase	0 (0)	0 (0)	1.02 (1)	225
Retinol-binding protein 4	0.93 (1)	1.92 (1)	1.02 (1)	94, 146, 244
Src substrate protein p85	0 (0)	0 (0)	1.02 (1)	141
Tubulin alpha-5 chain	0 (0)	0 (0)	2.04 (2)	172, 173
Tubulin beta-1 chain	0 (0)	0 (0)	1.02 (1)	172
Tubulin beta-5 chain	0 (0)	0 (0)	2.04 (2)	172, 173
Thioredoxin	0 (0)	0 (0)	1.02 (1)	246
Triosephosphate isomerase	1.87 (2)	1.92 (1)	1.02 (1)	79, 81, 145, 232
Ovotransferrin	15.89(17)	17.31 (9)	12.24 (12)	16, 17, 18, 26, 27, 28, 31, 32, 34, 35, 36, 37, 38, 39, 41, 52, 102, 114, 116, 117, 118, 119, 120, 121, 122, 123, 181, 185, 188, 189, 191, 192, 193, 194, 201, 203, 235, 252
Transthyretin	3.74 (4)	3.85 (2)	4.08 (4)	84, 85, 96, 97, 134, 148, 211, 237, 238, 245

described by manufacturers in 96-well multiscreen plates, albeit samples were ran as singlets. A 25 μL SF (acidified in case of TGF-βs) was used per sample well to measure the concentration.

Concentrations of TGF-β2, TGF-β3 and CXCL10 were further measured in pooled SF of each male (10 birds per breed) using a chicken-specific ELISA kit for TGF-βs (Nori™ Chicken TGF-β2 and TGF-β3 kit, Genorise Scientific, Inc., Glen Mills, PA, USA) and for CXCL10 (CXCL10 ELISA kit, Cat#MBS2505839, MyBiosource, Inc., San Diego, CA, USA), after preparation of a standard curve, following the manufacturer protocol. The 96-well microplate loaded with duplicate-samples was incubated, washed using an automatic microplate washer (Ref# 30022011, Hydroflex, Tecan Austria GmbH, Grödig, Austria) and the optical density of each well was determined using a microplate reader (Ref# 16039400, Sunrise, Tecan Austria GmbH) at 450 nm.

An independent *t*-test analysis was employed to compare the mean concentration values (expressed as pg/ml, mean ± SEM) of cytokines and chemokines in the SF of the different breeds. $P < 0.05$ was considered significant.

3. Results

A Principal Component Analysis (PCA) of the 2DE images of 12 individual SF samples (4 per group RJF, WL and AIL) (Fig. 1) was performed in order to visualize the 2DE data in terms of sample grouping and gain insights into their intra-group and inter-group variability. Fig. 1 (panel D) highlighted a clear intra-group similarity among individual roosters as well as a clear inter-group variability (e.g. between RJF, WL and AIL). Under such circumstances group/breed pools built by equal SF-amounts of 15 roosters per group were subjected to 2DE separation and compared using SameSpots, to analyze inter-group fold change of specific SF protein spots intensity between breeds.

3.1. The major conserved SF-proteins were serum albumin and ovotransferrin

Out of 107 (RJF), 52 (WL) and 98 (AIL) spots on the 2D gels analyzed (Fig. 2), a total of 28 (26.16%), 21 (40.38%) and 38 (38.78%) proteins were detected in SF (Table 1 and Supplementary Table S1). Around 50% of the detected SF-proteins (from the total number of spots analyzed per breed) were serum albumin (11.21%, 21.15%, and 13.27%) and ovotransferrin (15.89%, 17.31%, and 12.24%) in RJF, WL, and AIL, respectively (Table 1). Among the identified proteins, aspartate aminotransferase, annexin A5, arginosuccinate synthase, glutathione S-transferase 2 and L-lactate dehydrogenase-A were RJF-specific, while glyceraldehyde-3-phosphate dehydrogenase were found to be specific to the WL-SF. On the other hand, angiostensin-converting enzyme, γ-enolase, coagulation factor IX, fibrinogen α-chain, hemoglobin subunit α-D, lysozyme C, phosphoglycerate kinase, Src substrate protein p85, tubulins and thioredoxin were AIL specific (Table 1).

3.2. Eleven SF-proteins were down-expressed while eight proteins were over-expressed in RJF compared with WL and AIL

Compared to the WL, the down-regulated proteins (shown subsequently as fold changes, and spot number on the 2D gels) in the SF of the RJF were serum albumin (4.3, 126), ovotransferrin (2.1, 123), ovoinhibitor (1.9, 129), creatinine kinase B-type (2.6, 131), α-enolase (5.0, 132), L-lactate dehydrogenase B chain (5.3, 140), triosephosphate isomerase (9.1, 145), retinol-binding protein (3.4, 146) and apolipoprotein A-l (3.8, 147) and over-expressed proteins in the SF of RJF were malate dehydrogenase (1.7, 139), glyceraldehyde-3-phosphate dehydrogenase (3.0, 141), cystatin (2.7, 152) and gallinacin-9 (1.9, 157) (Fig. 3A-B and Supplementary Table S2A). A similar comparison revealed down-regulated proteins in RJF, including serum albumin (3.2, 214), ovotransferrin (4.0, 192), phosphoglycerate mutase 1 (2.1, 230) and tubulin β-1 chain (2.0, 172) while, the over-expressed proteins were transthyretin (2.6, 85), Ig λ-chain C (2.0, 243), protein NEL (2.3, 162) and arginosuccinate synthase (3.1, 62) as compared to the AIL (Fig. 3C-D and Supplementary Table S2B).

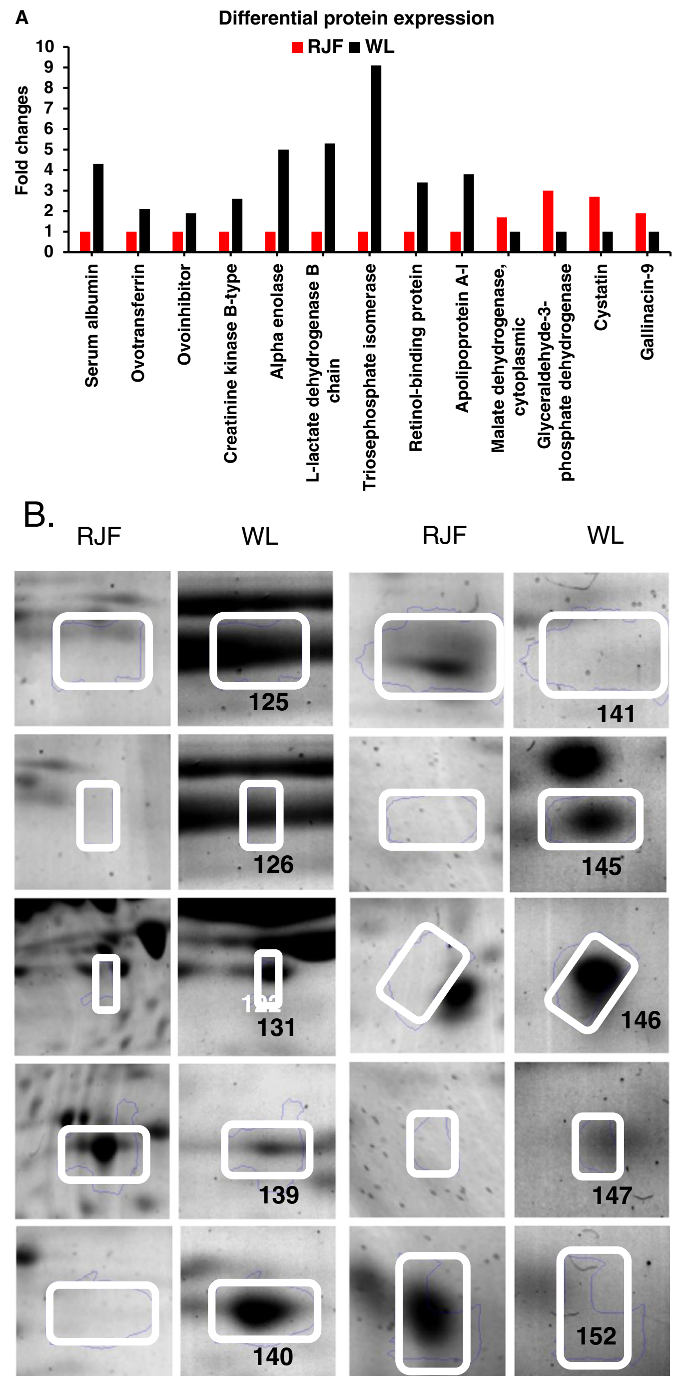
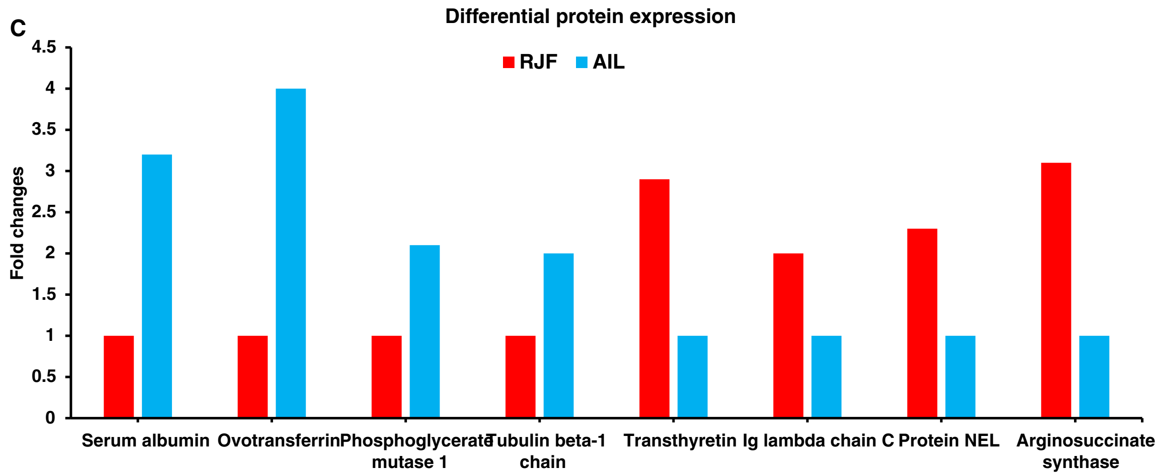


Fig. 3. Differentially expressed proteins (based on staining intensities of protein spots on 2D gels) in the seminal fluid (SF) of Red Junglefowl (RJF), White Leghorn (WL) and Advanced Intercross Line (AIL) roosters. A, fold changes of differentially expressed proteins in the SF between RJF (red column) and WL (black column); B, the protein spots on the 2D gels that had different staining intensities as shown in Fig. A; C, fold changes of differentially expressed proteins in the SF between RJF (red column) and AIL (light blue column); D, the protein spots on the 2D gels that had different staining intensities as shown in Fig. C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



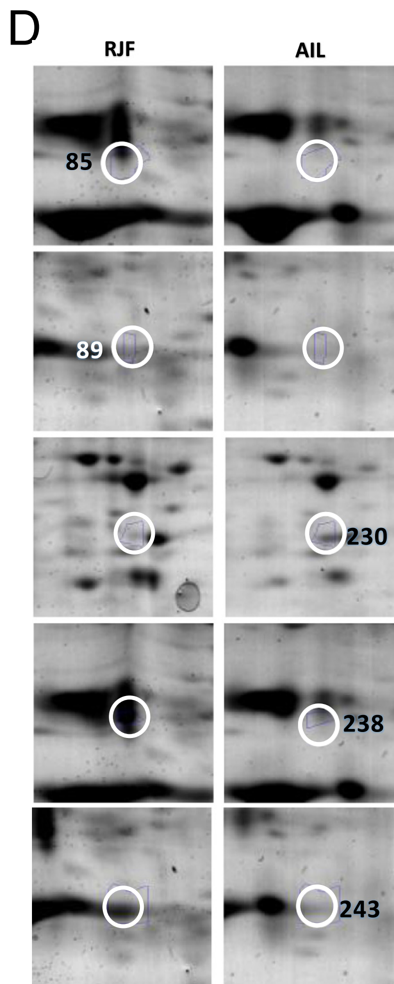


Fig. 3 (*continued*).

3.3. The SF of RJF contained fewer immune system process proteins compared to WL and AIL

The identified proteins were classified according to the 12 biological process categories for each breed of chicken (Fig. 4A and Table 2A). The highest number of proteins in the SF of RJF, WL and AIL were found in the metabolic process category (10, 8 and 13 respectively) while in the 'immune system process' category 3, 5 and 6 proteins were identified, respectively. The protein class analysis revealed 11, 15, and 18 types of proteins, respectively, in the seminal fluid of RJF, WL and AIL (Fig. 4B and Table 2B). Proteins were also screened in the EMBL-EBI Quick GO database to ascertain if any one of them were from sperm regions of *Gallus gallus*. We found that the trypsin inhibitor ClTI-1 (ISK1L_CHICK) is an acrosome membrane protein (GO: 0002080) and was detected in the SF of each breed.

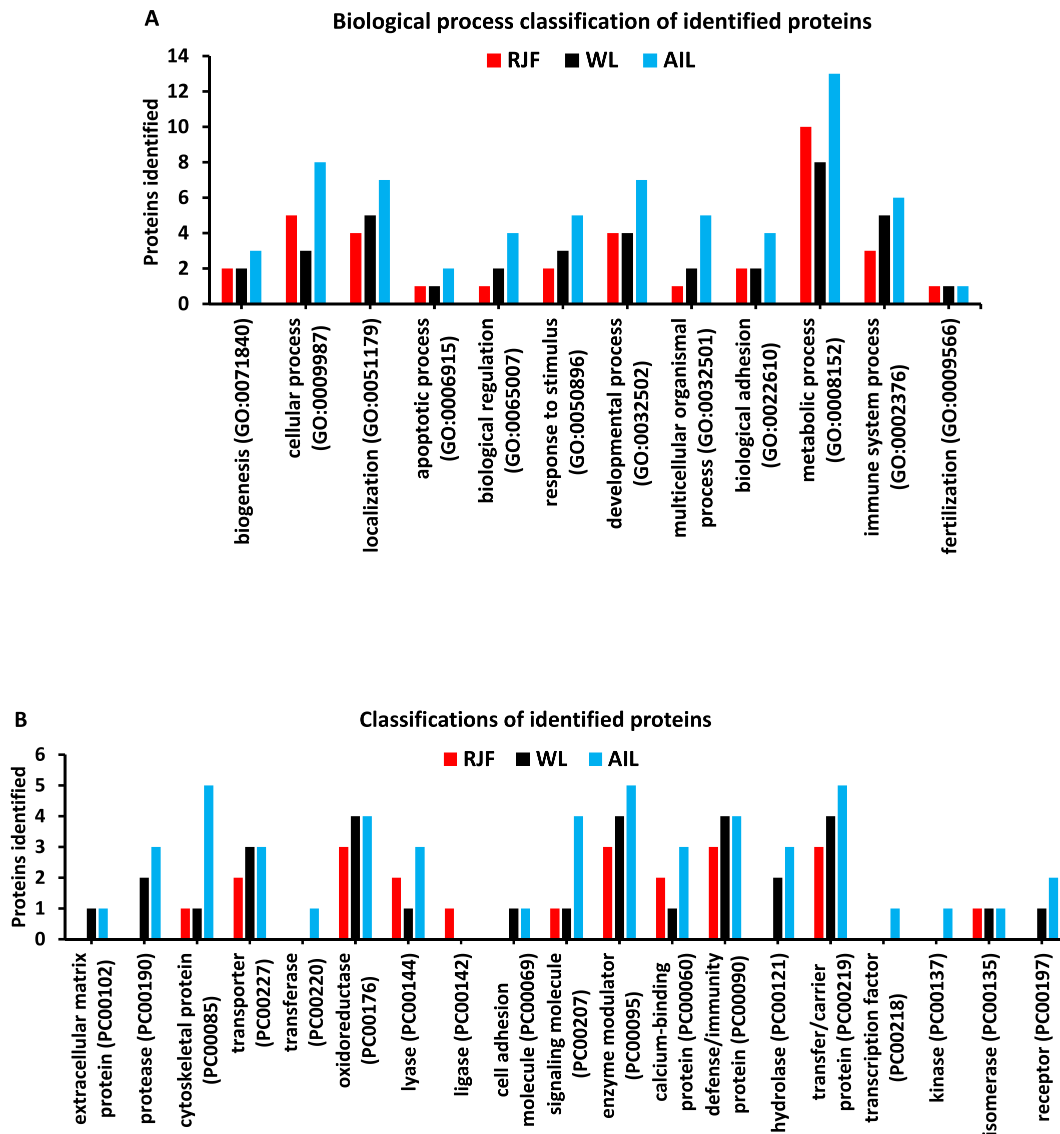


Fig. 4. Biological process (A) and protein class (B) analysis of identified proteins in pooled seminal fluid of roosters from the Red Junglefowl ($n = 15$), White Leghorn ($n = 15$) and Advanced Intercross Line ($n = 15$) breeds using PANTHER gene ontology classification following the protocol, described by Mi et al., 2016. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. The seminal fluid of RJF, WL and AIL contain TGF-ß2 and CXCL10

The Luminex screening solely detected TGF-β2 (RJF, 14,810.47 ± 1492.56; WL, 17,111.14 ± 1028.78; AIL, 13,706.36 ± 1318.50, pg/mL, mean ± SEM, Fig. 5A) and CXCL10 (RJF, 48.74 ± 5.75; WL, 0.00; AIL, 56.65 ± 14.93, pg/mL, mean ± SEM, Fig. 5B). The other cytokines or chemokines (IFNγ, CCL22, CXCL1, IL-17, GM-CSF, CCL2, IL-6, CXCL8, IL-15, IL-10, TGF-β1 and TGF-β3) were not in the detectable range when measured by Luminex. The following use of a chicken specific ELISA could not confirm the presence of TGF-β2 in RJF but could on WL and AIL samples (RJF: 0.00; WL: 2467.52 ± 731.93; AIL: 2844.30 ± 664.36, pg/mL, mean ± SEM, Fig. 5C). Regarding CXCL10, the ELISA confirmed its presence in the SF of all breeds in contrast to the Luminex (RJF: 107.65 ± 16.40; WL: 71.54 ± 5.97; AIL: 86.46 ± 7.36, pg/mL, mean ± SEM, Fig. 5D). ELISA measurements depicted further differences between breeds; thus, while TGF-β2 levels did not differ between WL and AIL, the cytokine was absent in RJF. The concentrations of CXCL10 differed significantly between RJF and WL ($P < 0.05$) with the SF of AIL having intermediate concentrations (n.s.).

4. Discussion

As it is the case in mammals, rooster seminal fluid has been considered to be a modulator of sperm functions, including fertilization (Douard et al., 2005; Froman et al., 2011; Marzoni et al., 2013). While even seminal plasma in mammals has been ascribed relevant roles in fertility including that of livestock selected for fertility, as pigs (Robertson, 2005; Song et al., 2016) such counterstudies are not available for chicken. Selection for fertility, marking differences in egg-laying capacity seemed to have affected sperm function. Murugesan et al. (2013) found high egg laying WL-lines had poorer sperm concentration and forward sperm motility than low-egg laying WL–lines. Uncertainty prevails, since other studies did not found any such differences (Frankham and Doornenbal, 1972; Niranjan et al., 2001). Progressive sperm motility appeared significantly higher in RJF compared to the domestic bantam chicken (Malik et al., 2013). Whether comparative changes have occurred in SF-components and whether eventual changes affect the endowement that SF provides the ejaculated spermatozoa with, as it is proven for mammals (Rodríguez-Martínez et al., 2011; Perez-Patiño et al., 2016) is yet to be explored. Chicken spermatozoa

Table 2A
Biological process analysis of identified protein in the seminal fluid of Red Junglefowl (RJF), White Leghorn (WL) and Advanced Intercross Line (AIL) male chicken.

	Category name (Accesssion)	(Number of protein identified) Identified protein symbols		
		RJF	WL	AIL
1	Cellular component organization or biogenesis (GO:0071840)	(2) Gelsolin Apolipoprotein A-I	(2) Gelsolin Apolipoprotein A-I	(3) Gelsolin Src substrate protein p85 Apolipoprotein A-I
2	Cellular process (GO:0009987)	(5) Gelsolin Protein NEL Argininosuccinate synthase Fibrinogen beta chain Apolipoprotein A-I	(3) Gelsolin Astacin-like metalloendopeptidase Apolipoprotein A-I	(8) Gelsolin Protein NEL Astacin-like metalloendopeptidase Fibrinogen alpha chain Thioredoxin Fibrinogen beta chain Src substrate protein p85 Apolipoprotein A-I
3	Localization (GO:0051179)	(4) Serum albumin Retinol-binding protein 4 Transthyretin Apolipoprotein A-I	(5) Serum albumin Astacin-like metalloendopeptidase Retinol-binding protein 4 Transthyretin Apolipoprotein A-I	(7) Hemoglobin subunit alpha-D Serum albumin Astacin-like metalloendopeptidase Retinol-binding protein 4 Coagulation factor IX Transthyretin Apolipoprotein A-I
4	Apoptotic process (GO:0006915)	(1) Trypsin inhibitor ClTI-1	(1) Trypsin inhibitor ClTI-1	(2) Coagulation factor IX Trypsin inhibitor ClTI-1
5	Biological regulation (GO:0065007)	(1) Apolipoprotein A-I	(2) Astacin-like metalloendopeptidase Apolipoprotein A-I	(4) Astacin-like metalloendopeptidase Coagulation factor IX Thioredoxin Apolipoprotein A-I
6	Response to stimulus (GO:0050896)	(2) Gallinacin-9 Apolipoprotein A-I	(3) Astacin-like metalloendopeptidase Gallinacin-9 Apolipoprotein A-I	(5) Astacin-like metalloendopeptidase Coagulation factor IX Thioredoxin Gallinacin-9 Apolipoprotein A-I
7	Developmental process (GO:0032502)	(4) Fatty acid-binding protein, brain Gelsolin Protein NEL Apolipoprotein A-I	(4) Fatty acid-binding protein, brain Gelsolin Astacin-like metalloendopeptidase Apolipoprotein A-I	(7) Fatty acid-binding protein, brain Gelsolin Protein NEL Astacin-like metalloendopeptidase Coagulation factor IX Src substrate protein p85 Apolipoprotein A-I
8	Multicellular organismal process (GO:0032501)	(1) Apolipoprotein A-I	(2) Astacin-like metalloendopeptidase Apolipoprotein A-I	(5) Hemoglobin subunit alpha-D Astacin-like metalloendopeptidase Fibrinogen alpha chain Coagulation factor IX Apolipoprotein A-I
9	Biological adhesion (GO:0022610)	(2) Apolipoprotein A-I Ovotransferrin	(2) Astacin-like metalloendopeptidase Ovotransferrin	(4) Astacin-like metalloendopeptidase Fibrinogen alpha chain Fibrinogen beta chain Ovotransferrin
10	Metabolic process (GO:0008152)	(10) Annexin A5 L-lactate dehydrogenase A chain Beta-enolase Malate dehydrogenase, cytoplasmic L-lactate dehydrogenase B chain Triosephosphate isomerase Argininosuccinate synthase Gallinacin-9 Alpha-enolase Apolipoprotein A-I	(8) Malate dehydrogenase, cytoplasmic L-lactate dehydrogenase B chain Triosephosphate isomerase Astacin-like metalloendopeptidase Gallinacin-9 Glyceraldehyde-3-phosphate dehydrogenase Alpha-enolase Apolipoprotein A-I	(13) Beta-enolase Malate dehydrogenase, cytoplasmic Phosphoglycerate kinase L-lactate dehydrogenase B chain Triosephosphate isomerase Astacin-like metalloendopeptidase Gamma-enolase Coagulation factor IX Thioredoxin Gallinacin-9 Src substrate protein p85 Alpha-enolase Apolipoprotein A-I
11	Immune system process (GO:0002376)	(3) Gallinacin-9 Ig mu chain C region Ig lambda chain C region	(5) Astacin-like metalloendopeptidase Gallinacin-9 Complement factor B-like protease Ig mu chain C region Ig lambda chain C region	(6) Astacin-like metalloendopeptidase Coagulation factor IX Gallinacin-9 Complement factor B-like protease Ig mu chain C region

(continued on next page)

Table 2A (*continued*)

Category name (Accesssion)	(Number of protein identified) Identified protein symbols		
	RJF	WL	AIL
			Ig lambda chain C region
12 Fertilization (GO:0009566)	(1) Trypsin inhibitor ClTI-1	(1) Trypsin inhibitor ClTI-1	(1) Trypsin inhibitor ClTI-1

are stored for days to weeks in the sperm-storage oviduct area (UVJ). Since spermatozoa (and the SF) are foreign to the hen, some sort of negotiation with the female immune system must be achieved to allow such long survival, with maintenance of potential fertilizing capacity. In mammals, the seminal plasma induces a state of immune tolerance (Robertson, 2005; Rodríguez-Martínez et al., 2011). Mating is capable of inducing changes in the expression of genes at the sperm storage region in the chicken oviduct, including genes involved in immune functions (Atikuzzaman et al., 2015b), indicating that similar mechanisms might be present in either animal classes. Ongoing studies replacing mating for infusion of seminal fluid in RJF and WL and exploring the utero-vaginal junction (UVJ) containing sperm-storage tubuli (SST) by oligonucleotide microarray has shown changes in the expression of immune-responsive genes (7 genes in RJF and 9 genes in WL) in this specific area of the oviduct (Atikuzzaman et al., 2015a and Atikuzzaman et al., unpublished [article in BMC]). The capacity of the seminal fluid to modulate immune responsive genes would therefore imply that a differential protein abundance in the seminal fluid might have a regulating capacity of the oviductal sperm storage area by modulating the local immune responsive genes. If so, selection might have changed this capacity, not affecting the spermatozoa but the SF proteome. Further studies are of course needed to explore this possibility. Differences in SF-proteins between the low egg-laying progenitor RJF, the high egg-laying modern domestic WL and an AIL— intercross between the two, are hereby made evident.

4.1. The SF of RJF lacks astacin-like metalloendopeptidase which is commonly found in most biological process and protein class categories

The present study showed that the numbers of identified SF-proteins from RJF, in the biological process categories and protein classes, were closer to WL, yet less than in the AIL (Fig. 4 and Tables 2A and 2B). Among the biological process categories, the RJF-SF contained stimulus responsive proteins (gallinacin-9 and apolipoprotein A-I) maintained in WL and AIL (Table 2A). However, the RJF-SF lacked several stimulus responsive proteins that were only detected in the SF of WL (i.e. astacin-like metalloendopeptidase) or AIL (i.e. astacin-like metalloendopeptidase, coagulation factor IX and thioredoxin). Astacin-like metalloendopeptidase, relevant in most of the biological processes and protein class categories (Tables 2A and 2B), is present in the seminal fluid of *D. melanogaster*, and is responsible for processing other reproductive proteins and for inducing a variety of post-mating responses in the female fly (Ram et al., 2006; Sirot et al., 2009; Ayroles et al., 2011; LaFlamme et al., 2012). Our results suggest this protein is also important in the chicken, reinforcing results previously reported (Labas et al., 2015). Interestingly, this protein was apparently absent in the SF of the ancestor RJF, calling for further studies to determine whether evolution has elevated the levels of this particular protein in modern chicken. Coagulation factor IX is an *anti*-hemophilic B single chain polypeptide with M_r 57,000 Da in human and bovine which circulates in blood plasma (DiScipio et al., 1978; Katayama et al., 1979). In humans, the coagulation factor IX has been detected in the seminal plasma of both fertile and infertile donors (Lwaleed et al., 2005). However, further research is necessary to disclose whether coagulation factor IX could be a biomarker for rooster fertility. Thioredoxin has been reported in the *Gallus gallus* seminal fluid being classified as defense- or immunity-protein (Marzoni et al., 2013) while in the present study it was classified as a stimulus responsive protein (Table 2A). Cytoskeletal proteins especially tubulins are strongly expressed in human normal sperm compared to sperm from infertile man (Salvolini et al., 2013). In the present study, cytoskeletal proteins were only detected in the AIL-SF, crossbred individuals without fertility deficiencies. Further analyses are needed to explore this exception.

4.2. The rooster seminal fluid conserves serum albumin and ovotransferrin, but they are over-expressed in modern WL and AIL, suggesting a role for production performance

There were a number of proteins conserved in modern chicken. Around 50 % of the identified proteins in the seminal fluid of all breeds from the 2D gels were ovotransferrin and serum albumin (Table 1), confirming recent reports (Castillo et al., 2011; Marzoni et al., 2013). Serum albumin (spots 126 and 214) was differentially expressed (with lesser expression in the RJF than in WL and AIL) (Fig. 3 and Supplementary Table 2). Serum albumin reported in both chicken (Marzoni et al., 2013) and sheep (Soleilhavoup et al., 2014), originates from the epididymis and prostate gland in humans (Elzanaty et al., 2007) but its origin is not determined as yet in chicken. Serum albumin has been classified as a defense or immunity protein (Marzoni et al., 2013) and it is used as an additive in animal semen handling industry (Matsuoka et al., 2006; Fukui et al., 2007; Hossain et al., 2007; Xia and Ren, 2009; Nang et al., 2012), since it appears to increase the percentage of motile spermatozoa and sperm velocity (Bakst and Cecil, 1992). The overall consideration is that the different expression levels between ancestors and modern breeds might relate to their documented differences in fertility.

Ovotransferrin (Spots 123 and 192), a member of iron-binding transferrin and metalloproteinases, has antimicrobial activity (Valenti et al., 1983) and is found in all roosters yet in lesser quantities (2 to 4 fold) in the SF of RJF than in that of WL and the AIL (Fig. 3 and Supplementary Table 2). In mammalian seminal plasma, transferrin originates in the Sertoli cells (Holmes et al., 1982; Gilmont et al., 1990) and thus been shown to be an important biomarker for spermatogenesis (Holmes et al., 1982; Bharshankar and Bharshankar, 2000). Ovotransferrin as a defensive protein (Marzoni et al., 2013) is also present in egg albumin, depicting a strong bactericidal effect (Baron et al., 2014). It is therefore possible that during chicken mating, the ovotransferrin contained in the SF acts as an antimicrobial during semen deposition in the female cloaca, thus decreasing pathogen overload during sperm colonization and storage in the oviduct reservoir (sperm-storage tubules). A lower amount of the protein in RJF-SF as compared to WL— and AIL— might therefore be related to production performance.

Another conserved protein is the cytoskeletal protein-gelsolin family, present in all roosters albeit in different types (Gelsolin, Src substrate protein p85, tubulin α-5 chain, tubulin β-1 chain and tubulin β-5 chain) (Table 2B). Among these cytoskeletal proteins, Gelsolin is reported as influencing the acrosome reaction during human fertilization (Finkelstein et al., 2010). Although it is unclear what role this protein plays in chicken fertilization, it is interesting that it is conserved in such a primitive phenomenon as internal fertilization.

Table 2B
Protein class analysis of identified proteins in the seminal fluid Red Junglefowl (RJF), White Leghorn (WL) and Advanced Intercross Line (AIL) male chicken.

	Category name (Accession)	(Number of protein identified) Identified protein symbols		
		RJF	WL	AIL
1	Extracellular matrix protein (PC00102)	–	(1) Astacin-like metalloendopeptidase	(1) Astacin-like metalloendopeptidase
2	Protease (PC00190)	–	(2) Astacin-like metalloendopeptidase Complement factor B-like protease	(3) Astacin-like metalloendopeptidase Coagulation factor IX Complement factor B-like protease
3	Cytoskeletal protein (PC00085)	(1) Gelsolin	(1) Gelsolin	(5) Gelsolin Src substrate protein p85 Tubulin alpha-5 chain Tubulin beta-1 chain Tubulin beta-5 chain
4	Transporter (PC00227)	(2) Transthyretin Ovotransferrin	(3) Astacin-like metalloendopeptidase Transthyretin Ovotransferrin	(3) Astacin-like metalloendopeptidase Transthyretin Ovotransferrin
5	Transferase (PC00220)	–	–	(1) Phosphoglycerate kinase
6	oxidoreductase (PC00176)	(3) L-lactate dehydrogenase A chain Malate dehydrogenase, cytoplasmic L-lactate dehydrogenase B chain	(4) Malate dehydrogenase, cytoplasmic L-lactate dehydrogenase B chain Astacin-like metalloendopeptidase Glyceraldehyde-3-phosphate dehydrogenase	(4) Malate dehydrogenase, cytoplasmic L-lactate dehydrogenase B chain Astacin-like metalloendopeptidase Thioredoxin
7	Lyase (PC00144)	(2) Beta-enolase Alpha-enolase	(1) Alpha-enolase	(3) Beta-enolase Gamma-enolase Alpha-enolase
8	Cell adhesion molecule (PC00069)	–	(1) Astacin-like metalloendopeptidase	(1) Astacin-like metalloendopeptidase
9	Signaling molecule (PC00207)	(1) Fibrinogen beta chain	(1) Astacin-like metalloendopeptidase	(4) Astacin-like metalloendopeptidase Fibrinogen alpha chain Coagulation factor IX Fibrinogen beta chain
10	Enzyme modulator (PC00095)	(3) Ovoinhibitor Cystatin Trypsin inhibitor ClTI-1	(4) Astacin-like metalloendopeptidase Ovoinhibitor Cystatin Trypsin inhibitor ClTI-1	(5) Astacin-like metalloendopeptidase Coagulation factor IX Ovoinhibitor Cystatin Trypsin inhibitor ClTI-1
11	Calcium-binding protein (PC00060)	(2) Gelsolin Protein NEL	(1) Gelsolin	(3) Gelsolin Protein NEL Coagulation factor IX
12	Defense/immunity protein (PC00090)	(3) Gallinacin-9 Ig mu chain C region Ig lambda chain C region	(4) Gallinacin-9 Complement factor B-like protease Ig mu chain C region Ig lambda chain C region	(4) Gallinacin-9 Complement factor B-like protease Ig mu chain C region Ig lambda chain C region
13	Hydrolase (PC00121)	–	(2) Astacin-like metalloendopeptidase Complement factor B-like protease	(3) Astacin-like metalloendopeptidase Coagulation factor IX Complement factor B-like protease
14	Transfer/carrier protein (PC00219)	(3) Serum albumin Retinol-binding protein 4 Transthyretin	(4) Serum albumin Astacin-like metalloendopeptidase Retinol-binding protein 4 Transthyretin	(5) Hemoglobin subunit alpha-D Serum albumin Astacin-like metalloendopeptidase Retinol-binding protein 4 Transthyretin
15	Transcription factor (PC00218)	–	–	(1) Src substrate protein p85
16	Kinase (PC00137)	–	–	(1) Phosphoglycerate kinase
17	Isomerase (PC00135)	(1) Triosephosphate isomerase	(1) Triosephosphate isomerase	(1) Triosephosphate isomerase
18	Receptor (PC00197)	–	(1) Astacin-like metalloendopeptidase	(2) Astacin-like metalloendopeptidase Coagulation factor IX
19	Ligase (PC00142)	(1) Argininosuccinate synthase	–	–

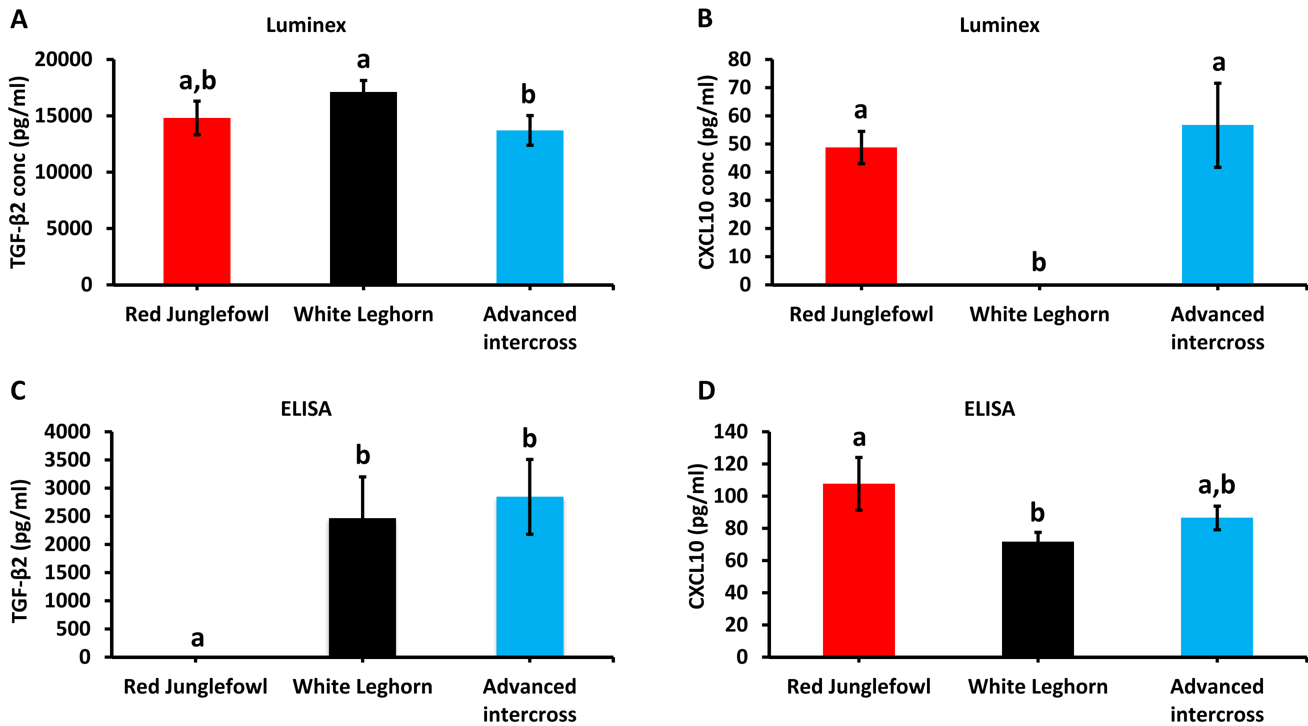


Fig. 5. Transforming growth factor β-2 and CXCL10 concentrations in the seminal fluids of RJF, WL and AIL roosters. In Luminex, individual ejaculates were used to measure the concentrations of TGF-β2 (A): RJF (ejaculates, $n = 40$), WL (ejaculates, $n = 79$), AIL (ejaculates, $n = 39$) and CXCL10 (B): RJF (ejaculates, $n = 45$), WL (ejaculates, $n = 79$), AIL (ejaculates, $n = 43$). In ELISA, ejaculates of individual birds were pooled and 10 pools (birds, $n = 10$) per breed were used to measure the concentrations of TGF-β2 (C) and CXCL10 (D). Different letters on the column represents values are significantly different ($P < 0.05$).

4.3. Other differentially expressed proteins in the seminal fluid might mirror egg-laying capacity evolved through selection pressure

In addition to serum albumin and ovotransferrin, ovoinhibitor (spot 129), creatinine kinase B-type (spot 131), α-enolase (spot 132), L-lactate dehydrogenase B chain (spot 140), triosephosphate isomerase (spot 145), retinol-binding protein (spot 146), apolipoprotein A-I (spot 147), malate dehydrogenase (spot 139), glyceraldehyde-3-phosphate dehydrogenase (spot 141), cystatin (spot 152) and gallinacin-9 (spot 157) were differentially expressed in the SF of RJF compared to WL, whereas, phosphoglycerate mutase 1 (spot 230), tubulin beta-1 chain (spot 172), transthyretin (spot 85), Ig λ-chain C (spot 243), protein NEL (spot 162) and arginosuccinate synthase (spot 62) were differentially expressed in the SF of RJF while compared with AIL (Fig. 3 and Supplementary Table 2).

Among these differentially expressed SF proteins between RJF and WL, ovoinhibitor, creatinine kinase B-type, α-enolase, L-lactate dehydrogenase B-chain, triosephosphate isomerase, retinol binding protein and apolipoprotein A-I were down-expressed in RJF compared to WL, whereas, glyceraldehyde-3-phosphate dehydrogenase, cystatin and gallinacin-9 were over-expressed in RJF compared to WL. Ovoinhibitor was reported as having antibacterial activity in turkey seminal fluid (Slowin'ska et al., 2014). Creatinine kinase B-type, α-enolase, triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase are reported chicken seminal fluid proteins, classified as energy metabolism proteins (Marzoni et al., 2013). This author also reported apolipoprotein A-I as a transport or binding protein and gallinacin-9 as a defense or immunity protein in chicken-SF. Malate dehydrogenase was reported to be 2.23 fold increased in the SF of infertile chicken (Labas et al., 2015). Our findings of down-expression of proteins with bactericidal (ovoinhibitor), sperm motility-enhancing (creatinine kinase B-type), energy metabolism (α-enolase and triosephosphate isomerase) and binding (apolipoprotein A-I) capacities; as well as the over-expression of defense or immunity protein (gallinacin-9) in the SF of low-laying RJF compared to high-laying WL (Fig. 3A and Supplementary Table S2A) suggest these proteins relate to their differential fertility.

Phosphoglycerate mutase 1 and tubulin β-1 chain are down-expressed in the SF of RJF compared to AIL (Fig. 2C and Supplementary Table S2B). Phosphoglycerate mutase 1 has been categorized into energy metabolism group (Marzoni et al., 2013) has also been identified in rooster sperm (Labas et al., 2015). In ruminant seminal plasma (bull, buck and ram) however, only phosphoglycerate mutase 2 has been identified (Druart et al., 2013). In a more recent report (Labas et al., 2015), predicted tubulin alpha-3 and tubulin β-3 chains were detected in rooster sperm while tubulin β-2C, isoform CRA-b was detected in the seminal plasma of rams (Soleilhavoup et al., 2014), indicating that differences are present between these classes. Four proteins (transthyretin, Ig λ-chain C, protein NEL and arginosuccinate synthase) were found over-expressed in the seminal fluid of RJF compared with AIL (Fig. 3C and Supplementary Table S2B). Transthyretin was found overexpressed in the yellow seminal fluid of sub-fertile turkey compared to fertile males (Slowinska et al., 2015). The Ig λ-chain C-a defense/immunity protein (Tables 2A and 2B), previously reported in rooster-SF (Labas et al., 2015), and protein NEL— is a developmental process and calcium ion-binding protein (Table 2A) found in bovine seminal plasma (Kelly et al., 2006) were over-expressed in RJF-SF compared to AIL-SF, in the current study, albeit reported before as down-expressed in infertile roosters (Labas et al., 2015). Argininosuccinate synthase seems to be involved in disposing excess ammonium in semen (Dietz and Flipse, 1969), and related to nitric oxide metabolism. However, it is not known whether the increment of this protein, as found in the present study, relates the enzyme to sperm survival in neither ancestor nor modern chicken. Its character as biomarker for sperm quality warrants further studies of this enzyme.

4.4. The seminal fluid of RJF lacks TGF-β2 but is rich in CXCL10: a relation to lower sperm survival?

Cytokines and chemokines are important modulators of the immune system process. They are necessary to help the female eliminate pathogens entering with the semen but at the same time allow

spermatozoa to survive in the sperm reservoir at the utero-vaginal junction (UVJ), as it happens in the mammalian tubal sperm reservoirs (Robertson et al., 2002). A bead-based immune-assay screening of a battery of cytokines/chemokines reported in mammals (Barranco et al., 2015) was initially done, which resulted in only few cytokines being detectable in rooster SF, i.e. the immune-suppressive TGF-β and the immune cell-chemoattractant CXCL10, those related to the above mentioned dual effects (Agostini et al., 2001; Dufour et al., 2002; Das et al., 2006). Since this initial detection in SF was not present in all breeds, a new attempt was done to confirm their presence by using a chicken-specific ELISA, where we could find that TGF-β2 levels did not differ between WL and AIL but that the cytokine was absent in the SF of RJF (Fig. 5C). The concentrations of CXCL10 differed significantly between RJF and WL ($P < 0.05$) with AIL values in between (Fig. 5D). The cytokine TGF-β2 is present in the seminal plasma of normal fertile men (Nocera and Chu, 1995; Srivastava et al., 1996). The presence of TGFβ in the seminal fluid is usually in latent form therefore, has to be activated in the female reproductive tract post-insemination (Robertson, 2005). In chicken, insemination increases the expression of TGF-β2 in the UVJ, which is apparently responsible for the immunosuppression as well as it helps sperm survival (Das et al., 2006). In contrast, the chemokine CXCL10 is a chemoattractant that recruits activated T-cells (Agostini et al., 2001). CXCL10$^{-/-}$ KO-mice show impaired T-cell responses (Dufour et al., 2002). Interestingly, the present study shows that immune-modulating cytokine/chemokine concentrations in the SF differ between low-laying and high-laying chicken breed, the RJF depicting high amounts of immune reacting CXCL10 and lack of the immunosuppressive cytokine-TGF-β2. The modern domesticated chicken has evolved from this common ancestor RJF, towards a higher egg-laying capacity. Such selection has, apparently, contributed to differential SF-cytokines, which may be key determinants of sperm survival in the UVJ.

5. Conclusion

We are aware that the lack of orthogonal quantitative validation of the comparative 2DE data represents a potential weakness of our study. However, the current lack of commercial antibodies with proven specificity, and the absence of SILAC chicken model for quantitative proteomics, relegate orthogonal validation beyond the scope of this study. Consequently, our work should be seen as preliminary, and the quantitative validation of relevant proteins should be pursued in future studies. Given this limitation, our findings suggest that several proteins (especially gallinacin-9 and Ig λ-chain C) and specific cytokines (TGF-β2 and CXCL10) are differentially expressed in the SF between ancestor-RJF and modern- (WL and AIL) chicken, presumably due to the selection for production traits during chicken breeding.

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Conflict of interest statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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