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SEMINAL INFLUENCE ON THE OVIDUCT

Mating and/or semen components induce gene expression changes in the pre-ovulatory functional sperm reservoir in poultry and pigs

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“Do not rest after your first victory because if you fail in the second, lips are waiting to say that your first victory was just luck”

- A. P. J. Abdul Kalam

To my Family

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ABSTRACT

Internal fertilization occurs in birds and eutherian mammals. Foetal development, however, is either extra- respectively *intra-corpore* (egg vs uterus). In these animal classes, the female genital tract stores ejaculated spermatozoa into a restricted oviductal segment; the functional pre-ovulatory sperm reservoir, where they survive until ovulation/s occur. Paradoxically, this immunologically foreign sperm suspension in seminal fluid/plasma, often microbiologically contaminated, ought to be promptly eliminated by the female local immune defence which, instead, tolerates its presence. The female immune tolerance is presumably signalled via a biochemical interplay of spermatozoa, as well as the peptides and proteins of the extracellular seminal fluid, with female epithelial and immune cells. Such interplay can result in gene expression shifts in the sperm reservoir in relation to variations in fertility. To further aid our understanding of the underlying mechanisms, this thesis studied the proteome of the seminal fluid (using 2D SDS-PAGE and mass spectrometry) including cytokine content (using Luminex and/or ELISA) of healthy, sexually mature and fertile boars and cocks. As well, gene expression changes (using cDNA microarray) in the oviductal sperm reservoirs of sexually-mature females, mated or artificially infused with homologous sperm-free seminal fluid/plasma were studied. Pigs were of commercial, fertility-selected modern breeds (Landrace), while chicken belonged to the ancestor Red Junglefowl (RJF, low egg laying-capacity), a selected egg-layer White Leghorn (WL) and of their Advanced Intercross Line (AIL). Ejaculates were manually collected as single sample in cocks or as the sperm-rich fraction [SRF] and the post-SRF fraction in boars to harvest seminal fluid/plasma for proteome/cytokine and infusion-studies. Oviducts were retrieved for gene-expression analyses via microarray immediately post-mortem (chicken) or at surgery (pig), 24 h after mating or genital infusion. In pigs, the protein-rich seminal plasma showed the highest amounts of cytokines [interferon- γ , interferon gamma-induced protein 10 (IP-10/CXCL10), macrophage derived chemokine (MDC/CCL22), growth-regulated oncogene (GRO/CXCL1), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemo-attractant protein-1 (MCP-1/ CCL2), interleukin (IL)-6, IL-8/CXCL8, IL-10, IL-15, IL-17 and transforming growth factor (TGF)- β_{1-3}] in the larger, protein-rich and sperm-poor post-SRF, indicating its main immune signalling influence. Chicken showed also a plethora of seminal fluid proteins with serum albumin and ovotransferrin being conserved through selection/evolution. However, they showed fewer cytokines than pigs, as the anti-inflammatory/immune-modulatory TGF- β_2 or the pro-inflammatory CXCL10. The RJF contained fewer immune system process proteins and lacked TGF- β_2 compared to WL and AIL, suggesting selection for increased fertility could be associated with higher expression of immune-regulating peptides/proteins. The oviductal sperm reservoir reacted *in vivo* to semen exposure. In chicken, mating significantly changed the expression of immune-modulatory and pH-regulatory genes in AIL. Moreover, modern fertile pigs (Landrace) and chicken (WL), albeit being taxonomically distant, shared gene functions for preservation of viable sperm in the oviduct. Mating or SP/SF-infusion were able to change the expression of comparable genes involved in pH-regulation (*SLC16A2*, *SLC4A9*, *SLC13A1*, *SLC35F1*, *ATP8B3*, *ATP13A3*) or immune-modulation (*IFIT5*, *IFI16*, *MMP27*, *ADAMTS3*, *MMP3*, *MMP12*). The results of the thesis demonstrate that both mating and components of the sperm-free seminal fluid/plasma elicit gene expression changes in the pre-ovulatory female sperm reservoir of chickens and pigs, some conserved over domestication and fertility-selection.

Key words: seminal plasma, proteome/peptidome, oviduct, gene expression, chicken, pig.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Intern befruktning, dvs fertilisering av ägg med spermier inne i honans genitalia, är ett evolutionärt särdrag hos en del olika djurklasser, såsom äggruvande fåglar eller däggdjur. Gemensamt för båda djurklasser är införseln av immunologiskt främmande celler: spermier, suspenderade i en äggvite-rik vätska: sädesvätskan. Båda borde därför vara föremål för utstötning av det honliga immunförsvaret, men spermier stannar inne hos honan under loppet av dagar eller t.o.m. veckor. Ännu mer komplicerat torde det vara hos däggdjur där fostret utvecklas inne i livmodern under en lång dräktighetstid och där moderkakan (placenta) garanterar dess tillväxt. Då såväl fostret som moderkakan emellertid innehåller för honan främmande proteiner från fadern, kan de också stötas bort. Väl känt är att utstötning normalt inte sker, utan honan "tolererar" dessa celler, vätskor, vävnader och organ som ur immunologiskt synpunkt är att betrakta som helt- eller delvis främmande.

Spermier och sädesvätskan deponeras i deponeras som sperma i det honliga könsorganet men elimineras till största delen snabbt genom avrinning via kloaka eller vagina, samt via bekämpning av honans lokala immunsystem, på samma sätt som honan bekämpar patogena mikroorganismer, via fagocytos. Även om denna bekämpning är mycket effektivt, kan en del spermier (1-2%) på något sätt "förhandla" med det honliga immunsystemet och överleva fram till ägglossningen, inne i ett särskilt segment i äggledaren; den funktionella spermiereservoaren (SR). Spermiereservoaren är en serie fördjupningar i äggledarens slemhinna, i den sk utero-äggledarövergången (UTJ) hos suga respektive den sk utero-vaginalaövergången (UVJ) hos höna. Här kan spermier lagras, behålla sin potentiella befruktningsduglighet upp till ägglossning (sent brunst) hos gris eller 1-4 veckor hos fjäderfä, tills de gradvis avancerar genom äggledaren för att befrukta de nyss ovulerade ägg, hos gris c:a 20-30 per ägglossning eller vanligen en gång per dygn hos höna.

Denna doktorsavhandling testade hypotesen att sperma, antingen spermier eller sädesvätskan, kan inducera ett förändring i genuttrycket hos de interna honliga könsorganen, främst i äggledarens spermiereservoar. Detta kan leda till att immuntolerans etableras och att spermier överlever med bibehållen befruktningsförmåga. Därutöver hypotetiseras att dessa grundläggande mekanismer behålls under evolutionen och att de har till och med förändrats till gagn för den högre fertilitet vi ser hos moderna tamgrisar eller höns.

Djurarterna valdes dels för deras reproduktiva skillnader, ägg- respektive livmoderutveckling, dels för deras ejakulatens särdrag. Medan tuppen saknar accessoriska könskörtlar, och lämnar ett koncentrerat ejakulat med liten volym, ejakulerar grisen, som lik mannen har samtliga accessoriska könskörtlar (sädesblåsor, prostata, Cowpers körtlar), ett fraktionerat, voluminöst ejakulat. I det senare kan distinkta fraktioner särskiljas vid manuell spermasamling; en initial spermie-rik fraktion och en efterföljande spermie-fattig, proteinrik fraktion. De studerade kycklingarna inkluderade den röda djungelfågeln (RJF), som är förfader till alla tamhöns, den vita Leghorn (WL) rasen, en för god äggproduktion selekterat modernt tamhöns, samt en avancerad korsning (AIL, 9:e generation) mellan RJF och WL; där genotypskillnader under evolutionen kan detaljstuderas. Grisarna hörde till den högfertila moderna Svensk lantras. Sperman samlades manuellt, via massage hos tuppar (hela ejakulat), och via handske-metod hos galtar (ejakulat fraktioner).

Studierna koncentrerades på innehållet av äggviteämnen och cytokiner i sädesvätskan hos galtar och tuppar, samt om parning eller sädesvätskeinseminering kunde förändra genuttrycket vid äggledaren hos suga respektive höns. Sädesvätskan separerades från spermier via

centrifugering och frystes till användning (infusering) eller analys. Äggviteanalyserna av sädesvätskan inkluderade identifiering av enskilda proteiner via tvådimensionell natriumdodecylsulfat- polyakrylamidgelelektrofores (2D SDS-PAGE) följt av masspektrometri. Cytokiner och kemokiner mättes med tekniker som utnyttjar specifika antikroppar, antigen via partikel-baserad multiplex immunanalys (Luminex s xMAP®) eller enzymkopplad immuno-sorbent assay (ELISA).

Honorna randomiserades för experimentell behandling via parning eller inseminering med spermiefri sädesvätska, respektive lämnades utan åtgärd (kontrolldjur). Honornas interna könsorgan (inkl kontroller) dissekerades post-mortem (höns) eller under narkos (suggor) 24 timmar efter respektive behandling. Specifika anatomiska segment dissekerades fram och frystes separat för senare analys. I synnerhet studerades spermiereservoarerna (UVJ resp UTJ), vilka även undersöktes med mikroskop för att bekräfta innehåll av spermier hos parade djur. De frysta vävnaderna analyserades avseende genuttryck och skillnader mellan grupper via två olika oligonukleotid-mikroarrayer. Mikroarray är ytbaserade tekniker där tusentals gensonder är inbäddade och som används för att se vilka gener som differentiellt uttrycks efter en behandling, samt för att statistiskt och bioinformatiskt analysera genotyp, genernas eventuella funktion/er och skillnader, i detta fall mellan höns och gris.

Resultaten visade att i galtens sädesvätska kunde 14 cytokiner/kemokiner mätas. De var antingen pro-inflammatoriska dvs som förstärker immunsvaret eller anti-inflammatoriska, dvs som minskar immunsvaret. Cytokin/chemokinkoncentrationer varierade mellan handjuren. De högsta cytokin-koncentrationerna visades i den spermiefattiga sista ejakulatfraktion, , en mycket äggviterika fraktion som främst innehåller spermadhesiner som den pro-inflammatoriska PSP-I/PSP-II proteinkomplex. Den mest spermierika portionen visade, å andra sidan, de lägsta cytotkinnivåerna. Dessa resultat verkar tyda på att en viss andel av spermier, de som finns i den första fraktion, kan nå spermiereservoaren utan risk för attack från immunförsvaret, i och med att dessa spermier når äggledarens spermiereservoar inom några minuter, medan inträde av inflammatoriska celler i livmoderslumen inte sker förrän 10 minuter senare. Kanske kan sädesvätskan, i synnerhet den senare fraktion, signalera spermieankomsten och därmed hjälpa immunförsvaret att "rensa" livmodern, vilken snart skall mottaga de nyss befruktade embryon. Därmed rensas såväl mikroorganismer som främmande celler/äggviteämnen bort.

Hos tupparna var förekomsten av cytokiner mycket magrare än hos gris. Endast det pro-inflammatoriska kemokinet CXCL10 och den immunmodulerande/anti-inflammatoriska tillväxtfaktorn TGF- β 2 var mätbara med speciesoberoende antikroppar, vilket bekräftades med kyckling-specifika antikroppar. Nivåerna skilde sig mellan de tre djurpopulationer som studerades. Koncentrationen av CXCL10 var högre hos RJF jämfört med moderna, hög-fertila WL och även gentemot AIL. Å andra sidan visade RJF ingen TGF- β 2. Sammantaget tyder resultaten på att TGF- β 2 ökade med evolutionen/selektionen för fertilitet, medan ur-rasen RJF kan använda sig av en starkare pro-inflammatorisk signal vid parning, vilket också torde ge ett starkare immunsvaret mot patogena mikroorganismer i det vilda.

Äggviteinnehållet i tupparnas sädesvätska visade sig vara ungefär 30-50% av galtarnas, sannolikt på grund av avsaknad av accessoriska könskörtlar hos fjäderfä. Den största mängden av äggviteämnen identifierades som serumalbumin och ovotransferrin hos alla de hönsraser som studerades. De moderna varianterna (WL och AIL) innehöll dock mer immunrelaterade proteiner jämfört med deras RJF förfäder, som i sin tur uppvisade tre mycket distinkta immunförsvarsproteiner. Sädesvätskan innehöll bland annat avian β defensin-9 (som ingår i en familj av medfödd immunitetsproteiner och bildar en ospecifik försvarsmekanism, vilken är

mycket snabb efter inträde av antigener) och Ig λ kedja C; dessa var överuttryckta i RJF jämfört med sädesvätskan från WL eller AIL.

Såväl parning eller seminering av homolog spermiefri sädesvätska förändrade genuttrycket i de honliga könsorganens spermiereservoarer hos både kyckling och svin 24 timmar efter behandling, jämfört med obehandlade kontroller. En stor del av dessa gener visade vara inblandade i immunreglering av spermieöverlevnad och i pH-reglering relaterad till spermierörlighet. Den aktuella undersökningen visade också att äggladarens spermiereservoarerna hos fertilitetsselektade tama höns och grisar delade vissa gemensamma genregleringsmekanismer som svar på parning och/eller sädesvätska-infusioner. Exvis påverkades uttrycket av immunreglerande gener via parning: *MMP27* hos kyckling, *ADAMTS3*, *MMP3*, *MMP12* hos gris eller via SF/SP infusion: *IFIT5* hos kyckling, *IFI16* hos gris. Även uttrycket av pH-reglerande gener kunde påverkas av parning: *SLC16A2*, *SLC4A9* hos kyckling eller *SLC13A1*, *SLC35F1* hos gris samt av infusionen av SF/SP: *SLC10A2*, *SLC4A9*, *ATP8B3* hos kyckling eller *SLC35F1*, *SLC7A7*, *ATP13A3* hos gris.

Sammanfattningsvis visar avhandlingen att ejakulatet hos så evolutionärt skilda klasser av djur som höns och grisar kan utgöra en signal via sädesproteiner och cytokiner till immunförsvaret hos hondjuret. Gensvaret kan vara snabbt för att skapa en momentant inflammation som renar könsorganen inte bara från accessoriska spermier utan också från mikroorganismer. Samtidigt kan en viss, liten andel av spermierna, som redan har koloniserat ett immunprivilegierat segment av äggladaren, den sk spermiereservoaren, leva vidare där tills tiden är inne för befruktningen av de nyss ovulerade ägg. Cellerna i dessa spermiereservoarer kan ändra uttrycket av vissa gener inblandade i immunmodulering och pH-reglering, när antingen parning eller t.o.m. inträde av spermie-fri sädesvätska sker i den interna honliga könsorgan. Intressant nog verkar denna signaleringen variera med evolutionen/selektionen för fertilitet bland höns, det vill säga ursprungliga RJF jämfört med högfertila WL. Dock behåller dessa nu vilt skilda artklasser gener med likartade funktioner inom immunmodulering av honans gensvar vid spermadeponering, som visades vid analyserna av högfertila hönsen och grisarna.

Återstår att studera och fastställa vilka är de specifika komponenter i spermierna eller i den medföljande sädesvätskan (äggviteämnen? cytokiner? cellmolekyler?) som kan utgöra enskild- eller kombinerad signaleringen från hanen till honan, för att påverka honans reaktivitet eller immunologisk tolerans. Framtiden verkar, i detta avseende, spännande!

LIST OF PAPERS

Paper I

Isabel Barranco, Marie Rubér, Cristina Perez-Patiño, **Mohammad Atikuzzaman**, Emilio A. Martinez, Jordi Roca, Heriberto Rodriguez-Martinez (2015) The seminal plasma of the boar is rich in cytokines, with significant individual and intra-ejaculate variation. *American Journal of Reproductive Immunology* **74**: 523-532.

Paper II

Mohammad Atikuzzaman, Libia Sanz, Manuel Alvarez-Rodriguez, Marie Rubér, Dominic Wright, Juan J. Calvete, Heriberto Rodriguez-Martinez. Selection for higher fertility reflects in the seminal fluid proteome of modern domestic chicken. *Comparative Biochemistry and Physiology - part D (accepted for publication)*.

Paper III

Mohammad Atikuzzaman, Ratnesh Mehta Bhai, Jesper Fogelholm, Dominic Wright, Heriberto Rodriguez-Martinez (2015) Mating induces the expression of immune- and pH-regulatory genes in the utero-vaginal junction containing mucosal sperm-storage tubuli of hens. *Reproduction* **150**: 473-483.

Paper IV

Mohammad Atikuzzaman, Manuel Alvarez-Rodriguez, Alejandro Vicente-Carrillo, Martin Johnsson, Dominic Wright, Heriberto Rodriguez-Martinez. Conserved gene expression in oviductal sperm reservoirs between birds and mammals in response to mating. (Manuscript).

ABBREVIATIONS

2D SDS-PAGE	Two-dimensional Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
2DE	Two-dimensional electrophoresis
AI	Artificial insemination
AIL	Advanced intercross line
AQN-1	Alanine-Glutamine-Asparagine-1 spermadhesin
AQN-3	Alanine-Glutamine-Asparagine-3 spermadhesin
AWN	Alanine-Tryptophan-Asparagine spermadhesin
BTS	Beltsville thawing solution
CCL2	C-C motif chemokine ligand 2
CCL22	C-C motif chemokine 22
CD4+	Cluster of differentiation 4 positive
CD8+	Cluster of differentiation 8 positive
cDNA	Complementary deoxy-ribonucleic acid
GRO (CXCL1)	Growth regulated oncogenes (Chemokine C-X-C motif ligand 1)
IP-10 (CXCL10)	Interferon γ -induced protein 10 (C-X-C motif chemokine 10)
IL-8 (CXCL8)	Interleukin-8 (Chemokine C-X-C motif ligand 8)
ELISA	Enzyme linked immuno sorbent assay
ESI	Electrospray ionization
FC	Fold change
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene ontology
IEF	Isoelectric focusing
IFN- γ	Interferon- γ
IL	Interleukin
IPG	Immobilized pH gradient
LC	Liquid chromatography
LN ₂	Liquid nitrogen
MS/MS	Mass spectrometry
PSP-I	Porcine seminal plasma protein-I (spermadhesin)

PSP-II	Porcine seminal plasma protein-II (spermadhesin)
Q	Quadrupole
qPCR	Quantitative polymerase chain reaction
PMN	Polymorphonuclear granulocyte
RJF	Red Junglefowl
RMA	Robust multichip average
RNA	Ribonucleic acid
SF	Seminal fluid
SNP	Single nucleotide polymorphism
SP	Seminal plasma
SR	Sperm reservoir
SRF	Sperm-rich fraction
SST	sperm-storage tubule
SWC-3+	Swine workshop cluster-3 positive (PMN-differentiation antigen)
TGF- β	Transforming growth factor- β
TOF	Time of flight
UTJ	utero-tubal junction
UVJ	utero-vaginal junction
WL	White Leghorn

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INTRODUCTION

In species with internal fertilization, the male deposits the ejaculate into the female genitalia but the internal female tract selects which spermatozoa are to fertilize the newly ovulated oocyte/s. This is true for taxonomically distant species such as poultry and pigs. The process is complex, since the ejaculate is foreign to the female reproductive tract and should thus be promptly eliminated after deposition by local female defence mechanisms. Instead, a subpopulation of deposited spermatozoa is somehow selected for fertilizing capacity and stored in specific segments of the female oviduct, the so-called pre-ovulatory sperm reservoirs, from where spermatozoa are continuously or sequentially released for fertilization in relation to ovulation. In hens, the reservoir is located in the utero-vaginal junction (UVJ), while in pigs it is present in the utero-tubal junction (UTJ). In these analogous reservoirs, functionality of the immunologically foreign spermatozoa is maintained for a couple of days in pigs or for weeks in poultry (Rodríguez-Martínez et al., 2005; Bakst, 2011; Sasanami et al., 2013), by ways yet to be fully understood.

Semen is a complex suspension of spermatozoa bathing in a heterogeneous composite fluid, the so-called seminal fluid (SF) or plasma (SP), built by species-specific contributions of the testis, the epididymis and/or the accessory sex glands (Mann, 1954). Some species, such as birds, produce single-shot ejaculates of small volume (Elagib et al., 2012; Malik et al., 2013); while others (as pig, horse or man) produce large, fractionated ejaculates delivered in spurts (Rodríguez-Martínez et al., 2009). These ejaculate differences correspond to specific characteristics in female genital anatomy and physiology (Lombardi, 1998). In most species, the protein-rich seminal fluid/plasma accounts for the largest part of the total ejaculate volume (Mann, 1969), containing specific proteins and peptides (Bentley et al., 1984; Caballero et al., 2008) relevant for sperm function (Caballero et al., 2012; Rodrigues et al., 2013) and as signals to the female (Robertson, 2007; Schubert et al., 2008). The signalling is seen as prerequisite for the establishment of a state of sperm selection and immune maternal tolerance post-insemination, through mechanisms yet to be fully disclosed (Rodríguez-Martínez et al., 2011).

Most proteins of the pig SP are of vesicular gland origin and 75-90% of them belong to the spermadhesin lectin family (Töpfer-Petersen et al., 1998; Rodríguez-Martínez et al., 2009). These proteins are involved in a variety of effects on sperm protection including membrane stabilization, capacitation, and sperm-oviduct/oocyte interactions (Calvete et al. 1997; Rodríguez-Martínez et al., 1998a; Töpfer-Petersen et al., 1998; Calvete et al., 2005; Caballero et al., 2006), as well as immune stimulation *in vivo* (Rodríguez-Martínez et al., 2010). In the boar, whose fractionated ejaculate sequence mimics that of human, its SP-proteome is rather well described (Rodríguez-Martínez et al., 2009; Rodríguez-Martínez et al., 2011; Patiño et al., 2016). Yet, the peptidome of cytokines and chemokines is less screened, with only few cytokines identified. Among these, the transforming growth factor- β (TGF- β_{1-2}), interferon- γ (IFN- γ) and interleukins (IL)-6 and IL-10, show quantitative variation between ejaculate fractions (O'Leary et al., 2011; Jiwakanon and Dalin, 2012). In chicken, proteomic/peptidomic studies of seminal fluid are scarce (Marzoni et al., 2013; Labas et al., 2015). Comparative cytokine/chemokine studies are also restricted to cytokine expression in the testis (Ocón-Grove et al., 2010; Michailidis et al., 2014) and the female genitalia. Owing to their signaling capacity for the attainment of female immune tolerance and its eventual relation to male fertility in various species (Robertson, 2005; Rodríguez-Martínez et al., 2011; Schjenken et al., 2015), the proteome and peptidome studies of the SP/SF in pigs respectively chicken ought to be followed

up. Of particular importance is to compare lines with different fertility, i.e. comparing ancestors (Red Junglefowl, RJF) vs fertility-selected poultry (as White Leghorn, WL) (Cheng, 2010).

Semen deposition elicits gene expression shifts in the female genital tract in mouse (Fazeli et al., 2004) and chicken (Das et al., 2006; Das et al., 2008 & 2009). This further calls for the identification of the pertinent signals involved, including components of the SF/SP-proteome that could be related to sperm survival and immunomodulation. Determination of gene expression changes in post-mated or post-AI oviducts, especially their sperm reservoirs have indicated roles of the poultry UVJ for sperm storage and survival (Das et al., 2006; Abdel-Mageed et al., 2008; Das et al., 2009; Huang et al., 2016). Use of holistic screening approaches such as microarray analysis of gene expression are yet scarce in chicken and in pig. In the latter, they have been general (isthmus/ampulla) or focused on the ampullary-isthmic junction, the site of *in vivo* fertilization (Georgiou et al., 2007; Almiñana et al., 2014; López-Úbeda et al., 2015). Moreover, none of the previous studies in chickens or pigs have determined whether it is the sperm-free SF/SP in itself or the entire semen (e.g. both spermatozoa and seminal fluid) that elicits gene expression shifts relevant for sperm storage and/or survival. Despite the UVJ of hens and UTJ of sows being functionally analogous, no study so far assessed whether these taxonomically distant animals share common mechanism(s) in oviduct sperm storage and survival.

REVIEW OF THE LITERATURE

Semen production and composition in poultry and pig

Semen is the fluid ejaculated by a male during mating. It is a suspension of cells (mostly mature spermatozoa from the epididymis, but also other cells as spermatogenic cells, genital tract epithelia or invading immune cells) in a fluid (the SF/SP) produced by the epididymis and the concerted secretion of accessory sex glands. Both volume and composition of the ejaculate varies among different animal classes/species owing to differences in reproductive anatomy, as exemplified in **Figure 1** for cocks and boars. Spermatozoa develop in the seminiferous tubules and those freed after spermatogenesis are excreted with testicular fluid via the rete testis and ducti excurrens to the epididymis (Lake, 1957; Mann & Lutwak-Mann, 1981). Spermatozoa mature throughout their transit in the ductus epididymis until being stored in its caudal segment (boar: Egbonike and Elemo, 1978) or in the proximal ductus deferens (cock: Lake, 1957; Razi et al., 2010). The general characteristics of the ejaculate in poultry (three species/lines studied) and boar (all ejaculate and fractions) are summarized in **Table 1**.

Figure 1. Schematic diagrammes of the reproductive organs in cock (left) and boar (right).

Table 1. General characteristics of the ejaculate of poultry (Elagib et al., 2012; Malik et al., 2013) and pigs (Rodríguez-Martínez et al., 2009).

SEMEN PARAMETER	POULTRY			PIG			
	RJF	WL	Whole ejaculate	Pre-SRF	Fractions of ejaculate		
					SRF	Post-SRF (P2)	
					P1	SRF-P1	
Volume (mL)	0.33	0.26-0.73	~300	~46	10	~37	~207
Sperm numbers (x10⁹/mL)	4.44	3.53-6.13	0.38	-	1.9	1.25	0.02
Total sperm motility (%)	72	75-83	75-96	-	83	89	na
Progressive sperm motility (%)	40	69	77.1	-	76	85	na
Sperm velocity (µm/sec)	na	81	63	-	73	63	na
SF/SP protein amount (mg/mL)	7.5-9	10	39.4	23	15.4	15.4	40
pH	7-7.4	7.0	~7.4	na	~7.1	~7.4	~7.4
HCO₃ (mM/L)	na	na	20-23	14.0	17.0	17.0	>30

RJF: Red Junglefowl; WL: White Leghorn; Pre-SRF: pre-sperm-rich fraction; SRF: sperm-rich fraction; P1: first 10 mL of the SRF; SRF-P1: SRF excluding P1; Post-SRF: post-sperm-rich fraction (including gel components); na: data not available; -: not existing.

The SF/SP is a heterogeneous fluid whose components interact with the suspended spermatozoa (Lake, 1957; Mann and Lutwak-Mann, 1981). Poultry lacks accessory sexual glands and so the SF derives from the testis, the rudimentary epididymis and the ductus deferens, as well as from the vascular bodies and lymph folds in the cloaca (Lake, 1957; Etches, 1996; Fujihara, 1992). In pigs, the SP is sequentially built by epididymal caudal fluid and the concerted secretion of the accessory sex glands: prostate, seminal vesicles and bulbo-urethral (Cowper) glands (Lavon and Boursnell, 1975; Mann and Lutwak-Mann 1981). While the cock ejects a single small volume ejaculate, the voluminous boar ejaculate is sequentially expelled in fractions, clearly defined by the amounts of spermatozoa present (Rodríguez-Martínez et al., 2009). The fractions are classically called the pre-sperm-rich fraction (Pre-SRF, with a clear sperm-free seminal fluid which contains mainly secretion of the urethral and bulbourethral glands, as well as the prostate), the sperm-rich fraction (SRF, composed by the emission of portions of the cauda epididymis contents, extended in vesicular and prostate gland secretions) and finally, the post sperm-rich fraction (Post-SRF, where the fewer emitted spermatozoa are largely extended in secretions of the vesicular glands, the prostate and, at the end, of the bulbourethral glands). Noticeably, an initial sperm-peak portion is present in the first 10 mL of the SRF, where a vanguard sperm sub-population of about 25 % of the total sperm numbers (Rodríguez-Martínez et al., 2009), seems to contain, *in vivo*, the first colonizers of the sperm reservoir in the oviduct (Wallgren et al., 2010).

The SF/SP contains electrolytes, hormones, sugars, and proteins/peptides, including enzymes. The SF of poultry is rich in electrolytes (Na, Ca, Mg, K, Cl) and nitrogen, as well as in steroid hormones, inositol, glycerylphosphoryl choline, glucose, fructose and enzymes (acid phosphatase, alkaline phosphatase, glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, lactic dehydrogenase, leucine amino peptidase) (Lake, 1957; Hammond et al., 1965; Anderson & Navara, 2011; Mohan et al., 2011; Getachew, 2016). The boar SP contains similar components, but with major differences among ejaculate fractions. The pre-SRF-SP is rich in electrolytes (mainly Na and Cl), the SRF-SP contains proteins, steroid hormones, glycerophosphoryl choline, fructose, glucose, inositol, citrate, bicarbonate and zinc; while the post-SRF-SP has the highest amounts of proteins, bicarbonate, zinc, Na, Cl and sialic acid (Lavon & Boursnell, 1975; Mann & Lutwak-Mann, 1981; Claus, 1990; Rodríguez-Martínez et al., 2009). The bulk amount of ejaculated proteins (mostly spermadhesins [Calvete et al., 1995]) and peptides (including cytokines/chemokines) in the boar ascends to 39.4±13.45 mg/mL (Rodríguez-Martínez et al., 2005; Rodríguez-Martínez et al., 2011). In contrast, the poultry SF contains a five-fold lower protein load (7.5-9.0 mg/mL) (Bentley et al., 1984; Mohan et al., 2011).

Sperm transport in the female

The internal reproductive tract in hens is built by a single oviduct with distinct anatomical and functional segments. Starting from the cloaca towards the ovary, these segments are called vagina, utero-vaginal junction (UVJ), uterus (shell gland), isthmus, magnum and infundibulum. In contrast, the internal reproductive tract of a sow is broadly divided in vagina, a single cervix, the uterus (a short uterine body and two long uterine horns) and the oviducts (each divided into the utero-tubal junction [UTJ], the isthmus, the ampullary-isthmic junction [AIJ], the ampulla and the infundibulum with its ovarian bursa). The anatomical structures of different segments of the reproductive tracts in hens and sows are shown in **Figure 2**.

Figure 2: Schematic diagrammes of hen (left) and sow (right) genitalia (not in scale). O: ovary; F: infundibulum; M: magnum; A: ampulla; I: isthmus; SR: sperm reservoir (UVJ/SST or UTJ); U: uterus (horn); V: vagina; CX: cervix; Cl: cloaca.

The site for semen deposition is also species-specific. In chicken, the erected ejaculatory duct protrudes into the urodeum of the cloacal chamber during the characteristic cloacal apposition between the cock and the hen at mating (Austin, 1984). The ejaculated spermatozoa are immediately transported by anti-peristaltic contractions of the female genital tract to the vagina where a subpopulation of spermatozoa enter the specialized sperm-storage tubuli (SST) present in the mucosa of the UVJ. Within one hour of intra-vaginal insemination in White Leghorn hens, the functional SST starts being filled with morphologically normal, live spermatozoa (Bakst, 1994) which are hereby stored up to 3-4 weeks (Das et al., 2006) and are released every day of oviposition (Allen and Grigg, 1957; Mero and Ogasawara, 1970; Romanoff, 1960). In sows, spermatozoa are deposited directly into the cervix (Hunter, 1981; Rodríguez-Martínez et al., 2005), and a small subpopulation of spermatozoa (10^5 - 10^8) reaches the functional UTJ-sperm reservoir that is colonized by those spermatozoa with normal morphology and motility, within 5-60 min of AI (Hunter, 1981; Rodríguez-Martínez et al., 2009), to be stored there during the pre-ovulatory period, before being gradually released for fertilization in relation to ovulation (Mburu et al., 1996).

However, the majority of deposited spermatozoa in either species does not reach the sperm reservoirs, facing another fate. In poultry, less than 1% of the total sperm deposited during natural mating or artificial insemination (AI) reaches the sperm reservoirs (Brilliard, 1993; Bakst et al., 1994). More than 80% of these are instead egressed from the vagina within 30 minutes (Bakst, 2011), and the remaining 15% are thought to be killed by local immune cells. In sows, about 20-25% of the cervically inseminated spermatozoa are rapidly (within 30 min) egressed by vaginal retrograde flow (Viring and Einarsson, 1981; Einarsson, 1985). The remaining spermatozoa in utero are phagocytosed by polymorphonuclear leukocytes (PMNs) migrating from the endometrial lamina propria (Lovell and Getty, 1968; Rozeboom et al., 1998; Rozeboom et al., 2000; Schuberth et al., 2008; Rodríguez-Martínez et al., 2009). On the other hand, neither PMNs nor sperm phagocytosis are seen in the functional pre-ovulatory sperm reservoirs in poultry (Holm and Wishart, 1998; Bakst, 2011) or pigs (Hunter et al., 1987; Rodríguez-Martínez et al., 1990).

Structure and function of the tubal sperm reservoir

In birds, the primary functional sperm reservoir is located in the UVJ and it is built as tubular invaginations (sperm storage tubuli, SST,) of the surface epithelium into the lamina propria (Fuji, 1963; Fuji and Tamura, 1963, Tingari and Lake, 1973; Bakst, 1992; 1998; 2011), either as simple- or branched tubuli, one cm-long by 70 μ m in diameter (Lake 1967; Burke et al., 1972); see **Figure 3A**.

Figure 3A-B. Histological sections of the sperm reservoir (SR) in a hen (A: UVJ/SST) and a sow (B, UTJ), e= mucosal epithelium, lp= lamina propria. Arrows indicate SR-spermatozoa.

The SST-epithelium is mainly built by non-ciliated columnar cells although ciliated cells are also observed, mainly at the neck of the tubules (Burke et al., 1972). The total number of SST in the UVJ of poultry varies between 4,000 and 10,000, numbers being directly related to fertility (Birkhead and Moller, 1992; Bakst et al., 2010); possibly because the total number of spermatozoa stored in the area increases with the numbers of available SST (Brilliard et al., 1998).

The spermatozoa that enter the SST become closely bundled to one another without apparent association with the SST epithelium (Tingari and Lake 1973; Van Krey et al., 1981). These SST-stored spermatozoa are subjected to several factors which suppress their motility, their metabolism, protects them from local immune attack and help them sustain their potential for fertilization. Poultry sperm motility is rapidly affected by changes in pH levels. *In vitro*, values of pH below 7.8 inhibit sperm motility, while raising pH by 0.2 units and higher induces vigorous sperm motility (Holm & Wishart, 1998). The enzyme carbonic anhydrase, responsible for changes in extra- and intra-cellular pH, is conspicuously present in the UVJ and particularly in the SST (Holm et al., 1996) possibly associated to low pH levels inside the reservoir. It is speculated that the SST provide nutrients to the resident spermatozoa as well as it removes metabolic waste products (Van Krey et al., 1967). In turkey SST, zinc has been found abundant in the mucosa (Bakst & Richards, 1985). *In vitro*, stored turkey spermatozoa exhibit reduced oxygen consumption and motility (Bakst, 1985). Several proteins, such as avidin (Long et al., 2003), aquaporins (Zaniboni & Bakst, 2004), and alkaline phosphatase (Bakst & Akuffo, 2007) have been identified in turkey SST where they are thought to play roles in maintaining resident spermatozoa alive. Huang et al. (2016) observed that the gene encoding *adipose triglyceride lipase (ATGL)* is expressed in SST epithelial cells, answering for the eventual release of oleic and linoleic fatty acids into the SST lumen, fatty acids that would support sperm survival. Das

et al. (2006) further demonstrated that TGF- β receptors are detected in the SST epithelia and their expression is increased when spermatozoa are stored in the lumen. The authors suggested that TGF- β might be involved in the suppression of the local immune response, which consequently would increase sperm survival.

The corresponding SR in pigs consists of the crypts and furrows of the UTJ (**Figure 3B**), structures that continue as deep furrows of the longitudinal primary folds of the isthmic endosalpinx. The tubal epithelium shows ciliated and non-ciliated cells, separated from the glandless lamina propria by a thin basal lamina (Johansson et al., 2000). Similar to poultry, this reservoir solely stores morphologically normal and potentially fertile spermatozoa, grouped immotile and intact (Rodriguez-Martinez et al., 1990; Mburu et al., 1996; 1997; Rodriguez-Martinez et al., 2001). Spermatozoa can be arrested in the pig SR by the interplay of several mechanisms. The most evident is the presence of a thick mucus (highly rich in hyaluronan, HA, Tienthai et al., 2000) during most of the pre-ovulatory period (Johansson et al., 2000), which impairs sperm transport beyond this tubal area (Hunter, 1984; Rodriguez-Martinez et al., 1990; Johansson et al., 2000). Other suggested mechanisms (Rodriguez-Martinez et al., 2005) include a lower pH in the SR, backed up by the detected rich presence of carbonic anhydrase (Rodriguez-Martinez et al., 1991), the low bicarbonate levels registered *in vivo* (Rodriguez-Martinez, 2007), the lowering of *in vivo* temperature in the SR (Hunter & Nichol, 1986) or the active prevention of calcium influx (see Chapter 4 of PhD dissertation by Machado, 2013). The concerted action of these mechanisms would, similarly to the situation in poultry, decrease sperm motility, as ostensibly evident in studies done after vascular perfusion of specific fixatives (Rodriguez-Martinez et al., 2005). In any case, the HA-rich fluid that embeds spermatozoa would help the resident spermatozoa (which bind to HA by their membrane CD44-receptor, Rodriguez-Martinez et al., 2016) to escape recognition by the female immune system, as well as increase their survival by delaying capacitation (Rodriguez-Martinez et al., 2001; Rodriguez-Martinez et al., 2005). As in poultry, sperm numbers in the UTJ (10^5) are positively related to fertility (Einarsson, 1985; Martinez et al., 2006).

In sows, SR-spermatozoa are continuously released to progress towards the fertilization site at the AIJ, mainly in relation to spontaneous ovulation but even occurring during the post-ovulatory period (Mburu et al., 1996; Mburu et al., 1997). Changes in the viscosity of the intraluminal mucus (perhaps with the influence of hyaluronidase activity, Johansson et al., 2000), the detachment of epithelium-bound spermatozoa (Fazeli et al., 1999), the increased ciliary movement of the epithelium, the flow of the intraluminal fluid as well as the conspicuous motility of the myosalpinx towards the AIJ (Rodriguez-Martinez et al., 1982; Rodriguez-Martinez et al., 1998b) could all act in a concerted way, perhaps under the influence of the peri-ovulatory surge of progesterone from the ovary (Hunter, 1995). After leaving the SR, the spermatozoa can be readily capacitated during transport by the increasing levels of bicarbonate in the upper oviductal fluid (see the review by Rodriguez-Martinez, 2007 and the references cited therein). Corresponding findings have been reported in poultry, with the release of SST-spermatozoa being slow, gradual and continuous, and without an absolute relation to the ovulation of the oocyte. Spermatozoa released from the SST are quickly transported to the infundibulum (possibly only by muscular contractions) where they can fertilize not only the newly ovulated oocyte but also the next-day oocyte (Sasanami et al., 2013). However, since each oocyte is then covered by several layers of secretions by the lower segments of the oviduct, forming the egg, the SST-spermatozoa do not leave again from the SR until the egg has been laid (Sasanami et al., 2013). This situation is basically different in pigs (as in most mammals) since the tubal lumen is not blocked. Progesterone has been

postulated, in both pigs and chicken, to act as a sperm-releasing factor in the SST respectively the UTJ, precluding ovulation (Hunter, 1995; Ito et al., 2011).

Semen is immunologically foreign to the female

The immune defence is carried out by two systems, the innate (in-born) and the adaptive (acquired) immunity. The innate immune response is rapid and it is usually governed by interferons, the complement system, as well as by cells (dendritic cells, macrophages, leukocytes and natural killer cells) using their inflammatory and phagocytic capacity. In contrast, the adaptive immune response is rather slow and it is governed by cytotoxic and helper T cells, by B cells and even by natural killer cells. The mechanisms included in each immune response depend on the nature of the pathogens or another immunologically foreign material. Immunological mechanisms differ between birds and mammals. Innate immunity mechanisms in the avian oviduct are poorly understood, while they are better described in mammals. Immunoglobulins (Ig)-IgM, IgA and IgG are homologous while poultry lack IgD and IgE (Higgins, 1975; Zheng et al., 1997; Davison et al., 2008). A schematic diagramme of the general distribution of immune cells in the female genital tract of the hen and the sow is shown in **Figure 4**. Morphologically, neutrophil granulocytes are absent in birds, where heterophil granulocytes replace their function, albeit through apparently different mechanisms (Penniall and Spitznagel, 1975; Montali, 1988). The distribution of the immune competent cells in the female genitalia varies between poultry and pigs as differences relate to the absence or presence of an oestrous cycle, or the site of semen deposition between these species. In sows, for instance, the entry of spermatozoa and the surrounding SP into the uterine cavity influences the PMN-leukocyte invasion from the lamina propria to the uterine lumen, as well as the transformation of monocytes towards intraepithelial macrophages, already 30 min after semen entry to the uterine cavity and sustained for few (around three) hours (Rodriguez-Martinez et al., 1990). Extravasation and accumulation of PMNs beyond the uterine and cervical epithelium occurs during pro-oestrus in consequence of the high oestrogen levels in this stage of the cycle (Lovell and Getty, 1968). The peak of PMN-entry seems caused by the presence of spermatozoa, but also by the presence of PSP-I/PSP-II in the SP (Rodriguez-Martinez et al., 2010). Noteworthy, the first spermatozoa (sperm-peak portion) bathe in very low concentrations of these spermadhesins (Rodriguez-Martinez et al, 2005).

The expression of local innate immunity between poultry and pig internal genitalia is of comparative interest (Das et al., 2008; Rodriguez-Martinez et al., 2009). Japanese quails have shown increased heterophil counts in their vagina and UVJ up to three hours after copulation (Higaki et al., 1995), similarly to the response seen in pigs (see above). Macrophages are distributed in the stroma and mucosal epithelium of laying hens and are increased in the vagina, magnum and infundibulum, but not in the SST (Zheng and Yoshimura, 1999), similarly to what is described in sows (Jiwakanon et al., 2005). The innate immunity in all segments of the oviduct in poultry is governed by β -defensins (av β D) with antimicrobial activity, namely av β D 1-5 and 7-12, that are overexpressed in the vagina of laying hens (Ohashi et al., 2005; Abdel-Mageed et al., 2008; Das et al., 2008).

The adaptive immune system is well represented in the female genitalia of poultry and pigs with both T cells (CD4+ and CD8+), B cells (IgA+, IgG+ and IgM+) and MHC class II positive antigen presenting cells (Withanage et al., 1997; Kaeoket et al., 2001c, Jiwakanon et al., 2005). The oviduct of the laying hen contains all sets of T lymphocytes largely in the lamina propria (Withanage et al., 1997), including CD8+ cells close to the epithelial lining, but rarely in the UVJ. The chicken oviduct also contains B lymphocytes scattered throughout the oviduct

mainly beneath the epithelium and associated with glands (Kimijima et al., 1990; Withanage et al., 1997). IgA+ lymphocytes are highly encountered in vagina, IgM+ cells in uterus and IgG+ cells in the isthmus, while the infundibulum and the UVJ contain very few immunoglobulins-positive cells (Withanage et al., 1997). Comparatively, the sow endosalpinx displays CD3+, CD14+ and CD79+ cells in the isthmus, ampulla and particularly in the epithelial layer of the infundibulum but they are not detected in the UTJ (Jiwakanon et al., 2005). Both CD14+ and CD79+ cells have also been observed in the sub-epithelial lamina propria, depicting a rich infundibular localization compared to isthmus and ampulla, and definitively contrasting that of the UTJ. Hussein et al. (1983) immunohistochemically examined IgA+, IgG+ and IgM+ B cells in the reproductive tract of sows. Of these, IgA+ and IgG+ B cells were predominant in the endometrial luminal surface epithelium, alongside the entire tract, particularly highest during oestrus.

Figure 4. Diagramme of the differential distribution of major immune competent cells in anatomical segments of the internal genital tract in hen (upper) and sow (lower). NK cells: natural killer cells, DC: dendritic cells, V: vagina, UVJ: utero-vaginal junction, U: uterus, I: isthmus, M: magnum, F: infundibulum, UTJ: utero-tubal junction, lp: lamina propria, lu: lumen, *: not clear, ?: the absence/presence of immune cells is unclear, besides the lack of neutrophils/heterophils in the sperm reservoirs (UVJ/UTJ).

Cells displaying major histocompatibility complex (MHC)-II are distributed in the vaginal and infundibular epithelium of poultry (Yoshimura et al., 1997; Zheng et al., 1998; Zheng et al., 2001) while in pigs they mainly appear in the endothelial cells but also in the lining and glandular epithelia of the endometrium (Kaeoket et al., 2001b). Noteworthy, very few MHC-II+ cells are present in the endosalpingeal epithelial layer but are conspicuous in the lamina propria of all tubal segments (Jiwakanon et al., 2005). In addition, cells bearing the PMN-antigen differentiation cluster SWC3+ were largely distributed in the infundibular and ampullar lamina propria compared to the isthmus and UTJ (Jiwakanon et al., 2005). The relative absence of immune cells (except of intraepithelial lymphocyte- and monocyte-like cells) in the oviductal sperm reservoir in poultry and pigs calls for defining this segment as an immunologically safe area for the spermatozoa (Bakst, 2011; Rodríguez-Martínez et al., 1990; Rodríguez-Martínez et al., 2001).

The latest two decades of research have shown that the introduction of semen into the female genitalia changes the nature of the innate and acquired local immunity in the female reproductive tract of chicken (Das et al., 2005; Das et al., 2006; Abdel-Mageed et al., 2008; Das et al., 2009) and pigs (Rozeboom et al., 1998; Robertson, 2007; Schuberth et al., 2008; Rodríguez-Martínez et al., 2009). The SF/SP signaling proteins and peptides -i.e. avian β -

defensins (Marzoni et al., 2013; Labas et al., 2015); or porcine spermadhesins (Rodríguez-Martínez et al., 2010; Perez-Patiño et al., 2016); cytokines/chemokines (pig: O'Leary et al., 2011; Jiwakanon and Dalin, 2012), and or sex hormones (progesterone, testosterone, dihydrotestosterone and estrogen) (chicken: Anderson and Navara, 2011; Oliveira et al., 2011; pig: Claus et al., 1983; Zdunczyk et al., 2011), are all considered to contribute to the orchestration of such local immune defence responses in the female genitalia.

Avian β -defensins (Av β D-9 and Av β D-10) have been identified in the SF as well as on the sperm-surface (Av β D-3) and are classified as immunity/defence proteins (Marzoni et al., 2013; Labas et al., 2015). Av β Ds 1-5 and 7-12 are important factors in the innate and adaptive immunity (Sugiarto and Yu, 2004; Das et al., 2008; Davison et al., 2008), expressed by the oviduct of hens (Abdel-Mageed et al., 2008; Shimizu et al., 2008). The β -defensins can directly kill pathogens as well as they act as chemoattractants to recruit immune-competent cells (mainly neutrophils, macrophage, monocytes and T-cells) as well as they can enhance degranulation of mast cells, all leading to pathogen phagocytosis and inflammation (Hancock and Diamond, 2000; Scott and Hancock, 2000). In pig, an endometrial inflammation is initiated after 30 minutes of semen deposition (Rodríguez-Martínez et al., 2005; Rodriguez-Martinez et al., 2009; Rodriguez-Martinez et al., 2011) to eliminate surplus spermatozoa (Rozeboom et al., 1998; Rozeboom et al., 1999) and foreign SP-proteins (O'Leary et al., 2004) within few hours. The major proteins in the boar ejaculate are the Alanine-Glutamine-Asparagine (AQN-1, AQN-3), the Alanine-Tryptophan-Asparagine (AWN) and the Porcine Seminal Plasma Protein (PSP-I, PSP-II) spermadhesins (Rodríguez-Martínez et al., 2009). The latter proteins recruit, once they are introduced intra-utero, different lymphocyte subsets (CD4⁺ and CD8⁺ T cells) and PMNs, the latter migrating through the uterine epithelial lining to the lumen of the uterus after 30 minutes of PSP:s infusion, with a sustained migration for around three more hours (Rodríguez-Martínez et al., 2010).

In addition to this protein-influenced immune reaction, sex hormones (either of ovarian or seminal origin) target the female reproductive tract tissues in birds and mammals to synergistically provoke the accumulation of immune competent cells in the lamina propria (Lutton and Callard, 2006; Wira et al., 2015). Oestrogens -in particular- influence the local female immune system both in poultry and pigs (poultry: Zheng et al., 1998; Zheng and Yoshimura, 1999; pigs: Hussein et al., 1983; Kaeoket et al., 2001a, b, 2003). Anderson and coauthors (2011) described the presence of progesterone (P₄), testosterone (T) and dihydrotestosterone (DHT) in the SF of cocks but failed to detect oestrogen (E₂). However, detection of oestrogen receptors in the cock testis and epididymis indicates presence of oestrogen in the testicular and epididymal fluid, which are added to the ejaculated semen (Oliveira et al., 2011). Oestrogen and testosterone are also present in the boar SP (Claus, 1990; Hess and Carnes, 2004; Frydrychová et al., 2007). Macrophages, antigen-presenting cells expressing MHC class II, CD4⁺ and CD8⁺ T cells as well as premature B and plasma cells are the immune competent cells that are increased by the influence of gonadal steroids in the vagina of laying hens (Zheng et al., 1998; Zheng and Yoshimura, 1999).

Cytokines and chemokines are directly linked to the regulation of the immune system, but they are only partly studied in cocks and boars. In pigs, those seminal cytokines examined were TGF- β ₁₋₂, IL-10 and IL-6 (O'Leary et al., 2011; Jiwakanon and Dalin, 2012). Chicken cytokines and chemokines have only 25-35% amino acid identity with their mammalian orthologues (Kaiser and Stäheli, 2008) and their repertoire in the SF is largely unknown, most likely due to the lack of proper bioassays for their determination.

Entry of semen induces gene expression changes in female genitalia

Since the antigenic semen is immunologically tolerated by the female during genital storage, scientists have been studying during the last two decades whether this paradox is mediated by the induction of changes in the expression of genes in the female genitalia that could allow the spermatozoa to remain alive and potentially fertile and to lead to a status of maternal immunological tolerance, as postulated by Robertson and Sharkey (2001). For this reason, many studies focused on the changes experienced by genes governing production of particular cytokines. For instance, polymerase chain reaction (PCR) studies revealed that cytokine expression in the female reproductive tract is modified by mating or artificial insemination (AI) in poultry (Das et al., 2006; Das et al., 2009) and pigs (O'Leary et al., 2004; Jiwakanon et al., 2010). For instance, AI of hens increased mRNA levels of *interleukin-1 β* and *lipopolysaccharide-induced TNF factor (LITAF)* in vaginal tissues as early as 1-6 hours post insemination, while such changes were not evident in other segments of the oviduct (Das et al., 2009). The authors suggested that an increase of these cytokines might lead to the degradation of those spermatozoa that fail to enter the SST and are, therefore, eliminated. Sperm residence in the UVJ of hens increased the mRNA expression of *transforming growth factor- β 2-4 (TGF β 5)* and *TGF β receptor 1-2 (T β Rs)*, suggesting they play a role in sperm survival (Das et al., 2006).

Not only entire semen or SF-free spermatozoa seem to have an effect. In pigs, intrauterine SP infusion into pre-pubertal gilts increased the mRNA expression of *granulocyte macrophage colony-stimulating factor*, *interleukin-6* and *monocyte chemo-attractant protein-1 (CCL2)* in the endometrium (O'Leary et al., 2004). The authors suggested these increments could influence cytokine synthesis and leukocyte trafficking, and play a beneficial effect in regulating embryonic pre-implantation. Moreover, Jiwakanon et al. (2010) found *TGF β 1* mRNA expression to be significantly increased in the isthmus 40 hours after SP infusion compared to controls, and suggested these changes might benefit immune modulation, as previously suggested by Robertson (2005).

Other genes were reported as changing their levels of expression after AI or mating in poultry and pigs, with suggested roles in sperm survival. These included the *MHC class II* gene whose mRNA was increased in the infundibulum of hens 24 hours after AI with fresh semen yet remaining unchanged in the UVJ. An influx of antigen-presenting cells expressing MHC class II in the infundibulum, where the anti-sperm immune response is strongest (Zheng et al., 2001), might decide the fate of the spermatozoa that do not participate in fertilization, as reported previously by Koyanagi and Nishiyama (1981). In laying hens, mRNA expression of *av β D 1-3* was strongest in the infundibulum and vaginal surface epithelia compared to other segments, suggesting a role for innate immunity to eliminate eventual microorganisms upon entry after mating or AI (Ohashi et al., 2005; Abdel-Mageed et al., 2008). Although the innate immunity of the hen oviduct is increased by the overexpression of *av β Ds* genes after semen deposition, the spermatozoa can protect themselves by β Ds attachment to the sperm surface, which apparently helps them hide from immune recognition (Shimizu et al., 2008). Lipases are also ascribed a role in sperm survival, and there is a relatively increased expression of *adipose triglyceride lipase (ATGL)* mRNA in the SST of AI-hens (Huang et al., 2016). As well, there is an increased mRNA expression of *avidin* and *avidin related protein-2 (AVR2)* in the SST of turkey (Long et al., 2003; Foye-Jackson et al., 2011), both related to the maintenance of spermatozoa in the oviductal sperm reservoir (for review see Sasanami et al., 2013). In pig endometrium, the mRNA expression of the *cyclooxygenase-2 (COX-2)* gene, which is responsible for prostaglandin synthesis, increases after intrauterine SP-infusion (O'Leary et al., 2004). López-Úbeda et al. (2015) studied, using microarray, whether AI of sows with semen

extended in Beltsville Thawing Solution (BTS) would change the gene expression of the ampullary-isthmic junction (AIJ, the site of fertilization in pigs) 48 hours after AI. They found that 17 genes were upregulated while 9 genes were downregulated, concluding they were involved in the preparation of this compartment for a successful fertilization. Gene expression changes in the pig oviduct have been reported after inseminating with X- or Y-bearing spermatozoa, which suggests that not only the entry of spermatozoa but also their chromosomal sex seems relevant, despite we do not know the mechanisms behind (Almiñana et al., 2014). Gene expression changes have also been reported in the internal genital tract of other species (Fazeli et al., 2004; Kodithuwakku et al. 2007; Mondéjar et al., 2012). Although the composition of the SF/SP proteome of boars and chickens might be relevant to disclose eventual signal molecules placed by the male at mating, this area has so far been only partially studied. In particular, whether and how SF/SP components may cause gene expression changes in the oviduct in relation to sperm storage and survival needs to be addressed.

HYPOTHESIS

Internal fertilization is a characteristic shared by different animal classes, where semen entry is considered to trigger responses by the female in terms of transient rejection or long-lasting tolerance of paternal components. In this thesis, I hypothesize that not only spermatozoa, but also components of the seminal fluid/plasma they bathe in (as proteins and cytokines/chemokines) might be relevant signals causing changes in gene expression at the oviductal sperm reservoir in poultry and pigs. Moreover, that this signaling has conserved elements across animal classes, including a relation to selection for fertility over domestication.

AIMS

The general aim of the thesis was to explore the proteome and peptidome of the SF/SP as well as the seminal influence on the female genitalia comparing domestic pigs (*Sus scrofa domestica*) with the taxonomically distant *Gallus gallus* (Red Junglefowl [RJF], the domesticated breed White Leghorn [WL] and an Advanced Intercross Line [AIL, RJFxWL]).

In particular, this thesis explored whether:

- the concentrations of pig SP-cytokine/chemokine varied between ejaculate fractions and boars (**Paper I**),
- the SF-proteome/peptidome varied between the ancestor poultry RJF, the modern selected egg-layer breed (WL) and their advanced line intercross (AIL) (**Paper II**),
- mating induced gene expression changes in the oviduct of AIL-hens (**Paper III**),
- entry of homologous semen or SF/SP induces changes in gene expression in the oviduct sperm reservoir in fertility-selected poultry or pig breeds (**Paper IV**).

METHODOLOGICAL CONSIDERATIONS

Animals, ethical considerations

Fertility proven commercial pure/cross-bred boars (AIM Iberica AI center, Calasparra, Murcia, Spain) and pure-bred boars and sows (parity 2-3) (Swedish Landrace, Flistad breeding farm, Östergötland, Sweden) were used. The boars were evaluated for semen characteristics and fertility records and sows were evaluated for normal reproductive performance and fertility before inclusion in the experiments. Chicken from three different lines were studied, namely the ancestor Red Junglefowl (RJF); the high-egg layer White Leghorn (WL) and an Advanced Intercross Line (AIL, 9th generation crossing between RJF and WL). The details of all these birds can be found elsewhere (Schütz & Jensen, 2001; Schütz et al., 2002). The Red Junglefowl original grandparent stock was brought to Sweden from Thailand in 1999, kept in captivity in a Zoological Park in northern Sweden (Frösö Zoo) and later kept in the chicken facility of Linköping University (Schütz & Jensen, 2001; Elfving et al., 2014). The modern layer breed White Leghorn was originally selected for egg production from an outbred mixture of breeds established in 1970 as reported elsewhere (Elfving et al., 2014). The AIL was produced from crossings between male Red Junglefowl and female White Leghorn. While a WL-hen lays more than 300 eggs in a year, a RJF-hen lays only 4 to 6 eggs per year in a wildlife condition (Cheng, 2010). Keeping RJF in captivity has improved their egg-laying performance (around 2 eggs per week), but it is still very low compared to WL (around 6 eggs per week) (Schütz et al., 2002). The production performance of AIL is closer to WL than to RJF (Schütz et al., 2002). For details of rearing and handling of the animals, see **Papers I-IV**.

All experiments were performed according to international guidelines and were approved either by the Bioethics Committee of Murcia University (research code: 639/2012) or by the Regional Committee for Ethical Approval of Animal Experiments (Linköpings Djurförsöksetiska nämnd) in Linköping, Sweden (permit no 75-12), in advance of the experiments.

Sample collection, assessment and handling

To achieve efficient manual collection of semen from cocks and boars, they were properly trained prior to the experiment. Cock semen was collected via gentle abdominal massage until cloacal eversion was obtained, followed by pressure on the phallus. The boars were trained to mount a stainless steel-made dummy and specific fractions of the ejaculate were manually collected using the gloved-hand method (Hancock, 1959).

Immediately after collection, the semen samples (whole ejaculate or fractions) were transferred to pre-warmed plastic tubes and extended using appropriate fluids (Dulbecco's or BTS) for evaluation of sperm concentration (using SP-100 NucleoCounter, ChemoMetec A/S, Allerød, Denmark) and kinematics (using ISASV1® CASA, Proiser R+D, Paterna, Spain)(**Paper I**) or using the Qualisperm™ software (**Papers II-IV**), before separation of the seminal fluid (SF) or seminal plasma (SP) by centrifugation and storage at -80 °C until analyses (**Papers I-II and IV**).

Internal genital tract tissues (hens: UVJ, uterus, magnum, isthmus and infundibulum; sow: cervix, uterine horn, UTJ, isthmus, ampulla and infundibulum) were collected either post-mortem in hens or at surgery in pre-ovulatory oestrus sows and immediately frozen by immersion in liquid nitrogen (LN₂) for following storage at -80°C until analyses. Samples of blood and follicular fluid were collected from sows during surgery for analyses of oestradiol

and progesterone (ELISA). A supplementary tissue sample from the UVJ or UTJ of each female was also fixed in 4% formaldehyde for histological confirmation of sperm presence.

Proteomics and cytokine analyses of the seminal fluid/plasma

One- and two-dimensional gel electrophoresis were used for the initial isolation of SP-proteins in the chicken seminal fluid; comprising one-dimension isoelectric focusing (IEF) and two-dimension sodium dodecyl sulphate-polyacrylamide gel electrophoresis (2DE SDS-PAGE). Eventual presence of interfering non-protein impurities was removed from the SF samples using 2-D clean-up kit before doing 2DE. Individual SF-proteins were separated using an Ettan IPGphor 3 isoelectric focusing system (GE Healthcare) comprising an immobiline DryStrip gel with an immobilized pH gradient (pH 3-10, IPG). The IPG strips were then loaded into the SDS-PAGE gels where the isoelectric point (PI)-based separated proteins were secondly separated by molecular weight, and later stained with Coomassie Brilliant Blue. The visualized protein spots were then picked up, digested with trypsin enzyme and analysed using liquid chromatography-electrospray ionization-quadrupole-time of flight-mass spectrometry (LC-ESI-Q-TOF-MS/MS). The proteins were then identified from their peptide sequences using MASCOT MS/MS ion search engine version 2.5 (Matrix Science, Boston, MA) along with the latest updated version of Swiss-Prot protein database (UniProtKB/Swiss-Prot).

The presence and relative concentration of a battery of cytokines and chemokines in pig SP (**Paper I**) and poultry SF (**Paper II**) was examined using the Luminex's xMAP® technology, a multiplexed microsphere-based flow cytometric assay with commercial kits (Cat#HCYTOMAG-60K-11 and Cat#TGFβ-64K-03, Merck Millipore, Billerica, MA, USA). Owing to the restricted cross-reactivity of the kits for chicken, commercial chicken-specific enzyme linked immune sorbent assay kits (ELISA, Nori™ Chicken TGF-β2 kit, Genorise Scientific, Inc., Glen Mills, PA, USA and CXCL10 ELISA kit, MyBiosource, Inc., San Diego, CA, USA) were also used.

Gene expression analyses

Total RNA was isolated using the TRIzol method and cDNA synthesised either by RevertAid Premium First-Strand cDNA synthesis kit for chicken oviduct tissues (**Papers III-IV**) or using GeneChip® WT PLUS reagent kit from Affymetrix for pig UTJ tissues (**Paper IV**). Two oligonucleotide microarrays were used to analyze the differential gene expression. For chicken, a custom-designed 12 X 135 k array from Roche NimbleGen was used (**Papers III-IV**). The custom-made probes were designed to avoid SNPs in the probe sequences, almost all known SNP positioned derived from the recent resequencing of RJF and domestic chickens (Rubin et al., 2010). Three 60-mer-oligonucleotide probes represented each transcript. A few differentially expressed genes in chicken oviduct were validated by qPCR using Maxima SYBR Green qPCR mastermix on a Rotor-Gene 6000 real-time cycycler (**Paper III**). For pig UTJ tissues, the Affymetrix GeneChip® PorGene 1.0 ST array was used (**Paper IV**). The array contains a total of 394,580 probes in a single chip comprising 22 probes (each containing 25-mer oligonucleotides sequence) per gene, for a total of 19,212 genes.

Statistics and bioinformatics

The variation of cytokine concentrations among boar and ejaculate fractions was analysed using mixed models of ANOVA (**Paper I**) and *t*-test for chicken breeds (**Paper II**). The gene

expression data were processed using the Robust Multichip Average (RMA) normalization, computing average expression values by background adjustment, quantile normalization between arrays and summarization, implemented by the oligo package of DEVA Software (Roche NimbleGen, Inc, DEVA 1.2.1)/Bioconductor. The statistical analysis of the normalized gene expression data was performed using the open source RStudio package (RStudio, Inc. Version 0.98.507). Linear models using the empirical Bayes' approach as implemented in the package 'limma' were used to calculate differentially expressed transcripts with Benjamini-Hochberg False Discovery Rate (FDR) and multiple testing correction to control type I errors. An enrichment analysis of differentially expressed genes ($p < 0.05$) was performed via a statistical overrepresentation test for gene ontology (GO) biological process, comparing the total number of reference genes in the genome of *Gallus gallus* (15,789) and of *Sus scrofa* (21,398) using bioinformatics of gene ontological classification and functional analyses (PANTHER, <http://www.pantherdb.org/>; Quick GO (<http://www.ebi.ac.uk/QuickGO-Beta>; European Bioinformatics Institute, EMBL-EBI). Top 200 differentially expressed genes (100 upregulated and 100 downregulated, based on log fold change at $p < 0.05$) in both animal classes were further selected for GO-Slim Biological Process category analyses. Bioinformatics included analyses of protein function (The UniProt Consortium, 2015) and functional pathway for GO-term category of immune system process using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. A $p < 0.05$ or FDR corrected $q < 0.05$ was set up as significant value.

Experimental design

Paper I

Cytokine and chemokine relative concentrations in two SP-fractions (SRF and post-SRF) of healthy, fertility-proven mature breeding boars were examined using Luminex. Boars had ejaculates that fulfilled the standards of sperm quantity and quality for the preparation of AI-doses ($>200 \times 10^6$ spermatozoa/mL, 70% of them motile and 75% depicting normal morphology). Each boar was weekly collected to retrieve fractionated ejaculates (SRF and post-SRF, $n=4$) from which, after centrifugation (1,500 $\times g$ for 10 min, twice, at room temperature), the SP was harvested and frozen (-80°C) until analysed.

Paper II

Semen was collected from a selected total of 84 male birds, centrifuged (21,000 $\times g$ for 10 min at $+5^\circ\text{C}$) and the SF harvested and frozen at -80°C until analysed. SF-samples from four males per group (RJF, WL and AIL, total= 12) were individually analysed by 2D-SDS-PAGE to determine the degree of individual variability within/between breed. As well, pools of SF from 15 males of each group (breed) were built to examine proteins using 2D-SDS-PAGE followed by LC-ESI-Q-TOF-MS/MS. The cytokine and chemokine SF-contents were first screened on individual ejaculates (RJF: $n=45$, WL: $n=79$, AIL: $n=43$) by a non-chicken specific Luminex commercial kit and with a subsequent chicken-specific ELISAs run on breed pools (10 birds per breed, including several ejaculates per male) to confirm Luminex-detected cytokines/chemokines.

Paper III

The gene expression of oviducts of 12 AIL hens retrieved post-mortem from either unmated (control, $n=4$) or individually mated with cocks delivering normal semen (treatment, $n=8$) was analysed after 24 h of treatment, by microarray and PCR.

Paper IV

The gene expression of the UVJ (WL hens) and the UTJ (Landrace sows of proven fertility, parity 2-3) was determined using microarray analyses and bioinformatics 24 h after treatment, and contrasted with un-mated/non-inseminated controls. Females were allotted to one of three separate groups: *natural-mating group* (n=4 for each species), where females were mated by a single male; *sperm-free SF/SP inseminated group* (n=4 for each species) where females were infused (similar to cloacal or cervical artificial insemination, AI) with the SF/SP harvested from the same males used for mating and finally, *control group* (n=4 for each species) of females that were neither mated nor infused with SF/SP. The oviduct functional sperm reservoirs (UVJ in hens and UTJ in sows) were collected from all females 24 hours after respective treatment/control either post-mortem (hens) or during surgery under narcosis (sows).

RESULTS

Cytokine/chemokine concentrations were highest in the seminal plasma of the sperm-poor fractions of the boar ejaculate

A total of fourteen measurable cytokines/chemokine comprising T-helper cells mediating type 1 response [interferon- γ (IFN- γ), interferon gamma-induced protein 10 (IP-10/CXCL10)], type 2 response [macrophage-derived chemokine (MDC/CCL22)], type 17 response [interleukin-17 (IL-17), growth regulated oncogene (GRO/CXCL1)], pro-inflammatory [interleukin (IL)-6 (IL-6), IL-8/CXCL8, IL-15, granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemo-attractant protein-1 (MCP-1/CCL2)], anti-inflammatory/immune tolerance related IL-10 and transforming growth factor- β (TGF- β_{1-3}) were detected in seminal plasma. The cytokines/chemokines were measurable in the SP of both fractions (SRF and post-SRF) except CXCL1, CXCL8 and IL-15, which did not show any measurable concentrations in the SRF. TGF- β_3 was measurable in the SP of either fraction in 4/8 boars. Concentrations of all detected cytokines/chemokines varied among boars ($p < 0.001$) and fractions of the ejaculate ($p < 0.05$).

Few seminal fluid proteins were conserved over poultry selection, but the WL and AIL showed presence of more immune system process proteins and of TGF- β_2 compared to the RJF ancestor

The SF-proteome of cocks of each breed showed a clear intra-group similarity as well as a clear inter-group variability (e.g. between RJF, WL and AIL, **Figure 5**), a fact that led to detailed analyses of breed pools built by equal SF-amounts of 15 males per group by mass spectrometry. The percentage of identified proteins from the total number of analysed spots on 2D gels (of the pools) is summarized in **Figure 6**.

Figure 5. Principal Component Analysis (PCA) of the 2DE proteome images of 12 individual SF samples (4 males per group; RJF [Red Junglefowl], WL [White Leghorn] and AIL [Advanced Intercross Line, RJF $\times$ WL]), showing the overall degree of intra- and inter-group variability. (Paper II).

Figure 6. Identified proteins in the SF of Red Junglefowl (RJF), White Leghorn (WL) and an Advanced Intercross Line (AIL, RJF x WL) (blue and green bars: number of proteins, orange bars: percentage of proteins).

The highest proportions of proteins in the SF of RJF, WL and AIL were represented by serum albumin (11.21, 21.15 and 13.27% respectively) and ovotransferrin (15.89, 17.31 and 12.24% respectively). Among other proteins, aspartate aminotransferase, annexin A5, arginosuccinate synthase, glutathione S-transferase 2 and L-lactate dehydrogenase were only detected in the SF of RJF. In contrast, glyceraldehyde-3-phosphate dehydrogenase was only detected in the SF of WL, 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 only detected in AIL.

The gene ontology (GO) and functional analysis revealed most proteins, irrespective of breed, belonged to the metabolic process group (**Figure 7**). The SF of WL and AIL, however, had more GO term category-immune system process protein compared to the RJF ancestor.

Figure 7. Biological process categories of SF-identified proteins in three different breeds of chicken (RJF-ancestor, WL-domestic layer, AIL (9th generation advanced crossline, RJF x WL). Modified from Atikuzzaman et al., Paper II).

Three immune system process SF-proteins were maintained in RJF, WL and AIL: avian β -defensin 9 (GLL9_CHICK), Ig mu chain C region (IGHM_CHICK) and the Ig lambda chain C region (LAC_CHICK). However, the immune system process proteins astacin-like metalloendopeptidase (ASTL_CHICK) and complement factor B-like protease

(CFBL_CHICK) were only present in the SF of WL and AIL, and the coagulation factor IX (FA9_CHICK) was only detected in the SF of AIL. The details of the biological process categories and protein classifications are presented in **Paper II, Table 2A-B**.

The chemokine CXCL10 was measurable in all breeds but its concentration remained higher in RJF compared to WL or AIL. The anti-inflammatory/tolerance/modulatory TGF- β_2 was, on the other hand, solely detected in the SF of WL and AIL.

Mating changed the expression of immune-modulatory and pH-regulatory genes in the chicken sperm reservoir (UVJ)

Fifteen genes were differentially expressed in the UVJ containing mucosal SST and seven genes were also differentially expressed in the uterus of mated hens while other segments of the oviduct (isthmus, magnum and infundibulum) did not show any significant differences compared to controls ($q \leq 0.05$, **Paper III**). Irrespective of treatment (hereby mating) intersegmental comparisons showed, the largest subpopulation of down-regulated genes belonged to the UVJ containing mucosal SST while the largest subpopulation of up-regulated genes was in the UVJ, the uterus and magnum (see **Table 3 in Paper III**). The UVJ depicted 1,712 up-regulated and 977 down-regulated genes that were unique (not differentially expressed in other segments) when comparing mated with control groups (a fold change of +0.45 or -0.45 had been taken into consideration).

The gene ontology and functional classification revealed 53.3% of the differentially expressed ($q \leq 0.05$) genes were immune modulatory, while 20% of the differentially expressed genes were pH-regulatory in the UVJ. Other differentially expressed genes in UVJ were referred to growth factor and few were uncharacterized genes. The differentially expressed ($q \leq 0.05$) genes in the uterus were involved in receptor activity, structural molecule activity, transporter, egg shell formation and few with unknown function. These genes are summarized in **Table 2**.

Table 2. Functional classification of differentially expressed genes at 5% FDR corrected P value ($q \leq 0.05$) in the UVJ and uterus of control versus mated hens (modified from **Paper III**).

Category	Gene symbols	Tissue
Immune-modulatory	<i>LMBRD2, CPAMD8, P450, PLA2G2E, RGS1, PDE7A, GZMA, PLCH1</i>	UVJ
pH-regulatory	<i>ATP13A3, SLC12A8, RHAG</i>	UVJ
Growth factor	<i>FGF18</i>	UVJ
Uncharacterized	<i>C17orf85, LOC417962, ENSGALG00000013955</i>	UVJ
Receptor activity	<i>ADORA2A</i>	uterus
Structural molecule activity	<i>IGFN1</i>	uterus
Transporter activity	<i>KCNV1</i>	uterus
Egg shell formation	<i>Ovocleidin 116, GKN2</i>	uterus
Unknown	<i>KNG1, CYP51</i>	uterus

Mating or SP/SF-infusion in high-fertile pigs (Landrace) and chicken (WL) changed the expression of comparable genes involved in pH-regulation or immune-modulation at the sperm reservoir (UTJ/UVJ)

The volcano plots of the probsets comparing between sperm reservoir tissues of control and treatments are presented in **Paper IV**. The gene expression changes were not significantly different ($q < 0.05$) in any comparisons except when comparing mating and control in pigs, where probsets for mated animals showed 3 upregulated and 25 down-regulated genes representing probes (**Figure 8**).

Figure 8. Volcano plot depicting differentially expressed probes in the pig oviductal sperm reservoir (UTJ) in response to mating. Each of the oligonucleotide probes is represented by a single dot. Red dots represent probes with log fold change (logFC) $> +1$ or < -1 at p -value < 0.05 . Green dots represent probes with logFC $> +1$ or < -1 at FDR adjusted p -value ($q < 0.05$). Dots above the horizontal broken line represent differentially expressed probes at p -value < 0.05 . The figure is modified from **Paper IV, Figure 1**.

Mating in chicken changed the expression of 303 genes (189 of them were upregulated and 114 were downregulated), while SF-insemination caused differential expression of 931 genes (513 of them were upregulated and 418 of them were downregulated) compared to controls ($p < 0.05$). In the pig, mating elicited expression changes in 1,722 genes (698 of them were upregulated and 1,024 of them were downregulated), while SP-infusion changed the expression of 1,148 genes (400 of them were upregulated and 748 of them were downregulated) at the same statistical threshold. The expression of 68 genes (37 of them were upregulated, 31 of them were downregulated) were commonly changed both by mating or SF-infusion in chicken, while, in pig, the expression of 592 genes (187 of them were upregulated and 405 of them were downregulated) were commonly changed both by mating and SP-infusion (for details see Venn diagrammes in **Figure 2** in **Paper IV**).

The gene ontology (GO) categories of immune system functions of the differentially expressed genes ($p < 0.05$) were overrepresented up to 35.72-fold enrichment (see **Tables 1-4** in **Paper IV**). The GO and functional classification using bioinformatics of the top 200 differentially expressed genes (equal subsets of upregulated and downregulated genes) revealed that large subsets of differentially expressed genes were referred to the GO term category of cellular and metabolic process in all comparisons (see **Figure 3** in **Paper IV**). However, the expression patterns (hereby depicted by the ratio of the upregulated and downregulated) of genes, which belonged to the GO term categories of stimulus response or immune system process, differed between animal classes after mating while followed similar patterns after sperm-free SF/SP infusion.

The bioinformatics analysis of the differentially expressed genes belonging to the GO term category of immune system process, revealed that both mating and sperm-free SF/SP-infusion resulted in the enhancement or suppression of the local oviductal immune defense mechanisms. These genes are summarized in **Table 3**.

Table 3. Differentially expressed immune-modulatory genes in the oviductal sperm reservoir (SR) of fertility-selected poultry and pigs (modified from **Paper IV**).

Treatment	Animal	Local Immune Defense (LID) at oviductal SR	Differentially expressed genes in the oviductal SR	
			UP	DOWN
Mating	Chicken	Enhancement	<i>CCR9, TNFSF4</i>	-
		Suppression	<i>LHX3</i>	<i>MASPI1, NPY6R, NRXN1, F2, TFPI, PTK2, HSPA13, NELL1</i>
	Pig	Enhancement	<i>GZMK, LY96, CD36, LOC100513220, PDZD2, DPP4</i>	-
		Suppression	<i>CSMD3, DRD2,</i>	<i>SELL</i>
SF/SP-infusion	Chicken	Enhancement	<i>DLK2, CCL1, CCR4, LIF, NOX3, ASTL,</i>	<i>BPIL3</i>
		Suppression	<i>ADCYAP1R1</i>	<i>DLL4</i>
	Pig	Enhancement	<i>GPRI16, F8, GZMK, PTK2B, LY96, SEMA6A,</i>	<i>NOR-1</i>
		Suppression	-	<i>TXNRD1</i>

The common functional category genes identified from the top 200 differentially expressed genes in each comparison (equal subsets of upregulated and downregulated genes) between chicken and pig oviduct are summarized in **Table 6** in **Paper IV**. In response to mating, the solute carrier family genes (*SLC16A2* and *SLC4A9* in chicken or *SLC13A1* and *SLC35F1* in pigs) were upregulated, while the metalloproteinase group genes (*MMP27* in chicken or *ADAMTS3*, *MMP3* and *MMP12* in pigs) as well as the Tata box gene family (*TBX4* in chicken or *TBX20* in pig) were downregulated. These genes are referred to as the common functional category. There were also common categorical genes that were differentially expressed in both animal classes after sperm-free SF/SP infusion but none of them was found in KEGG pathway analysis, while the mating-induced genes *SLC16A2* and *MMP3* were found in the KEGG pathway database. The pathway analysis indicated that the chicken *SLC16A2*, upregulated in the UVJ after mating, was involved in thyroid hormone signaling pathway while the *MMP3*, downregulated in the pig UTJ after mating, was involved in the TNF signalling pathway. Those differentially expressed genes in the solute carrier family belonged to the transmembrane transporter activity and those in the metalloproteinase group belonged to cellular recruitment and migration, as revealed by UniProt database in the category of biological process and molecular functions.

GENERAL DISCUSSION

The studies in this thesis have shown that the composition of the seminal fluid in chicken with differences in fertility (RJF vs WL/AIL) varies in relation to protein type and contents, similarly to what is already known for pigs (Perez-Patiño et al., 2016). Comparative analyses of a battery of cytokines/chemokines in the SP/SF of boars and cocks also showed major differences. While variations were seen between fractions of the ejaculate (highest in the protein-richest last fraction) and among boars (in apparent relation to individual fertility); the cock-SF revealed low amounts of two confirmed cytokines/chemokines, with TGF- β_2 being only present in the breeds selected for fertility/egg-laying. Mating or insemination with sperm-free seminal fluid/plasma induced changes in the expression of genes on the sperm reservoir areas in the oviduct, both in poultry and pig.

In this chapter, I shall discuss whether the oviductal response to mating or SF/SP infusion in domestic chickens and pigs share or kept common mechanisms to maintain sperm storage and survival in the oviductal functional sperm reservoir, thus avoiding rejection of seminal immunologically foreign cells/proteins, as part of the establishment of the phenomenon of internal fertilization during animal phylogeny.

The proteome of the seminal plasma (SP) in mammals, including humans, has been thoroughly studied (for a recent review see Rodriguez-Martinez et al., 2011). The proteome of the boar seminal plasma has been, very recently, characterized in detail for all ejaculated fractions and further for their relation with the fertility of the sires (Perez-Patiño et al., 2016). In pig, a convenient animal model for studies on species with fractionated ejaculates as human, the SP has been found to be involved in the induction of environmental changes in the female genitalia, previously considered as a sperm-related role, regarding pro- and anti-inflammatory phenomena (Lovell & Getty, 1968, Rodriguez-Martinez et al., 1990, Rozeboom et al., 1999). For instance, the intrauterine infusion of gel-free SP in pre-ovulatory gilts recruits MHC class II-activated macrophages and dendritic cells to the endometrium (O'Leary et al., 2004). The majority of the proteins in boar SP belong to the spermadhesin lectin family and comprises the alanine-glutamine-asparagine (AQN-1 and AQN-3), alanine-tryptophan-asparagine (AWNs) and the Porcine Seminal Plasma Proteins I and II (PSP-I and PSP-II) (Calvete et al., 1995; Töpfer-Petersen et al., 1998; Calvete et al., 2005). Insemination of pre-ovulatory sows with average concentrations of PSP-I/PSP-II induces a rapid (within 10 min) PMN migration into the uterine cavity as well as a later increasing amounts of CD4⁺ and CD8⁺ T cells (Rodriguez-Martinez et al., 2010), suggesting that the pig SP induces a dual defence response, firstly a transient inflammatory response followed by a re-organization of the female immune system towards a status of tolerance, as suggested for other species (Robertson, 2005; 2007).

The taxonomically distant chicken differs from a mammal such as the pig, also in relation to the ejaculate. The boar ejaculate is built by the concerted emission of the cauda epididymides and the temporal secretion of the sexual accessory glands (representing 95-98% of the total volume), ejaculating jets building an initial sperm-rich and later sperm-poor fractions. In contrast, the cock does not have any sexual accessory gland and barely a rudimentary epididymis (Lake, 1957), and thus the ejaculate is single and small in volume (<1 mL). Comparative examinations of the proteome of chicken are scarce and mostly identified major proteins in common commercial breeds (Marzoni et al., 2013; Labas et al., 2015). Moreover, eventual relationships of the SF proteome with fertility (egg-laying capacity) are yet to be studied in detail.

In the present thesis, the SF-proteome including cytokine/chemokine concentrations of three chicken lines with documented egg-laying capacity and reproductive performance were determined; namely of the ancestor breed RJF, the domestic layer WL and a 9th generation of an advanced cross line between RJF and WL (AIL) (Schütz and Jensen, 2001; Schütz et al., 2002; Cheng, 2010). In contrast to other species (human) or even laboratory animals where semen selection for quality has not been performed as tight as with chicken or pigs, there is an intrinsic variation between ejaculates and individuals, but the variation is low compared to breed and selected lines in chicken, as it could be demonstrated in the **Figure 1** in **Paper II**. Therefore proteins were identified in pools of SF samples of each chicken breed, owing to the interest in unveiling SF proteome differences that may underline a fertility-selected breed population fitness trait, rather than individual differences, and thus to minimize confounding individual intra- and inter-male variation.

The major proteins identified in the SF of all breeds were serum albumin and ovotransferrin, thus confirming previous studies (Marzoni et al., 2013; Labas et al., 2015). Expectedly too, spermadhesins were not present owing to the lack of sexual accessory producing glands in chicken, contrasting with the boars (and other mammals) where these proteins of seminal vesicle main origin dominate (Rodriguez-Martinez et al., 2011). Serum albumin in the SP of rams and humans is produced by the epididymis (Elzanaty et al., 2007; Soleilhavoup et al., 2014) and so this source might be common to cocks. Further studies ought to be done to confirm this source. The protein has been classified as defence or immunity protein by bioinformatics analysis reported by Marzoni et al. (2013). Serum albumin is widely used as an additive for semen handling in many species including turkey (Bakst and Cecil, 1992) and human (Elzanaty et al., 2007) since it maintains sperm motility (Matsuoka et al., 2006; Fukui et al., 2007; Hossain et al., 2007; Xia and Ren, 2009; Nang et al., 2012). However, the fact that serum albumin is overexpressed in the high egg-laying capacity domestic chicken might also apply to the positive correlation of serum albumin with egg-laying, since albumin is a major component of the egg. The other overexpressed protein, ovotransferrin, originates in the Sertoli cell (Holmes et al., 1982; Gilmont et al., 1990) and it is considered to act as defence protein (bactericidal), delivered in the albumin of the egg (Marzoni et al., 2013; Baron et al., 2014). Consequently, it is logical to suggest that the SF-ovotransferrin might help decreasing the pathogen overload during mating in chicken.

The SF-proteins measured in cocks varied between the ancestor (RJF) and domesticated modern chicken (WL and AIL), a variation that might be related to changes in fertility over selection/evolution. Sperm concentration and sperm forward motility are both higher in the ancestor RJF compared to domestic chicken (Malik et al., 2013). Moreover, intra-breed selection for high egg production (as a token for female fertility) has either not affected semen quality (Frankham and Doornebal, 1972; Niranjana et al., 2001), or negatively done so (White Leghorn, Murugesan et al., 2013). Such results on spermatozoa are now expanded to the seminal fluid, with results of **Paper II** being the first to reveal differences in SF-proteins among chicken with varying egg-laying capacity which might have signaling significance for sperm-storage and survival within the internal female tract.

The present thesis reports that serum albumin and ovotransferrin were overexpressed in the WL and AIL, contrasting with proteins holding immune regulating functions, as av β D-9 and Ig λ -chain. While av β Ds are considered involved in the innate immunity of the oviduct in poultry (for review see Das et al., 2008) the different members of this family appear related to different location and function. For instance, av β D-9 was identified in the SF of chicken (Marzoni et al., 2013). Alongside, the av β D-3 was present in spermatozoa, being assumed they are involved in protecting spermatozoa from immune recognition in the female genitalia (see

review Das et al., 2008). Other several av β Ds (1-5 and 7-12) were also identified in the oviduct, depicting antimicrobial activity (see review by Sugiarto and Yu, 2004). Findings of av β D (1-3) being overexpressed in the vagina and infundibulum in WL laying hens, suggested they constitute an increased first line of defence (Ohashi et al., 2005; Abdel-Mageed et al., 2008), with implications for fertility.

In contrast to the findings in the present studies (**Paper II**), the Ig λ -chain C has previously been identified in the least-fertile cock-SF where it was down-expressed (Labas et al., 2015). Differences of the line breeds between studies might reside behind these differences. In sum, it is evident that despite differential origin, the SF/SP of chicken or pig is equipped with proteins involved in microbiological defence but also intervening in the function of spermatozoa. Whether the latter is brief (for instance contributing to the preservation of sperm motility or membrane integrity before colonizing the sperm reservoirs in the female) or long-lasting (before spermatozoa are released for fertilization) is yet unknown, except for some events (poultry: Das et al, 2008; pigs: Caballero et al, 2006).

Cytokine/chemokine composition in the ejaculate has mostly been studied in humans (Carson et al., 1994; Maegawa et al., 2002; Politch et al., 2007) and in laboratory mice (Tremellen et al., 1998; Gopichandran et al., 2006), thus calling for attention in pigs and chicken in order to complete findings of the proteome of fractionated respectively single-bulk ejaculates in other species that could well serve as experimental models for biomedicine or domestication studies. Studies of the different fractions of the boar ejaculate (**Paper I**), demonstrated that the highest amounts of cytokines/chemokines were seen in the fraction with the largest amounts of proteins present (e.g. the post-SRF) a fraction composed mainly by the secretion of the vesicular glands (Einarsson, 1971; Mann & Lutwak-Mann, 1981). However, it was also clear that cytokines were present along all fractions, albeit in varying amounts. Interestingly, Th17-responsive chemokine CXCL1 and the pro-inflammatory cytokine IL-15 and the chemokine CXCL8 were not found in the SRF of any boar, e.g. they were not accompanying most spermatozoa emitted. Moreover, there were significant differences between males, presumably in relation to their inherent fertility. The study is highly relevant since it implies that the female is exposed to a higher load of SP-cytokines at the end of the mating, when the i.e. sperm-peak fraction has already reached the tip of the uterine horns and probably a fraction of these spermatozoa already entered the sperm reservoir in the UTJ. Thus, it is logical to expect that the cytokines play differential roles in interacting with the local immune system in the female genitalia (Rozeboom et al., 2001; O'Leary et al., 2004; Jiwakanon et al., 2010; Rodriguez-Martinez et al., 2011). Even though few cytokines were reported previously in the boar SP (O'Leary et al., 2011; Jiwakanon et al., 2012), a full battery of cytokines in the SP of well-identified fractions of the boar ejaculate have been lacking until **Paper I** was published. For instance, only presence of TGF- β_1 , TGF- β_2 , IL-6 and IL-10 were reported in the porcine SP (O'Leary et al., 2011; Jiwakanon et al., 2012). O'Leary et al. (2011) measured TGF- β_1 and TGF- β_2 in the bulk and fractionated seminal plasma with concentrations varying widely among boars. Jiwakanon et al. (2012) measured TGF- β_1 , IL-6 and IL-10 in SP-fractions, with TGF- β_1 and IL-10 concentrations varying most among boars, but IL-6 being low or non-detectable and TGF- β_1 concentrations being highest in the first 10 ml of SRF (the sperm-peak portion of the pig ejaculate) compared to other fractions. The variation shown for these particular cytokines among boars by the abovementioned studies was similarly found in **Paper I** but with clear differences in the concentrations recorded, which might be because of the different methods used. However, IL-6 appeared measurable in all ejaculate fractions of most boars and concentrations of TGF- β_1 appeared highest in the post-SRF in all boars studied, thus contrasting with the findings of Jiwakanon et al. (2012). Differences in concentrations between

studies might mirror different number of boars explored, their breeds or, with semen collection being manual, even to differences between operators in sampling the relevant fractions.

The same exploratory procedure was done with chicken SF, with individual and breed pools of the different lines, being used for proteomic analyses (e.g. RJF, AIL, WL). The results of the Luminex kits were, contrasting with the pig SP, showing barely a couple of cytokines present. The lack of sexual accessory glands in chicken might well explain absence of many other cytokines, but since “absence of evidence is not evidence of absence”, chicken specific ELISA-based kits were used to verify that these cytokines (TGF- β_2 and CXCL10) were actually present (**Paper II**). However, it must be remembered that presence of other non-detectable cytokines/chemokines cannot be ruled out, until the SF is further studied using assays specific for the species, once they become available. The TGF- β_2 , usually considered an immunosuppressing cytokine, was below the detection range in RJF, while the pro-inflammatory chemotactic chemokine CXCL10 showed higher concentrations in RJF than in the modern chicken lines explored. The presence of TGF- β isomers was definitely clearly limited to the TGF- β_2 . The presence of TGF- β isoforms in the SP of, for instance the boar, are considered related to the production of regulatory T cells which influence the female reproductive tract a state of immune tolerance to the spermatozoa as well as of the developing conceptus (see Rodriguez-Martinez et al., 2011 and the references used therein). The lack of some isoforms might relate to the differences between mammalian eutheria and egg-laying chicken, where the tolerance status is restricted to the spermatozoa in the oviduct. TGF- β_2 has been widely known has immunosuppressive function and considered one of the important factors in the female genitalia to tolerate immunologically foreign spermatozoa in mammals (Robertson et al., 2002) and poultry (Das et al., 2006). The lack of TGF- β_2 in the RJF-SF might therefore be in relation to the lower fertility of the ancestor in wildlife; although it is not clear whether high concentrations in the post-SRF ejaculate of the boar is beneficial or detrimental for the fertility. In contrast, the chemokine CXCL10 is widely considered as the chemoattractant that recruits activated T-cells (Agostini et al., 2001). CXCL10 knock-out mice shows impaired T-cell responses (Dufour et al., 2002). Interestingly, CXCL10 is found increased in the post-SRF compared to SRF in boar ejaculate (**Paper I**), while it seems decreased in the SF of fertility selected domestic breed cocks in the present study (**Paper II**). The low concentrations of CXCL10 in the pig or chicken might help spermatozoa to reach the oviduct sperm reservoir without being eliminated by a female immediate immune response.

The SF/SP-cytokines and chemokines originate in cells present in the cauda epididymides and the accessory sexual glands (seminal vesicles, prostate and bulbourethral glands) which constitutes the total volume of SP in the ejaculate of most mammalian species including interspecies variation (Beyler and Zaneveld, 1982). In the case of a boar, the larger portion of the SP (55-75%) is mainly contributed by the prostate and urethral glands, 15-20% comes from the vesicular glands and 10-25% from the bulbourethral glands (Pond and Houpt, 1978). The bulk volume of SRF SP is mainly contributed by the prostate glands, epididymides and in a lesser extent by the seminal vesicles while the post-SRF are constituted by the vesicular, prostate glands and at the end of the ejaculate by the bulbourethral glands (Einarsson, 1971; Mann & Lutwak-Mann, 1981). In rodents, TGF- β is synthesized by the vesicular gland (Tremellen et al., 1998) or by the prostate gland in human (Lee et al., 1999), while CXCL1 and CXCL10 are synthesized at the seminiferous tubules in rat (Aubry et al., 2000). The comparatively high concentrations of cytokines/chemokines in the SP of the sperm-poor post-SRF fraction in the boar suggests the vesicular glands make the major contribution of cytokines/chemokines to the SP a matter that should be followed up in future studies,

particularly in relation to the effect (stimulatory or suppressive) these cytokines might have on the female genital immune system.

In the present study, the chemokines CXCL1, CXCL8 and cytokine IL-15 were completely absent in the SRF fraction (**Paper I**). An *in vitro* study on human natural killer (NK) cells revealed that IL-15 is able to activate NK cells (Carson et al., 1994). It has also been reported that intranasal administration of CXCL1 variants to the mouse has been involved in neutrophil (PMN) migrations in the lung tissues (Sawant et al., 2015). PMN-chemotaxis is effectively influenced by the CXCL8 (Lin et al., 2004), while the concentration of CXCL8 in human seminal plasma is inversely related to sperm quality and positively correlated with leukocyte count in the ejaculate (Eggert-Kruse et al., 2001). These findings along with the current results indirectly support the ability of the pig spermatozoa, by-chance present in the SRF, to avoid immediate PMN-phagocytosis after deposition and find themselves entering the immune-privileged UTJ (Rodriguez-Martinez et al., 1990; Rodriguez-Martinez et al., 2005; Rodriguez-Martinez et al., 2009). In contrast, the high concentrations of pro-inflammatory proteins (PSP-I/PSP-II) and cytokines/chemokines of the post-SRF accessing the uterus shortly thereafter, provoke a massive infiltration of PMN to phagocytize and eliminate the rest of the spermatozoa and the SP proteins, thus cleansing the uterine lumen as a preparation for hosting the early embryos descending in few more days (Rodriguez-Martinez et al., 2010).

In chicken, such separated phenomena might be absent owing to the bulk, minute nature of the cock ejaculate, but in general the entry of the spermatozoa to the sperm reservoir follows a similar pattern e.g. few spermatozoa (less than 1%) reach the SST and the period of entry varies from minutes to one hour (Bakst et al., 1994). A backflow post-mating also occurs in chicken and eliminates >80% of the spermatozoa (Bakst, 2011). Those left in the vagina are thought to be eliminated by the temporal (1-3 h) overexpression of *IL1B* and *LITAF* (Das et al., 2009). The pro-inflammatory SF-proteins and cytokines found in **Paper II** are therefore possibly involved in stimulating infiltration of immunocompetent cells largely distributed in the vagina of laying hens, compared to immature, un-mated hens (Zheng et al., 1998; Zheng and Yoshimura 1999). For instance, entry of SF-protein av β D-9 might provoke oviduct production of local anti-bacterial β -defensin (Das et al., 2008) as these local increased levels were seen in the vagina after insemination (Ohashi et al., 2005; Shimizu et al., 2008). It is therefore assumed that in both pigs and chicken, those spermatozoa that fail to reach the immunologically privileged SR are eliminated by local inflammatory cells, and other local mechanisms triggered by some of the pro-inflammatory components in the SF/SP.

The major intention of the current thesis was to explore if entry of homologous semen (by mating) or of sperm-free SF/SP (by infusion similar to cloacal or cervical artificial insemination, AI) into the female genitalia could cause gene expression changes in the oviduct of chicken and pigs with a special reference to the oviductal sperm reservoir. Oligonucleotide microarrays (holistic gene expression analysis tools) were used; comparing chicken breeds, and also comparing fertility-selected chicken (WL) with fertility-selected pigs (Landrace). The studies (**Papers III and IV**) revealed that both mating and sperm-free SF/SP infusion caused a differential gene expression in the tubal sperm reservoir of hens and sows. The expression changes were greater when females were mated/AI with homologous semen compared to the AI-of homologous sperm-free SF/SP. The greater subpopulation of differentially expressed genes belonged to immune-modulation and pH regulation pathways, and few of these genes seemed to follow similar mechanisms in both domestic chicken and pigs, indicating a certain degree of conservation strategies throughout phylogenesis and selection during domestication.

For decades we had been aware that the oviductal pre-ovulatory functional sperm reservoir in birds and mammals is an immunologically privileged segment of the female genital tract, allowing the survival of potentially fertile foreign spermatozoa (Das et al., 2008; Rodriguez-Martinez et al., 2005). There are several review articles published describing a small subpopulation of spermatozoa reaching the oviduct sperm reservoir (UVJ in hens and UTJ in sows) where they remain alive without being eliminated by the immune attack, while the rest of the deposited ejaculates including spermatozoa are either egressed by backflow from the female genital tract or eliminated by phagocytosis both in chicken (Bakst, 1994; Das et al., 2008; Sasanami et al., 2013) and in pig (Schuberth et al., 2008; Holt, 2011; Tienthai, 2015). Only few immune-suppressing genes have been targeted and identified in the UVJ of poultry after insemination/mating using quantitative RT-PCR (Das et al., 2006; Abdel-Mageed et al., 2008; Huang et al., 2016) or including serial analysis of gene expression in oviduct SR in turkey (Long et al., 2003). On the other hand, no studies investigated gene expression changes after homologous sperm-free SF infusion in chicken. In pig, specific gene expression analysis of the SR, either after mating or after SP-infusion was lacking, except for the expression of the enzyme carbonic anhydrase, an enzyme relevant for pH regulation and hence control of sperm motility (Rodriguez-Martinez et al., 1991). However, other areas/segments of the female genital tract of chicken and mammals have been targeted (vagina in chicken, Das et al., 2009; oviduct in mouse, Fazeli et al., 2004; ampullary-isthmus junction in sows, López-Úbeda et al., 2015). Thus, focus was placed on the SR (UVJ/UTJ) after semen or SF/SP exposure in order to determine which components could be related to a differential gene expression in this segment, and in relation to sperm survival.

A small sperm subpopulation quickly reaches the oviductal SR and stays in there for either days (40-48 h in pig) or weeks (1-4 in poultry). In either species, an initial, transitory inflammation occurs to pass onto a second period of immunological tolerance to the paternal alloantigens. That such tolerance is needed in species with internal pregnancy, formation of placenta for long periods (114 days in pig) is almost obvious, but how about the egg-laying chicken? They have no pregnancy, nor placenta, but they have spermatozoa present for weeks. They do not show any long-lasting inflammation or sperm phagocytic events apart from immediately after mating, but spermatozoa are kept alive and fertile during this lengthy period.

The oviduct of AIL chicken was investigated 24 h after mating to see if the entry of semen would cause gene expression changes at the various segments of the genital tract. Significant changes were only observed in the UVJ and uterus. While differentially expressed genes in the uterus were mostly involved in egg shell formation or uterine elasticity; most differentially expressed genes in the UVJ were involved either in immune-modulation or pH-regulation, with the majority of the immune system process genes being involved in immune suppression (**Paper III**). In comparison, the RJF showed more than 50 times large subsets of gene expression changes in the UVJ and uterus after mating (Atikuzzaman et al., 2015), which suggests that domestication/selection for egg laying has greatly affected the female genitalia towards a more tolerant responsiveness to seminal exposure, perhaps leading to a longer lifespan of fertile spermatozoa in the SR and a higher fertility of the selected chicken. Future studies are needed to elucidate the extent of these findings.

In the present investigation (**Paper IV**), a large subpopulation of immune-processing genes was differentially expressed in the SR of chicken and pigs 24 h after mating, in agreement with studies in other species (Long et al., 2003; Fazeli et al., 2004). In turkey UVJ, expression of avidin and avidin-related protein-2 (AVR2) are increased, while the expression of progesterone receptor is decreased in response to the entry of spermatozoa compared to other oviductal segments, presumably leading to a longer sperm storage in the SST (Long et al., 2003; Foye-

Jackson et al., 2011). Gene expression changes issued by the entry of TGF- β isoforms to the chicken oviduct sperm storage area are considered necessary to tolerate the foreign spermatozoa and seminal proteins (Das et al., 2006). As well, lipase and lipid receptor mRNA expression changes have been related to SST-sperm-survival through increased fatty acid synthesis in chicken (Huang et al., 2016). In mice, entry of spermatozoa could provokes signal transduction pathways which lead to changes in gene expression, mostly involved in local immune responses (Fazeli et al., 2004). Most differentially expressed immune-modulatory genes in the oviductal sperm reservoir after mating or SF/SP-infusion of domestic chicken (AIL and WL) seem involved in immunosuppression (**Table 3**, and for AIL see **Paper III**) partly confirming previous studies in chicken (Das et al., 2008; Sasanami et al., 2013). Conversely, differentially expressed immune modulatory genes in pig UTJ are skewed to immune enhancement, which might be related to its comparatively shorter sperm storage. This relation should be elucidated in future studies.

Spermatozoa are quiescent within the SR in chicken (Holm and Wishart, 1998) and pig (Rodriguez-Martinez, 2007). pH-regulation should be highly relevant during sperm-storage in the oviduct SR since pH/bicarbonate levels regulate sperm motility *in vitro* both in pigs (Rodriguez-Martinez et al., 2005) and chicken. Sperm motility in chicken is inhibited by a pH below 7.8, while raising its value 0.2 units and higher provokes resumption of vigorous motility (Holm and Wishart, 1998). The enzyme carbonic anhydrase, present in the SST lumen of domestic hens (Holm et al., 1996) and the UTJ of sows (Rodriguez-Martinez et al., 1991) seems to be pivotal in controlling pH and bicarbonate levels. *In vivo* studies have shown that the pH in the oviduct SR tends to acidity [chicken: pH, 6.92-7.18 (Bakst, 1980); sows: pH, 6.7 (Rodriguez-Martinez, 2007)] compared to other segments [chicken: in the infundibulum, 7.02-7.21 and in the mid-vagina, 7.15-7.51 (Bakst, 1980); sows: pH, 7.5 in the ampullary-isthmic junction and pH, 8.3 in the ampulla (Rodriguez-Martinez, 2007)]. Alongside, the intraluminal content of bicarbonate (HCO_3) varies dramatically between the isthmus and the ampulla of pigs (10.0 vs 33.1 mM/L, respectively; Rodriguez-Martinez, 2007). The present studies in domestic chicken and pigs revealed that genes of the solute carrier family and ATPases are involved in the pH-regulation (see **Papers III** and **IV**) by exchanging protons, ions and bicarbonate between the intra- and extra-cellular space (Casey et al., 2010; Bublitz et al., 2011; Palmgren and Nissen, 2011; Liu et al., 2012).

Since mechanisms of immune and motility suppression seem present in chicken and pig SR:s as long as the spermatozoa are stored in this restricted oviduct segment, experiments (**Paper IV**) were attempted to discern whether hens and sows would share some common gene expression changes related to sperm function and survival. Interestingly, the bioinformatics investigation of the differentially expressed genes after mating and SF/SP-infusion (summarized in **Figure 9**) revealed that there were 30 genes involved in different molecular functions shared between these species. Among them, the immune modulatory (Parks et al., 2004) *matrix metalloproteinase (MMP)* genes (*MMP27* in chicken UVJ, and the *ADAM metalloproteinase with thrombospondin type 1 motif 3*, *MMP3* and *MMP12* in sow UTJ), were downregulated post-mating. The *MMP27* is expressed by the cycling human endometrial macrophages, suggesting a role on immune responses (Cominelli et al., 2014). The *MMP-3* and *MMP-12* knock-out mouse shows a reduced neutrophil influx and macrophage migration in the lung as well as a reduced TNF- α release from macrophages, which are also indicative of immunosuppression (Shipley et al., 1996; Silence et al., 2001; Warner et al., 2001a; Warner et al., 2001b; Churg et al., 2003). On the contrary, there were no common immunomodulatory genes upregulated in the avian or porcine SR after mating (see **Tabel 6** in **Paper IV**).

Figure 9. Semen and/or SF/SP proteins/cytokines could elicit changes (upregulation: red, downregulation: black) in the expression of pH-regulatory or immune-modulatory genes at the sperm reservoir of the hen or sow. The underlined genes are species equivalent. Lu: lumen, E: epithelium, Lp: lamina propria, arrows: stored spermatozoa. TGF- β ₂, transforming growth factor β ₂; CXCL10, C-X-C motif chemokine 10; ALB, serum albumin; TRFE, ovotransferrin; av β D-9, avian β defensin-9; IGHM, Ig λ chain C; PSP-I/PSP-II, porcine seminal plasma protein I and II, SLC, solute carrier family; RHAG, Rh-associated glycoprotein; ATP13A3, ATPase type 13A3; ATP8B3, ATPase aminophospholipid transporter Class I type 8B member 3; MMP, Matrix metalloproteinase; IFIT, Interferon induced protein with tetratricopeptide; IFI16, interferon gamma inducible protein 16.

However, genes controlling the interferon-induced proteins (hereby, *IFIT5* in chicken UVJ or *IFI16* in pig UTJ, involved in enhancing innate immune and inflammatory response; Baggetta et al., 2010; Zhang et al., 2013) were upregulated after sperm-free SF/SP-infusion (see **Table 6** in **Paper IV**). Noteworthy, considering that the SF/SP is a component of semen, why were these genes not upregulated by mating while this was the case after sperm-free SF/SP-infusion? Such differences indicate an interplay between spermatozoa and SF/SP of either species in the female genitalia where spermatozoa are able to positively balance the expression of immune-modulatory genes. Such interpretation is, however, early drawn, requiring future studies to unveil which are the specific components in spermatozoa or in the accompanying extra-cellular seminal fluid (proteins, cytokines, other cell molecules) that can be responsible, individually or in a concerted fashion, of the signalling from the male to the female that can modify her reactivity towards semen.

GENERAL CONCLUSIONS

The overall results of this thesis indicate that semen contains components that can influence gene expression changes in the oviduct sperm reservoir in evolutionarily divergent poultry and pigs, ultimately indicating sperm storage is an ancestral state. In particular, I could conclude that the:

- boar seminal plasma is rich in Th1, Th2, Th17, pro- and anti-inflammatory cytokines and chemokines, whose relative concentrations are highest in the sperm-poor post-SRF fraction of ejaculate, and varies among sires, possibly in relation to their fertility.
- seminal fluid proteome and peptidome vary greatly between ancestor and domestic modern chicken with respect to immunosuppression-related proteins and cytokines/chemokines, relevant for sperm survival in the female genitalia.
- expression of immune-modulatory and pH-regulatory genes in the UVJ has changed over domestication/selection towards higher egg-laying capacity, probably by making the modern female chicken immunologically more tolerant to the presence of foreign spermatozoa and SF-proteins.
- entry of homologous semen or SF/SP induces changes in gene expression in the oviduct sperm reservoir in fertility-selected poultry or pig breeds, sharing common functional genes relevant for sperm-storage and survival in the oviduct SR.

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Papers

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