Seminal fluid and sexual selection in the Red junglefowl, *Gallus gallus*

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Chapter 5: Seminal fluid proteomic profiles associated with male age and sperm velocity

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THESIS ABSTRACT

Sexual selection continues after copulation when the ejaculates from different males compete to achieve fertilization. Post-copulatory sexual selection is therefore a powerful evolutionary pressure, with seminal fluid emerging as an important factor. This thesis studies the role of seminal fluid in sexual selection in the polyandrous Red junglefowl, *Gallus gallus*. First, sperm Average Path Velocity (VAP) *in vitro* was higher over time when sperm were exposed to non-self, and particularly non-self related, seminal fluid. There was a tendency for sperm exposed to non-self seminal fluid to produce more hydrolysis points on the PVL membrane of the egg than sperm exposed to self seminal fluid. Second, males preferentially invested sperm in sexually novel females, by decreasing investment over successive copulations with the same female and increasing it again on encountering the new female. Seminal fluid volume allocation patterns mirror those of sperm. Seminal fluid protein allocation showed a status-specific pattern. While dominant males tended to allocate a constant amount of seminal fluid protein to successive copulations, subdominant males tended to increase their investment on the novel females. Third, I identified 1,141 proteins in the Red junglefowl seminal fluid, including proteins involved in immunity and antimicrobial defences, sperm maturation, metabolism, and fertilization. These proteins reveal a substantial contribution of blood plasma proteins that is conserved with humans. A comparison with the domestic fowl seminal fluid proteome, revealed qualitative and quantitative differences, likely associated with domestication and artificial selection. Fourth, by comparing the seminal fluid proteome of young and old males with fast and slow sperm velocity, I identified proteins associated with male reproductive ageing and sperm quality. This thesis shows that seminal fluid can play a potentially important role in sexual selection, and provides novel insights regarding male fertility and the molecular basis of reproductive divergence in seminal fluid proteins associated with domestication and artificial selection.

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CHAPTER 1

Sexual selection and seminal fluid - An Introduction

SEXUAL SELECTION

Sexual selection is the process that favors the expression of traits conveying an advantage to some individuals in relation to competition with their conspecifics of the same sex over access to reproductive opportunities. Traditionally, sexual selection was considered to operate through two interactive episodes. Firstly, sexual selection promotes traits enhancing individual success in intrasexual competition for mating opportunities. Secondly, sexual selection confers a reproductive advantage to individuals resulting more attractive to the members of the opposite sex (Darwin, 1871; Andersson, 1994). Sexual selection arose as a result of the transition from isogamous (involving gametes of similar size and morphology) to anisogamous sexual reproduction (involving the fusion of gametes of different size) and the evolution of sex roles ("sexual cascade", Parker, 2014). In a pristine state of isogamous sexual reproduction, an increase in gamete size is thought to have led to a selective advantage over smaller gametes since the larger the gamete the more nutrients it could provide to the zygote, thus enhancing zygote fitness. For this reason, large gametes were selected to fuse with each other. However, large gametes were produced in lesser numbers than small gametes. This generated the opportunity for individuals producing smaller gametes to exploit their bigger investment in resources enhancing zygote fitness. The predominance of small gametes together with their strong selection to fuse with large gametes allowed large gametes to invest fewer resources in mobility without increasing the time necessary to fuse with another gamete. Thereafter, large gametes could devote more resources to the zygote. Finally, the union between large gametes and small gametes became predominant, thus leading to the differentiation of sexes, namely, individuals producing many and small

gametes (males) and individuals producing few and large gametes (females) (Parker, Baker & Smith, 1972).

With the exception of species with sex-role reversal, anisogamous sexual reproduction results in males having a higher potential reproductive rate than females (Clutton-Brock & Vincent, 1991; Clutton-Brock & Parker, 1992) because males are able to fertilize eggs at a faster rate than females are able to produce them (Davies, Krebs & West, 2012). For this reason, males are often selected to pursue additional mating opportunities to maximize their reproductive success, thus expending lower resources in parental investment than females (Queller 1997; Kokko, Jennions & Brooks, 2006). Parental investment is any expenditure in an individual offspring that increases the offspring's chances of surviving at the cost of the parent's ability to invest in other offspring (Trivers, 1972). The asymmetries in parental investment between males and females are reflected in the operational sex ratio (Clutton-Brock & Parker, 1992), which is the average ratio of sexually receptive females to sexually active males at any given time (Emlen & Oring, 1977). The operational sex ratio is generally male-biased since males may spend more time in the mating pool after a breeding event (Clutton-Brock & Parker, 1992; Jennions & Kokko, 2010). High potential reproductive rate and subsequent short parental investment makes male reproductive success limited by mate availability. Thus, males are sexually selected to compete amongst each other to gain access to females. Consistent with this, there is widespread evidence that sexual selection for mating success is stronger in males than in females (Janicke *et al*., 2016). Conversely, short potential reproductive rate and subsequent selection for high maternal investment make female reproductive success limited by their ability to optimize the conversion of resources into eggs and offspring (Davies, Krebs & West, 2012).

Female mate selection models

Female mate choice may be operationally defined as any pattern of female behaviour that leads them to be more likely to mate with certain kind of males than with others (Halliday, 1983). The models explaining the evolution of female mate selection are based on the existence of a female preference towards certain male phenotypic traits. Female preference is any female trait that biases male mating and fertilization success towards certain male phenotypes (Arnqvist & Rowe, 2005). Such a preference may initially arise as a consequence of female sensory biases shaped by natural selection aimed to improve, for instance, foraging or predator avoidance efficiency. These preexisting sensory biases will be exploited by the displays of male phenotypic traits (Andersson 1994; Rodd *et al*., 2002; Arnqvist & Rowe, 2005; Andersson & Simmons 2006; Egger *et al*., 2011). The subsequent coevolution between female original preference and the displays of male phenotypic traits has been explained by two categories of models, namely, mutualistic and antagonistic coevolutionary models (Pizzari & Snook, 2003; Kokko, Jennions & Brooks, 2006).

Mutualistic coevolutionary models

These models state that the evolution of female preference towards male phenotypic traits increases female fitness (Andersson 1994; Andersson & Simmons, 2006) by obtaining direct or genetic benefits (Jones & Ratterman, 2009).

Mutualistic coevolutionary models based on direct benefits state that females evolve a preference towards male phenotypic traits that correlate positively with the ability of their bearer to provide immediate benefits to the female, such as, help with parental care, food or territory defense (Kokko, Jennions & Brooks, 2006; Jones & Ratterman, 2009).

Mutualistic coevolutionary models based on genetic benefits propose that female preference evolves towards male phenotypic traits that genetically covary with male viability or

attractiveness (Jones & Ratterman, 2009). Two scenarios explain female mate choice from this perspective, namely, the Fisherian runaway process and the indicator mechanisms (Andersson, 1994; Andersson & Simmons, 2006).

The Fisherian runaway process states that females choose males having phenotypic traits correlated with a selective advantage and, thus, evolved under direct selection. The intensity of the female preference increases due to having sons with high fitness and daughters with the preference for the selected male phenotypic trait (Fisher, 1930; Andersson, 1994). The alleles determining female preference will be in linkage disequilibrium with the alleles encoding the favored male phenotypic traits. Therefore, the alleles determining the female preference will be under indirect selection because of being correlated or in linkage disequilibrium with the alleles encoding the favored male phenotypic traits and will be established in the population as a consequence of this association (Kirkpatrick & Barton, 1997). Since males having the advantageous traits also increase their mating success they will be selected to evolve more elaborate displays of these traits until their expression become detrimental to survival and, eventually, natural selection halts their evolution (Fisher, 1930; Andersson, 1994).

The indicator mechanisms scenario presents two variants, namely, the “good genes” mechanism and the “handicap” mechanism which can be seen as two sides of the same coin. The “good genes” mechanism states that females choose males having conspicuous traits since they reflect the underlying additive genetic quality of their bearer (Andersson, 1994; Andersson & Simmons, 2006). The “handicap” mechanism states that these conspicuous traits make it more difficult to survive and, thus, that the bearer should be outstanding at certain crucial abilities to the ecological demands of the species (Zahavi, 1975). In both cases, mating with males having conspicuous traits will result in offspring with high viability or good condition as adults (Jones & Ratterman, 2009).

The Fisherian runaway process and the indicator mechanisms are not mutually exclusive and may operate interactively since the evolution of more elaborate male phenotypic displays eventually results in their expression reflecting the underlying additive genetic quality of their bearer (Kokko, Jennions & Brooks, 2006; Chandler, Ofria & Dworkin, 2012).

Antagonistic coevolutionary models

These models state that the evolution of female preference towards male phenotypic traits decreases (rather than increases) female fitness (Pizzari & Snook, 2003; Arnqvist & Rowe, 2005). In this scenario, the appearance of a mutation produces a rudimentary male trait that enables males to manipulate female reproductive decisions in a way that benefits male reproductive interests but reduces female fitness. This situation selects females to develop traits increasing resistance (or tolerance) towards male manipulation as a result of which female fitness increases at the expense of male fitness. Subsequently, males are selected to develop counteradaptions to regain control over female reproductive decisions within a recurrent "chase-away" process that may eventually be halted by natural selection when the exaggerated expression of traits in both sexes results detrimental to survival (Holland & Rice, 1998; Parker, 2006). This scenario establishes the causal link between sexual selection and sexual conflict (Pizzari & Snook, 2003).

SEXUAL CONFLICT

Sexual conflict arises whenever the fitness of males and females cannot be maximized simultaneously and is caused by a divergence between the evolutionary reproductive interests of both sexes (Pizzari & Snook, 2004; Parker, 2006). Sexual conflict can occur in two different variants depending on the underlying genetic mechanisms involved (Fricke, Bretman & Chapman, 2010), namely, intra-locus sexual conflict and inter-locus sexual conflict.

Intra-locus sexual conflict

Intra-locus sexual conflict occurs when the target of selection is determined by alleles at the same locus expressed in both sexes so that the expression of the trait in one sex prevents the other sex from achieving its optimal expression (Arnqvist & Rowe, 2005; Bonduriansky & Chenoweth, 2009). The human hip, for instance, is a conflict trait. This is because a wider hip is beneficial to females since it facilitates childbirth while a wider hip does not cause any benefit to males but a decrease in locomotion efficiency (Mulder & Rauch, 2009).

Intra-locus sexual conflict is modulated in three different ways, namely, migration of sexually antagonistic alleles into the sex chromosomes, genomic imprinting and sex-limited expression of sexually antagonistic alleles beneficial variants (Arnqvist & Rowe, 2005; Bonduriansky & Chenoweth, 2009).

Migration of sexually antagonistic alleles into the sex chromosomes

In a pristine scenario sexually antagonistic alleles were linked to the sex-determining gene coding for the sex to which they were beneficial. This linkage caused sex-biased gene transmission, resulting in the accumulation of alleles that were beneficial to one sex but detrimental to the other sex. The increasing accumulation of sexually antagonistic alleles on the primitive sex chromosomes caused mismatches between sexually antagonistic alleles and the sex-determining gene (Rice, 1984). For this reason, selection favored the suppression of the accumulation of sexually antagonistic alleles on the primitive sex chromosomes and the degradation of the non-recombining chromosomes, thus leading to the appearance of functional sex chromosomes (Arnqvist & Rowe, 2005; Bonduriansky & Chenoweth, 2009).

Genomic imprinting

Genomic imprinting limits the expression of alleles in the offspring inherited from the opposite-sex parent since these alleles would provide lower fitness that those inherited from the same-sex parent (Bonduriansky & Chenoweth, 2009). An illustrative example comes from the majority of mammal species in which the offspring obtains nutrients directly from the mother. As a result of sexual conflict over the rate at which maternal resources should be provided to the offspring genes coding for high rates of maternal provisioning are generally paternally expressed and maternally silenced while genes coding for low rates of maternal provisioning are generally maternally expressed and paternally silenced in the offspring (Arnqvist & Rowe, 2005).

Sex-limited expression of sexually antagonistic alleles beneficial variants

Sexually antagonistic alleles duplicate into two variants. Each variant is beneficial for one sex. Female beneficial variants are generally accumulated on the X chromosome while male beneficial variants tend to accumulate in the autosomes. This situation causes the evolution of genetic dimorphism for the expression of the conflicting male and female phenotypic traits (Bonduriansky & Chenoweth, 2009; Connallon & Clark, 2011).

Inter-locus sexual conflict

Inter-locus sexual conflict occurs when the target of selection is determined by alleles at different loci in males and females, causing a conflict over the outcome of the social interactions between males and females (Arnqvist & Rowe, 2005). Inter-sexual conflict may lead to sexual antagonistic coevolution, in which males are selected to evolve traits, manipulating females, e.g. to mate, while females are selected to evolve traits aimed to resist male manipulation. This situation results in an escalated “chase-away” coevolution between male manipulative traits and female resistance traits (Dawkins & Krebs, 1979; Holland & Rice 1998; Chapman, 2006; Davies, Krebs & West, 2012). A possible example of this process comes from the coevolution between male grasping

genitalia and female resistance in water striders of the genus *Gerris* (Heteroptera: Gerridae), where males are selected to evolve elongation of grasping genitalia to force matings while females are selected to evolve antigrasping structures in the form of elongation of the abdominal spines in order to avoid costly matings (Arnqvist & Rowe, 2002).

Sexual antagonistic coevolution gives rise to selection targeting multiple male and female traits and the evolution of a broad range of new traits in both sexes (Arnqvist & Rowe, 2005). This rapid inter-sexual coevolution may eventually result in males and females of a given species losing reproductive compatibility and becoming reproductively isolated from related populations, thus causing allopatric speciation (Martin & Hosken, 2003; Gavrilets & Hayashi, 2005).

POLYANDRY

An important source of sexual conflict may result from polyandry, defined here as a mating system in which females have several copulation partners during a single reproductive cycle (Birkhead & Pizzari, 2002). Polyandry causes potential for sexual conflict in the mating system when it leads to differences in fitness benefits in both sexes on a reproductive trait (Birkhead, 2000; Fricke, Bretman & Chapman, 2010; Parker & Birkhead, 2013). For instance, in the Red junglefowl the male age-dependent decline in fertilization success causes potential for sexual conflict over mating as old males that are dominant and monopolize access to females might be unable to fertilize all available eggs, thus causing fertility costs to females (Dean *et al*., 2010).

The advantages of polyandry to females are not immediately obvious (Simmons, 2001; Birkhead & Pizzari, 2002) because in many species a single insemination provides sufficient sperm to fertilize all the ova (Davies, Krebs & West, 2012). The potential benefits that females may gain from polyandry are divided into direct benefits (those improving female chances of reproductive success) and genetic benefits (those improving offspring fitness) (Birkhead & Pizzari, 2002)

although they are not necessarily exclusive (Simmons, 2001). For instance, in the fowl *G. domesticus* male ability to provide direct benefits (food and vigilance) to females may be used by females as an indicator of male genetic quality (Pizzari, 2003).

Direct benefits

Firstly, polyandry may provide females with very important resources to increase their chances of reproductive success, such as, valuable nutrients obtained through courtship or copulation (Thornhill & Alcock, 1983; Birkhead & Pizzari, 2002), good oviposition sites (Howard, 1978), lower risk of sexual harassment (Birkhead & Pizzari, 2002), and an increase in the amount of paternal care for the offspring (Burke *et al.*, 1989).

Secondly, polyandry may ensure fertility when males are inefficient at insemination or provide inviable sperm, when sperm degrade during long storage in female reproductive tract, and when females run out of sperm (Simmons, 2001).

Genetic benefits

Firstly, polyandry may improve the fitness of the offspring since the sperm from the most viable male will achieve fertilization precedence (Simmons, 2001; Birkhead & Pizzari, 2002). Females of several socially monogamous species of birds seek extra-pair copulations with males whose secondary sexual traits are more elaborated than those of their social partners since the elaboration of the secondary sexual traits might represent a reliable indicator of the genetic quality of their bearer. For example, in the blue tit extrapair offspring is more likely to survive than within pair offspring raised in the same nest (Kempenaers, Verheyen & Dhondt, 1997).

Secondly, social polyandry may increase the genetic diversity of sperm within female reproductive tract, thus decreasing potential risks of fertilization by genetically incompatible sperm (Zeh & Zeh, 1996, 1997; Birkhead & Pizzari, 2002).

POST-COPULATORY SEXUAL SELECTION

By inducing the ejaculates of several males to compete for fertilization polyandry generates post-copulatory sexual selection (Birkhead & Pizzari, 2002). Under this situation, copulation does not guarantee male fertilization success (Davies, Krebs & West, 2012). Post-copulatory sexual selection arises from variance in male fertilization success within the set of eggs of a female (Pizzari & Parker, 2009) and operates through two interactive processes, namely, sperm competition and cryptic female choice (Davies, Krebs & West, 2012).

Sperm competition

Sperm competition is the competitive process between the sperm from two or more males for the fertilization of a set of ova (Parker, 1970). Therefore, sperm competition selects males to evolve a broad range of behavioural, anatomical, and physiological strategies to increase their chances of achieving fertilization (Parker, 1984; Birkhead & Møller, 1992; Møller, 1998; Davies, Krebs & West, 2012). These strategies may be classified into three main categories, namely, defensive, offensive and rival exploitation strategies (Birkhead & Pizzari, 2002; Davies, Krebs & West, 2012).

Defensive strategies

Defensive strategies aim to prevent the mated female from remating with other males (Birkhead & Pizzari, 2002). Defensive strategies include closing female (Chapman, 2001; Poiani, 2006) or male genitalia area (Abele & Gilchrist, 1977), increasing female remating intervals (Poiani, 2006;

Fricke *et al.*, 2009) and discouraging other males from mating with the female by transferring antiaphrodisiacs to the female during mating (Gilbert, 1976; Schulz *et al.*, 2008).

Offensive strategies

Offensive strategies aim to decrease chances of fertilization of sperm from other males previously stored by the female (Birkhead & Pizzari, 2002). Offensive strategies include the removal of rival sperm from female storage organs (Waage, 1979; Watanasit, 1997; Fincke, 1984; Birkhead & Møller, 1992; Simmons, Parker & Stockley, 1999; Simmons, 2001), and its reposition in a disadvantageous position to achieve fertilization (Simmons, Parker & Stockley, 1999; Simmons, 2001).

Rival exploitation strategies

Rival exploitation strategies aim to exploit the ejaculates of potential competitors to gain a fertilization advantage (Hodgson & Hosken, 2006; Alonzo & Pizzari, 2010). While defensive and offensive strategies are widely widespread, rival exploitation strategies comprise a more limited set of mechanisms, and the empirical evidence about their operation is much more restricted.

In the flour beetle *Tribolium castaneum* the second male to mate with a female achieves fertilization precedence by removing the sperm previously ejaculated by another male. However, removed sperm from the first male might survive attached on the aedeagus of the second male for up to five minutes. For this reason, when the second male mates again, along with his ejaculate he may also inadvertently inseminate the removed sperm from the first male into a new female, and the first male may fertilize a considerable proportion of the eggs of a female by proxy (Haubruge *et al.*, 1999).

Patterns of ejaculate exploitation mediated by seminal fluid products have been recently demonstrated in *Drosophila melanogaster* males. When a male mates with a recently mated female it reduces strategically the allocation of ovulin (a seminal fluid protein boosting female fecundity) in order to take advantage of the ovulin transferred by the first male to mate with the female. This is because the transfer of ovulin would be redundant when mating with recently mated females so that the second male to mate with a female may reduce its mating costs and save resources for future mating events (Sirot, Wolfner & Wigby, 2011).

Ejaculate economics

The "raffle principle" in the models of sperm competition assumes that male fertilization success is proportional to the number of sperm transferred to the female and, thus, predicts that males should produce larger ejaculates when the risk of sperm competition increases (Pitnick & Hosken, 2010). This prediction has found sound empirical support since studies on several taxa show that males transferring larger ejaculates achieve higher reproductive success (Wedell, Gage & Parker, 2002; Parker & Pizzari, 2010; Pitnick & Hosken, 2010; Kelly & Jennions, 2011). Furthermore, comparative studies on primates, bats, mammals, birds, frogs, fish and insects found that testis size relative to body size is positively correlated to the level of sperm competition within the species (Wedell, Gage & Parker, 2002).

Nevertheless, sperm production is limited (Pizzari *et al*., 2003; Wedell, Gage & Parker, 2002; Parker & Pizzari, 2010) and spermatogenesis causes substantial costs both energetically and in the maintenance of mature sperm for future mating opportunities (Wedell, Gage & Parker, 2002). Males are therefore expected to allocate their ejaculates strategically (Wedell, Gage & Parker, 2002; Parker & Pizzari, 2010). A meta-analysis conducted across different species of several taxa found strong empirical evidence that males allocate strategically their ejaculates in response to the number of immediate competitors, female mating status and phenotypic quality. Firstly, males

produce larger ejaculates in the presence of one competitor. However, the presence of more competitors does not cause an increase in ejaculate size. Secondly, males produce larger ejaculates when mating with virgin females than when mating with mated females. Thirdly, males produce larger ejaculates when mating with higher quality females (Kelly & Jennions, 2011). These patterns may be modulated by male copulation order, male phenotypic traits (e.g. social status) and future mating opportunities (Parker & Pizzari, 2010).

Cryptic female choice

A second consequence of polyandry is cryptic female choice, which refers to the physiological or behavioural abilities of females to favor the sperm from a given male to achieve fertilization (Thornhill, 1983; Eberhard, 1996; Birkhead & Pizzari, 2002; Pitnick & Hosken, 2010). Cryptic female choice may cause two patterns of selection, namely, directional sexual selection and non-directional sexual selection (Birkhead & Pizzari, 2002).

Directional sexual selection

Directional sexual selection results from cryptic female choice favoring fertilization by the sperm from males with attractive phenotypes. Most females share the same preferences towards male phenotypes. For this reason, cryptic female choice may favor the selection of the alleles underlying the attractive male phenotypes (Birkhead & Pizzari, 2002).

Thus, cryptic female choice may cause directional sexual selection of the alleles encoding the preferred male phenotypes. For instance, in the fowl *G. domesticus* females tend to eject sperm from socially subdominant males right after insemination (Pizzari & Birkhead, 2000).

Non-directional sexual selection

Non-directional sexual selection may result from cryptic female choice favoring fertilization by the sperm from genetically compatible males, e.g. in order to avoid inbreeding (Birkhead & Pizzari, 2002). Inbreeding is the mating between genetically related individuals, which may decrease the fitness of offspring because it leads to an increase in the chances of expression of deleterious alleles and loss of heterozygosity (Tregenza & Wedell, 2000). Thus, cryptic female choice may favor the selection of the alleles reducing mating between genetically related individuals (Birkhead & Pizzari, 2002; Tregenza & Wedell, 2000).

For instance, in the field cricket *Gryllus bimaculatus* related and unrelated males transfer the same amount of sperm to females and the male with more sperm in female's spermathecae achieves paternity. The eggs of females mated in succession with two siblings have lower hatching success than the eggs of females mated with two non-siblings. However, the eggs of females mated in succession with two unrelated males and the eggs of females mated in succession with a sibling and an unrelated male and vice versa do not have significant differences in hatching success (Tregenza & Wedell, 2002). These results mean that females stored more sperm from unrelated males than from siblings in order to avoid inbreeding (Bretman, Newcombe & Tregenza, 2009).

In the guppy, *Poecilia reticulata*, females bias fertilization in favour of genetically compatible males by decreasing the velocity of the sperm from genetically related males through the action of the ovarian fluid (Gasparini & Pilastro, 2011). In the Red junglefowl, *Gallus gallus*, females retain fewer sperm when they are inseminated by siblings than when they are inseminated by unrelated males (Pizzari, Løvlie & Cornwallis, 2004; Løvlie *et al.*, 2013). In *Drosophila melanogaster*, females display a weak preference for previous mates and new mates sharing similarities to previous mates over novel males. However, it is not clear whether this preference

results from female behaviour to avoid potential costs of mating with phenotypically diverse males or from male manipulation through seminal fluid peptides to keep sperm competition within males more genetically related to the focal male than the average male in the population (Tan *et al*., 2013).

SEMINAL FLUID

Seminal fluid is the physiological non-sperm component of an ejaculate and consists of secretions produced by the male reproductive tract and associated accessory glands (Mann, 1964; Hill, Wyse & Anderson, 2004). Seminal fluid contains a great diversity of molecular components (such as, proteins, sugars, fatty acids, aminoacids, and steroids) with different functions (Poiani, 2006). Therefore, seminal fluid constitutes more than a medium to support the successful transit of sperm (Chapman, 2008), and can influence traits that are important in sexual conflict and post-copulatory sexual selection (Perry, Sirot & Wigby, 2013). These traits include sperm movement, sperm storage within female's sperm storage organs, sperm survival, and female propensity to remate in the short term (Pitnick & Hosken, 2010). The main seminal fluid effects have been studied in relation to antimicrobial properties, egg production and ovulation, and female life span.

Seminal fluid antimicrobial effects

Seminal fluid has important antimicrobial and immunostimulatory properties to differentially regulate female responses against both molecular components important for fertility and pathogens transferred during mating that can negatively affect the viability of the egg (Poiani, 2006; Pitnick, Wolfner & Suarez, 2009). These properties have been studied in insects and mammals.

Firstly, with regard to insects, in *Drosophila melanogaster* at least three seminal fluid proteins, transferred to females during mating, have antimicrobial activity on *Escherichia coli*. One of

these proteins is likely to be andropin (Samakovlis *et al.*, 1991; Lung, Kuo & Wolfner, 2001). Moreover, a comparison between extracts of male reproductive tracts without testes, extracts of testes, and the profile of reproductive tract proteins from unmated females showed that activity against *E.coli* is only present in the first case, which suggests that seminal fluid can assist in protecting the female's reproductive tract, the eggs, or the sperm against potential bacterial infections (Lung, Kuo & Wolfner, 2001). In addition to this, Mueller, Page and Wolfner (2007) showed that three *D.melanogaster* seminal fluid proteins (CG6168, CG9334, and CG10284) reduce a *Serratia marcescens* infection within females. Comparing the genes with different expression levels between mated and virgin females, and between females that were mated with males lacking sperm or unable to produce both sperm and seminal fluid proteins, McGraw *et al.* (2004) showed in *D.melanogaster* that seminal fluid proteins transferred during mating upregulate the expression of nine antimicrobial genes in females, namely, CG13422, *AttA*, *AttB*, *AttD*, *CecA1*, *CecA2*, *CecB*, *IM1*, and *IM2*. In turn, Peng, Zipperlen & Kubli (2005) demonstrated that Sex-Peptide, a seminal fluid peptide transferred to females during mating, is the main agent eliciting the transcription of Metchnikowin (which acts against gram-positive bacteria), Diptericins and Drosocins (both acting against gram-negative bacteria) antimicrobial peptides in *D.melanogaster*. Although not having shown similar antimicrobial peptide activity, the seminal fluid of the common bedbug, *Cimex lectularius*, contains lysozymes capable of degrading only bacteria (Otti *et al.,* 2009). Drosomycin, an antifungal protein expressed in *Drosophila* female sperm-storage organs, has also been found in the male ejaculatory duct and bulb, which means that it could be transmitted to females during copulation (Chapman, 2001).

Second, with regard to birds, seminal fluid in mallards shows significant antibacterial activity measured by the capacity to kill *in vitro E.coli* and *Staphylococcus aureus*. Furthermore, males with darker bills and lower reflectance in the UV range show greater antibacterial activity in their seminal fluid against *E.coli*. This mechanistic link between pre-and post-copulatory episodes of

sexual selection suggests that females may be under strong selection to mate with males having more colourful bills in order to avoid the costs of sexually transmitted diseases (Rowe *et al*., 2011). Similarly, the seminal fluid of the wild passerine *Malurus cyaneus* shows lysozyme antibacterial activity, likely to defend the sperm from bacterial-induced damage (Rowe *et al*., 2013).

Thirdly, with regard to mammals, seminal fluid increases the uterine inflammatory response to semen by increasing the number of polymorphonuclear neutrophils within 6 hours after insemination in mares (Portus, Reilas & Katila, 2005). Spermadhesins, a group of glycoproteins associated to the sperm surface, secreted by the male reproductive tract (Haase *et al.*, 2005), can exert uterine immunostimulatory effects in the boar, bull, buck, and stallion (Teixeira *et al.*, 2002). In the pig, where spermadhesins constitute more than 80% of the total seminal fluid proteins (Calvete *et al.*, 1995), seminal fluid also mediates a postmating inflammatory response in female reproductive tract (Hadjisavas, Laurenz & Bazer, 1994). In sows, the infusion of uterine horns with PSP-I/PSP-II in physiological saline solution induced a 4-7 fold increase in the lymphocyte CD2+T cells after 10 minutes, and a 3-fold increase in the lymphocyte CD8+T cells after 1 hour (Rodriguez-Martinez *et al.,* 2010). Spermadhesin PSP-I/PSP-II also enhances neutrophil migration into the peritoneal cavity of rats 2 hours elapsed from intraperitoneal injection (Assreuy *et al.*, 2002). The effects of the spermadhesin complex in the uterine immune activity might have evolved to favor reproductive success (Assreuy *et al.*, 2003). Furthermore, *in vitro* experiments in humans suggest that seminal fluid transforming growth factor beta 1 (TGF-beta 1) and interleukin 8 (IL-8) can stimulate the expression of pro-inflammatory cytokines by endometrial epithelial cells in female reproductive tract (Gutsche *et al.*, 2003).

Taken together, all these findings in insects, birds, and mammals strongly suggest that seminal fluid may play a role in protecting sperm, and clear pathogens introduced during copulation in

female reproductive tract, thus protecting the egg from suffering a potential infection (Chapman 2001; Poiani, 2006; Avila *et al*., 2011).

Seminal fluid effects on egg production and ovulation

Early work in *D. melanogaster* started to show that seminal fluid secretions transferred during copulation increase egg laying by the mated female (Kalb, DiBenedetto & Wolfner, 1993). Subsequent work showed that the seminal fluid protein ovulin (Acp26Aa), transferred to females during mating, increases the release of oocytes by the ovaries. In particular, *D. melanogaster* females that mated with males unable to produce ovulin lay significantly fewer eggs for 1 day after mating than females that mated with normal males (Herndon & Wolfner, 1995). Liu & Kubli (2003) found, also in *D.melanogaster*, that ovulin enhances fecundity for one day after mating.

Ovulin is generally processed sequentially upon entrance in the female reproductive tract, and two of its final forms (CP3N and CP3C) can independently induce ovulation in *D. melanogaster*. Interestingly, one of them, CP3N, includes regions of sequence similarity to *Aplysia* egg-laying hormones (Heifetz *et al.*, 2005). The fecundity-stimulating effects of ovulin allow males to tailor the composition of their ejaculates to take advantage of the effects of a previous male to mate with a female's ovulin. As males cannot increase the fecundity in recently mated females, they reduce the transfer of ovulin when mating with them, preventing an unnecessary investment in mating costs and, therefore, saving resources for future mating opportunities (Sirot, Wolfner & Wigby, 2011). Recent research has begun to elucidate the physiological pathways by which ovulin can regulate egg production. Rubinstein and Wolfner (2013) showed in *D.melanogaster* that the number of eggs in the lateral oviducts 3 hours after the start of mating was higher in normal females having mated with males that transferred ovulin than in normal females having mated with males unable to generate ovulin. Interestingly, experimental females, with genetically enhanced octopamine levels, showed elevated rates of ovulation regardless whether or not they

had received ovulin during mating. Previous research in *D.melanogaster* had shown that octopamine exerts differential effects on muscle contraction within female reproductive tract, promoting the contraction of ovarian muscles and blocking the contraction of oviduct muscles (Rodriguez-Valentin *et al.*, 2006; Middleton *et al.*, 2006). These effects on female reproductive tract muscle contractions might push the egg from the ovary into the oviduct (Middleton *et al.*, 2006).

Taken together, these findings strongly suggest that ovulin and octopamine might act together to increase ovulation rates by regulating muscle contractions within female reproductive tract. Ovulin elevates the number of synaptic release sites of neurons innervating oviduct muscles, although is not yet clear how it causes such effect (Rubinstein & Wolner, 2014). Interestingly, the influence of octopamine on the regulation of oviductal contractrions has also been reported in other insect species, such as, the locust, *Locusta migratoria*, (Lange, 2009a,b), the fly *Stomoxys calcitrans* (Cook & Wagner, 1992), and the field cricket *Grillus bimaculatus* (Sefiani, 1987).

Seminal fluid effects on female life span

The reduction of female life span as a consequence of mating constitutes a taxonomically widespread phenomenon within the animal kingdom (Bell & Koufopanou, 1986; Partridge & Harvey, 1988). The premature death of mated females in relation to virgin ones is due to a combination of three main factors, namely, the energetic requirements of offspring production, the continuous harassment exerted by males (that may prevent the female from eating and resting), and the action of seminal fluid components transferred by the males during mating (Wolfner, 1997).

Over the last two decades research in *D. melanogaster* has studied the negative impact of seminal fluid on female life span. In particular, Chapman, Hutchings & Partridge (1993) showed that

receiving sperm results costless to females since females that mated with males that transferred and did not transfer sperm during mating did not differ in their lifespan. However, the survival rates of females that mated with males that transferred seminal fluid but not sperm were significantly shorter than those of females that mated with males unable to produce seminal fluid or sperm. Chapman *et al.* (1995) compared female survival rates (keeping constant costly reproduction traits, such as, egg production, non-mating exposure to males, and rate of mating) after mating with males with no detectable seminal fluid main-cell products and also lacking sperm, males that produce normal seminal fluid but lack sperm, and the respective control groups of males (having their external genitalia microcauterized, so that their behaviour is normal but they are unable to mate). The authors found that females receiving seminal fluid main-cell products survived significantly shorter than females in the other three groups. Furthermore, the survival rates of females in the other three groups did not differ significantly. In addition to that, they found, in a second experiment, that the negative impact on female survival rates exerted by the seminal fluid main-cell components works in a dose-dependent way, since the higher their quantity the shorter the female survival rates. Wigby & Chapman (2005) demonstrated that the negative effect of seminal fluid on female life span is due to the single action of the accessory gland protein sex peptide (Acp70A) since females mated with males that produce no detectable sex peptide lived significantly longer than females mated with normal males. Since sex-peptide stimulates post-copulatory feeding in females, overfeeding was suggested to be related to the reduction of female life span, given the link between overfeeding and shortening of life span observed in many organisms (Carvalho *et al.*, 2006). Nevertheless, Barnes *et al.* (2008) demonstrated that, even though sex peptide transferred during copulation increases the feeding rate in fertile but not sterile females, the survivorship rates of females continuously exposed to mating males throughout their lifetimes were significantly lower than those of females exposed to mating males for just one day out of every four days cycle, regardless whether or not the females were fertile or sterile. This result proves that the decrease of female life span is not related to

feeding rate, thus supporting the fact that the direct action of sex peptide is the factor that results detrimental to female life span.

Seminal fluid in post-copulatory sexual selection

The role of seminal fluid in post-copulatory sexual selection has been mainly investigated by studying its effects on two major traits, namely, sperm fertilizing efficiency, and female propensity to remate in the short term. Moreover, the important role played by seminal fluid on sperm competition dynamics has led theoretical studies to highlight the importance of seminal fluid on ejaculate allocation strategies.

Seminal fluid effects on sperm fertilizing efficiency

The sperm incapacitation hypothesis states that the seminal fluid of a male inhibits the fertilizing efficiency of sperm from other males (non-self) to favour self sperm in sperm competition (Simmons, 2001). In principle, seminal fluid can help self and hamper non-self sperm.

There is some empirical support for this hypothesis. Early work in *Drosophila melanogaster* suggested that the seminal fluid of the second male to mate with a female might displace the sperm previously inseminated by another male (Gilchrist & Partridge, 1995). Moreover, the seminal fluid of the second male to mate with a female was suggested to harm non-self sperm previously stored by a female (Price, Dyer & Coyne, 1999). The inhibition of sperm of the first male to mate with a female by the seminal fluid of the second male to mate with the female was also found in *Drosophila simulans* (Price, Dyer & Coyne, 1999). In addition, in the stalk-eyed fly *Cyrtodiopsis whitei* sperm fertilize dramatically fewer eggs when exposed to non-self seminal fluid inside the female's spermathecae (Fry & Wilkinson, 2004). However, more recent studies were unable to replicate these effects. In *Drosophila melanogaster* (Holman, 2009) and in the field cricket *Teleogryllus oceanicus* (Simmons & Beveridge, 2011) seminal fluid enhances (rather

than reduce) the viability of non-self sperm. In some ants and bees the effect of seminal fluid over non-self sperm depends on the mating system. In monandrous species, the bumblebee *Bombus terrestris* and the fungus-growing ant *Trachymyrmex zeteki*, non-self seminal fluid does not significantly affect sperm survival. However, in polyandrous species, the honey bee *Apis mellifera* and the leafcutter ants *Acromyrmex echinatior* and *Atta colombica*, non-self seminal fluid significantly shortens sperm survival. Interestingly, females *Atta colombica* have the potential to prevent the negative effects of seminal fluid over non-self sperm from happening through the spermathecal fluid (den Boer, Baer & Boomsa, 2010).

The sperm incapacitation hypothesis also found empirical support when it comes to heterospecific sperm. When a *Drosophila simulans* female mates successively with both, a conspecific male and a *D. mauritiana* male (regardless of mating order), the conspecific male fertilizes the great majority of the eggs because its seminal fluid substantially reduces the effectiveness of heterospecific sperm (Price, 1997). However, the mechanisms underpinning these seminal fluid effects depend on the order of mating. If a *simulans* male, producing normal seminal fluid but no sperm, mates firsts females produce fewer offspring than when they mate only with a *mauritiana* male. The number of sperm stored by females does not differ significantly in both cases, which indicates that *simulans* male's seminal fluid incapacitates *mauritiana* male's sperm. However, if the *simulans* male mates second, its seminal fluid displaces *mauritiana male's* sperm from the female sperm storage organs (Price *et al.*, 2000).

Seminal fluid effects on female propensity to remate in the short term

A decrease in female sexual receptivity following mating is likely to be a universal phenomenon within the animal kingdom, although the responsible factors vary considerably across taxa (Pitnick, Wolfner & Suarez, 2009). For example, mounting by the male independently of insemination in *G. domesticus* (Løvlie, Cornwallis & Pizzari, 2005), the spermathecae being filled

with sperm in *Anopheles gambie* female mosquitos (Klowden 2001, 2006), and the action of seminal fluid components, more specifically the sex-peptide (Acp70A), which has been studied predominantly in *Drosophila* (Chapman & Davies, 2004).

The pioneering work of Maynard-Smith in *Drosophila suboscura* indicated that some components of the ejaculate cause an increase in female remating intervals (Maynard-Smith, 1956). Later work in *D. melanogaster* started to show that seminal fluid can decrease female propensity to remate in the short term (Kalb, DiBenedetto & Wolfner, 1993). Subsequent research in *D. melanogaster* demonstrated that sex peptide is the seminal fluid component that decreases female sexual receptivity in the short term. In particular, females that mated with males transferring sex-peptide showed a strong reduction of sexual receptivity elapsed from 48 hours after mating, whereas females that mated with males unable to produce sex-peptide remated only after 4 hours (Liu & Kubli, 2003). Chapman *et al.* (2003) reported similar results. They compared the receptivity to further matings within one hour of females that mated with two lines of sex-peptide knockdown males, carrying the sex-peptide inverted repeat and *Gal4* transgenes (Acp26Aa-P-Gal4;UAS-SP-IR1, and Acp26Aa-P-Gal4;UAS-SP-IR2), with that of females that mated with normal males, at 24 and 48 hours after their first matings. Females that mated with both lines of sex-peptide knockdown males were significantly more receptive than females that mated with normal males. 24 hours after their first matings, the sexual receptivity of females that mated with sex-peptide knockdown males was intermediate between that of females that mated with normal males and that of virgin females. 48 hours after their first matings, the sexual receptivity of females that mated with sex-peptide knockdown males (Acp26Aa-P-Gal4;UAS-SP-IR1) was only slightly lower than that of virgin females, whereas the sexual receptivity of females that mated with sex-peptide knockdown males (Acp26Aa-P-Gal4;UAS-SP-IR2) did not differ significantly with that of virgin females. Overall, 48 hours after their first matings, the sexual receptivity of females that mated with sex-peptide knockdown males was similar to that of virgin females.

Consistent with the aforementioned results, further work, also in *D. melanogaster*, showed that females that mated with two independently produced types of males lacking sex peptide (via sex-peptide gene knockouts and knockdowns) remated more frequently and sooner, within a 6 and 24 hours interval, than females that mated with the respective control males (Fricke *et al.*, 2009). The transfer of sex-peptide during mating provides a significant competitive advantage to the male that mates first with a female since the reduction of female receptivity to remating delays the onset of potential sperm competition to fertilize the ova, thus allowing the male to maximize the number of offspring sired. *D. melanogaster* males selected for larger accessory glands produced more sex-peptide and sired more progeny over a 10 days multiple mating competition assay despite the fact that they did not mate more frequently than their competitors (Wigby *et al.*, 2009). It should be noted that sex peptide also regulates other important female post-mating responses by controlling female gene expression in processes related to egg development, early embryogenesis, feeding (Gioti *et al.*, 2012), and eliciting female immune response by increasing the transcription of Mtk and other antimicrobial peptides (Peng, Zipperlen & Kubli, 2005; Domanitskaya *et al.*, 2007).

Female inhibition of remating due to the action of seminal fluid has been reported in other species, such as, the fruit flies *Drosophila sechellia* (Chen & Balmer, 1989) and *D. suzukii* (Ohashi, Haino-Fukushima & Fuyama, 1991), the grasshopper *Gomphocerus rufus* (Hartmann & Loher, 1999), the bumblebee *Bombus terrestris* (Baer, Morgan & Schmid-Hempel, 2001), the moth *Helicoverpa zea* (Raina, Kingan & Giebultowicz, 1994), and in Wistar rats (Forsberg *et al.*, 1990).

The knowledge about the effects of seminal fluid in post-copulatory sexual selection is based mainly on investigations conducted on insects, whereas very little is known about their operation in other taxa. In particular, despite the fact that birds have been widely used as a model system for

sexual conflict, paradoxically, relatively little is known about seminal fluid in birds and its effects on sexual selection.

Seminal fluid and strategic patterns of ejaculate allocation

Seminal fluid plays a significant role on sperm competition dynamics by manipulating female remating intervals, female fecundity, and affecting sperm quality. For this reason, recent theoretical studies have begun to highlight that research on ejaculate allocation patterns should take this potential role of seminal fluid into account.

Alonzo and Pizzari (2010) propose a model according to which, when males have information about their mating roles, the second male to mate with a female may exploit the seminal fluid fecundity-enhancing effects of the first male to mate with the female. For this reason, the second male to mate with the female may invest more resources to transfer more sperm to the female. This hypothesis has found empirical support. *Drosophila melanogaster* males can tailor strategically the composition of their seminal fluid in order to exploit the ejaculate of a previous male to mate with a female. In particular, when a male mate with a recently mated female he reduces the allocation of ovulin (a seminal fluid protein boosting female fecundity) in order to take advantage of the ovulin transferred by the first male to mate with the female. The transfer of ovulin would be redundant when mating with recently mated females so that males may reduce their mating costs and save valuable ejaculate resources for future mating events (Sirot, Wolfner & Wigby, 2011). Furthermore, when males lack information about their mating roles, both males are predicted to invest more resources in sperm production than in seminal fluid fecundity-enhancing compounds when the female has a high baseline fecundity, and in seminal fluid fecundity-enhancing compounds when the female has a low baseline fecundity. In turn, females are predicted to gain fitness benefits from seminal fluid fecundity-enhancing effects and, thus, to be selected to shorten their remating intervals, which may eventually increase the levels of sperm

competition within the population, thus selecting the males to invest more resources in sperm production than in seminal fluid (Alonzo & Pizzari 2010, 2013).

Cameron, Day & Rowe (2007) analyzed theoretically patterns of ejaculate allocation depending on the effects of seminal fluid and sperm in fertilization success when males have full information about their mating roles. Their model shows that when fertilization success is mainly dependent on seminal fluid fecundity-enhancing compounds, favored males are predicted to invest more in seminal fluid fecundity-enhancing compounds than disfavored males, while disfavored males are predicted to invest more resources in sperm production (in order to increase their chances of fertilization) and less resources in seminal fluid fecundity-enhancing compounds (since this would be more beneficial to the rival males than to themselves) than favored males. On the other hand, if fertilization success is mainly dependent on sperm investment, favored males are predicted to invest more resources in both seminal fluid fecundity-enhancing compounds and sperm than disfavored males.

In accordance with the predictions of the theoretical models, empirical evidence backs up the fact that males are able to allocate strategically their seminal fluid compounds with great levels of sophistication in order to increase their reproductive success. For example, *Drosophila melanogaster* males are able to adjust their ejaculates in response to the potential level of sperm competition by transferring significantly more sex peptide (a protein that decreases female propensity to remate) and ovulin (a fecundity-stimulating compound) when a rival male was present before and during mating (Wigby *et al*., 2009). In line with this fact, *Drosophila melanogaster* males kept with rivals prior to mating increased significantly the duration of their matings, which is positively related to the amount of seminal fluid transferred in this species. In addition to that, females mated with these males increased significantly their remating latency,

and laid significantly more eggs that, moreover, have an average three per cent higher survival to adulthood (Bretman, Fricke & Chapman, 2009).

THE RED JUNGLEFOWL, *GALLUS GALLUS*, AS STUDY SYSTEM

The overarching goal of my thesis is to study the role of seminal fluid in sexual selection in the Red junglefowl. The Red junglefowl belongs to the Phasianidae family (Delacour, 1951) and is one of the ancestors of the domestic chicken (Eriksson *et al*., 2008). The geographical distribution of the wild Red junglefowl extends from northeast and central India, extreme South of China, and Southeast Asia, especially Sumatra, Java, and Bali (Delacour, 1951).

The Red junglefowl live in socially structured groups with some levels of polyandry, which cause opportunity for sperm competition and cryptic female choice of sperm (Pizzari, Froman & Birkhead, 2002). This is consistent with the fact that male Red junglefowl have large relative testes mass compared to other galliformes (Pizzari, 1999), which indicates an evolutionary response to sperm competition and sperm depletion (Pizzari, Froman & Birkhead, 2002). The processes involved in sperm competition dynamics in the fowl are reasonably well characterized.

Firstly, under sperm competition, the fertilization potential of an ejaculate changes significantly over time depending on sperm numbers and sperm mobility (a measure of sperm swimming performance). Low mobility-ejaculates achieve higher fertilization success over the first days after artificial insemination if their sperm outnumber the sperm in high-mobility ejaculates. The rate at which this fertilizing advantage decreases over successive days is more pronounced as the sperm numerical advantage is shorter. In particular, in engineered heterospermic ejaculates containing $40x10^6$ of sperm from low-mobility ejaculates and $10x10^6$ of sperm from high-mobility ejaculates, low mobility-ejaculates achieve a fertilizing advantage over the five days following artificial insemination. However, when the engineered ejaculates contain $20x10^6$ of sperm from

low mobility-ejaculates and $10x10^6$ of sperm from high-mobility ejaculates, this fertilizing advantage is restricted to the first day following artificial insemination. In turn, high mobility-ejaculates achieve higher fertilization success in the long term, even if outnumbered by low-mobility ejaculates (Pizzari *et al*., 2008b).

Secondly, males are able to allocate sperm strategically in response to the perceived risk of sperm competition. This differential behaviour is modulated by male social status. In particular, as the number of rival males increases from one to three, dominant males increase the allocation of sperm. In turn, subdominant males maximize their sperm investment in the presence of one rival male. Dominant males have preferential control over females so that an increase in the presence of rival males does not necessarily translate into a more intense sperm competition and, thus, an increase in sperm investment is worthwhile. However, in the case of subdominant males, the presence of rival males is more likely to result in a more intense sperm competition. For this reason, subdominant males may save sperm for future less competitive mating situations (Pizzari *et al*., 2003).

Thirdly, females may exert a strong influence on the outcome of sperm competition through the differential ejection of unwanted sperm. In particular, in the absence of any type of male manipulation, females tend to eject sperm from socially subdominant males. Dominance is heritable and, thus, females may gain genetic benefits for their offspring when the sperm from a dominant male fertilize the ova. Since subdominant males may force copulations, the differential ejection of their sperm allows females to favor fertilization by the sperm of dominant males (Pizzari & Birkhead, 2000).

The male reproductive tract and seminal fluid in the fowl

Male fowl has paired reproductive tracts, each of which including a testis, an epididymis, and a deferent duct. The testes are located inside the body cavity, and regulate the process of spermatogenesis. The left testis is larger than the right one. The testes are surrounded by a layer of fibro-elastic tissue, called the tunic albuginea. Their network of seminiferous tubules contains the germ cells (spermatogonia, primary spermatocytes, secondary spermatocytes, and spermatids), and the Sartori cells that control the process of spermatogenesis by regulating the successive transformations of the germ cells in response to follicle-stimulating hormone and androgens, the latter secreted by the Leydig cells (situated in the testicular interstitium between adjacent seminiferous tubules) in response to the luteinizing hormone. Once spermatogenesis is completed, immotile sperm are release into the excurrent ducts of the epididymis. The rete testis connects the seminiferous tubules in the testis with the excurrent ducts of the epididymis. The epididymis consists of three excurrent ducts situated upon the surface of the testis, namely, efferent ducts (the main duct, consisting of 60% of the epididymal ducts), connecting ducts (linking the distal efferent ducts with the epididymal ducts), and epididymal duct (the last excurrent duct). The epididymis plays a critical role in sperm maturation since the passage of sperm through the excurrent ducts, where they are mixed with seminal fluid proteins, results critical in order for them to acquire fertility. Finally, the deferent duct constitutes a temporary storage site for sperm where they are held within the receptaculum prior to their ejaculation through the papilla (Al-Aghbari 1992; Froman, 1995; Etches, 1996).

Fowl seminal fluid consists of two components, namely, seminal plasma and transparent fluid. Seminal plasma is produced by the testes and vas deferens, and is mixed with the sperm as it goes down through the vas deferens. Transparent fluid exudates of the lymphatic folds surrounding the cloaca, and is mixed with the sperm during the ejaculation (Lake, 1966, 1984; Fujihara, 1992; Etches, 1996).

Fowl seminal fluid has a great diversity of molecular components. Sodium is the main component with a concentration of 145 milimoles per litre (mmol/L). Glutamate (75 mmol/L) accounts approximately for 88% of the aminoacids (Lake & Wishart, 1984), and facilitates the initiation of sperm motility upon ejaculation by acting as a motility agonist through N-methyl-D-aspartate receptor channels (Froman, 2003). The rest of the aminoacids are aspartic acid, threonine, serine, glycine, and alanine (Lake & Wishart, 1984).

Fowl seminal fluid antioxidants (Vitamin C, Vitamin E, Selenium, and the enzymes Glutathione peroxidase and Superoxide dismutase) might protect sperm from lipid peroxidation, caused by the high amount of polyunsatured fatty acids in sperm membranes, which may lead to a detrimental effect on sperm motility and damage in sperm membranes (Fujihara & Howarth, 1978; Khan, 2011).

The amount of proteins is 8 grams per litre. In turn, glucose, if present, is only in trace amounts (0.19 mmol/L) (Lake & Wishart, 1984). Other components of fowl seminal fluid are Chloride (46 mmol/L), Potassium (13 mmol/L), Calcium (1.4 mmol/L), Magnesium (2.3 mmol/L), Lactate (3.73 mmol/L), Pyruvate (0.28 mmol/L), α-Ketogutarate (0.4 mmol/L), Carnitine (3.18 mmol/L), and Acetyl carnitine (2.1 mmol/L) (Lake & Wishart, 1984).

Role of seminal fluid in sperm fertilizing efficiency in the fowl

The effects of seminal fluid in sperm fertilizing efficiency in the fowl have been the subject of considerable investigations. Lake (1956) and Nishiyama, Ogawa & Nakanishi (1971) showed that sperm fertilizing performance does not differ when they are diluted in transparent fluid (a main component of seminal fluid that is added to sperm during ejaculation) or in a different but suitable solution. Nishiyama Ogawa & Nakanishi (1971) pointed out that transparent fluid can exert a detrimental effect on sperm fertilizing potential when the concentration of sperm in the ejaculate

is low. Several studies indicate that a prolonged storage with transparent fluid causes a rapid decline in sperm fertilizing ability (Nishiyama, 1955; Nishiyama, Ogawa & Nakanishi, 1971; Lake 1956, 1971, 1981). In turn, Ashizawa & Okauchi (1984) showed that sperm motility is affected by the concentration of seminal plasma (obtained by diluting seminal plasma with sodium phosphate buffer containing 20 mM-glucose). In particular, when the concentration of seminal plasma was below 20% sperm rapidly lost motility. However, as the concentration of seminal plasma increases so does sperm motility. Sperm incubated with seminal plasma at concentrations higher than 40% barely lost motility over a 15 minutes interval. Lake & Ravie (1987) investigated the effect of heterospecific seminal fluid on sperm fertilizing efficiency. To do so, Lake & Ravie (1987) diluted fowl sperm in an aqueous medium, natural homologous seminal fluid, turkey seminal fluid, and turkey whole semen. Lake & Ravie (1987) observed the best fertilizing efficiency when the sperm were highly diluted in homologous seminal fluid. On the other hand, the dilution with either turkey seminal fluid or turkey whole semen caused similar results in sperm fertilizing efficiency than the dilution in an aqueous medium when the dose of sperm was 5x5 or $10x10^6$.

A more recent study carried out in the Red junglefowl, provided indirect evidence that seminal fluid affects sperm velocity and, thus, sperm competition dynamics. Cornwallis and O'Connor (2009) found that sperm from larger ejaculates is faster than sperm from smaller ejaculates. Moreover, when the sperm are mixed with seminal fluid from a different ejaculate of the same male the change in sperm velocity is positively related to the volume of the ejaculate from which the seminal fluid was taken. Collectively these findings suggest that males tend to produce high quality seminal fluid in large ejaculates and this has an important effect on sperm velocity (Cornwallis & O'Connor, 2009).

Taken together, the results of these investigations point out that seminal fluid can have an important effect on sperm fertilizing efficiency and sexual selection in the fowl, but to date the specific nature of this effect as well as its underlying mechanisms remains inconclusive.

THESIS AIMS

My thesis seeks to investigate the effects of seminal fluid in sexual selection in the Red junglefowl with special emphasis on proteomics and other potentially important traits whose role, so far, remains poorly understood. In particular:

(1) Chapter 2 investigates the influence of seminal fluid on sperm fertilizing efficiency within a sperm competition context from an *in vitro* and *in vivo* perspective.

(2) In Chapter 3 I study patterns of strategic ejaculate allocation in relation to male social status and female sexual novelty.

(3) Chapter 4 characterizes the Red junglefowl seminal fluid proteome in relation to that of the domestic fowl.

(4) Chapter 5 investigates seminal fluid proteomic profiles associated with variation in male age and sperm quality (i.e. swimming velocity).

CHAPTER 2

Seminal fluid and sperm fertilizing efficiency in a sperm competition context

ABSTRACT

Females of many species mate with multiple males during a single reproductive cycle (polyandry), causing their ejaculates to compete to fertilize a set of ova (sperm competition), and giving rise to sexual selection after mating. Recent evidence suggests that seminal fluid, the non-cellular physiological component of a male's ejaculate, might play a key role in determining the outcome of sperm competition by differentially modulating the fertilizing efficiency of self and non-self (i.e. competing) sperm. This study aims to test this idea, by investigating the effect of seminal fluid on sperm fertilizing efficiency and whether these effects are modulated by the genetic relatedness between the seminal fluid and sperm donor males. To do so, I conducted an *in vitro* experiment where I mixed sperm with seminal fluid from: the same male (self), a full-sib brother (non-self related), or a genetically unrelated male (non-self unrelated), and measured sperm average path velocity (VAP) at three ten-minutes intervals. While VAP declined over time, this decline was slower when sperm were exposed to non-self, and particularly non-self related, seminal fluid. To test whether these effects translate into differential fertilization success, I conducted an *in vivo* experiment where I artificially inseminated females with engineered ejaculates containing sperm mixed with self, non-self related, or non-self unrelated seminal fluid, and quantified the number of sperm reaching the eggs ovulated over successive days following insemination. There was a tendency for non-self inseminations to outperform self inseminations, and a significant interaction between seminal fluid treatment and initial sperm VAP: relatively more sperm from high-VAP ejaculates reached the eggs in the non-self unrelated treatment, while more sperm from low-VAP ejaculates reached the eggs in the self treatment. Taken together, these results suggest that seminal fluid affects sperm fertilising efficiency differentially, according to whether the sperm belong to the same male or a different male, suggesting that seminal fluid effects might modulate the outcome of sperm competition in this species. These findings may also have relevant practical applications in the fields of assisted reproduction, animal production, and captive breeding of endangered populations.

INTRODUCTION

Darwin envisioned sexual selection as a process that acts exclusively on male mating success, through male-male competition for access to females and female choice of mating partner (Darwin, 1871), and this view has dominated sexual selection theory until recently (Anderson, 1994). During the last few decades however, it has been shown that females of many species mate with multiple males during a single reproductive cycle (polyandry), which gives rise to sexual selection after mating (Parker, 1970; Birkhead, 2000; Simmons, 2001; Davies, Krebs & West, 2012; Perry, Sirot & Wigby, 2013). This is because sperm from several males coexist within female reproductive tract, causing sperm competition to fertilize the ova and cryptic female choice of sperm. These postcopulatory episodes generate variance in a male's paternity share of the ova of individual females (Parker, 1998; Birkhead & Pizzari, 2002; Pizzari & Parker, 2009).

Postcopulatory sexual selection should therefore target ejaculate features associated with competitive fertiziling efficiency, such as sperm motility and swimming velocity (Pizzari & Parker, 2009). Mounting evidence indicates that seminal fluid may mediate the operation of both episodes of post-copulatory sexual selection (Wolfner, 1997; Chapman, 2001; Perry, Sirot & Wigby, 2013). Seminal fluid is the physiological non-sperm component of a male ejaculate, and contains a great diversity of molecular components, such as, steroids, antioxidants, proteins, and carbohydrates (Poiani, 2006). For example, sperm motility is associated with seminal fluid biochemistry in many taxa, including insects (flies (Tram & Wolfner, 1999; Chapman, 2001), moths (Friedlander, Jeshtadi & Reynolds, 2001), and water striders (Miyata *et al.*, 2012)), fish (the ray-finned fish (Lahnsteiner *et al*., 1996), the rainbow trout (Lahnsteiner *et al*., 1998), the Caspian brown trout (Hajirezaee, Amiri & Mirvaghefi, 2010), and the European eel (Perez *et al*., 2003)), birds (domestic fowl (Froman, 2003)), and mammals (cattle (Vanha-Pettula, Ronkko & Lahtinen, 1990), horses (Watson *et al*., 1999), deer *Capreolus capreolus*, (Goeritz *et al*., 2003), and humans (Poiani, 2006).

For these seminal fluid effects to play a direct role in sperm competition dynamics, a differential response to self and non-self sperm is required. Such differential response to self and non-self sperm would enable seminal fluid to favour the sperm from the same ejaculate, and decrease the chances of fertilization by sperm from competing ejaculates (Simmons, 2001; Pitnick & Hosken, 2010; Davies, Krebs & West, 2012; Perry, Sirot & Wigby, 2013). This differential response may occur through a number of ways, including mechanisms through which seminal fluid recognises the genetic relatedness of sperm. Evidence for such differential response is however highly ambiguous. Simmons & Beveridge (2011) found no evidence for differential effects in the field cricket *Teleogrillus oceanicus*, in which low-quality ejaculates (i.e. low sperm viability) can exploit the seminal fluid of high-quality rival ejaculates (i.e. high sperm viability). However, when sperm from high-quality ejaculates were combined with seminal fluid of low-quality rival ejaculates the viability of the sperm was unaffected, thus showing no evidence for differential effects when the sperm are exposed to seminal fluid from a different male. Similarly, Holman (2009) found in *D.melanogaster* that seminal fluid improves sperm survival, even when it comes from a different male. On the other hand, some evidence consistent with such differential effects has been reported in the externally fertilizing Grass goby, *Zosterisessor ophiocephalus*. In this species, seminal fluid effects on sperm performance depend on alternative male mating tactics. In particular, seminal fluid from sneakers males exerts a detrimental effect on the sperm velocity of territorial males, whereas seminal fluid from territorial males increases the sperm velocity of sneakers males. These results translate into differential fertilization efficiency determined *in vitro*. However, when sperm are exposed to a mixture of seminal fluid from territorial and sneaker males, sperm from sneaker males are faster than sperm from territorial males, which suggests that the detrimental effect of seminal fluid from sneaker males on sperm velocity from territorial males may not be caused by seminal fluid differential effects on self and non-self sperm but by a variation in seminal fluid components (Locatello, Poli & Rasotto, 2013). Similarly, recent research in the externally fertilizing species Artic charr, *Salvelinus alpinus*, has shown that over

time sperm from an aliquot of an untreated ejaculate maintain higher velocity when they are activated in a solution of water either with self or non-self seminal fluid than when they are activated just in a solution of water. This suggests that seminal fluid, regardless of its source, exerts a beneficial effect on sperm swimming performance. Interestingly, 10 seconds after activation in a solution of water with self seminal fluid the percentage of motile sperm cells was 25% fewer than when the sperm were activated in either a solution of water or in a solution of water with non-self seminal fluid. This finding also suggests the existence of a differential recognition mechanism of self and non-self sperm in seminal fluid yet, in this case, with negative effects on self sperm (Rudolfsen, Serrano & Folstad, 2015).

Finally, more convincing evidence for seminal fluid differential effects was detected across three species of polyandrous eusocial insects, *Apis mellifera*, *Acromyrmex echinatior*, and *Atta colombica*, in which seminal fluid reduces survival of non-self sperm. The negative effect of seminal fluid on non-self sperm survival may result from recognition of self and non-self sperm, and suggests that seminal fluid contains substances that are favourable to self sperm survival, and other substances that may incapacitate rival sperm (den Boer, Baer & Boomsma, 2010). Moreover, in two of these three species (*Apis mellifera*, *Acromyrmex echinatior*), there was a tendency for the negative impact on non-self sperm to be weaker when the competing males (i.e. seminal fluid and sperm donors) are brothers than when they are unrelated. This pattern may be consistent with kin selection theory, according to which individuals are selected to maximise their inclusive fitness by competing less intensively with individuals that are more genetically related then the population average (Hamilton, 1964). Therefore, seminal fluid might be expected to be less detrimental to non-self related sperm than to non-self unrelated sperm since, in the first case, the donor of seminal fluid may gain some degree of inclusive fitness if related sperm achieve fertilization.

This chapter seeks to establish differential sperm: seminal fluid interactions in the red junglefowl, *Gallus gallus*, and their role in sperm competition. The red junglefowl represents an adequate study system due to the intensity of sperm competition faced by males in polyandrous social groups (Pizzari, Froman & Birkhead, 2002), and the information available on reproductive physiology and postcopulatory sexual selection in this species and the related domestic fowl, *G. domesticus* (Pizzari & Birkhead, 2000; Pizzari *et al.,* 2003; Cornwallis & Birkhead, 2007; Pizzari, Cornwallis & Froman, 2007; Dean, Nakagawa & Pizzari, 2011). Sperm fertilizing efficiency in this system is largely determined by sperm motility and particularly by sperm swimming velocity (Birkhead *et al*., 1999; Pizzari *et al*., 2008b).

Several studies have attempted to shed light on the effects of seminal fluid in sperm fertilizing efficiency in domestic fowl. Lake (1956), and Nishiyama, Ogawa & Nakanishi (1971) showed that sperm fertilizing performance does not differ when sperm are diluted in transparent fluid (a main component of seminal fluid that is added to sperm during ejaculation) or in a different but suitable solution. Nishiyama, Ogawa & Nakanishi (1971) point out that transparent fluid, a lymph-like component of fowl seminal fluid produced by the cloacal folds, can exert a detrimental effect on sperm fertilizing potential when the concentration of sperm in the ejaculate is low. Several studies indicate that a prolonged storage with transparent fluid causes a rapid decline in sperm fertilizing ability (Nishiyama, 1955; Nishiyama, Ogawa & Nakanishi, 1971; Lake 1956, 1971, 1981). In turn, Ashizawa & Okauchi (1984) showed that sperm motility is positively affected by the concentration of seminal plasma, the component of seminal fluid produced by the male vas deferens (obtained by diluting seminal plasma with sodium phosphate buffer containing 20 mM-glucose). In particular, when the concentration of seminal plasma was below 20%, sperm rapidly lost motility. However, as the concentration of seminal plasma increases so does sperm motility. Sperm incubated with seminal plasma at concentrations higher than 40% barely lost motility over a 15 minutes interval. Lake & Ravie (1987) investigated the

effect of heterospecific seminal fluid on sperm fertilizing efficiency. To do so, Lake & Ravie (1987) diluted fowl sperm in an aqueous medium, natural homologous seminal fluid, turkey, *Meleagris gallopavo*, seminal fluid, or turkey whole semen. Lake & Ravie (1987) observed the best fertilizing efficiency when the sperm were highly diluted in homologous seminal fluid. On the other hand, the dilution with either turkey seminal fluid or turkey whole semen caused similar results in sperm fertilizing efficiency than dilution in an aqueous medium when the dose of sperm was 5x5 or $10x10^6$. Similarly, recent evidence suggests that self seminal fluid may influence sperm swimming velocity in a population of domestic feral fowl. In this population sperm from larger ejaculates are faster than sperm from smaller ejaculates of the same male. In addition, when sperm are mixed with seminal fluid from a different ejaculate of the same male the change in sperm velocity is positively related to the volume of the ejaculate from which seminal fluid is taken. Collectively, these findings suggest that males tend to produce high quality seminal fluid in large ejaculates and this can influence sperm velocity (Cornwallis & O'Connor, 2009). Overall however, the effects of seminal fluid in fowl sperm fertilizing efficiency remain little understood (Fujihara, 1992).

The aims of this chapter are to investigate: (1) whether seminal fluid affects non-self sperm velocity over time *in vitro*, and fertilization efficiency *in vivo* within a live female; and (2) whether seminal fluid effects are modulated by genetic relatedness between the competing males. To address these aims, I experimentally exposed sperm to seminal fluid from the same male (self), from his brother (non-self related), or from an unrelated male (non-self unrelated), and conducted two experiments. Firstly, to know whether seminal fluid has an effect on non-self sperm velocity I conducted an *in vitro* experiment. Secondly, to investigate whether any possible effect of seminal fluid on non-self sperm quality translates into a differential fertilization success, I conducted an *in vivo* experiment.

MATERIALS AND METHODS

In vitro experiment

I carried out the investigation at the John Krebs Field Station (Wytham, Oxfordshire) during the 2013 breeding season (May-July). All sampling of birds was conducted in accordance with approved experimental protocols under Home Office licence (PPL 30/2931). I conducted 56 trials. In each trial, I engineered two ejaculates by mixing sperm from the focal male with self seminal fluid (self, 56 trials) along with either seminal fluid from a full-sib brother (non-self related, 28 trials) or from a genetically unrelated male (non-self unrelated, 28 trials). To do so, I collected semen samples from both males by abdominal massage (Burrows & Quinn, 1935; Etches, 1996), and placed each semen sample in a micro test tube within a water bath set at physiological body temperature (41ºC). I diluted 2.5µl of semen from the focal male into 197.5µl of Phosphate Buffered Saline (PBS), and transferred 70µl of the mixture to a spectrophotometer set at 595 nm in order to calculate the concentration of sperm within the semen sample (C) through the light absorbance. I measured the light absorbance several times until getting the same value on two successive attempts. Subsequently, I split the focal male's semen sample into two micro test tubes containing equal volume of semen. I transferred a similar amount of semen from the donor of seminal fluid's semen sample to a third micro test tube. Then, I transferred these three micro test tubes to a centrifuge, separated sperm from seminal fluid by centrifugation at 3500 ×*g* for 1 minute at 4ºC, pipetted in the seminal fluid from the top of the supernatant, and transferred it into two new different micro test tubes (one containing the seminal fluid of the focal male, and the other containing the seminal fluid of the seminal fluid donor). I standardized the amount of seminal fluid (V) to be added to the sperm across trials by setting a fixed value of C = 0.6 in the formula C × V = A. To determine the volume of seminal fluid to be added to the two micro test tubes containing sperm from the focal male, I calculated A (i.e. amount of sperm) from C (i.e. concentration of sperm within the original semen sample) and V (i.e. amount of semen from the original sample transferred into each of the two micro test tubes). "C" indicates the intensity of

light or amount of photons absorbed by the ejaculate (that is, the percentage of light transmitted by the substance relative to the reference blank) and is therefore used as an estimate of the proportion of the ejaculate that contains sperm. “A” summarizes the relationship between “C” and “V” but does not have units as it is not an empirical measure of sperm. For instance, if C = 0.598 and V = 45, then A = 26.9 (0.598 × 45). Then, I calculated V in the formula C × V = A given the constant value C = 0.6 and the value of A previously calculated (26.9). From that it follows, that V = 26.9 / 0.6 = 44.8 µl, which is the amount of seminal fluid from the focal male and from the seminal fluid donor to be added to each of the two micro test tubes containing the sperm from the focal male. I pipetted the mixture in and out and shook gently the two micro test tubes to ensure that sperm and seminal fluid were properly mixed in the two engineered ejaculates. I diluted 1µl of semen from each of the two engineered ejaculates into a culture medium containing 180µl of Dulbecco’s Modified Eagle’s Medium (DMEM) and 20µl of Standard Fetal Bovine Serum (FBS). In each case, I transferred 10µl of this mixture to a microscope slide placed on a heated microscope stage set at 41ºC (the body temperature of the Red junglefowl) and connected to a digital video camera set at 10 × magnifications. I measured sperm average path velocity (VAP), in all trials within 40 minutes after collection of semen samples, three times at 10 minutes intervals using the Sperm Class Analyzer: SCA v. 3.0.3. I set the SCA to capture 25 images/sec. from 50-200 sperm tracking particles (in microns^2) in Ph- within a Leja 10 counting chamber. I only included in the analysis the engineered ejaculates where, at least, 200 sperm could be tracked with the SCA over 3-5 videos from different positions. 3 videos were sufficient in the great majority of cases to track (and generally to exceed by far) the required 200 sperm (only in a very few cases 4 or 5 videos were necessary). See Figure 1 for an illustration of the main steps described.

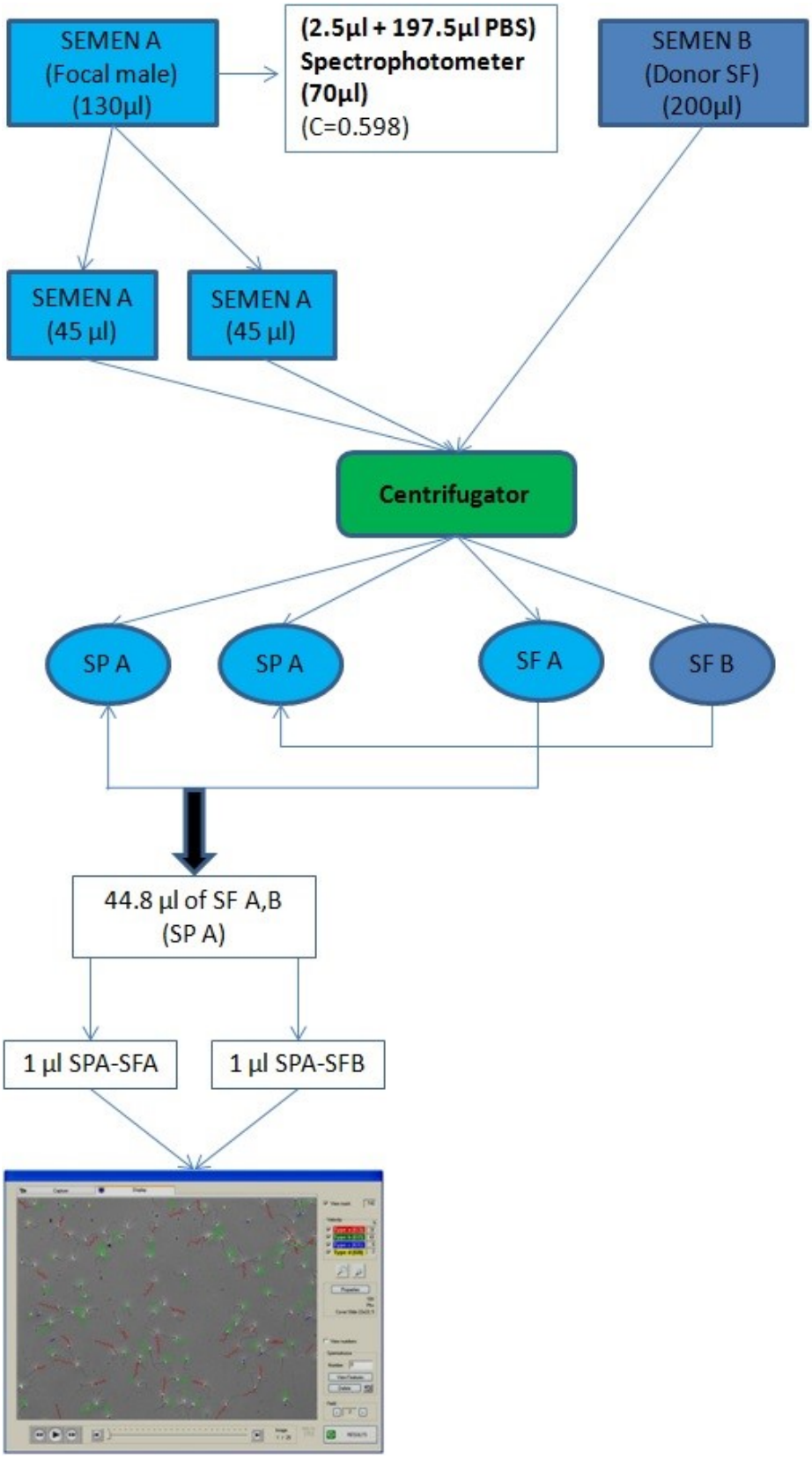


Figure 1. Pipeline preparation of samples for the *in vitro* experiment. After measuring the concentration of sperm within the sample from the focal male this sample was split into two samples of equal volume. These two samples and the sample from the seminal fluid donor were centrifuged and the seminal fluid supernatant from the focal male and from the donor of seminal fluid was transferred into two different micro test tubes. This seminal fluid from the focal male and from the donor of seminal fluid was mixed separately with the sperm from the focal male previously kept into two different micro test tubes in order to measure sperm VAP in both experimental conditions (i.e. sperm in self and in non-self seminal fluid).

***In vivo* experiment**

I carried out this experiment at the John Krebs Field Station (Wytham, Oxfordshire) during the 2013 (July-September) and 2014 (April-May) breeding seasons. All sampling of birds was conducted in accordance with approved experimental protocols under Home Office licence (PPL 30/2931). I collected data from 37 trials. In each trial, I used a protocol similar to the one utilized in the *in vitro* experiment to engineer three ejaculates by exposing sperm from the focal male to the self seminal fluid treatment (N=12), the non-self related (N=13) and the non-self unrelated treatment (N=12). To do so, I collected semen samples from the focal male and from the seminal fluid donor by abdominal massage (Burrows & Quinn, 1935; Etches, 1996), and placed them in two different micro test tubes within a water bath set at 41ºC (the body temperature of the Red junglefowl). Then, I diluted 2.5µl of semen from the focal male into 197.5µl of PBS, and transferred 70 µl of the mixture to a spectrophotometer set at 595 nm to calculate the concentration of sperm within the focal male's semen sample (C) through the light absorbance. I measured the light absorbance several times until getting the same value on two successive attempts. Inseminated ejaculates with very low concentrations of sperm tend to have reduced chances of fertilization. This fact was confirmed during preliminary trials in the current study, where inseminated ejaculates with $C < 0.2$ hardly produced sperm hydrolysis points in the PVL of the egg. For this reason, I did not include in the study the samples with $C < 0.2$.

I standardized the amount of sperm from the focal male to be artificially inseminated throughout the trials by setting a fixed value of $A = 25$ in the formula $C \times V = A$, where C is the concentration of sperm within the focal male's semen sample previously described, V is the volume of semen from the focal male to be artificially inseminated, and A is an estimate of the amount of sperm within the semen from the focal male's sample (the value $A = 25$ in this formula was set after performing several pre-trials aimed to determine the suitability of the relationship between the volume of semen usually collected by abdominal massage and the volume of semen

required to perform adequately the trials). “C” indicates the intensity of light or amount of photons absorbed by the ejaculate (that is, the percentage of light transmitted by the substance relative to the reference blank) and is therefore used as an estimate of the proportion of the ejaculate that contains sperm. “A” summarizes the relationship between “C” and “V” but does not have units as it is not an empirical measure of sperm. For instance, if C = 0.6, then V = 25 / 0.6 = 41.6, which means that 41.6 µl of semen from the focal male’s sample would be transferred into a different micro test tube as part of the engineered ejaculate. Subsequently, I added, in all trials, 50µl of seminal fluid from the donor of seminal fluid’s sample to the focal male’s semen already in the engineered ejaculate micro test tube in order to standardize the amount of donor’s seminal fluid throughout the experiment. I pipetted in and out and shook gently the micro test tube to ensure that the sperm and seminal fluid were properly mixed (to separate the seminal fluid from the sperm within the seminal fluid donor’s sample, I centrifuged it at 3500 ×*g* for 1 minute at 4ºC, and took the seminal fluid from the top of the supernatant). Then, I diluted 1µl of semen from the engineered ejaculate into a culture medium containing 180 µl of Dulbecco’s Modified Eagle’s Medium (DMEM) and 20 µl of Standard Fetal Bovine Serum (FBS). I transferred 10 µl of the mixture to a microscope slide placed on a heated microscope stage set at 41ºC, and connected to a digital video camera set at 10×magnification. I measured sperm average path velocity (VAP) using the Sperm Class Analyzer: SCA v. 3.0.3, being sure that, in all trials, I tracked at least 200 sperm overall in 3-5 videos from different positions. I set the SCA to capture 25 images/sec. from 50-200 sperm tracking particles (in microns^2) in Ph- within a Leja 10 counting chamber. Finally, I artificially inseminated females in the uterovaginal junction between 17:00-20:30h. I only used the trials in which the female retained all the semen after withdrawing the insemination pipette (Etches, 1996). See Figure 2 for an illustration of the main steps described.

I collected eggs daily for the seven days following artificial insemination, although I excluded the eggs laid on the first day after insemination because they lacked sperm hydrolysis points having

been ovulated before the sperm could have reached them (Wishart, 1987; Etches, 1996). I therefore analysed only the eggs collected from the second day onwards. I cracked gently the eggs, and separated the yolk from the albumen by passing the eggs very carefully between hands. Then, I cut with scissors a portion of the outer perivitelline membrane (approximately of 2×2 cm.), and cleaned it of residual yolk by brushing it with a hairpin loop within petri dishes filled with PBS. Once clean, I transferred the membrane onto a microscope glass slide and placed a cover slip over it. Finally, I placed the slide under a microscope set at 10×magnification to count the number of sperm-induced hydrolysis points on the perivitelline (PVL) membrane of the egg. The number of sperm hydrolysis points on the PVL of the egg is an accurate indicator of the amount of sperm stored by the female and, thus, of the probability of fertilization of the egg. For this reason, the variation in PVL sperm-induced hydrolysis points provides a sensitive measure of the fertilizing performance of an ejaculate (Wishart, 1987; Brillard, 1993). I waited for at least 10 days from the artificial insemination day to use the female in a new trial in order to ensure that she would be sperm depleted (in one trial I waited for just 8 days since the female had laid her last 3 eggs without sperm, which indicates that she was sperm depleted).

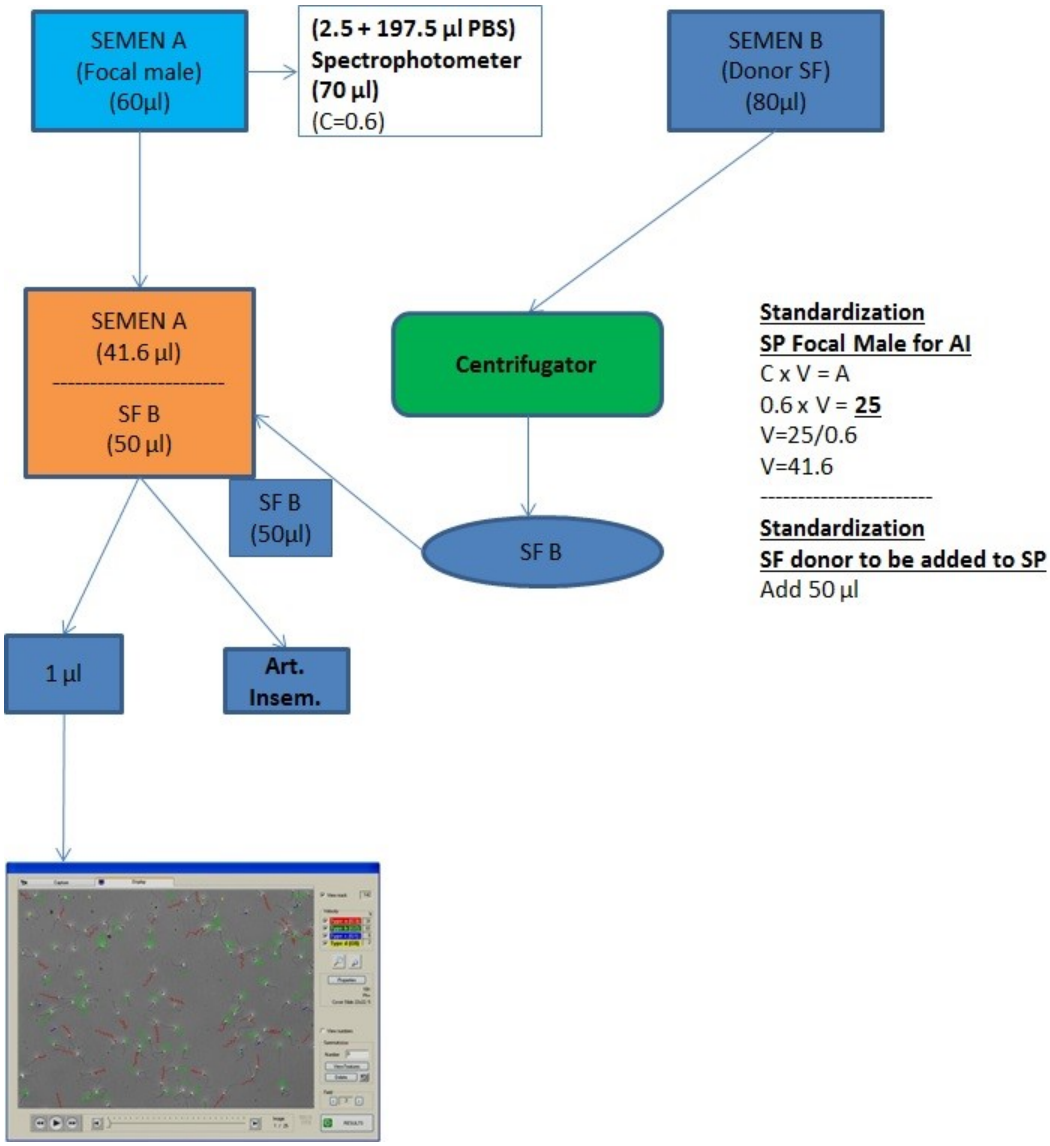


Figure 2. Pipeline preparation of samples for the *in vivo* experiment.

Statistical analysis

I conducted the analyses using the statistical software R with the lme4 package.

In vitro experiment

I characterised temporal variation in sperm velocity in the self treatment in two ways. First, to test whether the mere passage of time has an effect on sperm velocity I fit a generalised linear mixed effects model, with sperm VAP as the response variable, time interval (0', 10', and 20' after preparation of the ejaculate) as fixed effect, and male ID as a random effect. Second, I tested if variation in rate at which sperm velocity declines over time is predicted by initial VAP (i.e. VAP

at time 0'). I therefore fit another generalized liner mixed effect model, with sperm VAP (at times 10' and 20') as the response variable, time interval (10' and 20' after preparation of the ejaculate), VAP at time 0' and its interaction as fixed effects, and male ID as a random effect. In both cases, time interval was modelled as a three-level factor.

Secondly, to test whether the experimental seminal fluid treatments (i.e. the genetic relatedness between focal male and seminal fluid donor) have an effect on temporal declines in sperm velocity I fit a generalised linear mixed effects model, with standardized VAP (sperm VAP in non-self seminal fluid *minus* sperm VAP in self seminal fluid) as the response variable, seminal fluid treatments, time interval (0',10', and 20' after preparation of the ejaculate) and its interaction as fixed effects, and competing males IDs as random effects. As above, time interval was modelled as a three-level factor. In all three cases, I calculated the Minimal Adequate Model (Crawley, 2005), testing the significance of the 3-way and 2-way-interactions, and that of the single factors. To do so, I conducted the simplification process of the full model in a stepwise fashion comparing in each step a model with an update version lacking a term (i.e. interaction or single factor) using the R function "anova" for comparing two models. The likelihood ratio tests caused by dropping terms from the models were reported as chi-square tests of significance of each relevant term. In each step of the simplification process, I dropped the non-significant term with the highest p-value until reaching the Minimal Adequate Model. In all three cases, the normality of the data was confirmed by the straight line of the "quantile-quantile plots", indicative of the normality of the residuals, obtained using the R functions "qqnorm" and "qqline" (Crawley, 2005). The summaries of the GLMMs are presented in the appendix.

***In vivo* experiment**

First, to test whether time elapsed from artificial insemination affects the probability of finding sperm hydrolysis points on the PVL membrane of the egg I fit a generalised linear mixed effects

model to the data, using the R command “glmer”, with a binomial error structure, with presence of hydrolysis points on the PVL membrane (“0”=absence, “1”=presence) as the response variable, oviposition day as fixed effect, and ID of the inseminated female as a random effect. I checked for overdispersion dividing the deviance of the full model by its residual degrees of freedom in order to obtain the dispersion parameter. As the dispersion parameter was very close to 1 (122/117=1.04) there is no evidence of overdispersion in the model. Second, to test whether time elapsed from artificial insemination and genetic relatedness between competing males affect the probability of finding sperm hydrolysis points on the PVL membrane of the egg I fit a generalised linear mixed effects model to the data, using the R command “glmer”,with a binomial error structure, with presence of hydrolysis points on the PVL membrane (“0”=absence, “1”=presence) as the response variable, oviposition day, seminal fluid treatments and its interaction as fixed effects, and identity of the inseminated female as a random effect. I checked for overdispersion dividing the deviance of the full model by its residual degrees of freedom in order to obtain the dispersion parameter. As the dispersion parameter was very close to 1 (110.5/113=0.98) there is no evidence of overdispersion in the model.Third, I analysed variation in the number of sperm hydrolysis points on the PVL membrane of individual eggs. As the data were not normally distributed I log-transformed them as follows: log (number of PVL sperm hydrolysis points + 1), which enabled me to include eggs with zero hydrolysis points. I fit a generalized linear mixed effects model to the data, using the R command “lmer”, with log PVL membrane sperm hydrolysis points as the response variable, seminal fluid treatments, oviposition day, and sperm VAP of the engineered ejaculate as fixed effects, and ID of the inseminated female as a random effect. The data met the assumption of normality as they were log-transformed.

In all three cases, I calculated the Minimal Adequate Model (Crawley, 2005), testing the significance of the 3-way and 2-way-interactions, and that of the single factors. To do so, I conducted the simplification process of the full model in a stepwise fashion comparing in each

step a model with an update version lacking a term (i.e. interaction or single factor) using the R function "anova" for comparing two models. The likelihood ratio tests caused by dropping terms from the models were reported as chi-square tests of significance of each relevant term. In each step of the simplification process, I dropped the non-significant term with the highest p-value until reaching the Minimal Adequate Model. The summaries of the GLMMs are presented in the appendix.

RESULTS

In vitro experiment

The model investigating whether the mere passage of time has an effect on sperm velocity lost explanatory power after removing "time interval" ($X^2_1 = 16.723$, $p < 0.001$). This was largely due to the fact that sperm in the self treatment slowed down over time (Fig. 3A).

The model investigating if variation in rate at which sperm velocity declines over time is predicted by initial VAP (i.e. VAP at time 0') lost explanatory power after removing "VAP at time 0'" ($X^2_1 = 74.112$, $p < 0.001$). This indicates that ejaculates with higher initial VAP suffered a higher rate of decline in VAP over time (Fig. 3B). Importantly, in addition to temporal effects there was a significant effect of the seminal fluid treatment. Temporal decline in VAP was significantly slower in non-self treatments, and particularly in the non-self related, where sperm are mixed with the seminal fluid of the focal male's full-sibling (Fig. 3A). This means that over time sperm go faster in non-self seminal fluid, particularly so in seminal fluid from a full-sibling (Fig. 3C). This pattern was confirmed by the fact that the model investigating whether the experimental seminal fluid treatments have an effect on temporal declines in sperm velocity lost explanatory power after removing "seminal fluid treatments" ($X^2_1 = 4.5389$, $p = 0.03$) and "time interval" ($X^2_1 = 15.255$, $p < 0.001$). Finally, there was a marginally non-significant trend for the interaction between the relatedness of competing males and time interval, whereby relative to the

self treatment, non-self VAP did not decline over time when sperm were exposed to the seminal fluid a full sibling male (i.e. non-self related), while it declined marginally when sperm were exposed to the seminal fluid of an unrelated male (i.e. non-self unrelated; removal of the interaction “seminal fluid treatments: time interval”, $X^2_1 = 3.2667$, $p = 0.07$).

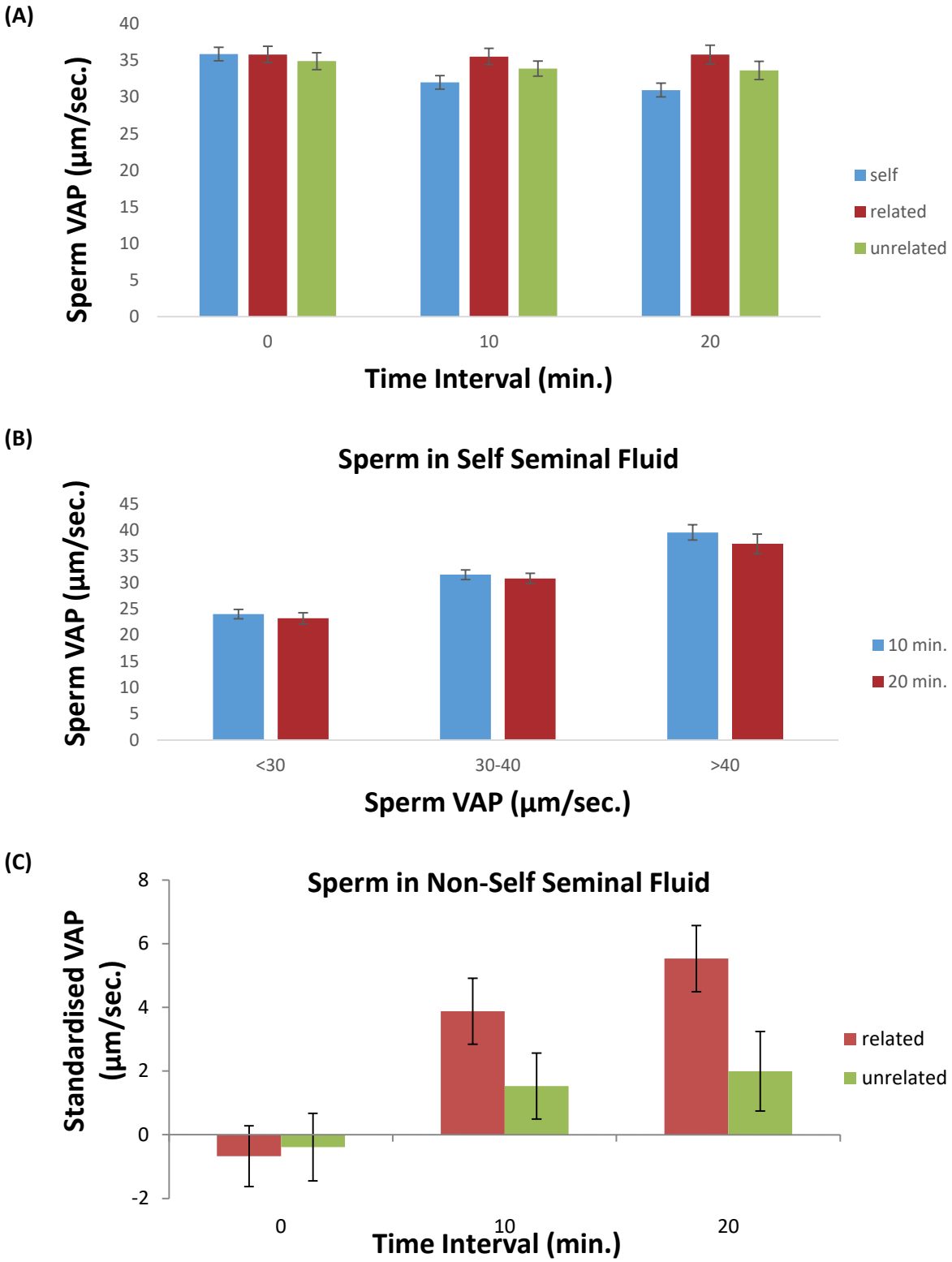


Figure 3. (A) Changes in sperm VAP over three time intervals, across the three seminal fluid treatments representing different degrees of genetic relatedness between focal male and seminal fluid donor. **(B)** Changes in sperm VAP in self seminal fluid over two time intervals in relation to sperm VAP right after sample preparation (0 min.). **(C)** Changes in sperm VAP in the non-self seminal fluid treatments relative to sperm VAP in self at different time intervals (standardised VAP = non-self VAP – self VAP). Vertical bars = SE.

***In vivo* experiment**

As expected, there was a marked temporal decline in the probability of detecting sperm-induced hydrolysis points on the PVL membrane of eggs laid over successive days (Fig. 4A), as indicated by the fact that the model investigating whether time elapsed from artificial insemination affects the probability of finding sperm-induced hydrolysis points lost explanatory power after removing "oviposition day" ($X^2_1 = 16.197$, $p < 0.001$).

However, although this probability declined more markedly in the self than in either non-self treatments, seminal fluid treatment did not have an overall significant effect, independently or through an interaction with oviposition day (the model investigating whether time elapsed from artificial insemination and genetic relatedness between competing males affect the probability of finding sperm-induced hydrolysis points did not lose explanatory power after removing "seminal fluid treatments": $X^2_2 = 20.881$, $p = 0.103$).

In contrast, the model investigating variation in the number of sperm-induced hydrolysis points over successive oviposition days lost explanatory power after removing "oviposition day" ($X^2_1 = 51.815$, $p < 0.001$) and the interaction "seminal fluid treatments : VAP of the engineered ejaculate" ($X^2_2 = 20.881$, $p < 0.001$). The first result may be accounted for by the fact that there was a marked decline in the number of hydrolysis points over successive oviposition days on the PVL membrane of the eggs (Fig. 4B). The second result indicates that slower sperm tend to produce more hydrolysis points in the PVL membrane of the egg when mixed with self seminal fluid or with seminal fluid from a full-sibling, whereas faster sperm tend to produce more hydrolysis points in the PVL membrane of the egg when mixed with seminal fluid from a genetically unrelated male (Fig. 4C).

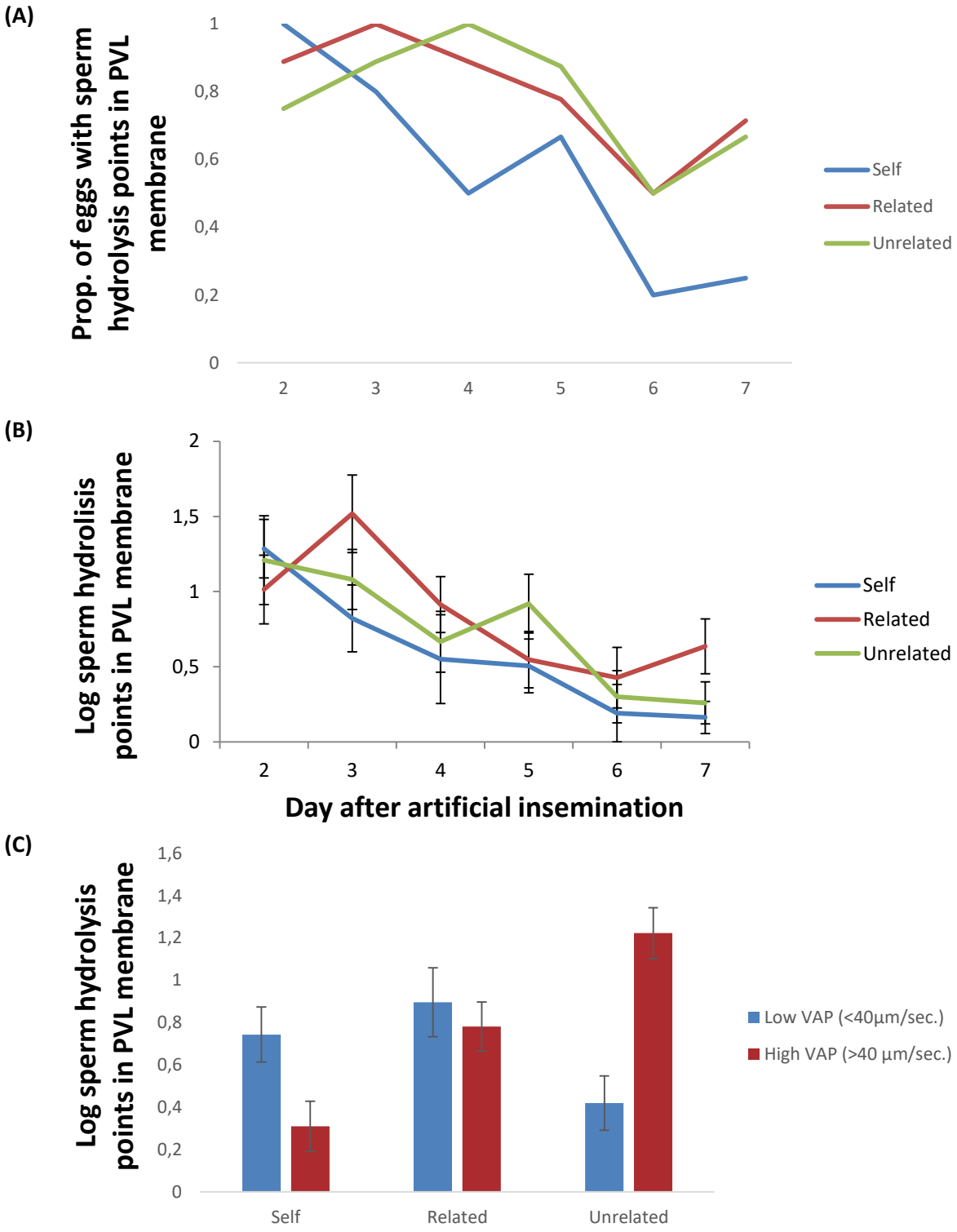


Figure 4. (A) Presence of sperm hydrolysis points in the PVL membrane of the egg over successive days following artificial insemination, calculated as the proportion of eggs with sperm out of all eggs laid on each oviposition day. **(B)** Log sperm hydrolysis points in the PVL of the egg over successive days elapsed from artificial insemination. **(C)** Average log sperm hydrolysis points in the PVL of the egg throughout a week elapsed from artificial insemination when the sperm are mixed with seminal fluid of different degrees of genetic relatedness. The benchmark of 40 µm/sec. for illustrative purposes to categorise sperm VAP as either low or high was chosen as the mean sperm VAP of the engineered ejaculates used to perform artificial insemination in the experiment. Vertical bars = SE.

DISCUSSION

This chapter investigates the effect of seminal fluid on sperm fertilizing efficiency and whether these effects are modulated by the genetic relatedness between the sperm and seminal fluid donor males (i.e. competing males). The results show that sperm VAP tend to decline over time *in vitro*. This decline however was lower when sperm were exposed to non-self, and particularly non-self related, seminal fluid. There was a tendency for non-self inseminations to outperform self inseminations *in vivo*, and a significant interaction between seminal fluid treatment and initial sperm VAP, that is, relatively more sperm from high-VAP ejaculates reached the eggs in the non-self unrelated treatment (compared with sperm from low-VAP ejaculates), while more sperm from low-VAP ejaculates reached the eggs in the self treatment (compared with sperm from high-VAP ejaculates). I discuss the significance of the results of the *in vitro* experiment first, and then consider the results of the *in vivo* experiment.

In vitro experiment

Sperm slowed down over time. The extent of this decline depended on the initial VAP, and was prevented by exposure to seminal fluid from a full-sibling and reduced by exposure to seminal fluid from a genetically unrelated male. One possible scenario to explain the beneficial effect of non-self seminal fluid on sperm VAP might be that sperm react to the presence of rival (i.e. non-self) seminal fluid by increasing VAP. The degree to which this can be achieved might be influenced by the genetic relatedness between the sperm and seminal fluid donors. When the seminal fluid comes from a male related to the sperm donor the increase in sperm VAP can be higher that when the sperm are exposed to non-self seminal fluid from a genetically unrelated male. This might have inclusive fitness consequences, possible favouring cooperation between genetically related ejaculates. A mechanism for this scenario is based on the idea of "copulation complementation" (Xue & Noll, 2000), according to which the exposure to non-self seminal fluid may have allowed for any deficiencies in self-seminal fluid, causing a decline in sperm VAP over

time, to be compensated. Related male seminal fluid would be better than unrelated seminal fluid because the sperm and seminal fluid proteomes act synergistically and therefore related seminal fluid is more likely to match the focal male's sperm proteome in a manner analogous to blood types and organ transplants. The fact that sperm VAP in related seminal fluid over time was almost identical than in self seminal fluid at time 0' is consistent with this mechanism.

Recent work in domestic fowl showed that the sperm and seminal fluid proteomes do not fully overlap (Labas *et al.*, 2015), thus providing support for this scenario. Sperm proteins absent in the seminal fluid might react upon detection of rival ejaculates, triggering an increase in VAP in order to outcompete them. Seminal fluid proteins absent in the sperm might react differentially to this increase in sperm VAP depending on the degree of genetic relatedness with the sperm, thus being less detrimental to genetically related than genetically unrelated sperm. Seminal fluid immunological components (Poiani, 2006) could mediate this response by discriminating different degrees of genetic relatedness with the sperm, leading to immune tolerance towards non-self but genetically related sperm. Previous *in vitro* work in the Red junglefowl showed that sperm VAP in seminal fluid of a Major Histocompatibility Complex (MHC) similar male was higher than in seminal fluid of a MCH dissimilar male (Gillingham, 2007). As genetically related males tend to be more similar in their MHC than genetically unrelated males, this result is consistent with sperm VAP being faster over time in non-self related than in non-self unrelated seminal fluid.

Future work should explore to what extent the degree of genetic relatedness between the sperm and seminal fluid affects sperm VAP over time. Measuring VAP when sperm are experimentally exposed to engineered seminal fluid having different degrees of genetic relatedness with them will shed light on this matter. For example, sperm would be exposed to 1) self seminal fluid mixed with non-self related seminal fluid, 2) self seminal fluid mixed with non-self unrelated

seminal fluid, and 3) non-self related seminal fluid mixed with non-self unrelated seminal fluid. According to my data, sperm VAP over time is predicted to be higher as the degree of genetic relatedness between the sperm and the engineered non-self seminal fluid increases.

The process of centrifugation might have affected sperm VAP. However, as all sperm used throughout the experiment were subjected exactly to the same conditions is very unlikely that it could have a significant impact on the results. Another potential methodological limitation of this experiment is that sperm were separated from seminal fluid at 3500 ×*g* for just a minute. This protocol allows separation of the great majority of sperm cells from the seminal fluid while minimizing the potential damage that centrifugation can cause to the molecular components of seminal fluid and to the sperm. The potential effect that the possible presence of some sperm cells within the seminal fluid donor might have had on the results was kept to a minimum due to their reduced number confirmed by phase contrast microscopy.

***In vivo* experiment**

Consistently with previous work in the fowl (Wishart, 1987), the probability of finding sperm hydrolysis points in the PVL membrane of the egg declined over successive days elapsed from artificial insemination. This result may be accounted for by the progressive loss of sperm from female sperm storage tubules.

Relatively more sperm from high-VAP ejaculates reached the eggs in the non-self unrelated treatment, while more sperm from low-VAP ejaculates reached the eggs in the self treatment. The fact that sperm from high-VAP ejaculates are more competitive in the non-self unrelated treatment may constitute an adaptive response to outcompete genetically unrelated rival ejaculates within the female reproductive tract. In turn, the fact that sperm from low-VAP ejaculates are more competitive in the self treatment suggests that high sperm VAP only results in a competitive

edge in the presence of competition. Future work is required to understand the reasons of these differences.

The fact that sperm fertilizing ability throughout the week elapsed from artificial insemination tended to be higher when exposed to non-self seminal fluid is consistent with the *in vitro* experiment result that over time sperm go faster in non-self seminal fluid, as sperm VAP correlates with fertilization success in the fowl (Wishart & Palmer, 1986).

Previous *in vivo* work in the Red junglefowl showed that sperm fertilization success was not affected by being experimentally mixed with self or non-self seminal fluid (Collet, 2010). However, the insemination of centrifuged sperm in this work may account for this result, as the centrifugation process might have damaged the sperm cells, thus limiting their capacity to perform adequately within the female reproductive tract.

Conclusion

Taken together, the results of the *in vitro* and *in vivo* experiments show that sperm fertilizing efficiency increases when exposed to non-self seminal fluid. This might be explained by an increase in VAP when sperm detect the presence of a rival ejaculate and, in the case of the *in vitro* experiment, a differential seminal fluid response to related and unrelated sperm. However, it is very early stages to draw any functional conclusions from these results and future research should aim to understand the underlying molecular mechanisms that make sperm more competitive in non-self seminal fluid.

Appendix

Table 1. Summary of GLMM to test whether the mere passage of time has an effect on sperm velocity.

Model: lmer (VAP ~ Time + (1|FocalMale))

Random effects:

Groups	Name	Variance	Std. Dev.
FocalMale	(Intercept)	10.57	3.250
Residual		38.78	6.228

Fixed effects:

	Estimate	Std. Error	t-value
(Intercept)	35.412	1.154	30.686
Time	-0.246	0.058	-4.189

Table 2. Summary of GLMM to test if variation in rate at which sperm velocity declines over time is predicted by initial VAP.

Model: lmer (VAP ~ VAP0 * Time + (1|FocalMale))

Random effects:

Groups	Name	Variance	Std. Dev.
FocalMale	(Intercept)	4.79	2.189
Residual		20.20	4.494

Fixed effects:

	Estimate	Std. Error	t-value
(Intercept)	-1.025	7.271	-0.141
Time	0.469	0.452	1.036
VAP0	0.949	0.198	4.785
Time:VAP0	-0.016	0.012	-1.291

Table 3. Summary of GLMM to test whether the experimental seminal fluid treatments have an effect on temporal declines in sperm velocity.

Model: lmer (standardizedVAP ~ Treatment * Time + (1|FocalMale) + (1|DonorMale))

Random effects:

Groups	Name	Variance	Std. Dev.
DonorMale	(Intercept)	1.109	1.053
FocalMale	(Intercept)	1.118	1.057
Residual		31.744	5.634

Fixed effects:

	Estimate	Std. Error	t-value
(Intercept)	-0.080	1.049	-0.077
TreatmentUnrelated	-0.138	1.413	-0.098
Time	0.310	0.075	4.122
TreatmentUnrelated:Time	-0.191	0.106	-1.798

Table 4. Summary of GLMM to test whether time elapsed from artificial insemination affects the probability of finding sperm hydrolysis points on the PVL membrane of the egg.

Model: glmer (PVL points ~ Day + (1|Female), family=binomial)

Random effects:

Groups	Name	Variance	Std. Dev.
Female	(Intercept)	0.882	0.939

Fixed effects:

	Estimate	Std. Error	z-value
(Intercept)	3.863	0.916	4.217
Day	-0.566	0.156	-3.608

Table 5. Summary of GLMM to test whether time elapsed from artificial insemination and genetic relatedness between competing males affect the probability of finding sperm hydrolysis points on the PVL membrane of the egg.

Model: glmer (PVL points ~ Day * Treatment + (1|Female), family=binomial)

Random effects:

Groups	Name	Variance	Std. Dev.
Female	(Intercept)	2.122	1.457

Fixed effects:

	Estimate	Std. Error	z-value
(Intercept)	5.436	1.741	3.122
Day	-1.026	0.319	-3.210
TreatmentRelated	-0.660	2.138	-0.309
TreatmentUnrelated	-2.730	2.024	-1.349
Day:TreatmentRelated	0.473	0.418	1.132
Day:TreatmentUnrelated	0.862	0.428	2.013

Table 6. Summary of GLMM to analyse variation in the number of sperm hydrolysis points on the PVL membrane of individual eggs.

Model: lmer (log PVL points ~ Treatment * Day * VAPEE + (1|Female))

Random effects:

Groups	Name	Variance	Std. Dev.
Female	(Intercept)	0.093	0.305
Residual		0.174	0.418

Fixed effects:

	Estimate	Std. Error	t-value
(Intercept)	5.485	1.595	3.439
TreatmentRelated	-4.865	2.053	-2.370
TreatmentUnrelated	-8.921	2.070	-4.309
Day	-0.576	0.282	-2.043
VAPEE	-0.097	0.040	-2.407
TreatmentRelated:Day	0.476	0.409	1.164
TreatmentUnrelated:Day	1.056	0.385	2.741
TreatmentRelated:VAPEE	0.119	0.051	2.332
TreatmentUnrelated:VAPEE	0.215	0.050	4.230
Day:VAPEE	0.008	0.007	1.188
TreatmentRelated:Day:VAPEE	-0.009	0.010	-0.967
TreatmentUnrelated:Day:VAPEE	-0.024	0.009	-2.576

CHAPTER 3

Patterns of differential sperm and seminal fluid investment and the Coolidge effect

ABSTRACT

Competitive ejaculates are costly to produce. For this reason, males are selected to tailor strategically their ejaculates depending on the value of a particular mating event. A complete understanding of ejaculate expenditure patterns requires studying male investment in the different constituents of an ejaculate: i.e. sperm and seminal fluid. However, most studies have focused on either total ejaculate volume or sperm numbers, overlooking the possible role of seminal fluid and its components, such as proteins. Here, I investigate differential patterns of male ejaculate expenditure in relation to female sexual novelty in socially dominant and subdominant male Red junglefowl. To do so, I collected natural ejaculates by allowing the males to copulate up to five times with the same female followed by once with a novel female in quick succession. I measured sperm numbers, seminal fluid volume, and seminal fluid protein concentration. Regardless of their social status, males preferentially invested sperm and seminal fluid in sexually novel females, by decreasing sperm and seminal fluid investment over successive copulations with the same female and increasing it again on encountering the new female (a pattern known as the Coolidge effect). Patterns of seminal fluid proteins however, changed markedly with male status. While seminal fluid protein content in subdominant males varied with female sexual novelty consistent with a Coolidge effect, dominant males tended to allocate a constant amount of seminal fluid proteins to each successive copulation. Collectively, these results suggest that strategic ejaculate expenditure in favour of sexually novel females (i.e. the Coolidge effect) reported in a range of species, is mediated by differential seminal fluid allocation. Furthermore, allocation of proteins to seminal fluid may be subject to status-specific strategies or constraints, possibly related to status-specific levels of sperm competition. Further research is required to investigate the effects of potential trade-offs between ejaculate components, to develop a more comprehensive understanding of patterns of male strategic ejaculate allocation.

INTRODUCTION

Competitive ejaculates are costly to produce and males are selected to invest resources strategically in their ejaculates depending on the value of a mating event in order to maximize their reproductive success. A number of factors are therefore predicted to dictate patterns of male ejaculate expenditure, including the level of sperm competition likely experienced by an insemination and female phenotypic traits (Wedell, Gage & Parker, 2002; Parker & Pizzari, 2010).

Sperm competition risk (i.e. probability that a male ejaculate will compete with rival ejaculates to fertilize the egg) and intensity (i.e. number of rival ejaculates competing with a particular ejaculate) are predicted to affect male ejaculate expenditure and to result in conditional dependent strategies. Across species male ejaculate expenditure in a mating event is expected to increase as the number of ejaculates competing to fertilize the egg increases. Comparative studies provide empirical evidence to support this prediction. In taxa where sperm competition is common, males show greater ejaculate expenditure, as indicated by their relative testis size, both at species and population levels. Within species, the reverse pattern is predicted as males are expected to reduce ejaculate expenditure as the number of competitors increases above one as they would get diminishing returns for costs in sperm investment (Parker *et al*., 1996; Wedell, Gage & Parker, 2002; Pizzari & Parker, 2009; Parker & Pizzari, 2010). Supporting this prediction, gobiid fishes sneaker males increase sperm expenditure in the presence of one competitor sneaker and decrease it as the number of competitor sneakers rises above one (Pilastro, Scaggiante & Rasotto, 2002).

Female reproductive quality can also affect male ejaculate expenditure. When females vary in reproductive quality an implicit prediction from the raffle models of sperm competition is that males will achieve greater reproductive payoffs by preferentially allocating their ejaculate resources to the most fecund females. Studies across several taxa confirm this prediction as males

transfer significantly larger ejaculates to higher quality females. However, such preferential response may be modulated by a trade-off between present and future mating opportunities and by the way female reproductive quality is associated with sperm competition levels across different mating systems (Wedell, Gage & Parker, 2002; Pizzari & Parker, 2009; Parker & Pizzari, 2010). A female attribute that may play a particularly important role is a female's sexual novelty to a male. Male responses to female sexual familiarity are explained by strategic ejaculate allocation and the Coolidge effect.

The Coolidge effect is a response to female sexual novelty that may be defined as the restoration of mating behaviour and ejaculate investment in males that have reached sexual satiation with a female when this female is replaced with a novel one (Wilson, Kuehn & Beach, 1963; Dewsbury, 1981; Pizzari, 2002; Wedell, & Gage, Parker, 2002; Pizzari *et al*., 2003; Gage, 2003). The Coolidge effect has been described in rodents (Dewsbury, 1981) as a progressive decline in a male's sexual interest in a familiar sexual partner and the subsequent resumption of his mating propensity on encountering a sexually novel female. More recently, an increasing number of studies have shown that the Coolidge effect occurs in a wide range of species and is manifested not only though differential male mating propensity, but importantly also in preferential ejaculate expenditure in sexually novel partners.

In the burying beetle *Nicrophorus vespilloides*, male time to mating (an indicator of male sexual interest) increases continuously over the course of successive encounters with the same female and, when exposed to a novel female, males mate significantly faster than on their last encounters with the familiar female and as fast as in their first encounter with the familiar female (Steiger *et al.,* 2008). In the birch catkin bug, *Kleidocerys resedae*, males re-paired with a novel female one hour after their first mating mated for significantly longer than those males re-paired with the same female. Assuming that copulation duration accurately reflects ejaculate investment in this

species (as often happens in insects) males would be investing more sperm numbers and/or seminal fluid in novel females (Reinhold *et al*., 2015). An analogous male response to female sexual novelty, in this case modulated by male social status, has been reported in the externally fertilizing fish species European bitterling (*Rhodeus amarus*). Females place their eggs in the gills of freshwater mussels through their exhalant siphon. Males fertilize the eggs by allocating sperm over the inhalant siphon of the mussel during the course of a mating. For this reason, the mussel, rather than the female, represents a new fertilizing opportunity for the males. Dominant males increase the number of ejaculations, used as a proxy to measure sperm investment, on encountering a new mussel compared with a familiar one. However, subdominant males keep a consistently high level of ejaculates, allocating most sperm to the first mussel encountered regardless of its status. As subdominant males spend more time inspecting novel mussels than familiar mussels, it is very likely that they are able to recognise mussel novelty. Therefore, their ejaculate allocation behaviour may be an opportunistic strategy to deal with their reduced number of fertilizing opportunities since dominant males aggressively defend territories to monopolize their access to both mussels and females (Spence, Reichard & Smith, 2013). The Coolidge effect has also been shown in hermaphrodites. In particular, when mating in the male role, the pond snail, *Lymnaea stagnalis*, significantly inseminates more novel than original partners (Koene & Ter Maat, 2007). Finally, male Red junglefowl, *Gallus gallus*, and males from a feral population of domestic fowl, *G. domesticus*, display a marked preference to mate with and invest sperm in sexually novel females, in a way entirely consistent with the Coolidge effect (Pizzari *et al*., 2003).

Collectively, these studies suggest that the Coolidge effect may represent an adaptive strategy that enables males to reduce their sperm investment in a particular female according to the number of sperm previously inseminated into her in order to conserve sperm resources for future mating opportunities. Therefore, males may be able to distribute their limited sperm supplies more evenly and adaptively across multiple females (Pizzari, 2002; Wedell, Gage & Parker, 2002). This

strategy makes sense in promiscuous mating systems but not in monogamous species. Consistent with this expectation, monogamous species of rodents (Dewsbury, 1981) and crickets (Gershman & Sakaluk, 2009; Thomas & Simmons, 2015) do not exhibit a Coolidge effect.

Most studies of strategic ejaculate expenditure by males have focused on sperm allocation (Wedell, Gage & Parker, 2002; Parker & Pizzari 2010; Kelly & Jennions, 2011), ignoring seminal fluid and its interactions with sperm allocation. Yet, it is now well established that seminal fluid can play a significant role in fertility and sperm competition dynamics, for example by manipulating female remating intervals, female fecundity, and affecting sperm quality (Wolfner 1997; Chapman, 2008; Pitnick & Hosken, 2010; Perry, Sirot & Wigby, 2013). Recent theoretical studies have shown that explicit consideration of potential seminal fluid effects can drastically change predictions of evolutionarily stable strategies of male ejaculate expenditure, and have called for empirical research to elucidate patterns of male seminal fluid allocation (e.g. Cameron, Day & Rowe 2007; Alonzo & Pizzari 2010; 2013). Empirical evidence has begun to emerge indicating that in some species males might be capable of sophisticated strategies of seminal fluid allocation. For example, consistent with theoretical predictions (Alonzo & Pizzari 2010), *Drosophila melanogaster* males are able to adjust their ejaculates in response to the potential level of sperm competition by transferring significantly more sex peptide (a protein that decreases female propensity to remate) and ovulin (a fecundity-stimulating compound) when a rival male was present before and during mating (Wigby *et al*., 2009). In line with this, *D. melanogaster* males kept with rivals prior to mating increased significantly the duration of their matings, which is positively related to the amount of seminal fluid transferred in this species. In addition, females mated with males kept with rivals prior to mating had signicantly higher latency to remating, and laid significantly more eggs that, moreover, had an average three per cent higher survival to adulthood (Bretman, Fricke & Chapman, 2009). Despite this recent work in *D. melanogaster* however, patterns of male seminal fluid allocation remain largely unexplored.

Here, I study patterns of sperm and seminal fluid allocation in response to female sexual novelty in a population of Red junglefowl, *Gallus gallus*. I used an experimental protocol based on male repeated exposure to a particular female followed by the substitution of this female by a new one in quick succession. I collected natural ejaculates by allowing the males to copulate up to five times with the familiar female and once with the novel female in order to understand patterns of male investment in the familiar and new (i.e. sexually novel) females. I measured male propensity to mate; and the allocation of: i) sperm numbers, ii) seminal fluid volume; and iii) seminal fluid protein content to successive copulations with a progressively more sexually familiar female and to a new, sexually novel female. Because ejaculate expenditure strategies can be influenced by the social status of a male in this species (Pizzari *et al*., 2003), I explored these patterns in both dominant and subdominant males separately.

MATERIALS AND METHODS

Male social status

I studied a population of Red junglefowl at the John Krebs Field Station (Oxfordshire) during the breeding season 2014 (May-August). I selected 20 males in order to create 10 pairs so that each male belonged to a unique pair. I released each pair into different new pens of 324 x 606 cm. All the males used were in good physical condition. I monitored their social interactions over two consecutive days between 16:00-19:00h, starting one hour after the birds were released in the new pen, to allow the males to acclimatize to the new social and physical environment of the new pen. Social status between pair members was determined based on established protocols (Froman *et al*., 2002; Cornwallis & Birkhead, 2006, 2007, 2008; Pizzari, Cornwallis & Froman, 2007; Tan, 2012). The subdominant member of a pair was the male, which most frequently avoided (defined as a movement of at least one body length away from the approaching male) and was chased away or attacked by the other male (i.e. the dominant). In the two male pairs where the social

status was not clear after the first two days of observations, I monitored their social interactions the next day during 20 minutes between 14:00-16:00h, and, as the hierarchy was still unclear, I repeated observations the following day while introducing a small amount of valuable resources in the pen (lettuce and egg yolk), in order to induce competition between the birds, after which the social status was always noticeable.

Ejaculate allocation trials

All sampling of birds was conducted in accordance with approved experimental protocols under Home Office licence (PPL 30/2931). A male pair was moved to another new experimental pen, where each of the two males was kept in one half of the pen physically separated from the other by a square mesh, which kept them in visual contact. I allowed the males 30 minutes to acclimatize to the new pen before starting the ejaculate allocation trials. I conducted the trials between 14:00-20:00h, following an established protocol (Pizzari *et al*., 2003; Løvlie, Cornwallis & Pizzari, 2005; Cornwallis & O'Connor, 2009; Tan, 2012). A female was held for one minute facing the focal male in order to enable the male to inspect her. The female was then turned around and held in a soliciting position, placed on a plastic substrate and with a harness covering her cloaca to facilitate ejaculate collection without contact with the female reproductive tract. In order to prevent the male from being inhibited by the experimenter, I sat behind a black plastic curtain with a square hole in its central bottom part through which the female was presented to the male.

The aim of this first part of the experiment was to habituate males to copulate with a female under experimental conditions. If a male did not copulate with a female for the first 10 minutes, I replaced the female and repeated the procedure. In total, each male was given the opportunity to copulate with up to 5 females, with a maximum of two females presented on a given day. When a male copulated with a female, I considered this female his first female and I started the trial.

Males required an average exposure to 2.05±0.27 (mean±SE) females to start mating. Two dominant and five subdominant males did not copulate with any of the 5 females to which they were presented during the habituation phase and, thus, were not included in the trials.

Following the first copulation, the male was given 10 more minutes to copulate a second time with the first female. If the male copulated a second time, he was given another 10 minutes to copulate a third time and so on for a total of up to 5 consecutive successful copulations with the first female. At the end of this session, the first female was immediately replaced by a new female, which was considered the second (sexually novel) female of the trial. Similarly, if following previous copulation(s) with the first female, the male failed to copulate with this female again, or copulated three consecutive times without delivering semen, the first female was replaced by the second female. Again, the second female was presented for one minute facing the male, and then turned around and held in the soliciting position, placed on a plastic substrate and with a harness covering her cloaca. The male was given up to 10 minutes to copulate once with the second female, after which the trial was ended. I repeated this protocol with both members of each male pair (Fig. 1). Because males are known to bias ejaculate expenditure in response to female comb size (Pizzari *et al*., 2003; Cornwallis & Birkhead 2006, 2007), I reduced variation in female comb size so that there was no significant difference in comb size between the familiar and novel females (i.e. first and second females) used during a trial (oneway ANOVA, $F_{1,24}$ = 0.014, p=0.906, Fig.1 Appendix).

After each copulation I collected the ejaculates and measured their volume using a Gilson pipette. Subsequently, I diluted 2.5µl of semen into 197.5µl of Phosphate Buffered Saline (PBS) and transferred 70 µl of the mixture to a spectrophotometer set at 595 nm to measure the concentration of sperm within the ejaculate through the light absorbance. Then, I separated the sperm from the seminal fluid by centrifugation at 3500×*g* for 1 minute at 4ºC, took the seminal fluid from the top

of the supernatant, and froze it at -20ºC for further protein quantification. I measured the volume of the seminal fluid using a Gilson pipette at the end of the experiment to avoid slowing down the trials. Sperm numbers were calculated from the ejaculate light absorbance based on an established standard curve (C. Tan, unpublished).

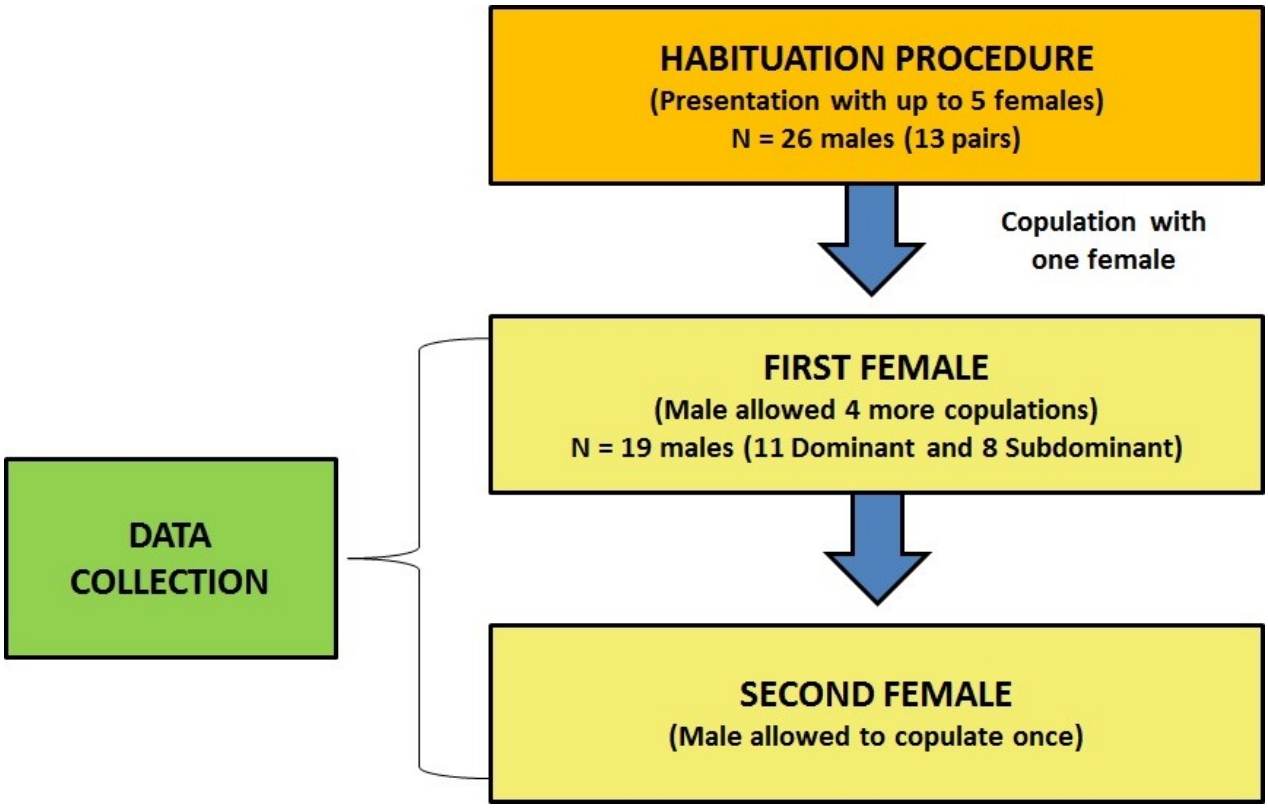


Figure 1. Experimental protocol outline.

Seminal fluid protein quantification

For a subset of trials, I quantified the protein content of the seminal fluid of each sample (N males = 7, 4 dominant and 3 subdominant, 33 samples overall). Solubilization buffer (6M Urea, 2M Thiourea, 4% Chaps, 5mM Mg Acetate, 10mM Tris pH 8.5) was added to each seminal fluid sample and protein concentrations were quantified using the Quick Start Bradford Assay from Bio-Rad and a spectraMax M2 microplate reader. Bovine Serum Albumin (BSA) was used as a standard (scale for protein estimation from 0-1 mg/ml) and samples were sonicated using a bioruptor to disrupt residual protein interactions.

Statistical analyses

I conducted the analyses using the statistical software R with the lme4 package.

a) Male propensity to mate

I investigated the effect of sperm depletion through successive copulations and sexual familiarity on the propensity of a male to mate again. To do this, I tested whether the order of cumulative copulations during a trial with both first and second female (i.e. from copulation 1 up to copulation 6; "copulation order"), and female familiarity (i.e. first familiar female *vs* second novel female; "familiarity") affect male propensity to mate. I fit a generalised linear mixed effect model to the data, using the R command "glmer", with a binomial error structure, with male propensity to mate ("0" = male did not mate, "1" = male mated) as the response variable, copulation order, familiarity, and its interaction as fixed effects, and male ID as a random effect. I calculated the Minimal Adequate Model (Crawley, 2005), testing the significance of the 2-way-interaction, and that of the single factors. To do so, I conducted the simplification process of the full model in a stepwise fashion comparing in each step a model with an update version lacking a term (i.e. interaction or single factor) using the R function "anova" for comparing two models. The likelihood ratio tests caused by dropping terms from the models were reported as chi-square tests of significance of each relevant term. In each step of the simplification process, I dropped the non-significant term with the highest p-value until reaching the Minimal Adequate Model.

Secondly, I used a chi-squared test to test whether the probability of mating with the second (i.e. novel) female differs between dominant and subdominant males. To do so, I performed the calculation using the 19 males (11 dominant and 8 subdominant) that mated with the first female (i.e. familiar) regardless of the number of matings they had performed with this female.

b) Sperm numbers

Similarly, I tested the influence of sperm depletion and female novelty on the number of sperm invested by a male in successive copulations with a generalised linear mixed effects model to the data, using the R command "lmer",with sperm numbers as the response variable, copulation order, female familiarity, and its interaction as fixed effects, and male ID as random effect. I log-transformed the response variable of sperm numbers (using "sperm numbers" + 1) to reduce variability in sperm numbers and meet the assumption of normality. The data met the assumption of normality as they were log-transformed. I calculated the Minimal Adequate Model (Crawley, 2005), testing the significance of the 2-way-interaction, and that of the single factors. To do so, I conducted the simplification process of the full model in a stepwise fashion comparing in each step a model with an update version lacking a term (i.e. interaction or single factor) using the R function "anova" for comparing two models. The likelihood ratio tests caused by dropping terms from the models were reported as chi-square tests of significance of each relevant term. In each step of the simplification process, I dropped the non-significant term with the highest p-value until reaching the Minimal Adequate Model.

c) Seminal fluid volume allocated

I tested the influence of sperm depletion and female novelty on the amount of seminal fluid invested by a male in successive copulations with a generalised linear mixed effects model to the data, using the R command "lmer", with seminal fluid volume as the response variable, copulation order, female familiarity and its interaction as fixed effects, and male ID as random effect. I log-transformed the response variable of seminal fluid volume (using "row data" + 1) to reduce variability and meet the assumption of normality. The data met the assumption of normality as they were log-transformed. I calculated the Minimal Adequate Model (Crawley, 2005), testing the significance of the 2-way-interaction, and that of the single factors. To do so, I conducted the simplification process of the full model in a stepwise fashion comparing in each

step a model with an update version lacking a term (i.e. interaction or single factor) using the R function “anova” for comparing two models. The likelihood ratio tests caused by dropping terms from the models were reported as chi-square tests of significance of each relevant term. In each step of the simplification process, I dropped the non-significant term with the highest p-value until reaching the Minimal Adequate Model.

d) Seminal fluid protein content allocated

I investigated patterns of variation in the amount of protein (µg/µl) allocated to the seminal fluid of individual ejaculates, and tested the influence of sperm depletion and female novelty. Using an approach similar to what described above, I therefore fit a generalised linear mixed effect model to the data, using the R command “lmer”,with amount of seminal fluid protein allocated (µg/µl) as the response variable, copulation order, female familiarity, male social status and their interactions as fixed effects, and male ID as random effect. I calculated the Minimal Adequate Model (Crawley, 2005), testing the significance of the 3-way-interaction, the significance of the 2-way interactions, and that of the single factors. To do so, I conducted the simplification process of the full model in a stepwise fashion comparing in each step a model with an update version lacking a term (i.e. interaction or single factor) using the R function “anova” for comparing two models. The likelihood ratio tests caused by dropping terms from the models were reported as chi-square tests of significance of each relevant term. In each step of the simplification process, I dropped the non-significant term with the highest p-value until reaching the Minimal Adequate Model. The normality of the data was confirmed by the straight line of the “quantile-quantile plots”, indicative of the normality of the residuals, obtained using the R functions “qqnorm” and “qqline” (Crawley, 2005). I focused on a subset of males, for which I quantified protein concentration in the seminal fluid (N males = 7, 33 samples overall, see above).

A female familiarity effect whereby a male's sexual interest (responses (a)-(d), above) in the second, novel female is higher than one would predict based on the pattern of decline over the previous copulations with the familiar female is consistent with the Coolidge effect. To further confirm preferential investment in the novel female, I generated an expected average male response at the 6th copulation based on male behaviour in the previous five copulations with the familiar female. In other words, I predicted how males would behave if the 6th copulation was with the same first, familiar female rather than the second, novel female. To do this, I used the Excel function "Forecast" based on the linear model "y=a + bx" with the standard errors (using the data from the copulations with the familiar female) in order to predict the probability of copulation, log sperm numbers allocated, log seminal fluid volume allocated, and seminal fluid protein content expected to be allocated to the second (i.e. novel) female based on a putative sixth copulation with the familiar female. There was interindividual variability in the number of copulations performed with the familiar female so that these analyses assume that if all males had copulated five times with the familiar female the trend would have been the same. The aim of these analyses is to test whether any of those four traits showed the Coolidge effect. If this were the case, the observed value with the new female should be higher than the predicted one for a putative sixth copulation with the familiar female. In a), b), c), and d) I conducted a one sample t-test on the differences between the observed and predicted values. The summaries of the GLMMs are presented in the appendix.

e) Status-specific sperm numbers and seminal fluid protein content allocation

Subdominant males are expected to have fewer reproductive opportunities than dominant males. On the other hand, ejaculate proteins have been shown to improve sperm quality (Lahnsteiner *et al*., 1996; Poiani, 2006; Hajirezaee, Amiri & Mirvaghefi, 2010) and to decrease female propensity to remate in the short term (Pitnick & Hosken, 2010; Perry, Sirot & Wigby, 2013). Taken together, these three facts make it reasonable to think that subdominant males might need to

allocate proportionally more seminal fluid protein to their ejaculates than dominant males. This possible difference between dominant and subdominant males suggests that seminal male investment in fluid protein content and sperm numbers across different ejaculates may covary in a status-specific pattern. To explore this idea, I standardized sperm and seminal fluid protein content in dominant and subdominant males against the maximum investment in each of these two ejaculate traits for a male in each of his copulations with the familiar and novel females (i.e. investment in a particular copulation / maximum investment in a copulation over the trial). The standardization of sperm numbers and seminal fluid protein content allows testing the existence of a trade-off between sperm and seminal fluid protein content allocated by dominant and subdominant males by comparing their investment in both ejaculate traits, controlling for inherent inter-male variation. In order to reduce variability in sperm numbers and meet the assumption of normality I log transformed the data (using "row data + 1"). I then conducted a two-way ANOVA using standardized sperm numbers as the response variable, and standardized seminal fluid protein content, social status, and its interaction as the explanatory variables.

RESULTS

a) Male propensity to mate

The model investigating male propensity to mate lost significantly explanatory power after removing "copulation order" ($X^2_1 = 3.8549$, $p = 0.049$). This may be accounted for by the fact that males tended to decrease their propensity to mate as their number of copulations performed went by (Fig. 2). There is a marginally non-significant difference between dominant and subdominant males in their probability to mate with the second female ($X^2_1 = 3.841$, $p = 0.051$). Dominant males had a higher probability of mating with the second and novel female than subdominant males (Fig. 2). However, neither dominant nor subdominant males were more likely to copulate with the novel female than expected based on their probability of copulation with the familiar

female (dominant: one sample t-test, $t_{10} = 1.1348$, $p = 0.283$; subdominant: one sample t-test, $t_7 = -1.1024$, $p = 0.306$, Fig. 2).

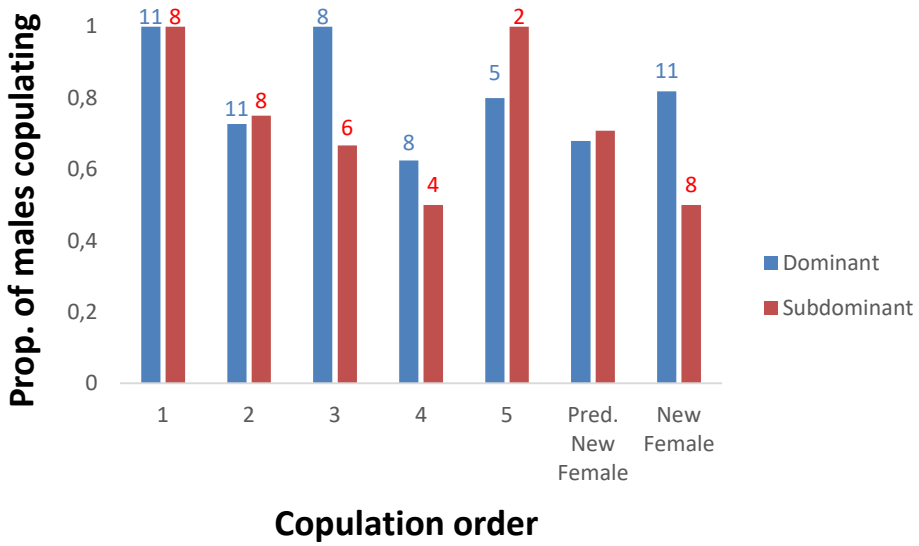


Figure 2. Proportion of males copulating with a familiar (1 to 5) and new female. The proportion of copulations in a given attempt with the familiar female is calculated using the number of males that copulated in the previous attempt. In the case of the new female, it is calculated using all the males that copulated with the first female. Vertical bars = SE. Numbers above vertical bars = Sample size.

b) Sperm numbers

The model investigating sperm allocation to females lost significantly explanatory power after removing "copulation order" ($X^2_1 = 6.8403$, $p = 0.008$). This may be accounted for by the fact that males produced fewer sperm over successive copulations with the familiar female. There was a marginally non-significant tendency for "female familiarity" to influence pattern of sperm numbers ($X^2_1 = 3.2929$, $p = 0.069$). This tendency was confirmed by the t-test which showed that males invested more sperm on encountering the novel female compared to the predicted value for a putative sixth copulation with the familiar female (one sample t-test, $t_{12} = 3.6041$, $p = 0.003$, Fig.3).

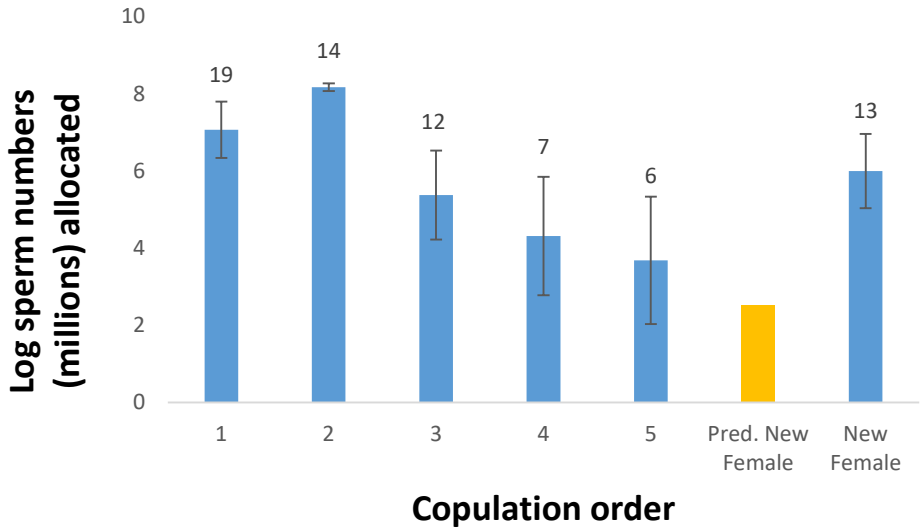


Figure 3. Predicted and observed log sperm numbers (millions) allocation with a new female based on log sperm numbers allocation in previous copulations (1 to 5) with a familiar female. Vertical bars = SE. Numbers above vertical bars = Sample size. The trials where the male did not copulate are not included.

c) Seminal fluid volume allocated

The model investigating seminal fluid allocated to females lost significantly explanatory power after removing "copulation order" ($X^2_1 = 10.927$, $p < 0.001$) and "familiarity" ($X^2_1 = 4.2175$, $p = 0.040$). The volume of seminal fluid declined over successive copulations with the familiar female, and increased on encountering the novel, second female. This preferential investment of seminal fluid in the novel female was further confirmed by the t-test, showing that males invested more seminal fluid with the novel female than one would predict males to invest in a putative sixth copulation with the first, familiar female (one sample t-test, $t_{12} = 8.6977$, $p < 0.001$, Fig.4).

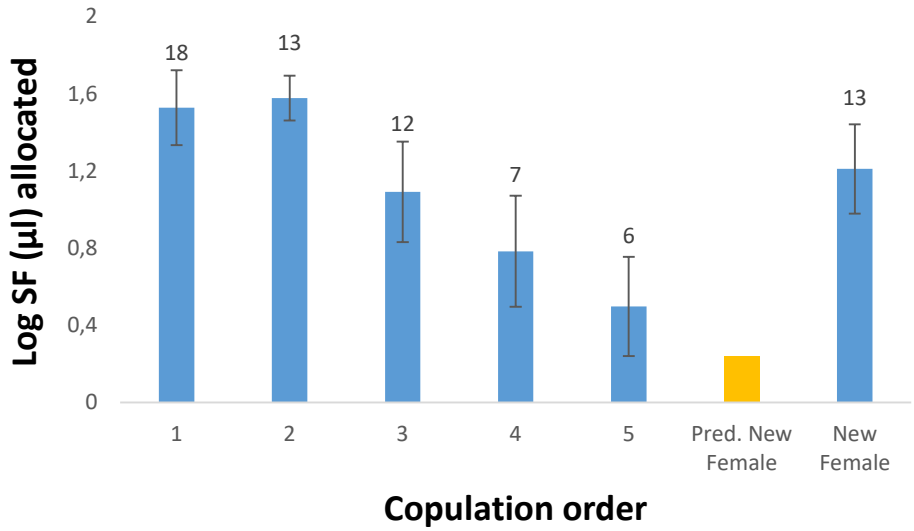


Figure 4. Predicted and observed log seminal fluid volume (µl) allocation with a new female based on log seminal fluid volume allocated in previous copulations (1 to 5) with a familiar female. Vertical bars = SE. Numbers above vertical bars = Sample size. The trials where the male did not copulate are not included. The sample size in copulations 1 and 2 differs from the sample size in these copulations in the previous figure because two samples (one sample in copulation 1 and another sample in copulation 2) did not have enough seminal fluid to be stored after having measured their concentration of sperm.

d) Seminal fluid protein content allocated

The model investigating seminal fluid protein content allocated to females lost significantly explanatory power after removing "copulation order: status" ($X^2_1 = 6.0058$, $p = 0.014$). This status-specific response to female sexual familiarity arises because subdominant males preferentially invested seminal fluid protein based on the sexual novelty of the female (Coolidge effect), while dominant males tended to invest seminal fluid protein more evenly over their successive copulations regardless of female novelty. Dominant males did not invest more seminal fluid protein on encountering the novel female compared to their predicted value for a putative sixth copulation with the familiar female (one sample t-test, $t_3 = 0.85829$, $p = 0.453$, Fig. 6). However, subdominant males invested more seminal fluid protein on encountering the novel female compared to their predicted value for a putative sixth copulation with the familiar female (one sample t-test, $t_2 = 4.7696$, $p = 0.041$, Fig. 5).

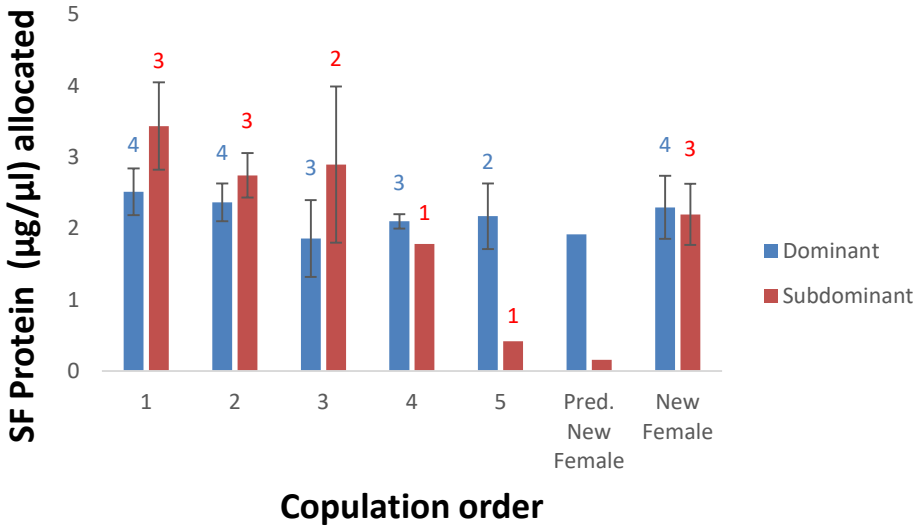


Figure 5. Predicted and observed seminal fluid protein concentration (µg/µl) allocation with a new female based on seminal fluid protein concentration (µg/µl) allocation in previous copulations (1 to 5) with a familiar female. Vertical bars = SE. Numbers above vertical bars = Sample size.

e) Status-specific sperm numbers and seminal fluid protein content allocation

The results of the two-way ANOVA show that standardized investment in seminal fluid protein content has a significant effect on standardized investment in sperm numbers. As investment in seminal fluid protein content increases so did sperm investment ($F_{1,29} = 9.779$, $p = 0.004$, Fig.6). However, contrary to my prediction, the interaction between seminal fluid protein content investment and male social status does not significantly affect the standardized investment in sperm numbers ($F_{1,29} = 0.808$, $p = 0.376$).

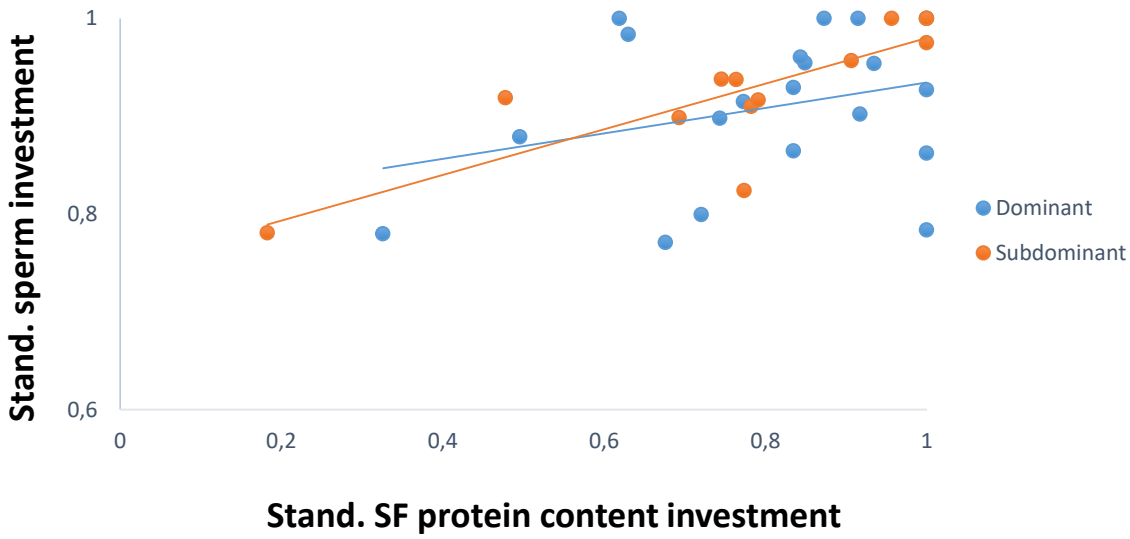


Figure 6. Relationship between standardized sperm and seminal fluid protein content investment in relation to male social status over different copulations with familiar and novel females.

DISCUSSION

This study set out to investigate male sexual behaviour (i.e. propensity to mate and strategic ejaculate allocation) in relation to male social status and female sexual novelty. Seminal fluid plays a significant role in sperm competition dynamics by affecting female remating intervals, female fecundity, and sperm quality in a range of species (Wolfner 1997; Chapman, 2008; Pitnick & Hosken, 2010; Perry, Sirot & Wigby, 2013). For this reason, recent theoretical studies have begun to highlight that research on strategic ejaculate allocation should take this potential role of seminal fluid into account. To the best of my knowledge, this is the first study that simultaneously analyses the allocation of three ejaculate traits, namely, sperm numbers, seminal fluid volume, and seminal fluid protein content.

I found the existence of a Coolidge effect for sperm and seminal fluid allocation, and a status-specific response to female sexual novelty. Subdominant males preferentially invested seminal fluid protein based on the sexual novelty of the female (Coolidge effect), while dominant males tended to invest seminal fluid protein more evenly over their successive copulations.

More dominant than subdominant males contributed to the trials, which reflects a higher propensity of dominant males, compared with subdominant males, to mate in the experimental conditions. The fact that subdominant males were less likely to mate than dominant males could be explained by the presence of the dominant males in the other half of the pen during the trials keeping very close visual contact with them. The presence of dominant males could have restrained subdominant males due to the aggression they had suffered from dominant males during the days they were paired.

Consistently with a previous study in the fowl (Pizzari *et al*., 2003) males decreased their sperm investment in a familiar female and increased it, in relation to their last copulations with the familiar female, on encountering a novel female. The progressive decrease in sperm allocation in a familiar female might result from progressive depletion in male sperm reserves. The increase in sperm allocation on encountering a new female suggests that the progressive loss of sexual interest in the familiar female may also account for the decrease in sperm allocation in the successive matings with the familiar female. As sperm investment in the new female is lower than on the first copulations with the familiar female it is reasonable to think that both sperm depletion and renewed sexual interest on encountering the novel female may explain the pattern of sperm allocation to the novel female.

A novel contribution of the present study is the finding that seminal fluid allocation patterns also display a strong Coolidge response which mirrors patterns of male sperm allocation to familiary and novel females. As seminal fluid investment in the novel female is lower than on the first copulations with the familiar female it is reasonable to think that depletion of seminal fluid reserves and renewed sexual interest on encountering the novel female interact to explain the pattern of seminal fluid allocation to the novel female. An increase in seminal fluid investment on encountering the novel female may compensate for the inability of males to allocate more sperm

to her due to the depletion of their sperm reserves. In this way, males would be able to optimize their ejaculate resources and to maximize their reproductive returns by filling female sperm storage tubules with seminal fluid compounds that may favour the fertilising performance of self sperm, decrease the fertilization chances of rival sperm, and/or increase female remating intervals.

As I showed in the previous chapter seminal fluid exerts a significant effect on sperm fertilizing efficiency. For this reason, it is reasonable to think that males could have evolved seminal fluid allocation specific responses in order to maximize their reproductive returns according to their favoured or disfavoured mating roles. According to this expectation, subdominant males invested more seminal fluid protein than dominant males in the ejaculates allocated to novel females. Dominant males allocated the seminal fluid protein content of their ejaculates more evenly over successive copulations with the familiar and novel females. Ejaculates from subdominant males tend to experience higher levels of sperm competition within female reproductive tract than ejaculates from dominant males since subdominant males are less likely to prevent other males from copulating with females than dominant males. Moreover, ejaculates from subdominant males are more likely to be ejected by females than ejaculates from dominant males (Pizzari & Birkhead, 2000). Taken together, these mechanisms substantially reduce the fertilization chances by subdominant males. As seminal fluid proteins may improve sperm competitiveness and decrease female propensity to remate, subdominant males may have evolved to maximize the reproductive payoffs out of their limited and disfavoured matings opportunities by investing a substantial amount of seminal fluid protein in the presence of a new mating opportunity, therefore diminishing the risk of their ejaculates being outcompeted within female reproductive tract. Although dominance can change, there is a heritable component to social status (Craig, Ortman & Guhl, 1965), which could have led subdominant males to face selection pressure to evolve the capability to modify strategically their seminal fluid protein composition in order to compensate for their disfavoured mating position. Consistent with this status-specific response, subdominant

male fowl of a free-ranging population that is behaviourally and morphologically very similar to the Red junglefowl consistently allocate high numbers of sperm to their initial copulations, regardless of whether females were presented singly or as a pair (Cornwallis & Birkhead, 2006). A similar pattern of status-specific ejaculate allocation was reported in the European bitterling (*Rhodeus amarus*). Subdominant males allocate most sperm to the first mussel encountered regardless of its familiarity. In this species, this response is very likely to be an opportunistic strategy to deal with their reduced number of fertilizing opportunities since dominant males aggressively defend territories to monopolize their access to both mussels and females (Spence, Reichard & Smith, 2013). The experimental setup of this study involves a competitive scenario as males have the opportunity to mate in the presence of a potential competitor. In the future, it would be interesting to replicate the experiment keeping the males in isolation in order to determine whether their patterns of ejaculate allocation differ.

Comb size constitutes a strong indicator of female sexual attractiveness that may significantly affect male propensity to mate and ejaculate investment. Females with larger combs have superior condition and reproductive investment since they lay larger eggs with more yolk, and are more likely to be socially dominant with higher lifetime reproductive success than subdominant females (Collias, Collias & Jennrich, 1994; Pizzari *et al*., 2003; Cornwallis 2004; Cornwallis & Birkhead, 2006; 2007). For these reasons, males are expected to invest more ejaculate resources in females with larger combs. The absence of systematic significant differences in the comb size of the first and second females used during a trial indicates that the results of this study are not affected by the potential effect of female sexual attractiveness.

Finally, the results of this study should be taken with caution due to its reduced sample size. Future work should focus on the trade-offs between the allocation of different molecular components of the ejaculate within and across successive matings with familiar and novel females

towards a comprehensive understanding of how males tailor strategically their ejaculates to maximize their reproductive payoffs.

Appendix

a) Female comb length

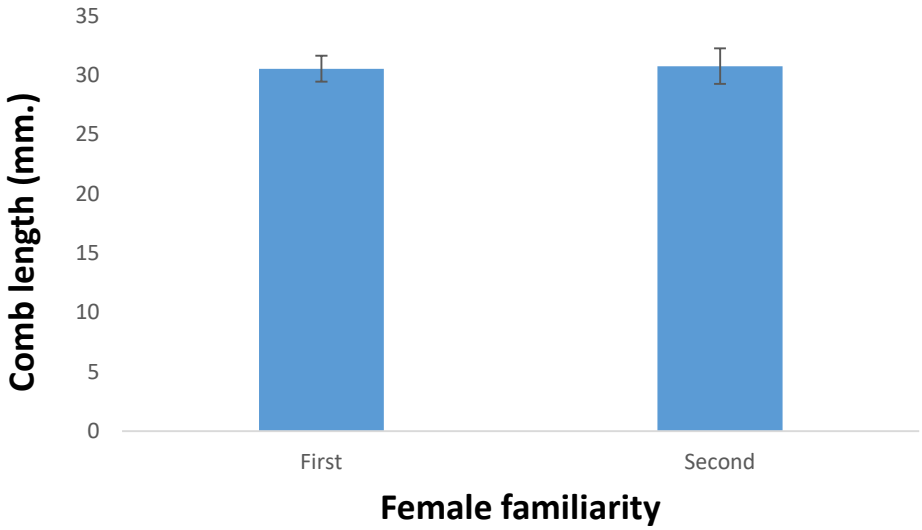


Figure 1. Comb length (mm.) of first (i.e. familiar) and second (i.e. new) females used during a trial.

b) Relationship between ejaculate traits allocated

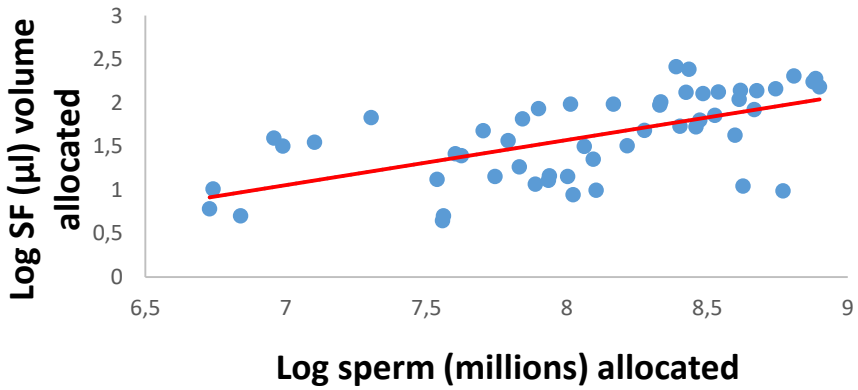


Figure 2. Relationship between log sperm numbers and log seminal fluid volume allocated to familiar and novel females (including dominant and subdominant males).

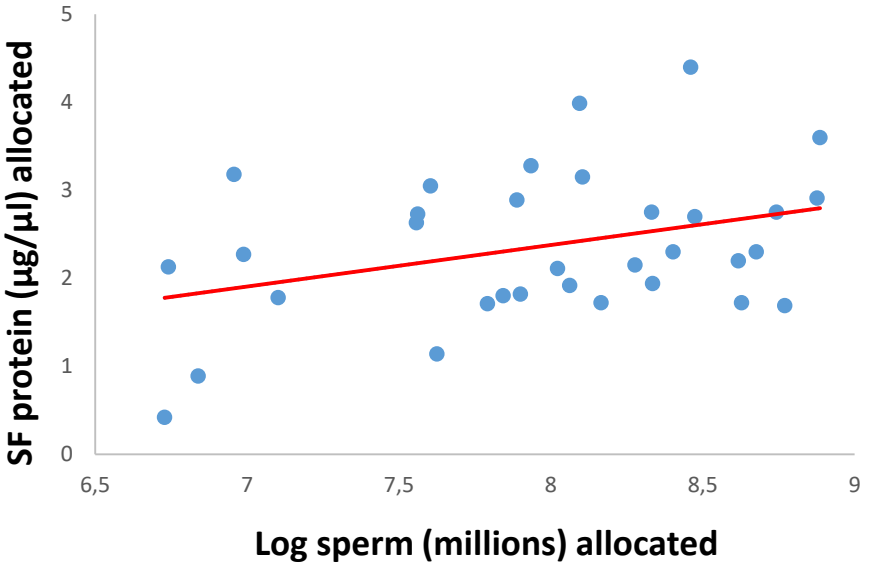


Figure 3. Relationship between log sperm numbers and seminal fluid protein content allocated to familiar and novel females (including dominant and subdominant males).

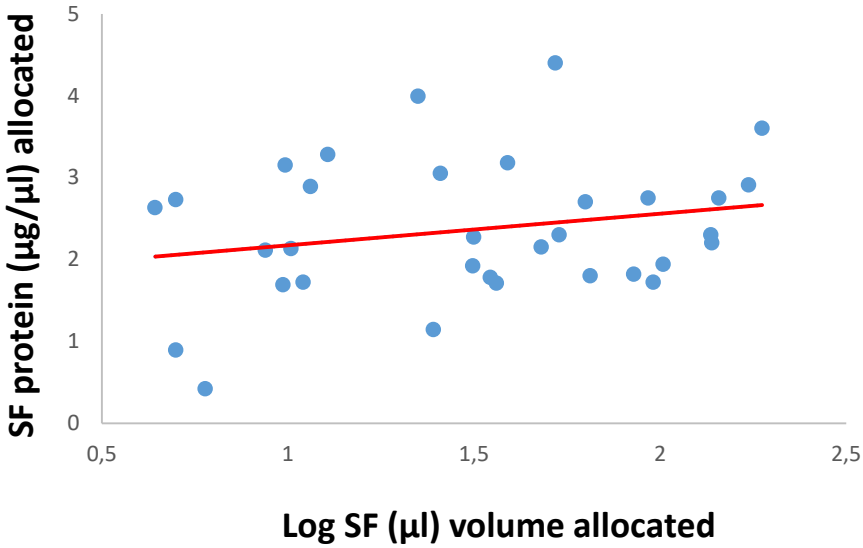


Figure 4. Relationship between log seminal fluid volume and seminal fluid protein content allocated to familiar and novel females (including dominant and subdominant males).

Table 1. Summary of GLMM fit to investigate male propensity to mate.

Model: glmer (copulation ~ exposures * female + (1|maleID), family = binomial)

Random effects:

Groups	Name	Variance	Std. Dev.
MaleID	(Intercept)	0.644	0.8025

Fixed effects:

	Estimate	Std. Error	z-value
(Intercept)	6.190	2.737	2.261
exposures	-1.329	0.700	-1.899
female	-3.026	2.733	-1.107
exposures:female	0.746	0.541	1.380

Table 2. Summary of GLMM fit to investigate sperm numbers allocation.

Model: lmer (log sperm ~ exposures * female * status + (1|maleID))

Random effects:

Groups	Name	Variance	Std. Dev.
MaleID	(Intercept)	3.89	1.975
Residual		7.176	2.679

Fixed effects:

	Estimate	Std. Error	t-value
(Intercept)	11.580	4.321	2.680
exposures	-2.357	0.982	-2.401
female	-3.128	3.952	-0.791
status(Sub.)	-8.262	10.625	-0.778
exposures:female	1.242	0.812	1.528
exposures:status(Sub.)	2.546	2.162	1.178
female:status(Sub.)	8.583	10.331	0.831
exposures:female:status(Sub.)	-2.108	1.972	-1.069

Table 3. Summary of GLMM fit to investigate seminal fluid volume allocation.

Model: lmer (log SF ~ exposures * female * status + (1|maleID))

Random effects:

Groups	Name	Variance	Std. Dev.
MaleID	(Intercept)	0.103	0.321
Residual		0.133	0.364

Fixed effects:

	Estimate	Std. Error	t-value
(Intercept)	2.248	0.707	3.179
exposures	-0.223	0.163	-1.369
female	-0.453	0.655	-0.692
status(Sub.)	0.660	1.648	0.401
exposures:female	0.119	0.130	0.909
exposures:status(Sub.)	-0.242	0.352	-0.687
female:status(Sub.)	-0.203	1.605	-0.127
exposures:female:status(Sub.)	0.139	0.317	0.440

Table 4. Summary of GLMM fit to investigate seminal fluid protein allocation.

Model: lmer (SF protein ~ exposures * female * status + (1|maleID))

Random effects:

Groups	Name	Variance	Std. Dev.
MaleID	(Intercept)	0.400	0.632
Residual		0.217	0.466

Fixed effects:

	Estimate	Std. Error	t-value
(Intercept)	-1.497	3.663	-0.409
exposures	0.541	0.630	0.859
female	4.043	3.638	1.111
status(Sub.)	9.084	4.174	2.176
exposures:female	-0.644	0.624	-1.031
exposures:status(Sub.)	-1.827	0.753	-2.425
female:status(Sub.)	-7.789	4.115	-1.893
exposures:female:status(Sub.)	1.497	0.729	2.051

CHAPTER 4

Characterization of the Red junglefowl seminal fluid proteome

ABSTRACT

Seminal fluid proteins (SFPs) are emerging as fundamental contributors to sexual selection given their role in post-mating reproductive events, particularly in polyandrous species where the ejaculates of different males compete for fertilisation. SFP identification however remains taxonomically limited and little is known about avian SFPs, despite extensive work on sexual selection in birds. We characterize the SF proteome of the polyandrous Red junglefowl, *Gallus gallus*, the wild species that gave rise to the domestic chicken. We identify 1,141 SFPs, including proteins involved in immunity and antimicrobial defences, sperm maturation, and fertilisation, revealing a functionally complex SF proteome. This includes a predominant contribution of blood plasma proteins that is conserved with humans. SFP comparisons with domestic chickens revealed both qualitative and quantitative differences likely associated with domestication and artificial selection. Collectively, these results shed light onto the functional complexity of avian SF, and provide a platform for molecular studies of fertility, reproductive ageing, and domestication.

INTRODUCTION

Seminal fluid (SF), the physiological, non-cellular component of an ejaculate, contains a diversity of molecules including peptides and proteins (Perry, Sirot & Wigby, 2013). A growing body of empirical work has demonstrated the involvement of several such molecules in different stages of reproduction, including sperm fertilising efficiency and longevity, antimicrobial defences, female sperm utilisation, the coordination of ovulation and oviposition in females, maternal allocation, and female remating propensity (McGraw, Suarez & Wolfner, 2015). Given their modulation of postcopulatory reproductive events, much research has focused on the role of seminal fluid proteins (SFPs) in polyandrous species, where females obtain ejaculates from multiple partners, prolonging sexual selection on males after copulation via processes such as sperm competition and cryptic female choice (Perry, Sirot & Wigby, 2013).

Increasing evidence indicates that SFPs can play a key role in postcopulatory sexual selection by promoting the fertilisation success of an ejaculate through distinct (although not necessarily mutually exclusive) functionalities. First, some SFPs can have a direct influence on the fertilising performance of self-sperm by modulating sperm motility and successful sperm storage within the female sperm storage organs. For example, mammalian SFPs may increase sperm velocity by accelerating the conversion of glucose into ATP (Storey, 2008). In *Drosophila melanogaster*, the SFP Acp36DE facilitates post-copulatory sperm storage. Females mated to Acp36DE mutant males store only 15% of sperm and produce 10% of progeny relative to those mated to wild-type males (Neubaum & Wolfner, 1999). Not surprisingly, Acp36DE mutant males are disadvantaged in sperm competition when competing against wild-type males with normal expression of Acp36DE (Chapman *et al.*, 2000). Second, SFPs may have ‘offensive’ effects, for example by reducing the fertilising efficiency of competing (i.e. non-self) ejaculates (den Boer, Baer & Boomsma, 2010), or ‘defensive’ effects by reducing the future risk of sperm competition, for example through the formation of mating plugs (Dean, 2013). These ‘defensive’ effects can be

achieved by manipulating female responses to the male's advantage – e.g. by reducing female propensity to remate or accelerating female oviposition rate. Amongst *D. melanogaster* SFPs, Sex Peptide (Acp70A) delays female remating (Wolfner, 1997), while ovulin (Acp26Aa) enhances short term egg production by the female (Herndon & Wolfner, 1995). These functionalities, while adaptive to males within the context of postcopulatory sexual selection, can also have detrimental repercussions on the fitness of females, and have been put forth as potential examples of sexual conflict (Wigby & Chapman, 2005).

SFP genes often experience accelerated rates of adaptive evolution, potentially due to their contribution to sexual selection. For example, the *SEMG2* gene has experienced accelerated evolution in polyandrous species of primates in relation to monandrous species. This gene encodes a predominant component of the copulatory plug, a trait crucial to the fertilisation success of competing males participating in subsequent copulations (Dorus *et al.,* 2004). Similarly, the molecular mass of the SFP SVSII, which is responsible for the formation of the copulatory plug in muroid rodents, correlates positively with the level of polyandry across species (Ramm *et al.,* 2009), and the gene encoding this protein is subjected to positive selection in lineages with higher levels of sperm competition (Ramm *et al.,* 2008).Thus, polyandry can drive the rapid evolution of SFPs and this, in turn, can contribute to the establishment of reproductive barriers and, eventually, to speciation (Gavrilets, 2000; Swanson & Vacquier, 2002).

However, the study of SFPs remains taxonomically restricted and largely limited to populations that have long adapted to captivity or domestication. In particular, little is known about SFPs and their functional significance in wild birds (but see Finseth, Bondra & Harrison, 2014), despite the fact that birds have long served as a paragon of sexual selection and more recent evidence that SF may influence reproductive fitness (Rowe & McGraw, 2008; Rowe *et al.,* 2011). Avian SFP biology also has the potential to be unique because birds lack accessory tissues dedicated to the

production of SFPs, such as the seminal vesicle in mammals and accessory gland present in some insects. Here, we begin to address this gap in knowledge by characterizing the SF proteome of a population of Red junglefowl (RJF), *Gallus gallus ssp*. The RJF is an appropriate model for the study of avian SF for the following reasons. First, RJF populations are typically polyandrous and the mechanisms of pre- and post-copulatory sexual selection are reasonably well understood (Zuk *et al.*, 1990; Pizzari & Birkhead, 2002; McDonald & Pizzari, 2016). Second, because the RJF is the wild species that gave rise to the domestic chicken, *G. domesticus* (Fumihito *et al*., 1996), information on the physiology of chicken SF can be used to infer the functional significance of the RJF SF proteome, and, in turn, the RJF proteome can shed light on the influence of artificial selection associated with domestication. Domestication has had a profound impact on chicken reproductive biology (Etches, 1996), and information on domestic SFPs (Labas *et al*., 2015) provides a unique opportunity to investigate the molecular basis of reproductive divergence between domesticated and undomesticated species.

Physiological studies of domestic chickens indicate that the SF of ejaculates consists of two components: seminal plasma and transparent fluid. Seminal plasma is produced by the testis and vas deferens, and sperm are expected to be continuously exposed to these secretions as they leave the epididymis. Transparent fluid, a plasma-like secretion produced from the tumescent lymphatic folds surrounding the cloaca, has a similar composition to a dialysate of blood plasma, and is mixed with sperm upon ejaculation (Lake & Wishart, 1984; Fujihara, 1992).

The aims of this study were to: (i) systematically characterize the SF proteome of RJF; and (ii) compare and contrast these data with published SFP data in domestic chickens.

MATERIALS AND METHODS

Semen collection and SF isolation

We studied a population of RJF at the John Krebs Field Station (Oxfordshire), which was founded in 2006 from other captive populations. All sampling of birds was conducted in accordance with approved experimental protocols under Home Office licence (PPL 30/2931). In July-September 2013 semen samples were obtained from 12 sexually mature males by abdominal massage, which captures a representative sample of extragonadal semen reserves of a male (Etches, 1996). Males were isolated from females for 48 hours before being massaged for the first time. Two age classes of males were selected: "Young" (1 year old, n=6), and "Old" (4, 5, and 7 years old, n=6). Males were kept sexually rested and isolated from females for 48h. For each male, semen samples were collected every four days to allow full replenishment of extragonadal sperm reserves, between 12:00-17:00 h. In total, 157 semen samples were collected (77 from young, and 80 from old males). Immediately following collection, samples were placed in a water bath at RJF physiological temperature (41C).

We separated sperm from SF by centrifugation at 3,500 g for 1 minute at 4C. The SF supernatant was transferred to a sterile tube for storage at -20C. This protocol was designed to purify SF from sperm cells, while reducing damage (i.e. flagellum or midpiece separation) and protein contamination from damaged cells. To conduct an independent assessment of the presence of integral sperm components in SF in samples in which whole sperm were confirmed to be absent by microscopy, we purified semen samples from four separate ejaculates of a single male using a more extensive centrifugation protocol. This included centrifugation at 600g for 10 minutes at 4C, and, then, centrifuging the supernatant twice more at 600g for 10 minutes and 3,000g for 30 minutes at 4C (Labas *et al*., 2015). The absence of sperm cells and flagella in this SF samples was confirmed by phase contrast microscopy. Mass spectrometry analyses of all samples were conducted using the same methodology (see below).

Protein preparation and 1-dimensional SDS-PAGE

Solubilization buffer (4% Chaps, 7M Urea, and 2M Thiourea) was added to each SF sample and protein concentrations were quantified using the Quick Start Bradford assay and a spectraMax M2 microplate reader. Bovine Serum Albumin (BSA) was used as a standard (scale from 0-2 mg/ml) and samples were sonicated using a bioruptor to disrupt residual protein interactions. To account for variation between ejaculates, 15µg of protein were combined from each of three ejaculates per male and diluted appropriately to obtain equal protein concentrations across samples from each of the 12 samples. 45ug of total protein from each sample was separated for the maximum duration possible (without running small proteins off the gel) on 4-12 % Nupage Bis-Tris gels running on an XCell SureLock Mini-Cell PowerEase 200 system (Life-Technologies). Gels were then fixed in 45% methanol and 1.0% acetic acid for 1 h and immediately stained with Coomassie (0.1%w/v Coomassie, 34% methanol, 17%w/v ammonium sulfate, and 0.5% acetic acid). Gels were transferred to a gel slicer where each lane was cut horizontally into 12 slices (Fig. 1), resulting in total of 144 samples for MS/MS analysis. To reduce spectra derived from the highly abundant SFP serum albumin and increase proteomic coverage, a narrow region centered on the predominant serum albumin band was not included amongst the fractionated gel slices. Gel slices were subjected to proteolytic digestion using an Automated Preparation Station (Perkin Elmer). Proteins were first reduced (1hr at 37C in 15.4mg dithiothreitol in 10ml 100mM ammonium bicarbonate solution) and alkylated (1hr at room temperature in 102mg iodoacetamide in 10ml 100mM ammonium bicarbonate solution) followed by digestion with trypsin (500 ng/µl, Promega) at 37C for 16 h.

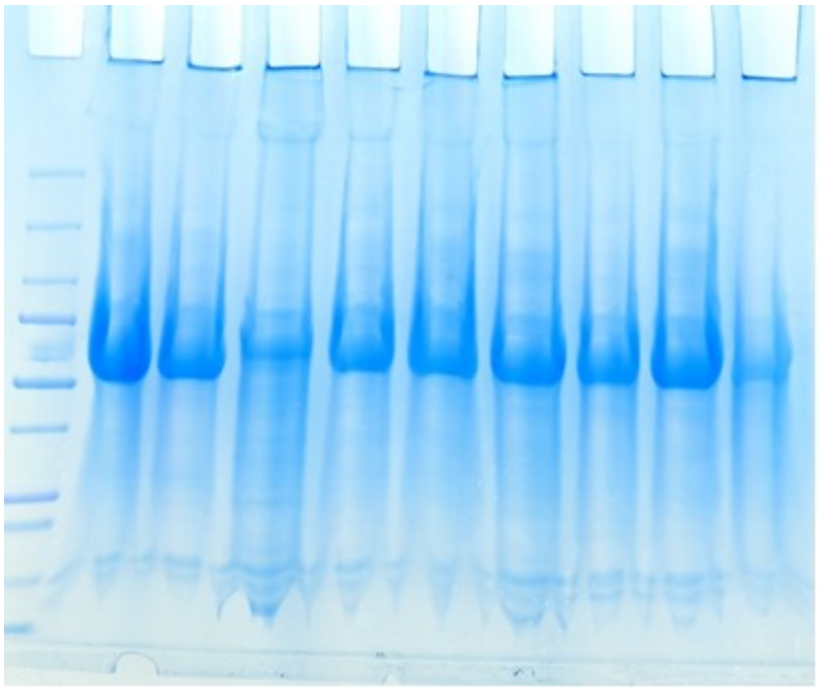


Figure 1. 1D gels used for the RJF SF proteome characterization, including the cutting areas applied.

Tandem mass spectrometry analysis

Tandem mass spectrometry (MS/MS) experiments were performed using a Dionex Ultimate 3000 RSLC nanoUPLC (Thermo Fisher Scientific Inc, Waltham, MA, USA) system and a QExactive Orbitrap mass spectrometer (Thermo Fisher Scientific Inc, Waltham, MA, USA). Separation of peptides was performed by reverse-phase chromatography at a flow rate of 300 nL/min and a Thermo Scientific reverse-phase nano Easy-spray column (Thermo Scientific PepMap C18, 2µm particle size, 100A pore size, 75µm i.d. x 50cm length). Peptides were loaded onto a pre-column (Thermo Scientific PepMap 100 C18, 5µm particle size, 100A pore size, 300µm i.d. x 5mm length) from the Ultimate 3000 autosampler with 0.1% formic acid for 3 minutes at a flow rate of 10µL/min. After this period, the column valve was switched to allow elution of peptides from the pre-column onto the analytical column. Solvent A was water + 0.1% formic acid and solvent B was 80% acetonitrile, 20% water + 0.1% formic acid. The linear gradient employed was 2-40% B in 30 minutes. The LC eluant was sprayed into the mass spectrometer by means of an Easy-spray source (Thermo Fisher Scientific Inc.). All *m/z* values of eluting ions were measured in an Orbitrap mass analyzer, set at a resolution of 70000. Data dependent scans (Top 20) were

employed to automatically isolate and generate fragment ions by higher energy collisional dissociation (HCD) in the quadrupole mass analyser and measurement of the resulting fragment ions was performed in the Orbitrap analyser, set at a resolution of 17500. Peptide ions with charge states of 2+ and above were selected for fragmentation. Post-run, the data was processed and peak-lists generated using Protein Discoverer (version 1.3, ThermoFisher).

Peptide identification and protein annotation

Raw data from each tandem mass spectrometry (MS/MS) run was analyzed by X!Tandem (Craig & Beavis, 2004) and Comet (Eng, Jahan & Hoopmann, 2013) against the complete *Gallus gallus* refseq protein database (June 2013 release, 32134 proteins), supplemented with characterized, but yet to be annotated, immunoglobulin sequences. A fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 10.0 PPM were used. Iodoacetamide derivative of cysteine was specified as a fixed modification, whereas oxidation of methionine was specified as a variable modification. Peptides were allowed up to two missed trypsin cleavage sites. All downstream analyses were conducted using the Trans-Proteomic Pipeline (TPP v4.7 POLAR VORTEX rev 1 (Deutsch *et al*., 2010)). False Discovery Rates (FDRs) were estimated with a randomized decoy database using PeptideProphet, employing accurate mass binning model and the nonparametric negative distribution model. X!Tandem and Comet PeptideProphet results were combined using iProphet, to provide more accurate and conservative peptide identification probabilities. Peptide identifications were accepted if they could be established at greater than 95.0% iProphet probability and protein assignations were accepted if they could be established at greater than 99.0% probability. Proteins that contained identical peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The same analysis pipeline was used to reanalyze available raw MS/MS data from fractionated domestic chicken SF samples (Labas *et al.,* 2015). SF proteomes were compared to the chicken blood plasma proteome (Ma *et al*., 2014), the exosome ExoCarta high quality protein marker dataset (Keerthikumar *et al*.,

2016), human blood plasma (Farrah *et al*., 2011), human sperm (Wang *et al*., 2013), mouse epididymal sperm (Chauvin *et al*., 2012) and human seminal plasma (Pilch & Mann, 2006) using orthology relationships in Ensembl Genes 84 (Galgal4). Composition of protein datasets were analyzed using the DAVID Bioinformatics Resource (6.7) (Huang, Sherman & Lempicki, 2009 a,b), including statistical analyses of functional enrichment using the *G. gallus* genome as the background dataset for comparisons.

Protein quantitation and analysis

After consideration of serum albumin depletion options and the potential impact of this highly abundant protein on various quantitative approaches, a semi-quantitative spectral counting approach was employed. Protein quantitation was conducted using the APEX Quantitative Proteomics Tool (Braisted *et al*., 2008). Fifty SFPs previously identified using the same MS/MS methodology (data not shown) were utilized for the training dataset based on having the highest numbers of spectral counts per protein and the highest protein identification probabilities. The 35 physicochemical properties available in the APEX tool were used for prediction of peptide detection/non-detection in the construction of a training dataset file. Protein probabilities (O_i) were computed using the Random Forest classifier algorithm trained with the data set generated in the previous step. APEX protein abundances per male and as a combined dataset were calculated using the protXML file generated by the ProteinProphet. The same approach was used to calculate Apex scores for the domestic *G. gallus* SF proteome (Labas *et al*., 2015). Given the skew in the distribution of peptide matches for the lower coverage domestic chicken dataset (see Results), proteins exhibiting abundance differences between subspecies were identified as those with the greatest abundance rank shift between proteomes, in conjunction with quantitative differences based on their Apex estimate.

RESULTS

The Red junglefowl SF proteome

Tandem mass spectrometry analyses were conducted on SF from 12 male RJF, where each sample was the product of 3 pooled SF samples from the same male. Analysis of 144 protein fractions in total resulted in 200,622 high-quality spectral matches to peptides and the identification of 1141 proteins (Table S1, Supporting Information). Proteins were required to be identified by no fewer than 2 unique peptides and of these proteins, 24.8% were identified in all 12 males and 55.9% of proteins were identified in at least half of the males surveyed (Fig. 2a). The proteomic complexity of RJF SF is therefore much greater than those in other well-studied invertebrates, such as *Drosophila* and mosquito, *Aedes aegypti*, where 138 and 93 ejaculated SFPs have been identified, respectively (Findlay *et al*., 2008; Sirot *et al*., 2011) but comparable in size to the SF proteome in humans (Pilch & Mann, 2006).

To examine the contribution of transparent fluid to the RJF SF proteome, we investigated overlap with existing proteomic data from domestic chicken blood plasma (Ma *et al*., 2014). This revealed that 52.1% (137 out of 263) of identified blood plasma proteins were present in RJF SF (Table S1, Supporting Information). These proteins were more commonly identified across most or all SF samples analysed than the remainder of the SF proteome (Fig. 2a) and a substantial number of these (46.7%; 64 out of 137) are amongst the most abundant 20% of SFPs. Gene ontology analysis of SF/blood plasma proteins identified numerous biological process enrichments (Table S1, Supporting Information), including response to stress (3.2E-19), defense response (3.26E-16), hemostasis (8.1E-12), regulation of peptidase activity (1.1E-12) and immune system process (8.4E-14). Predominant components of SF and blood plasma include serum albumin, transthyretin and ovotransferrin. Quantitative analysis indicated that 29% of SFPs by mass are accounted for by proteins also found in blood plasma and that on average these proteins are significantly greater in abundance than the non-blood plasma components of the SF proteome ($p < 0.0001$; Fig.2b).

These observations confirm that plasma secretions within the male reproductive track are a major component of RJF SF and likely represent the contribution of transparent fluid. To assess if these abundant SF components are evolutionarily conserved blood plasma proteins, we then compared our proteome to that of human blood plasma (Farrah *et al*., 2011). This revealed that the vast majority of SF proteins in chicken blood plasma (122 of 134) were present in the human plasma proteome and identified an additional 349 SF proteins present in human blood plasma. We note that the proteomic coverage of human blood, compiled from 91 high-quality MS/MS data sets, far exceeds that in chicken. Based on this observation it was then logical to determine if the prevalence of blood plasma proteins in the RJF might be a common feature of vertebrate ejaculates. A comparison of our SF proteome to that of humans, a dataset that was generated in a similar fashion using SF purified from whole ejaculates (Pilch & Mann, 2006), identified 343 proteins in common, including 244 (71.1%) also present in the blood plasma proteome (Fig. 2c). Therefore, blood plasma proteins comprise a large fraction of SF proteins identified in whole ejaculates of RJF and humans, and accounts for most of the proteins identified in both species.

A diverse repertoire of immunity proteins function in reproduction and several impact fertility through direct or indirect influences on sperm (Dorus, Skerget & Karr, 2012). A survey of the SF proteome revealed a wide array of molecules which function in immunity, such as (i) innate immunity proteins, including 10 proteins in the classical complement pathways (Table 1), (ii) a diverse set of adaptive immunity proteins, including all four annotated chicken immunoglobulin proteins, and (iii) the avian antimicrobial proteins gallinacin 9 and 10, which are highly abundant and comprise 0.73 and 0.93% of the total protein in SF, respectively (Table 2). It is noteworthy that several abundant immunity proteins are absent from the domestic chicken SF proteome (see below).

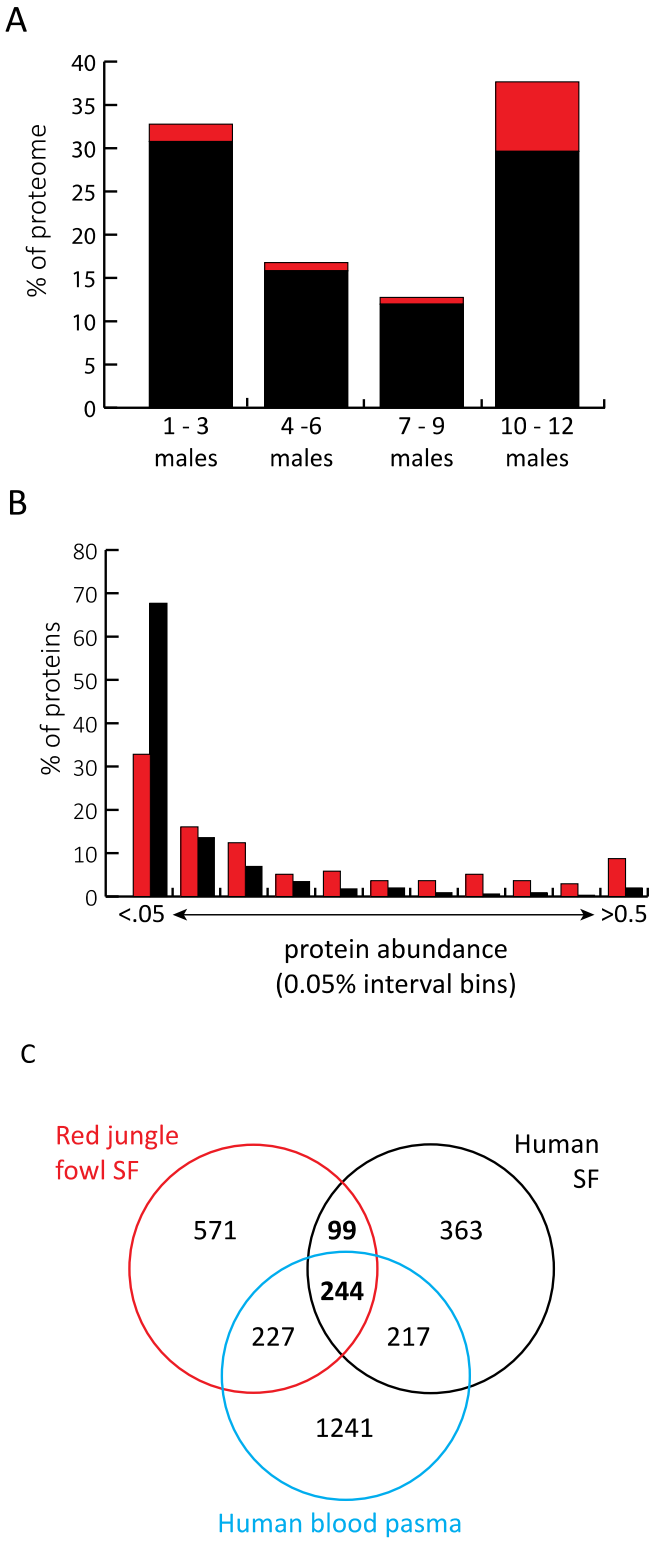


Figure 2. Red junglefowl seminal fluid protein identification and composition. (A) Histogram displaying the proportion of the seminal fluid proteome identified as a function of the number of males in which proteins were identified.Proteins also identified as components of blood plasma are indicated in red. (B) Histogram comparing the distribution of protein abundance amongst seminal fluid proteins identified (red) and not identified (black) as components of blood plasma. (C) Venn diagram displaying the protein overlap between Red junglefowl SF (red), human SF (black) and human blood plasma (blue). The vast majority of conserved SF proteins are also present in the blood plasma proteome.

Table 1. Innate immunity proteins of the Red junglefowl SF proteome.

GI #	Protein Name	# of males	Abundance percentile	Not identified in domestic chicken*
46048795	vitronectin	12	93.2	
118098426	polyubiquitin-C	11	89.8	
363744121	complement C7	12	88.0	
363740281	complement C8 gamma chain	12	86.9	
363736454	complement factor H	12	86.4	**X**
363729287	complement C4-A	12	84.0	
513213068	complement C5	12	76.9	
363740226	macrophage migration inhibitory factor	12	76.1	
221325664	complement C6	12	82.2	
513220989	complement C2 isoform X2	10	71.6	**X**
57529999	toll-interacting protein	10	60.4	
433282973	complement factor I	12	59.5	
118094713	complement C8 alpha chain	11	56.2	**X**
118094715	complement C8 beta chain	2	22.0	**X**
326633216	soluble mannose-binding lectin	1	9.6	**X**

* Note: These differences may be attributable to methodological differences between this study and Labas *et al.* (2015).

Table 2. Anti-microbial and adaptive immunity proteins of the Red junglefowl SF proteome.

GI #	Protein Name	Abundance Percentile	Not identified in domestic chicken*
	Anti-microbial/viral proteins		
48976029	gallinacin- 10	99.3	
48675889	gallinacin-9	98.8	
	Adaptive immunity proteins		
CAA30161.1	Immunoglobulin gamma chain	99.6	
513225677	alpha-2-macroglobulin	97.0	
AAB22614.2	Immunoglobulin alpha heavy chain	96.6	
AAA48833.1	Immunoglobulin heavy chain	94.1	
P01875.2	Immunoglobulin mu chain C	91.1	**X**
513200587	beta-2-microglobulin isoform X1	82.6	
52345405	Leukocyte surface antigen CD47	71.5	
52138689	Lymphocyte antigen 86	70.5	**X**
403224973	cathelicidin-B1	68.8	**X**
448261627	ATP synthase subunit beta, mitochondrial	55.3	
113206156	N-acetylmuramoyl-L-alanine amidase	50.1	
513211517	class II histocompatibility antigen, B-L beta chain	46.5	**X**
48675879	cathelicidin-1	26.6	**X**

* Note: These differences may be attributable to methodological differences between this study and Labas *et al.* (2015).

SF proteome and post-spermatogenic sperm maturation

Fowl sperm do not complete maturation in the epididymis (Froman & Kirby, 2005) and, consistent with the idea of continued maturation in the vas deferens, research in domestic chickens has shown that sperm from the testis, epididymis, and three segments of the vas deferens experience a progressive increase in survival, fertilising ability, motility, acrosomal proteolytic activity, sperm penetration of the egg perivitelline layer, and binding capacity to the uterovaginal junction sperm storage tubules and infundibulum (Ahammad *et al*., 2011 a,b). To explore the possibility that SF (or secreted vesicles therein; see below) might contribute to extragonadal maturation, we assessed the overlap between sperm proteins previously identified in a population of domestic broiler chickens (Labas *et al*., 2015) and two independent RJF SF datasets derived from SF purified using distinct protocols to control for potential sperm contamination (see Methods). The first dataset represents the combined proteome of 12 RJF samples described above, of which 9.55% were also specifically identified in domestic chicken sperm (Table S1, Supporting Information). The second dataset, which included SF samples derived from 4 independent ejaculates from a single male, exhibited an almost identical overlap with sperm proteins (9.78%). Importantly, 78.9% of these SFPs also identified in sperm were present in both of our SF samples, strongly supporting a relationship between SF and sperm composition. This pattern is also evident in Labas *et al*. 2015, which identified a substantial number of proteins (n = 234) common to both domestic chicken SF and sperm. A survey of these 234 proteins revealed that 92.3% and 94.4% of these proteins were also identified in our RJF SF datasets, respectively. We then asked if these predicated sperm proteins identified in our SF dataset represent evolutionary conserved sperm components present in human (Wang *et al*., 2013) and mouse (Chauvin *et al*., 2012) sperm. Of the sperm proteins present in our dataset, 81 (74%) and 68 (62%) were also present in the human and mouse sperm proteomes, respectively, further supporting the potential importance of these proteins in sperm maturation and function.

Gene ontology analysis of SFPs also identified in chicken sperm further suggests a predicted role for SFPs in flagellum development. A significant enrichment of cytoskeletal (1.0E-22), microtubule (5.6E-15), cilia (2.5E-6) and flagella (7.2E-5) proteins was observed, including subunits of chaperonin containing TCP-1 complex, which is responsible for actin and tubulin folding. Enrichment of protein complex subunits involved in axoneme elongation were also identified, including proteasome core complex, alpha-subunit complex (1.3E-9) and microtubule organizing centre (5.1E-6). The identification of 12 flagellum proteins amongst the 284 protein identified in all 12 males (4.2%) exceeds the proportion of mouse (1 out of 69) (Dean *et al*., 2011) and *Drosophila* (3 out of 138) (Findlay *et al*., 2008) SFPs with predicted subcellular localization in the flagellum. Although we cannot entirely rule out the possibility of sperm protein contamination during SF purification, the highly reproducible results obtained using alternative purification techniques supports the presence of integral sperm components in RJF SF.

Proteomic support for SF exosomes

Sperm modification after spermatogenesis has been well documented in mammals, where sperm undergo complex modifications within the epididymis (Liu *et al*., 2014; Skerget *et al*., 2015). This process is governed, in part, by extracellular vesicles, termed epididymosomes, which are believed to deliver molecular components to sperm (Girouard, Frenette & Sullivan, 2009). To explore the possibility of a similar mechanism in RJF, we investigated the presence of the most common protein markers of exosomes (Keerthikumar *et al*., 2016) within our SF dataset. This revealed the presence of 85.6% (60 out of 70) of the top exosome markers with one-to-one orthology between chickens and mammals (Table S1, Supporting Information). This included 7 members of the ras-related Rab protein family, which regulate vesicle formation, trafficking and membrane fusion, 6 annexin proteins, which are membrane scaffolding proteins that regulate vesicle formation, and 6 members of the 14-3-3 proteins family, which regulate vesicle targeting through cytoskeleton interactions. Exosome markers in SF were also found to be significantly

more abundant on average than the remainder of the proteome (non-parametric Kolmogorov-Smirnov test; D = 0.523; $p < 0.0001$), suggesting that exosome-like vesicles may be abundant in SF.

Comparative analysis of the RJF and domestic chicken SF proteome

A direct proteomic comparison, based on the reanalysis of raw MS/MS data from domestic chicken SF samples, revealed that 94.4% of domestic SFPs were identified in our RJF proteome. 31 proteins were identified as unique to the domestic SF proteome (Table S4, Supporting Information), while 585 RJF proteins were absent from the domestic proteome (Fig. 3a). Domestic SFP identification was significantly enriched amongst more abundant RJF proteins ($X^2 = 459$; $p < 0.0001$), indicative of limited coverage of low abundance proteins in the domestic chicken (Fig. 3b). Given the more comprehensive nature of the RJF proteome, proteins uniquely identified in the domestic chicken are likely to represent *bona fide* SF proteome differences between domestic chickens and RJF. Notable amongst these 31 proteins are a significant enrichment in cell recognition proteins (e.g. Cadm1 and Arsa). The relevance of these proteins to fertility is indicated by mammalian phenotypes, including the role of Arsa as an acrosome vesicle zona pellucida binding protein and sperm maturation defects and infertility in Cadm1 mutants. Gst3, a detoxifying enzyme and potential mediator of ROS damage to sperm, was also identified uniquely in the domestic chicken. The substantial overlap (>93%) between the RJF and domestic chicken proteomes amongst the most abundant protein class (Fig. 3b) supports the possibility that some highly abundant proteins not identified in the domestic chicken may be RJF-specific (Table S4, Supporting Information). Gene Ontology analyses of predicted RJF-specific proteins reveals a significant enrichment of proteins predicted to be involved in humoral immunity (3.6E-4) and complement activation (5.7E-3), amongst a diverse set of immunological process categories. Complement factor H, C8 gamma and immunoglobulin mu chain are of particular note as putative RJF-specific SFPs given their high abundance and consistent identification across individuals.

Epididymal secretory protein E1, which has been implicated in mammalian sperm maturation, is also noteworthy.

A significant correlation in protein abundance was observed amongst the 538 proteins identified in both the domestic chicken and RJF proteomes (Fig. 3c), despite the systematic differences in protein abundance estimates revealed by comparing cumulative abundance distributions (Fig. 3d). Direct abundance comparisons would therefore be predicted to result in many low-to-moderate abundance proteins appearing to be of greater abundance in RJF and a smaller set of highly biased protein as greater in abundance in the domestic chicken. To minimize this issue, an approach was implemented that assessed the shift in both a proteins abundance rank and abundance estimate between species. This analysis successfully identified quantitative differences in a largely unbiased bidirectional pattern between taxa, including proteins with coherent functions (Table S4, Supporting Information). A significant enrichment was confirmed in the following biological processes: defense response (1.6E-4), response to stress (3.1E-4), inflammatory response (3.8E-3) and regulation of immune system process (3.9E-2). Three of these proteins (IgM, IgG and IgGFc-binding protein) were found to be quantitatively more abundant in RJF than domestic chicken, consistent with the identification of other immunity proteins putatively specific to the RJF SF proteome.

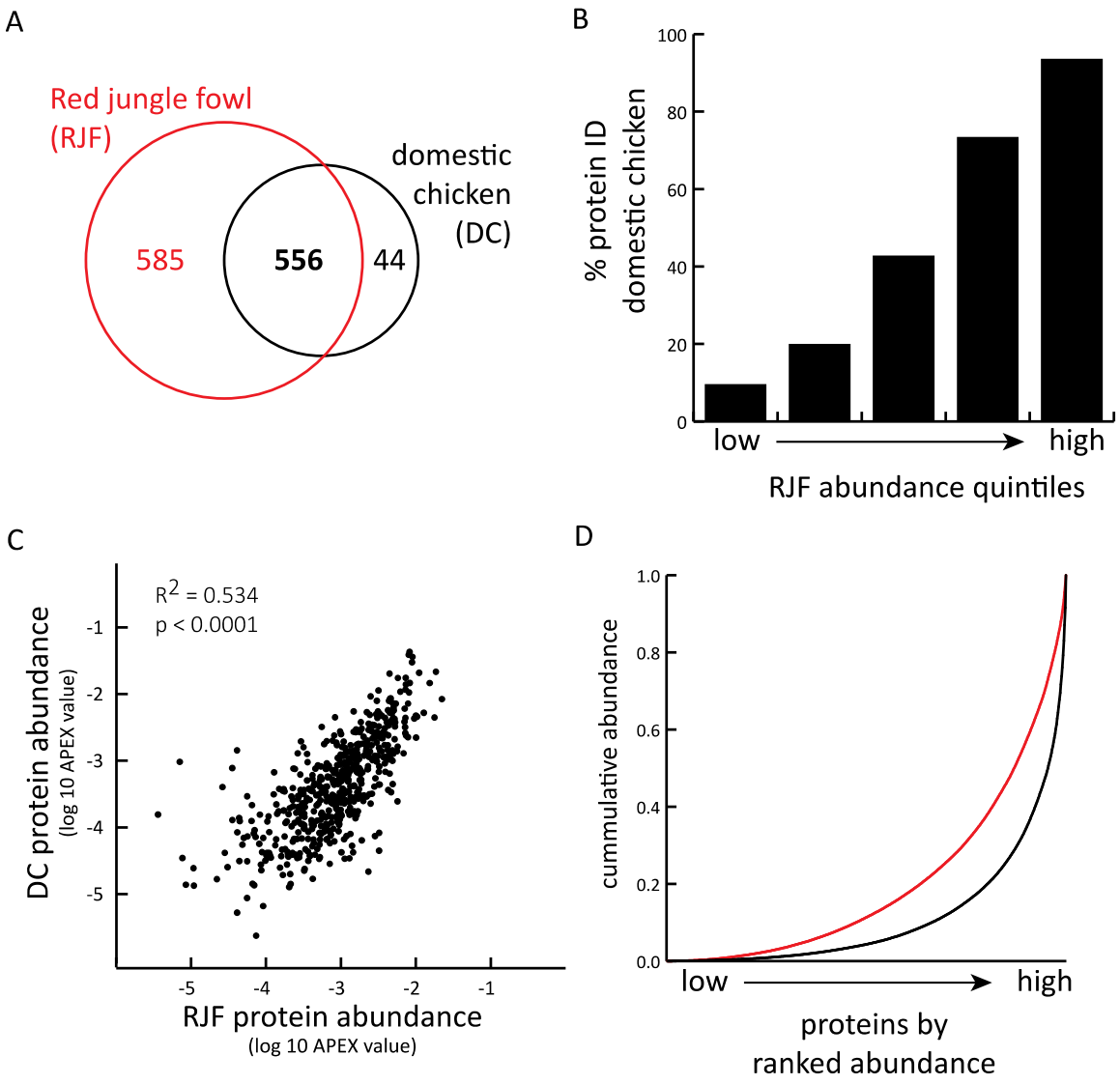


Figure 3. **Comparison of the Red junglefowl and domestic chicken seminal fluid proteomes.** (A) Venn diagram indicating the size and overlap between the seminal fluid proteomes of the Red junglefowl and domestic chicken. (B) Overlap in proteins identified in the domestic chicken in relation to protein abundance in the Red junglefowl proteome. (C) Linear regression identifies a significant correlation in the abundance of proteins identified in both the domestic chicken and Red junglefowl proteomes. Protein abundance is plotted as log transformed APEX values, which represent normalized estimates of protein abundance in each sample. (D) Cumulative protein abundance distribution of the domestic chicken (black) and Red junglefowl (red) proteomes.

DISCUSSION

This study presents a systematic SF proteome analysis of a non-domestic, polyandrous bird population. While the functions of SFPs have received substantial interest (McGraw, Suarez & Wolfner, 2015), much of this work has focused on a few lab-adapted or domestic study organisms. Information on SFPs is particularly patchy in birds, despite the importance of this

group to the study of sexual selection. Information on avian SFPs is largely restricted to a recent study of a domestic broiler chicken population (Labas *et al*., 2015). Our study contextualizes this earlier work, by conducting a more comprehensive analysis of a population that has not been exposed to artificial selection. Below we discuss the key aspects of: (i) the composition of the RJF SF proteome, and (ii) the signature of domestication and artificial selection.

In mammals and insects, specific reproductive tissues are responsible for the production of SFPs. Analogous dedicated tissues have yet to be identified in birds and many birds, such as passerines, produce little SF (Fujihara, 1992). Fowl, on the other hand, produce substantial quantities of SF (Etches, 1996). Our comparative analysis of the SF and blood plasma proteomes reveals that plasma-like secretions represent a major component of both fowl and human SF. Although the functional significance of this transparent fluid in fowls remains unclear, it may contain SFPs that contribute to ROS buffering and the protection of sperm from oxidative damage during prolonged periods of sperm storage in the male extragonadal reserves and in the female storage tubules. The present study also indicates that RJF SF proteome has a compositional complexity that exceeds that of insects and is likely to be more comparable to that of the characterized human SF proteome (Pilch & Mann, 2006), indicative of diverse range of functions (see below). The substantive contribution of broadly expressed plasma proteins (with putative “housekeeping” roles), appears to stand in stark contrast to the highly specialized repertoire of male-specific SFPs in other species. However, our understanding of gene expression across both male and female reproductive tissues in birds is very limited. Additional proteomic and transcriptomic studies will be required to determine if avian SF also contains highly specialized, male-biased reproductive proteins and, if so, where they originate from in the male reproductive tract.

A diversity of innate and adaptive immunity proteins, as well as antimicrobial proteins, were identified in RJF SF. Immunity proteins are widespread in both male and female reproductive

systems (Dorus, Skerget & Karr, 2012) and some SFPs are known to target microbes that pose a threat to sperm viability and clear pathogens introduced during copulation, thus protecting the egg from potential infections (Lung, Kuo & Wolfner, 2001; Otti *et al*., 2009; Avila *et al*., 2011; Rowe *et al*., 2013; Peng *et al*., 2016). Evidence suggests that avian semen can harbour bacteria (Westneat & Rambo, 2000; White *et al*., 2010) and that the SF of some birds may have antibacterial properties (den Boer, Baer & Boomsma, 2010; Rowe *et al*., 2011, 2013). Our results confirm that antimicrobial proteins gallinacin 9 and 10, of the defensin protein family, are amongst the most abundant proteins in the RJF SF proteome, a finding consistent with SF analysis in domestic chickens (Labas *et al*., 2015) and of particular relevance for promiscuous populations of RJF, where ejaculates host diverse bacterial communities (R. Eccleston & T. Pizzari, unpublished). Immunological proteins may also play a key role in genetic recognition, such as kin-biased interactions between ejaculates of different males or interactions between semen and the female reproductive tract. For example, SF can affect sperm fertilising efficiency differentially based on the genetic similarity between sperm and SF donor males in some taxa (e.g. den Boer, Baer & Boomsma, 2010; Rudolfsen, Serrano & Folstad, 2015). Polyandrous RJF females may also select against sperm of closely related males following mating, a response that appears to be associated with male-female similarity at the Major Histocompatibility Complex locus (MHC) (Løvlie *et al*., 2013). Two proteins belonging to the MHC Class I (beta-2-microglobulin, and ATP synthase subunit beta, mitochondrial precursor), and one to the MHC Class II (class II histocompatibility antigen, B-L beta chain) were identified in our study. Both MHC Class I proteins are also present in the domestic chicken SF. Interestingly, the class II histocompatibility antigen, B-L beta chain was not present in domestic chicken SF (see discussion of domestication below). Our proteomic results are consistent with the proposition that MHC variation may provide a molecular cue for post-copulatory genetic recognition between rival ejaculates and between mates.

In many species, sperm are not mature or fertilisation competent following spermatogenesis and there is evidence that fowl sperm may also acquire fertilisation capacity progressively through their passage through the vas deferens. (Munro, 1935; Howarth, 1983; Esponda & Bedford, 1985; Ahammad *et al*., 2011a, b). Consistent with this idea, a comparison of two independently derived RJF SF proteomes with the domestic chicken sperm proteome revealed considerable overlap and a significant enrichment of SFPs that are structural components of the flagellum. An alternative, non-mutually exclusive explanation of this overlap is that sperm proteins are released into SF as sperm cells age and degrade. While the present study cannot explicitly distinguish between these alternatives, a careful inspection of our proteomic data reveals the presence of numerous marker proteins that are commonly associated with exosomes. Exosomes produced in the epididymis of mammals are believed to play a prominent role in post-spermatogenic sperm maturation and their molecular cargo (*e.g.* protein or RNA) have been the focus of investigation (Girouard, Frenette & Sullivan, 2009). A similar process has also been characterized in *Drosophila*, where exosomes produced in the secondary cells of the accessory gland bind and fuse with sperm (Corrigan *et al*., 2014). Although it is not possible to make firm conclusions based on our data alone, our analysis is consistent with the continued maturation of sperm during transit, and points to the presence of secreted vesicles that could sustain the final stages of maturation in a manner analogous to those described in other systems. Members of the 14-3-3 protein family, which are diverse exosome markers in our dataset, influence mammalian sperm maturation and motility through their interactions with sperm-specific protein phosphatases (Fardilha *et al*., 2011). Importantly, 14-3-3 eta and zeta are decreased in abundance in SF of subfertile domestic chickens (Labas *et al*., 2015) and 3 distinct protein phosphatases were identified in our study as differentially abundant based on age and velocity (see next chapter).

Genetic homogenization during domestication of the chicken is likely to have resulted from a combination of intense artificial selection, life-history trade-offs and bottlenecks. The impact of

this at key genomics regions, such as those important contributions to immunity and fertility (Callaway, 2014), may underlie the well documented reductions in performance amongst domestic chickens and resultant impact on the poultry industry (Laughlin, 2009). This loss of function is particularly pronounced in broiler breeders where extreme artificial selection for growth rate has resulted in a trade-off with traits associated with immunity and fertility (Hocking *et al*., 2001). Our comparative results, although preliminary, highlight divergence between the SF proteome of RJF and that of a population of broiler breeders. We note that methodological differences, including the analysis of larger quantities of proteins and substantially greater numbers of MS/MS runs per male in the current study, has resulted in a far greater depth of coverage in the RJF relative to the domestic chicken. Nonetheless, we show that predicted RJF-specific SFPs are functionally coherent, being more likely to be involved in immunological defenses, consistent with a loss of genetic diversity associated with domestication. A number of factors may underpin this selective disappearance in domestic chickens. For example, genes associated with immune responses may have been "lost" during domestication due to a trade-off with genes supporting different functionalities promoted by artificial selection (*e.g.* production traits), possibly catalyzed by the protection of domestic populations through artificial means (*e.g.* vaccines and antibiotics) which may relax selection on immunity genes and pathways (Decuypere, 2010; Husby, Ekblom & Qvarnstrom, 2011).

The observation that several SFPs associated with male age and sperm velocity (see next chapter) have yet to be identified in the domestic chicken SF proteome is also consistent with the notion that domestication may have led to reductions in male fertility through changes in SF composition. While morphological and behavioural traits, as well as sperm traits, have been evoked to explain reduced male broiler breeder fertility, the results of the present study indicate that SFPs may also play a key role. Additionally, in populations under natural conditions, such as in our study RJF population, males are sexually selected throughout their lifespan, which often

extends past their prime (Collias & Collias, 1996). Genes that encode SFPs conferring fertilisation efficiency late in life will be favoured by postcopulatory sexual selection. In domestic populations, which are typically female-biased and where males are culled early in life, sexual selection on late life fertilising efficiency will be relaxed, favoring the evolution of early reproductive senescence. Therefore, our study creates a platform for the identification of SFPs "swept away" by the domestication process, some of which might explain fertility deficits in domesticated chickens. Given that our study was based on semen samples obtained through manual abdominal massage, a related area of future research will be to establish the way in which individual males modulate allocation of different SFPs across natural ejaculates, which are notoriously plastic due to strategic ejaculate expenditure in response to multiple socio-sexual factors (Pizzari *et al*., 2003).

CHAPTER 5

Seminal fluid proteomic profiles associated with male age and sperm velocity

ABSTRACT

Sperm swimming velocity is a fundamental trait to determine variance in male fertilization success in polyandrous species in which the ejaculates of different males compete for fertilization. Sperm velocity can be affected by ageing, causing a decline in male reproductive potential. Mounting evidence indicates that seminal fluid proteins may play a key role in determining sperm velocity. A complete understanding of post-mating reproductive events therefore requires studying how seminal fluid proteins and male ageing interact to affect sperm velocity. However, such studies are lacking. Here, we establish seminal fluid proteomics profiles associated with male age ("old" and "young") and sperm velocity ("slow" and "fast") in the Red junglefowl, *Gallus gallus*. To do so, we combined multivariate ordinal approaches with the analysis of individual proteins. The results of the PCA and NMDS show that the "old fast" group emerged as different from the other three groups. Two-way ANOVAs of individual proteins identified 24 proteins significantly associated with ageing and/or sperm velocity. Of these proteins, eight are expressed exclusively in all males of the group "old fast". Old males that retain high sperm velocity have proteome characteristics that distinguish them from other males. Collectively, these results shed light onto the effects that seminal fluid proteins and ageing may have on male reproductive potential, providing a platform for the identification of fertility markers at the protein level in Galliformes, and towards a more comprehensive understanding of post-copulatory sexual selection.

INTRODUCTION

Sperm motility and swimming velocity play a key role in determining sperm fertilizing success and the outcome of sperm competition (Snook, 2005; Pizzari *et al*. 2008b; Pizzari & Parker, 2009; Wishart 2009). These traits can be critically affected by ageing, a widespread phenomenon within the animal kingdom, defined as the accumulation of damage to body components that results in the progressive loss of physiological functions and reproductive potential. Over the last decades, there has been a growing interest on the evolutionary repercussions of declines in sperm fertilizing potential in relation to male ageing, as increasing evidence suggests the existence of a strong link between sexual reproduction and male ageing. The ageing of the diploid genome of a male causes sperm senescence, which results in older males experiencing a decline in sperm production, sperm fertilizing efficiency, sperm genetic quality, and subsequent offspring viability (Kirkwood & Austad, 2000; Kirkwood, 2001; Partridge & Gems, 2002; Pizzari *et al*., 2008a; Nussey *et al*., 2013; Jones *et al*., 2014).

Age-related declines in male fertilizing potential attributed to sperm senescence have been widely reported across different organisms, such as, mites (Radwan, Michalczyk & Prokop, 2005), swallows (Møller *et al*., 2009), beetles, (Carazo, Molina-Vila & Font, 2011), spider monkeys (Hernandez-Lopez *et al*., 2012), honeybees (Stürup *et al*., 2013), and bustards (Chantepie *et al*., 2015). This kind of studies have typically focused on the correlation between different parameters of sperm quality measured upon ejaculation (motility, viability, and swimming velocity) and quantitative measurements of male fertility. However, this approach can lead to an incomplete view of the intrinsic factors underlying male reproductive senescence as it overlooks the role played by the molecular components of seminal fluid. Empirical evidence from domestic chickens indicates that seminal fluid modulates aspects of sperm motility and fertilising efficiency (Fujihara, 1992; Etches, 1996; Cornwallis & O'Connor, 2009), although the molecular nature of these effects remains unclear (Etches, 1996). The role of seminal fluid in sperm motility is

particularly significant, given that swimming velocity is a major determinant of fertilisation success under sperm competition (Pizzari *et al*., 2008b; Wishart, 2009). Importantly, sperm velocity tends to decline as individual males age past their prime (Dean *et al*., 2010; Cornwallis, Dean & Pizzari, 2014), and evidence from Red junglefowl populations suggests that this is mediated by changes in the antioxidant capacity of seminal fluid (Noguera *et al*., 2012). Collectively, these data suggest that seminal fluid proteins may modulate aspects of male fertilising efficiency and reproductive ageing.

The fact that fowl high-mobility sperm have double ATP and stearoylcarnitine content than average-mobility sperm (Froman & Feltmann, 1998; Froman *et al.*, 1999) began to highlight the critical importance of studying sperm swimming performance in relation to the underlying semen proteome towards a more comprehensive understanding of the traits determining sperm quality, and, thus, the potential outcome of sperm competition.

The pioneering work of Froman *et al*. (2011) laid the foundations for this novel approach. Comparing lines of low and high-sperm mobility of New Hampshire roosters, they found that three proteins affecting sperm energy metabolism were differentially expressed in both lines of males, namely, brain-type creatine kinase (an isozyme that enables regeneration of ATP in the axoneme), aldolase (a glycolytic enzyme), and phosphoglycerate kinase 1 (the first enzyme in the glycolytic pathway used to produce ATP). A follow-up experiment showed that 596 proteins were more abundant in high-mobility sperm roosters, while 298 proteins were more abundant in low-mobility sperm roosters. Of particular interest is the fact that proteins involved in different stages of ATP metabolism were upregulated in the high-mobility males. Following this work, the study of the effects of seminal fluid proteins on sperm performance became relatively more frequent across different groups of organisms.

In the Atlantic cod (*Gadus morhua*) the sperm swimming velocity parameters (VCL, VSL, VAP, ALH, and DANCE μm2/s) are significantly higher in wild cod sperm than in sperm from cultivated males hatched in commercial hatcheries. Interestingly, seminal plasma lactate dehydrogenase (a protein involved in energy production that regulates the conversion of pyruvate into lactate and vice versa) and superoxide dismutase (an important antioxidant in all living cells) activity in wild males were significantly higher than in cultivated males (Butts *et al*., 2011).

In rams, proteins with low molecular weight were found to protect sperm motility and velocity parameters (VCL, VSL, VAP) during incubation at low temperature, 5ºC (Ivanova-Kicheva & Dimov, 2011). Subsequent work in rams associated seminal fluid proteomic profiles with high-motile (containing more than 80% motile sperm cells) and low-motile ejaculates (containing less than 80% motile sperm cells). Two proteins were expressed with more intensity in the group with high-motile ejaculates, namely, arysulfatase A, and zinc-alpha-2-glycoprotein. Arysulfatase A is acquired by sperm upon epididymal passage and helps the sperm to reach the oocyte. Zinc-alpha-2-glycoprotein is a lipid-mobilizing factor to stimulate lipolysis in adipocytes (Bing *et al*., 2004), and was found to stimulate forward sperm motility from the epididymal caput in humans (Ding *et al*., 2007). In turn, two proteins were expressed with more intensity in the group with low-motile ejaculates, namely, RSVP-22, and bodhesin-2 (Rodrigues *et al*., 2013). RSVP-22 belongs to the binder of sperm protein family that binds to sperm during ejaculation and remains bound until the sperm reach the oviduct epithelium (Souza *et al*., 2008) where they form a storage reservoir (Gwathmey *et al*., 2006). Bodhesin-2 belongs to the spermadhesin family that appears to be involved in sequential steps of fertilization, such as, sperm capacitation and sperm-egg interaction (Töpfer-Petersen *et al*, 1998; Teixeira *et al*., 2006).

The seminal fluid proteomes of the Bali bull, *Bos javanicus*, associated with high-quality and low-quality sperm (determined by general motility, viability, and normal morphology) showed

significant differences in the regulation of three proteins, namely, binder of sperm 1, binder of sperm 5, and seminal ribonuclease. Binders of sperm coat the sperm surface by interacting with phospholipids to prevent a premature acrosome reaction. In turn, seminal ribonuclease enhances oocyte-penetration capacity. The binder of sperm 5 and seminal ribonuclease are upregulated in the high-quality sperm bulls. As the binder of sperm 1 is upregulated in the low-quality sperm bulls the authors suggest its potential use as a fertility marker (Sarsaifi *et al*., 2015). Collectively, all these studies point out the critical effect that some seminal fluid proteins may potentially exert on sperm quality and, therefore, on the outcome of sperm competition. They may also contribute to identify male fertility markers in the seminal fluid at a protein level, which would allow high-throughput evaluations of male fertility and the subsequent effective development of better targeted fertility improvement treatments.

In the fowl, sperm mobility affects male fertility (Froman *et al*., 1999), competitive ability (Birkhead *et al*., 1999), and fertilization success in the long term (Pizzari *et al*., 2008b). In addition to that, sperm VAP correlates to fertilization success (Wishart & Palmer, 1986). Therefore, given the importance of ageing on male reproductive potential and the critical effects that some seminal fluid proteins may exert on sperm swimming performance, the association between different phenotypes of male age, and sperm quality with their underlying seminal fluid proteomic profiles constitutes a promising research avenue that, surprisingly, given the extensive use of birds in studies of sexual selection and the importance of the fowl in the animal industry, remains largely unexplored.

This study therefore aims to address this gap in the literature by comparing the seminal fluid proteome of young and old males with fast or slow sperm swimming velocity in order to identify genes and proteins associated with reproductive ageing and/or sperm velocity in the Red junglefowl, *Gallus gallus*.

MATERIALS AND METHODS

Semen collection and SF isolation

We studied a population of RJF at the John Krebs Field Station (Oxfordshire), which was founded in 2006 from other captive populations. All sampling of birds was conducted in accordance with approved experimental protocols under Home Office licence (PPL 30/2931). In July-September 2013 semen samples were obtained from 16 sexually mature males by abdominal massage, which captures a representative sample of extragonadal semen reserves of a male (Etches, 1996). Males were isolated from females for 48 hours before being massaged for the first time. Two age classes of males were selected: "Young" (1 year old, n=7), and "Old" (4, 5, and 7 years old, n=9), which collectively represent a relevant dataset to detect age-dependent declines in fertility (Dean, Nakagawa & Pizzari, 2011; Cornwallis, Dean & Pizzari, 2014). Males were kept sexually rested and isolated from females for 48h. For each male, semen samples were collected every four days to allow full replenishment of extragonadal sperm reserves, between 12:00-17:00 h. In total, 157 semen samples were collected (77 from young, and 80 from old males). Immediately following collection, samples were placed in a water bath at RJF physiological temperature (41C). We then diluted 1µl of semen into a culture medium containing 180 µl of Dulbecco's Modified Eagle's Medium (DMEM) and 20 µl of Standard Foetal Bovine Serum (FBS). We transferred 20 µl of this mixture to a microscope slide placed on a heated microscope stage set at 41C and connected to a digital video camera set at 10x magnification, and measured sperm average path velocity (VAP) using the Sperm Class Analyzer: SCA v. 3.0.3 (Microptics) set for chicken with the following specifications: capture 25 images/sec; tracking objects between 50 and 200 microns; Optics: Ph-; Chamber: Leja 10; Scale: 10x. A minimum of 200 individual sperm cells were tracked for each sample, across at least three different fields.

Immediately following VAP measurement, we used the remainder of a sample to separate sperm from SF by centrifugation at 3,500 g for 1 minute at 4C. The SF supernatant was transferred to a

sterile tube for storage at -20C. This protocol was designed to purify SF from sperm cells, while reducing damage (i.e. flagellum or midpiece separation) and protein contamination from damaged cells. To conduct an independent assessment of the presence of integral sperm components in SF in samples in which whole sperm were confirmed to be absent by microscopy, we purified semen samples from four separate ejaculates of a single male using a more extensive centrifugation protocol. This included centrifugation at 600g for 10 minutes at 4C, and, then, centrifuging the supernatant twice more at 600g for 10 minutes and 3,000g for 30 minutes at 4C (Labas *et al*., 2015). The absence of sperm cells and flagella in this SF samples was confirmed by phase contrast microscopy. Mass spectrometry analyses of all samples were conducted using the same methodology (see below).

To determine SF proteomic profiles associated with male age and sperm VAP, we created four categories: young males with high VAP (Young Fast), young males with low VAP (Young Slow), old males with high VAP (Old Fast), and old males with low VAP (Old Slow). To do so, we measured the average VAP of each male monthly (Fig. S5a, Supporting Information), and selected the three samples with the highest monthly VAP for each of the three young males with the highest average monthly VAP, and the three samples with the lowest VAP for each of the three young males with the lowest average VAP. We performed a similar selection among the old males. Two males were represented in two different categories (Old Fast - Old Slow, and Young Fast - Young Slow, respectively) as they produced highest and lowest average VAP in two separate months. Pearson correlations within these two males that were replicated twice and represented in both the Slow and Fast categories, indicated that these males had a high overall consistency in their SF proteomic profile (correlation coefficients of 0.85 for the Old male, and 0.84 for the Young male). Therefore, we worked on a dataset of 12 samples from 10 males. This selection resulted in significant differences in VAP across the Young and Old, and the Slow and Fast categories (Fig. S5b, Supporting Information).

Protein preparation and 1-dimensional SDS-PAGE, *Tandem mass spectrometry analysis*, *Peptide identification and protein annotation*, *Protein quantitation and analysis*

See descriptions in previous chapter as the methods (excluding the analysis involving other datasets, which were not used in this chapter) and samples used were the same.

Analysis of male age and sperm swimming velocity (VAP)

In order to establish SF proteomic profiles associated with male age (Old and Young) and sperm quality (Slow and Fast), we combined multivariate ordinal approaches with the analysis of individual proteins. First, we asked whether variation in the proteomic profile of individual samples could be explained by the sperm velocity and/or the male age of a sample. We therefore summarized variation in APEX-corrected protein spectral counts across the four specified categories (i.e. Old Slow, Old Fast, Young Slow, Young Fast). To do this, Principal Component Analysis, as implemented by the FactoMine R package, was used to compare the protein spectral counts across the four categories (PCA1) after normalization (i.e. equal total number of spectral counts across all males). We focused on an 'inclusive' dataset, which included all the proteins (840) identified in a minimum of 3 and up to all 12 samples (a "0" was entered when no spectra were detected for a protein in a given sample). Because each age/velocity category was comprised of 3 males, we chose a minimum of 3 males to identify situations in which a protein was expressed in all samples of a category but nowhere else. For each protein, we calculated the average spectral count for each of the four categories of males. We positioned these categories of males in a variables factor map to visualize their correlations with the two main dimensions of the PCA. To refine the interpretation of the variables factor map, we calculated for each protein over the four dimensions: a) its coordinates on the dimension; b) its squared cosine, which measure the quality of the projection of the protein on the dimension; c) and its contribution (%) to the construction of the dimension. We confirmed these results using between-groups PCA (PCA2) (Krzanowski, 1979) using the ade4 R package, which distinguishes variance within and across categories. This optimizes group variability, instead of between sample variability, by

transforming the abundance data by averaging across the individuals belonging to each age/speed group. The protein and sample weightings, measuring distances between the proteins and samples, respectively, do not change from PCA1. Four groups were used for the PCA2 analysis, representing each of the age by velocity categories. As in PCA1, a "0" was entered when no spectra were detected for a protein in a given sample. Because PCA can be sensitive to transformation, we verified the robustness of these results with the results from Nonmetric multidimensional scaling (NMDS) in the vegan R package, using the 840 proteins of the 'inclusive' dataset. NMDS is a non-eigenvector technique that summarizes the variance in a N-dimensional ordination space that best represents the differences in protein ranks (Paliy & Shankar, 2016). Unlike the PCA, NMDS ranks the proteins based on their abundance, therefore eliminating potential problems (e.g., sensitivity to transformation) associated with the use of absolute distances between their spectral counts. NMDS iteratively repositioned the proteins into the ordination space to minimize the disagreement (i.e. stress) between their original distances and their final dimensional representation. We plotted the relationship between the original protein distances and their dissimilarities in the final two-dimensional ordination space using a Shepard stress plot (Figure S3, Supplemental Information).

Second, we performed a third PCA (PCA3), using the 840 proteins of the 'inclusive' dataset, to characterize the 12 samples from the point of view of these proteins (i.e. the spectral count of each SFP is a variable). Thus, PCA3 uses the spectral count of individual protein as variables and summarises this variation across the 12 samples, in a way similar to a PCA of ecological communities in which the abundance of each species (i.e. SFP spectral count) is a variable and the PCA summarises variation in species abundances across a number of sites (i.e. samples). This analysis explores whether any of the main dimensions of the PCA summarizes biologically relevant differences in the proteomics profiles associated with male age and/or sperm quality, by positioning the 12 samples of individual males in the multidimensional space. The rationale of

this approach is that, should individuals belonging to a given category have different loadings on a particular dimension than individuals belonging to a different category, the functional analysis of the proteins differentially expressed in these categories will shed light on the biological significance of that dimension. As in PCA1 and PCA2, a "0" was entered when no spectra were detected for a protein in a given sample.

Finally, we complemented the multivariate approach outlined above with an in-depth analysis on each individual protein of the inclusive dataset. To do so, we conducted a two-way ANOVA, with "male age" and "sperm quality" as fixed factors, and "spectral counts" as a response variable. We conducted the Shapiro test for each individual protein. Those proteins that were not normally distributed ($p<0.05$) were log 10 transformed (Log_{10} (corrected spectral counts + 0.001)). The model fitted in R was: model<-aov (spectral~age*velocity). If the 2-term interaction was not significant, we repeated the procedure by fitting a new model: model1<-aov (spectral~age+velocity). When the ANOVA detected significant effects across the four categories, we performed pair-wise comparisons to determine which categories differed from each other. We used t-tests with a 2-tailed distribution assuming unequal variances (heteroscedasticity) to perform pairwise comparisons of the spectral counts across the 8 possible comparisons, and used the Benjamini-Hochberg formula to control the false discovery rate over the multiple comparisons. This approach identified genes that were differentially up- or down-regulated with respect to male age, sperm velocity or both. In addition, we made use of the fact that two males were represented in two different categories (Old Fast - Old Slow, and Young Fast - Young Slow, respectively). We used Pearson correlations to measure the strength of the relationship of the spectral counts between the two measurements of each male (that is, when belonging to each of the two categories) to quantify overall intra-male consistency in SF proteome, and to identify individual proteins with abundance changes associated with changes in categories that deviated from the overall trend (i.e. proteins with the largest residual).

RESULTS

We first performed PCA1 to summarise the variance in protein abundances across the four categories. The first dimension explained 95% of the variance in protein abundance across categories (Fig. 1a). The four categories loaded strongly with this dimension, indicating that it captures patterns of protein abundance variation that are consistent across the four categories (Fig. 1a; z-transformed protein abundance yields qualitatively identical results (data not shown)). Thus, dimension 1 captures the dynamic range of relative protein abundance within samples rather than absolute changes across samples. The values of the squared cosine for dimension 1 reflect the major contribution of abundant proteins to this dimension since the 30 most abundant proteins in the dataset have squared cosines scores between 0.83 and 1. The five proteins with the highest weighting on dimension 1 (i.e. the most abundant) were: serum albumin precursor, transthyretin precursor, fatty acid-binding protein brain, pendrin isoform X2, and gallinacin-10 preproprotein; collectively they explain more than 42% in the construction of dimension 1 (Table S2, Supporting Information). Conversely, for example, amylase (alpha 2A) is low in abundance and one of the lowest coordinates. Dimension 2 of PCA1 explained 3.23% of the variance in protein abundance across categories (Fig. 1a). Old Fast was the only category that had a significant positive loading with dimension 2, while Old Slow and Young Fast both had significant negative loadings (Table S2, Supporting Information), indicating that this dimension may reflect protein abundance variation that is distinct in the ejaculates of old males with fast sperm (Fig. 1a). The between-category PCA2 confirmed this pattern, revealing that Old Fast samples were tightly clustered together and away from the other categories in dimension 1, with clear separation from Old Slow and Young Fast (Fig. 1b). We also note that Young Slow samples occupy a unique space, particularly in relation to dimension 2, but do not cluster as tightly as Old Fast males. Finally, the NMDS analysis summarized the variance in a 2-dimensional ordination space with high fit with the original protein ranks (stress = 0.116; Table S2, Supporting Information). Once again, the Old

Fast category emerged as different from the other three categories (NMDS dimension 1; Figure S3, Supplemental Information), confirming the results of PCA1 and PCA2.

PCA3 summarized variation in the profile of individual SFPs across the 12 samples. This analysis identified 11 dimensions, of which the first 3 explained 51.2% of the cumulative variance (Table S2, Supporting Information). Importantly, dimension 1 (eigen value=219.56, 26.14% of variance explained) captures a gradient in sperm velocity, discriminating Old Slow from Old Fast samples (Fig. 2a). Consistent with this, two of the three old slow males occupy negative coordinates in dimension 1 while all three old fast males occupy positive coordinates in this dimension (Table S2, Supporting Information). We next analyzed dimension 1 weightings of proteins exhibiting significant abundance differences within the single replicated old male (#308) who was sampled with both Fast and Slow sperm velocity. This revealed a striking association between the distribution of dimension 1 weightings and directional changes in protein abundance between Fast and Slow samples from this male (Fig. 2b). To explore the functional basis for dimension 1 weighting associations, we compared the weightings of blood plasma and exosome marker proteins identified above. This revealed a strong disparity between these functional classes, including (i) an enrichment for blood plasma proteins on the negative side of this dimension (Old Slow), (ii) a deficit of blood plasma protein on the positive side (Old Fast) and (iii) the inverse pattern for exosome-related components (Fig. 2c). Consistent with this observation, a substantial number of blood plasma proteins were significantly more abundant in the Slow sample of the replicated Old male (#308) relative to his Fast sample. Interestingly, a somewhat opposite pattern for differentially abundant blood plasma proteins is observed for the young male (#H28) sampled twice (i.e. both Slow and Fast sperm velocity; data not shown). Consistent with the colocalization of Old Fast and Young Slow on dimension 1, 26 of 43 blood plasma protein are more abundant in the H28 Fast sample. Interestingly, these proteins are largely distinct from the blood plasma proteins identified as differentially abundant in male #308. Taken together, these observations

suggest that variation in sperm velocity across males is determined by the abundance of blood plasma and exosome proteins in SF. The detection of distinct sets of proteins in the young and old males outlined above further suggests that the proteins and molecular pathways governing sperm velocity may be age-specific.

Two-way ANOVAs of individual proteins identified 9 cases in which, after controlling for false discovery rates, a protein was differentially expressed with respect to male age, sperm velocity or their interaction (Table 1; top section). Of these 9 proteins, five are proteins more abundant in Old Fast samples. Interestingly, patterns of differential protein expression associated with sperm velocity across males were recapitulated in changes within the two males that were replicated twice and represented in both the Slow and Fast categories. This included three proteins up-regulated in the Old Fast category (fibulin 2, proto-oncogene protein kinase ROS and receptor-type tyrosine phosphatase eta precursor) and two proteins exhibiting differential abundance between the Young Fast and Young Slow categories (beta-galactoside-binding lecting and inosine-5'-monophosphate dehydrongenase 2). A total of 12 proteins were also identified exclusively and consistently amongst males of a single age (1), velocity (1) or age-velocity category (10) (Table 1; bottom section).Of these, 8 were found in the category Old Fast, which is consistent with the distinct nature of this category as identified by the PCA and NMDS analyses (above). The number of proteins identified exclusively in Old Fast males is unique within the dataset, as the identification of multiple proteins in a specific subset of 3 males was otherwise rare. Lastly, we re-ran PCA1 of the 'inclusive' proteome (above) excluding those proteins identified here by ANOVA and restricted to specific age-velocity categories. This analysis yielded nearly identical results, indicating that the loading of Old Fast samples on dimension 2 of PCA1 is not solely driven by a subset of SFPs, but reflects a general property of SF proteome profiles.

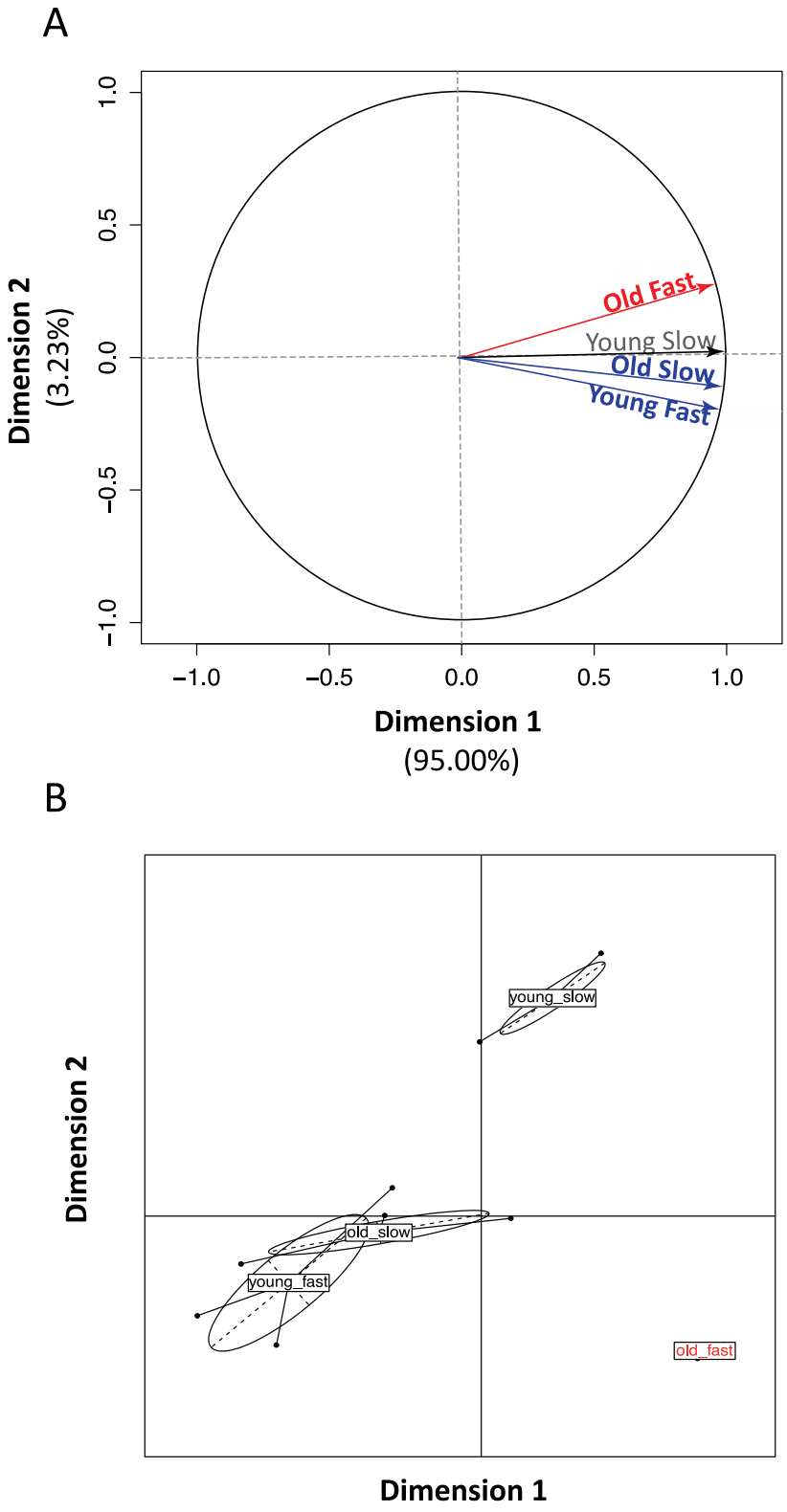


Figure 1. **Multivariate ordination of seminal fluid protein abundance with respect to male age and sperm velocity**. (A) Principle Component Analysis (PCA1) variables factor map. The direction of the arrows represents the relative loadings of the four categories (Old Fast, Old Slow, Young Fast, Young Slow) on the first and second dimension. All four categories have strong positive loadings on dimension 1; Old Fast has a significant positive loading and Old Slow and Young Fast have significant negative loadings on dimension 2, respectively. (B) Between group PCA (PCA2) biplot. First and second dimension loadings for the four categories of males are displayed, as well as the extent of within category variance (ovals) and loadings of individuals within each category (points at the end of solid lines).

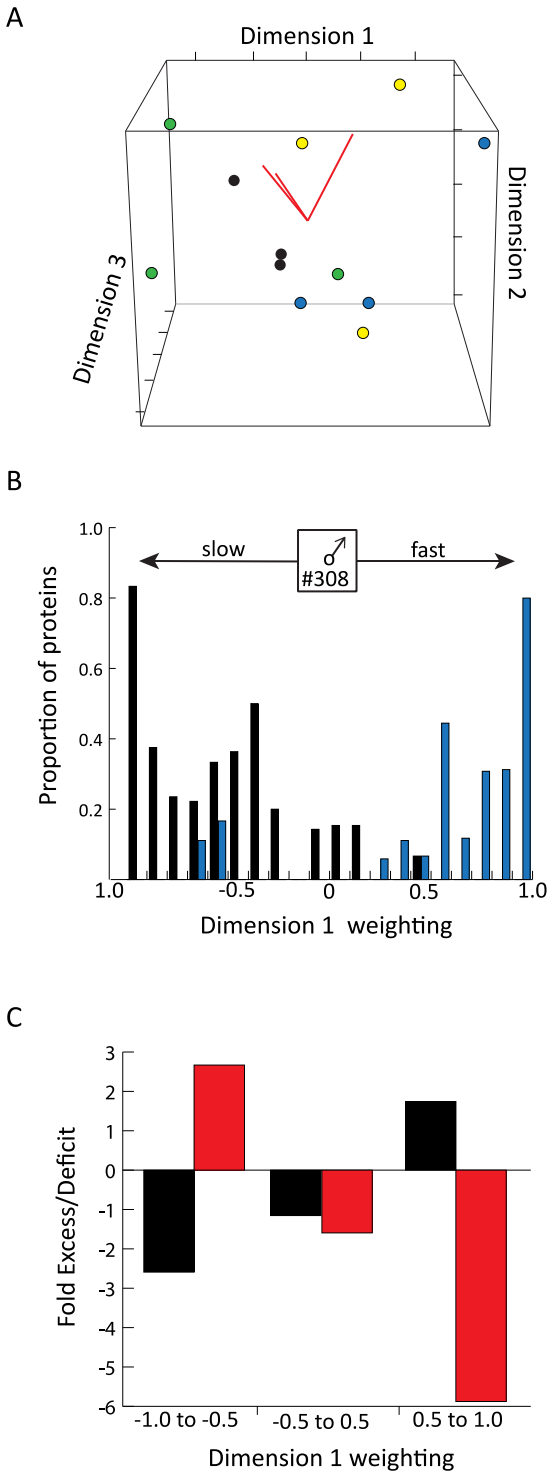


Figure 2. Seminal fluid protein abundance variation associated with sperm velocity in old males. (A) 3-dimension visualization of Principle Component Analysis (PCA3), using the inclusive protein dataset as variables (Old Fast, blue; Old Slow; black; Young Fast; green; Young Slow, yellow). Dimension 1 captures variance associated with old male sperm velocity, including negative weightings for Old Slow and positive weightings for Old Fast. (B) Histogram displaying the distribution of first dimension weightings for proteins exhibiting significant abundance differences between the Slow and Fast sample of the replicated old male (#308) (abundance increase in fast sample, blue; abundance increase in slow sample, black). Differential abundance was calculated using Z-score test with Bonferroni correction to the p-values. (C) Histogram displaying the excess or deficit of blood plasma proteins (red) and exosome markers (black) across first dimension weight ranges. Fold enrichment was calculated as follows: log2 of (exosome markers or plasma proteins in bin / total genes in bin) / (total exosome markers or plasma proteins / total genes).

Table 1. SFPs differentially expressed with respect to male age, sperm velocity or age: velocity interaction

Category	Gi-number	Protein name	Comparison or Age-velocity category
Significant in FDR-corrected 2-way ANOVAs			
Age : Velocity	46048932	sodium-dependent phosphate transport protein 2B	OF > YF; OF > YS
Age : Velocity	71895709	glycine-tRNA ligase	OF > YF
Age : Velocity	45382907	receptor-type tyrosine-protein phosphatase eta precursor	OF > YF
Age : Velocity	513204938	fibulin-2	OF > OS; YS > OS
Age : Velocity	160333842	proto-oncogene tyrosine-protein kinase ROS precursor	OF > OS; OF > YF; OF > YS
Age : Velocity	45382785	beta-galactoside-binding lectin	YF > YS
Age	45383337	SPARC precursor	Y > O; YF > OS; YS > OS; YS > OF
Age	303227902	acetyl-CoA acetyltransferase, cytosolic	Y > O
Age	60302752	ras-related protein Rab-11B	Y > O; YF > OS
Identification restricted by age and/or velocity			
Age	56605916	protein tyrosine phosphatase type IVA 1	Old only
Age : Velocity	513237884	neuroblast differentiation-associated protein AHNAK-like	Old Fast only
Age : Velocity	45383816	protein S100-A6	Old Fast only
Age : Velocity	45384028	protein S100-A11	Old Fast only
Age : Velocity	118085711	acyl-CoA-binding protein	Old Fast only
Age : Velocity	160358333	neo-calmodulin	Old Fast only
Age : Velocity	326368309	guanine nucleotide-binding protein (gamma-10)	Old Fast only
Age : Velocity	363730875	myosin regulatory light chain 2,	Old Fast only
Age : Velocity	363745926	guanine nucleotide-binding protein subunit gamma-7-like	Old Fast only
Age : Velocity	478247057	microsomal glutathione S-transferase 3	Young Fast only
Age : Velocity	363742947	nitrilase homolog 1 isoformX1	Young Fast only
Velocity	71895387	inosine-5'-monophosphate dehydrogenase 2	Slow only

OF= Old Fast; YS= Young Slow; YF= Young Fast; O= Old; S= Slow

DISCUSSION

Sperm velocity is a key determinant of fertilising efficiency in domestic chickens (Froman & Kirby, 2005), particularly under sperm competition when ejaculates with relatively high sperm mobility progressively achieve a competitive edge over female sperm storage time (Pizzari, 2008 b). However, sperm velocity tends to decline as males age (Dean *et al*., 2010; Cornwallis, Dean & Pizzari, 2014), and these age-dependent effects are associated with changes in SF composition (Noguera *et al*., 2012). This study set out to provide the first characterization of the SF proteomic profiles associated with male age and sperm velocity in a wild species of bird. However, we urge caution in the interpretation of our results and stress that additional studies, using complementary quantitative techniques, will ultimately be required to achieve a refined understanding of SF proteomic variation.

Multivariate analyses distinguished the proteome of Old Fast samples from that of the other three categories of males. These results suggest that high sperm velocity in older males is associated with higher abundance of exosome markers, potentially linked to sperm maturation. Eight proteins were identified exclusively in the samples belonging to the three males of this category. Although it would be premature to draw any specific functional conclusions, many of these proteins are involved in calcium metabolism. For example, calmodulin, a calcium-binding messenger that controls sperm activation and hyperactivation in mammals, activates kinases in chicken sperm that influence both viability and motility (Nguyen *et al*., 2016 a, b). It will be of great interest to determine if and how secreted calmodulin in SF might influence RJF sperm maturation and the acquisition of motility. Additionally, the S100 proteins are involved in calcium homeostasis and can function in concert with various growth factors following extracellular secretion (Donato *et al*., 2013). Interestingly, neuroblast differentiation-associated protein AHNAK-like, also identified exclusively in Old Fast males, is implicated in calcium regulation and constitutes a calcium-dependent S100B target protein (Gentil *et al*., 2001). In domestic chickens, calcium

constitutes a primary determinant of sperm mobility (Froman & Feltmann, 2005). Excessive calcium uptake can result in mitochondrial degeneration, as was found to be the case in roosters with low sperm mobility (Froman, Wardell & Feltmann, 2006). It is possible that these proteins may ameliorate the decline in sperm velocity associated with ageing, through the mediation of calcium uptake. One possibility is that only some males may be able to retain high rates of sperm maturation in old age. Conversely, semen of Old Slow males (with low rates of sperm maturation) is characterized by relatively high amounts of blood plasma proteins, which likely originate from transparent fluid. These patterns could be due to two alternative mechanisms. First, plasma proteins may cause a reduction in sperm velocity. Second, given that many abundant plasma proteins have either buffering or antioxidant properties, it is tempting to speculate that ageing males with slow rates of sperm maturation may partially ameliorate the fertilizing efficiency of their ejaculates by preferentially investing in transparent fluid. The former mechanism would suggest that in the absence of elevated plasma proteins in their SF, the ejaculates of Old Slow males may display higher sperm velocity while the latter mechanism would predict even lower sperm velocity and/or faster rates of sperm mortality. At present the specific role of transparent fluid in fertilizing efficiency in fowl remains inconclusive (Lake & Wishart, 1984), and more research is required to test these hypotheses. In light of the conserved contribution of blood plasma proteins to SF in fowl and humans (see previous chapter), it is interesting to speculate that blood plasma protein abundance might also influence sperm quality characteristics across a wide range of vertebrates, possibly including humans.

CHAPTER 6

General Discussion

This thesis explores the role of seminal fluid in sexual selection, with special emphasis on seminal fluid proteomics, in the Red junglefowl, *Gallus gallus*. Firstly, I found that sperm swimming velocity increases when the sperm are exposed to non-self seminal fluid over time. This effect translates into differential fertilization success within an alive female. Secondly, I found that males tend to decrease sperm and seminal fluid investment over successive copulations with a familiar female and to increase it on encountering a new female, a pattern known as the Coolidge effect. Patterns of seminal fluid protein allocation changed markedly with male status. While seminal fluid protein content in subdominant males varied with female sexual novelty consistent with a Coolidge effect, dominant males tended to allocate a constant amount of seminal fluid protein to each successive copulation. Thirdly, I identified 1,141 seminal fluid proteins, including proteins involved in immunity and antimicrobial defences, sperm maturation, metabolism and fertilization. These proteins include a substantial contribution of blood plasma proteins that is conserved with humans. There are qualitative and quantitative differences between the Red junglefowl and domestic fowl seminal fluid proteomes, likely associated with domestication and artificial selection. Fourthly, I identified seminal fluid proteins associated with male age and/or sperm quality. Here I discuss the most relevant implications of the key aspects of these findings.

(i) Seminal fluid differential response to non-self sperm

Seminal fluid may affect sperm competition dynamics through a differential response to self and non-self sperm, which requires seminal fluid to recognise both types of sperm. Such differential response to self and non-self sperm constitutes an important trait in sperm competition dynamics because, in principle, seminal fluid may favor the sperm from the same ejaculate, and decrease fertilization chances of sperm from competing ejaculates (Simmons, 2001; Pitnick & Hosken,

2010; Davies, Krebs & West, 2012; Perry, Sirot & Wigby, 2013). Thus, seminal fluid can differentially affect the fertilizing efficiency of self and non-self sperm. However, the empirical evidence for the existence of such recognition mechanism remains highly ambiguous.

I found that sperm VAP *in vitro* declined over time, and that this decline was slower when the sperm were exposed to non-self, and particularly non-self related, seminal fluid. Furthermore, there was a tendency for non-self inseminations to outperform self inseminations, and a significant interaction between seminal fluid treatment and initial sperm VAP, that is, relatively more sperm from high-VAP ejaculates reached the eggs in the non-self unrelated treatment, while more sperm from low-VAP ejaculates reached the eggs in the self treatment.

Collectively, the results of the *in vitro* and *in vivo* experiments show that sperm fertilizing efficiency increases when exposed to non-self seminal fluid. This might be explained by an increase in VAP when sperm detect the presence of a rival ejaculate in order to outcompete it and, in the case of the *in vitro* experiment, a differential seminal fluid response to non-self related and unrelated sperm with seminal fluid being more harmfull to genetically unrelated sperm, which might have inclusive fitness consequences possible favouring cooperation between genetically related ejaculates. However, it is very early stages to draw any functional conclusions from these results.

(ii) Plasticity in the seminal fluid proteome

Recent research has shown that males from different taxa are able to adjust some components of their seminal fluid proteome depending on the perceived risk of sperm competition. In particular, *Drosophila melanogaster* males exposed to a high intensity sperm competition treatment exhibited a significant decline 72 hours after adult eclosion in the expression of genes coding two seminal fluid proteins (Acp 26Aa and Acp 62f) compared with the males exposed to a low

intensity sperm competition treatment (Fedorka, Winterhalter & Ware, 2011). Moreover, males exposed to a high intensity sperm competition treatment maintained sperm production levels. To some extent, these observations are consistent with theory as males in a disfavoured mating position are expected to decrease their seminal fluid investment, in order to prevent other males from exploiting its fecundity-enhancing properties, while keeping constant their sperm allocation (Cameron, Day & Rowe, 2007).

Ramm *et al*. (2015) studied in the house mouse (*Mus musculus domesticus*) how males tailor the composition of their ejaculates in response to their social environment (i.e. cues of sperm competition risk and cues of potential mating rate). 7 of 31 secreted seminal fluid proteins (Cathepsin D, SVS 6, Annexin A5, CEACAM 10, Phosphoglycerate kinase, SVS 5, and Peroxiredoxin-6) show consistent differences in relative abundance levels when comparing high *versus* low sperm competition treatments. Interestingly, 3 of these 7 proteins (SVS 6, SVS 5, and CEACAM 10), having potential roles in sperm competition, were upregulated in the males exposed to potential high sperm competition. Furthermore, the three sperm production parameters considered in this study (testes mass, epididymal sperm numbers, and daily sperm production rate), and seminal fluid production (estimated from the seminal vesicles mass) where higher in the males exposed to high intensity sperm competition. Taken together, the results of these studies indicate that the seminal fluid proteome, far from being a static trait, has the potential to exhibit very sophisticated plasticity in order to maximize male reproductive success.

The third chapter of this thesis suggests that subdominant males may have faced selection pressures to evolve the capability to modify their seminal fluid proteome in order to get the most value out of their limited reproductive opportunities and to compensate for their disfavoured mating positions.

Further work should investigate seminal fluid proteomic plasticity in the ejaculates allocated over successive and quick copulations with familiar and novel females. This kind of approach will eventually allow the identification of male fertility proteins that are more costly to produce and have critical importance in determining male reproductive success. As discussed in *(iii)*, this thesis laid the foundations for this kind of studies by demonstrating simultaneously for the first time not only the existence of sophisticated plasticity in the two main components of the ejaculate (i.e. sperm and seminal fluid) but also in its seminal fluid protein content in relation to male social status.

(iii) Sophisticated patterns of ejaculate allocation

Competitive ejaculates are costly to produce. For this reason, males are selected to tailor strategically their ejaculates depending on the value of a given mating event. Males of several species are sensitive to cues indicative of potential sperm competition and are able to allocate their limited ejaculate resources accordingly in order to maximize their reproductive payoffs. For instance, in the polyandrous Small White butterfly *Pieris rapae* mated males increase the spermatophore size and the number of sperm produced when mating with a female having a mating history indicative of higher sperm competition intensity (Wedell & Cook, 1999a,b). Furthermore, an increase in sperm investment when mating in the presence of a competitor compared to mating in isolation was reported in males of several species, such as, the Mediterranean fruit fly (Gage, 1991), meadow voles (del Barco-Trillo & Ferkin, 2004), Norway rats (Pound & Gage, 2004), mice (Preston & Stockley, 2006; Ramm & Stockley, 2007), and crickets (Simmons *et al*., 2007).

Sperm competition theory predicts that males should progressively decrease sperm allocation as the number of competitors rises above one since they would get diminishing returns for costs in sperm investment (Parker *et al*., 1996). According with theoretical predictions, gobiid fishes

sneaker males increase sperm expenditure in the presence of one competitor sneaker and decrease it as the number of competitor sneakers rises above one, i.e., from two to four (Pilastro, Scaggiante & Rasotto, 2002). In the bushcricket *Kawanaphila nartee* males reduce ejaculate expenditure when mating with larger females as larger females have higher mating success than smaller females and, thus, are indicative of high sperm competition intensity. In the house mice, males do not adjust ejaculate resources on detection of odour-cues indicating an immediate risk of sperm competition (Ramm & Stockley, 2009b) but are able to adjust sperm investment according to the average levels of sperm competition within the population. In particular, males exposed to social cues from three rivals (i.e. "high competition" group) have higher daily sperm production and sperm numbers in the caput epididymis than males exposed to the same type of social cues from just one competitor (i.e. "low competition" group) (Ramm & Stockley, 2009a).

Consistently with previous work in the fowl (Pizzari *et al*., 2003), the results of my study show that males tend to decrease sperm and seminal fluid investment as their copulations with a familiar female went by. However, they increase sperm and seminal fluid investment, compared with their last copulations with the familiar female, on encountering a new female. This strategy, known as the Coolidge effect, would allow males to allocate more evenly their limited ejaculate resources across several females in order to maximize their reproductive payoffs. Interestingly, I found that patterns of seminal fluid protein allocation changed markedly with male social status. While subdominant males allocated more seminal fluid protein content to novel females consistent with a Coolidge effect, dominant males tended to allocate a constant amount of seminal fluid proteins to each successive copulation. There is a heritable component to social status (Craig, Ortman & Guhl, 1965). Therefore, assuming enough consistency across generations, subdominant males could have faced selection pressures to evolve the capability to tailor their seminal fluid protein composition.

Taking into account that the ejaculates of subdominant males are more likely to experience high levels of sperm competition and to be ejected by females than the ejaculates of dominant males (Pizzari & Birkhead, 2000), this strategy would allow subdominant males to increase the competitiveness of their ejaculates, thus maximizing the reproductive payoffs out of their limited and disfavoured mating opportunities.

(iv) Domestication, and genetic and phenotypic variation

Domestication has exerted a profound impact on chicken reproductive biology and the availability of a seminal fluid proteome from *G. domesticus* (Labas *et al.*, 2015) provides a unique opportunity to investigate the molecular basis of divergence between domesticated and undomesticated subspecies.

Domestication involves a close relationship between the humans and non-human animals under their control and constitutes an excellent scenario to study the selective forces associated with remarkable genetic and phenotypic evolution. This results from the organisms evolving rapid adaptations to strong human selection and the need to adapt to a very different environments, generally the farm. Surprisingly, the majority of studies on domestication have focused on dogs, cows, and pigs, therefore overlooking the fowl, which is the most moulded domestic animal by human selection (Callaway, 2014). In the context of artificial selection and animal industry, *in vitro* assays do not generally show significant correlations with *in vivo* fertility. Proteomic studies are therefore of critical importance to explain male fertility, as they potentially allow identification of proteins associated with each stage of male fertility (Park *et al*., 2013), and to understand the underlying mechanisms of genetic and phenotypic variation in domestic subspecies.

For instance, modern genetic work comparing DNA from domestic fowl with Red junglefowl's identified two striking selective sweeps in all domestic fowls studied. The first sweep occurs at the locus for thyroid stimulating hormone receptor (TSHR), which has a critical role in metabolic regulation and photoperiod control of reproduction in vertebrates. A non-conservative amino acid substitution, glycine to arginine at residue 558, might have been the target for this sweep. The presence of at least one copy of the sweep TSHR haplotype in all domestic fowls may have resulted from their lack of clearly defined breeding seasons as compared with the wild fowl (Rubin *et al.*, 2010). The second sweep is caused by a recessive allele of the β-carotene dioxygenase 2 (BCDO2) that, when present in two copies, causes the yellow skin phenotype characteristic of the great majority of Western domestic fowl (Flink *et al.*, 2014). This recessive allele has probably been acquired through hybridization with the Gray junglefowl (Eriksson *et al*., 2008).

The very rapid selection to which the domestic fowl has been subjected should have led to strong inbreeding and, thus, to whittle down immune genes, reducing their ability to combat pathogens (Callaway, 2014). Consistent with this expectation, I showed that several innate immunity proteins abundant in the Red junglefowl seminal fluid proteome were not identified in the domestic fowl. Such loss of immune function is very likely to have resulted from a trade-off with genes involved in different functions being pushed forward, and the protection of the domestic fowl by artificial means, such as, vaccines.

(v) Seminal fluid and sperm maturation

In many species, sperm are not mature or fully fertilization competent following spermatogenesis. In mammals, sperm undergo critical maturational changes upon passage through the epididymis, which allow them to acquire the ability for survival and fertilization. A strong line of evidence

suggests that fowl sperm gain progressively their fertilization capacity through their passage down the male reproductive tract.

Munro's classical investigations (1935, 1938) showed that sperm from the testis, epididymis, and vas deferens have, respectively, low, medium, and high levels of motility. Using intravaginal insemination, he found that sperm from the testis are infertile and acquire their fertilizing capacity in the epididymis and vas deferens. However, as the uterovaginal junction, used by Munro to perform the artificial insemination, constitutes a very selective and hostile medium for the sperm, subsequent studies aimed to bypass this junction by performing the insemination in close proximity to the infundibulum (i.e. the site of fertilization) in order to reassess the fertilizing ability of testicular sperm. Howarth (1983), consistently with Munro's results, found that the percentage of motile sperm from the testis was low but increases in the epididymis and, specially, in the vas deferens. The percentage of dead sperm was higher in the testis and the epididymis than in the vas deferens and the ejaculated semen. He did not find any significant differences between the sperm from the vas deferens and the ejaculated sperm. Moreover, performing intravaginal insemination, he showed that sperm from the testis do not produce fertile eggs. Interestingly, by contrast, performing intramagnal insemination of testicular, epididymal, vas deferens, and ejaculated sperm he did not find significant different results in both fertility and hatchability of fertile eggs through the first week elapsed from artificial insemination.

Subsequent molecular work provided more evidence to support the hypothesis that fowl sperm experience a gradual maturational process in the male reproductive tract. Esponda and Bedford (1985) found that testicular sperm did not bind to purified IgG against Wolffian duct (i.e. epididymis + vas deferens) seminal fluid. However, sperm recovered from the most proximal segment of the Wolffian duct (i.e. the epididymis) and, more intensely, from its middle or terminal segments were bound to the IgG. This fact is indicative of a different protein profile from

the initial and terminal regions of the Wolffian duct, as different proteins appear either de novo or in higher concentrations as sperm go through the Wolffian duct. However, the authors did not name these putative proteins. Following up this study, Morris *et al*. (1987) confirmed that testicular sperm do not bind the IgG but that sperm in the lower vas deferens display an intense immunospecific staining. They identified four proteins bound to sperm surface upon their passage through the Wolffian duct, of which those of Mr 30,000-40,000 and Mr 13,000 are similar in size, respectively, to the heavy and beta-2-microglobulin chains of the class 1 major histocompatibility (MHC) antigens (this protein was identified in both Red junglefowl and domestic fowl seminal fluid). Moreover, 48 hours following vaginal or direct intramagnal artificial insemination the authors reported that some of these proteins are still retained in the sperm present in the uterovaginal and infundibular tubal glands.

Kirby *et al.* (1990) found that roosters having morphological anomalies in the ductus efferents proximales presented reduced fertility following intravaginal artificial insemination when compared with unaffected roosters. Furthermore, following intravaginal artificial insemination of testicular sperm, neither affected nor unaffected roosters achieved fertilization. By contrast, when testicular sperm were inseminated in the magnum the fertility achieved by both kinds of roosters was high and equivalent. Based on these results, the even epithelial surface and greater luminal area in the ductus efferents proximales characteristic of the affected roosters, and the fact that the sperm appear to be mixed with seminal fluid components in the ductus efferents, the authors concluded that the reduced fertilizing capacity of sperm from affected roosters could be caused by their incapacity to get mixed with an optimal concentration of seminal fluid components within this region of the male reproductive tract. In line with these observations, seminal plasma from roosters carrying the sperm degeneration allele (i.e. mutant roosters) shows significant differences compared to seminal plasma from normal roosters, which are attributable to the existence of a dysfunction in the excurrent ducts of mutant roosters. In particular, seminal plasma from mutant

roosters contains 1.5 more protein concentration, a deficiency in polypeptides of 36 and 81 kDa (which suggests a relationship between sperm survival and seminal fluid proteins), a higher concentration of the electrolytes sodium, potassium, and chloride, a higher concentration of the aminoacids alanine, aspartic acid, glycine, histidine, serine, and threonine, and a lower concentration of glutamic acid, the most abundant aminoacid in fowl seminal plasma (Al Aghbari, Engel & Froman, 1992). Furthermore, the fertilizing ability of sperm from mutant roosters increases significantly after supplementation with albumin-depleted seminal plasma proteins from normal roosters prior to artificial insemination when compared to either supplementation with Bovine Serum Albumin or absence of supplementation (Al Aghbari, Engel & Froman, 1993). Interestingly, supplementation of sperm from normal roosters with seminal plasma proteins from mutant roosters did not cause an effect on fertility (Al Aghbari, 1992), indicating that the effect of seminal plasma protein supplementation results from a protein deficiency in the seminal plasma of mutant roosters (Al Aghbari, Engel & Froman, 1993). Janssen *et al.* (2000) found that males with stones in the epididymis present reduced fertility following both natural mating and artificial insemination, which suggests, according to the authors, that the process of sperm maturation may be disrupted by the epididymal stones and the associated inflammation.

Recent research strongly support the fact that, unlike mammalian sperm, fowl sperm do not undergo a complete maturation in the epididymis as this process continues during their passage through the proximal, middle, and distal vas deferens. In particular, sperm from the testis, epididymis, and the three segments (proximal, middle, and distal) of the vas deferens experience a gradual increase in survival under *in vitro* and *in vivo* conditions, fertilizing ability (Ahammad *et al.*, 2011c), motility, acrosomal proteolytic activity, sperm penetrating ability in the inner perivitelline layer of the egg (Ahammad *et al.*, 2011b), and binding capacity to both the uterovaginal junction and infundibular sperm storage tubules under both *in vitro* and *in vivo*

conditions (Ahammad *et al.*, 2011a). Interestingly, in all cases there are not significant differences in the ejaculated sperm (collected by massage) compared to the distal region of the vas deferens.

The results of my work are consistent with the idea of a continuous maturation of fowl sperm during their transit down the male reproductive tract as they revealed a considerable overlap with the domestic fowl sperm proteome (Labas *et al*., 2015) and a significant enrichment of seminal fluid proteins that are structural components of sperm. Moreover, they point to the possible presence of secreted vesicles (i.e. exosomes) that could sustain the final stages of sperm maturation in a manner analogous to those described in mammalian and insect models.

I also assessed the presence of Red junglefowl seminal fluid proteins predicted to contribute to sperm maturation in the sperm proteome of mice and humans. This analysis showed that a substantial number of these proteins are conserved components of sperm across birds and mammals, lending further support to the potential importance of seminal fluid-mediated sperm maturation.

(vi) Contribution of blood plasma proteins to seminal fluid

The vast majority of seminal fluid proteins in domestic chicken blood plasma (122 of 134) were present in the human plasma proteome. Moreover, 349 domestic chicken seminal fluid proteins were present in the human blood plasma. The comparison between the Red junglefowl and human seminal fluid proteomes revealed that 30% of the Red junglefowl seminal fluid proteome has related proteins in the human seminal fluid proteome (343 of 1,141). 71% of these proteins (244) were also identified in human blood plasma (Farrah *et al*., 2011). Therefore, blood plasma proteins comprise a large fraction of seminal fluid proteins identified in Red junglefowl and humans. Collectively, all these facts support the argument that the contribution of blood plasma

proteins to seminal fluid is both prevalent and likely to be a conserved feature of seminal fluid production in vertebrates.

(vii) Seminal fluid proteins associated with male age and/or sperm velocity

My study set out to provide the first characterization of the seminal fluid proteomic profiles associated with male age and/or sperm quality in a wild species of bird. The results of the PCA and NMDS showed that the group "old fast" emerges as different from the other three groups. The divergence of this group may be explained, to some extent, by the presence of 8 proteins that are expressed exclusively in all three males belonging to it. Of particular relevance is the fact that none of these proteins were found in the "old slow" samples of the replicated male.

Although it would be premature to draw any specific functional conclusions from these observations, interestingly, all 8 proteins expressed exclusively in all individuals of the "old fast" group are involved in processes related to calcium metabolism. In particular, the S100 proteins are calcium binding proteins involved in calcium homeostasis. Furthermore, this family of proteins is involved in the regulation of protein phosphorylation, transcription factors, dynamics of cytoskeleton constituents, enzymatic activities, cell growth and differentiation, and inflammatory response (Ji *et al*., 2014; Bresnick, Weber & Zimmer, 2015; Zackular, Chazin & Skaar, 2015). Calmodulin is a calcium messenger (Chin & Means, 2000) that controls sperm activation and hyperactivation in mammals, and activates kinases in chicken sperm that influence both viability and motility (Nguyen *et al*., 2016 a, b). The Guanine nucleotide-binding proteins (G proteins) are involved in the activation of ion channels, and in the regulation of the intracellular concentrations of secondary messengers, such as, cAMP, diacylglycerol, sodium or calcium cations (Svoboda *et al*., 2004). The myosin regulatory light chain proteins are involved in phosphorylation, and in calcium and magnesium binding (Morimoto & Harrington, 1974; Szczesna *et al*., 2001). The neuroblast differentiation-associated protein AHNAK-like, partial is implicated in calcium flux

regulation and constitutes a specific calcium dependent S100B target protein. Therefore, S100B and AHNAK may interact to regulate cellular calcium homeostasis (Gentil *et al*., 2001). Acyl-CoA-binding protein also has the potential to modulate calcium metabolic activity (Fulceri *et al*., 1997; Melloni *et al*., 2000). This protein also binds chain acyl-CoA esters with very high affinity and may function as an intracellular carrier of acyl-CoA esters (Rose, Schultz & Todaro, 1992).

Calcium constitutes a primary determinant of sperm motility in the fowl (Froman & Feltmann, 2005). Seminal plasma contains high levels of glutamate (Lake & Wishart, 1984), which is likely to determine the sperm mitochondrial uptake of calcium when the sperm are moving through the excurrent ducts of the testis (Froman & Kirby, 2005), thus facilitating the initiation of sperm motility upon ejaculation by acting as a motility agonist through the NMDA receptors channels, which are permeable to calcium and sodium ions (Aidley & Stanfield, 1996; Waxham, 1999; Froman, 2003). In fowl domesticated populations, sperm swimming velocity tends to decline as individual males age past their prime (Dean *et al*., 2010; Cornwallis *et al.*, 2014). As the average sperm VAP for the group "old fast" is intermediate between the group "young slow" and the group "young fast", it is reasonable to think that the exclusive expression of the aforementioned 8 proteins in old males could contribute to ameliorate the decline in sperm velocity associated with ageing by allowing the sperm to keep an adequate calcium metabolism. In addition to that, the first dimension of the PCA discriminates between ejaculates abundant in transparent fluid and sperm maturation proteins. This dimension shows that males of the "old fast" group retain relatively more sperm maturation proteins than males of the "old slow" group who produce a higher proportion of transparent fluid, enriched for blood plasma proteins (prothrombin, heparin, gelsolin, plasminogen, haemoglobin, transthyretin, apolipoprotein, and fibrinogen), in their ejaculates. Thus, this dimension can be interpreted as a gradient in transparent fluid: seminal plasma ratio, with negative loadings indicating a high proportion of transparent fluid in the ejaculates and positive loadings indicating an enrichment in exosome-related proteins that may

contribute to sperm maturation. This pattern would also contribute to explain why some males retain the capacity to produce relatively high quality sperm into old age. Furthermore, it can be argued that exosome and serum plasma components in the seminal fluid reflect two different functionalities (i.e. sperm maturation and “cheap filler”, respectively).

The proteins tyrosine phosphatase type IVA 1 and inosine-5'-monophosphate dehydrogenase 2 are also of particular interest since they are expressed exclusively in all 6 individuals belonging to one of the four widest groups (“old” and “slow sperm”, respectively). The protein tyrosine phosphatase type IVA 1 only appears in all old males, regardless of their sperm quality. For this reason, this protein seems to be associated with ageing, especially considering that it could be implicated in the development of tumors (Lu *et al*., 2012; Dong *et al.,* 2014; Jin *et al*., 2014). The gene controlling the expression of this protein is involved in cellular and metabolic processes, and catalytic activity. In turn, the protein inosine-5'-monophosphate dehydrogenase 2 is expressed exclusively in all 6 individuals with slow sperm, regardless of their age. Of particular relevance is the fact that the replicated old and young males only express this protein when belonging to a group with slow sperm, thus recapitulating the pattern of differential protein expression associated with low sperm VAP. Therefore, this protein seems to be associated with sperm velocity and, more specifically, with slowing down the sperm. Unfortunately, no hits for ontology categories were found for the gene controlling its expression. This protein participates in the synthesis of guanine nucleotides and plays a prominent role in the regulation of cell growth (Zhou *et al*., 2014; Zou *et al*., 2015).

Moreover, two different proteins involved in metabolic process were expressed exclusively in all young males with fast sperm (microsomal glutathione S-transferase 3) or in all three young males with slow sperm (nitrilase homolog 1 isoform X1). The glutathione S-transferases provide protection against oxidative stress and lipid peroxidation (Sharma *et al*., 2004; Singhal *et al*.,

2015). The nitrilase homolog 1 is involved in nitrogen compound metabolic process (GO: 0006807), and in hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds (GO: 0016810). Again, of particular interest is the fact that the replicated male only expresses the protein microsomal glutathione S-transferase 3 when belonging to the group "young fast", and the protein nitrilase homolog 1 isoform X1 when belonging to the group "young slow". These variations recapitulate the differential protein expression associated with sperm velocity across males in changes within these males.

Conclusions

The results of my thesis show that (1) sperm increase their fertilizing efficiency when exposed to non-self seminal fluid; (2) males tend to decrease ejaculate investment on a familiar female and to increase it on encountering a novel female consistent with the Coolidge effect; (3) subdominant males allocate more seminal fluid protein content on encountering novel females; (4) 1,141 proteins were identified in the Red junglefowl seminal fluid, including proteins involved in immunity, antimicrobial defences, sperm maturation, metabolism and fertilization, with quantitative and qualitative differences with the domestic fowl seminal fluid proteome, including a predominant contribution of blood plasma proteins that is conserved with humans; and (5) 8 proteins involved in calcium metabolism were found to be exclusively expressed in old males with fast sperm, which may contribute to explain the maintenance of relatively high quality sperm into old age.

To sum up, my thesis contributes to increase our understanding in sexual selection (and, more specifically, in sperm competition dynamics), avian seminal fluid, and the molecular basis of fertility, reproductive ageing, and domestication. These basic contributions set the stage for the development of techniques and functional investigations in applied fields, such as, assisted

reproduction, animal production, animal livestock, and captive breeding of endangered populations

REFERENCES

Abele, L.G. & Gilchrist, S. 1977. Homosexual rape and sexual selection in acanthocephalan worms. *Science* 197: 81-83.

Ahammad, M.U. *et al*. 2011a. Maturational changes in binding capacity of fowl sperm to the epithelium of the sperm storage tubules during their passage through the male reproductive tract. *Asian-Aust. J. Anim. Sci.* 24: 1199-1203.

Ahammad, M.U. *et al*. 2011b. Maturational changes in motility, acrosomal proteolytic activity, and penetrability of the inner perivitelline layer of fowl sperm, during their passage through the male genital tract. *Theriogenology*. 76: 1100-1109.

Ahammad, M.U. *et al*. 2011c. Maturational changes in the survivability and fertility of fowl sperm during their passage through the male reproductive tract. *Anim. Reprod. Sci.* 128: 129-136.

Aidley, D. & Stanfield, P. 1996. *Ion channels: molecules in action*. New York: Cambridge University Press.

Al-Aghbari, A.M. 1992. Demonstration of a Link Between Seminal Plasma Proteins and Male Fertility in the Domestic Fowl (Gallus Domesticus). PhD dissertation. University of Oregon.

Al-Aghbari, A.M., Engel Jr., H.N. & Froman, D.P. 1992. Analysis of seminal plasma from roosters carrying the *Sd* (sperm degeneration) allele. *Biol. Reprod.* 47: 1059-1063.

Al-Aghbari, A.M., Engel Jr., H.N. & Froman, D.P. 1993. Modulation of the fertilizing ability of spermatozoa from roosters carrying the *Sd* (sperm degeneration) allele. *Biol. Reprod*. 48: 308-312.

Alonzo, S.H. & Pizzari, T. 2010. Male fecundity stimulation: Conflict and cooperation within and between the sexes: Model analyses and coevolutionary dynamics. *Am. Nat.* 175: 174-185.

Alonzo, S.H. & Pizzari, T. 2013. Selection on female remating interval is influenced by male sperm competition strategies and ejaculate characteristics. *Philos. T. Roy. Soc. B*. 368: 20120044.

Andersson, M. 1994. *Sexual selection*. Princeton University Press, Princeton, NJ.

Andersson, M. & Simmons, L.W. 2006. Sexual selection and mate choice. *T.Ecol.Evol.* 21: 296-302.

Arnqvist, G. & Rowe, L. 2002. Correlated evolution of male and female morphologies in water striders. *Evolution* 56: 936-947.

Arnqvist, G. & Rowe, L. 2005. *Sexual conflict*. Princeton University Press, Princeton, NJ.

Ashizawa, K. & Okauchi, K. 1984. Stimulation of sperm motility and oxygen consumption of fowl spermatozoa by a low molecular weight fraction of seminal plasma. *J. Reprod. Fertil.* 71: 593-598.

Assreuy, A.M.S. *et al*. 2002. Spermadhesin PSP-I/PSP-II heterodimer and its isolated subunits induced neutrophil migration into the peritoneal cavity of rats. *Biol. Reprod*. 67: 1796-1803.

Assreuy, A.M.S. *et al*. 2003. Porcine spermadhesin PSP-I/PSP-II stimulates macrophages to release a neutrophil chemotactic substance: Modulation by mast cells. *Biol. Reprod*. 68: 1836-1841.

Avila, F.W. *et al*. 2011. Insect seminal fluid proteins: Identification and function. *Annu. Rev. Entomol.* 56: 21-40.

Baer, B., Morgan, E.D. & Schmid-Hempel, P. 2001. A nonspecific fatty acid within the bumblebee mating plugs prevents females from remating. *Proc. Natl. Acad. Sci. U.S.A.* 98: 3926-3928.

Barnes, A.I. *et al.* 2008. Feeding, fecundity and lifespan in female *Drosophila melanogaster*. *Proc. R. Soc. B*. 275: 1675-1683.

Bell, B. & Koufopanou, V. 1986. The cost of reproduction. In: Dawkins, R. & Ridley, M. *Oxford surveys in evolutionary biology, vol. 3* (pp. 83-131). Oxford: Oxford University Press.

Bing, C. *et al*. 2004. Zinc-α2-glycoprotein, a lipid mobilizing factor, is expressed in adipocytes and is up-regulated in mice with cancer cachexia. *Proc. Natl. Acad. Sci. U.S.A.* 101: 2500-2505.

Birkhead, T.R. 2000. *Promiscuity*. Faber and Faber, London, UK.

Birkhead, T.R. & Møller, A.P. 1992. *Sperm competition in birds: evolutionary causes and consequences*. London: Academic Press.

Birkhead, T.R. & Pizzari, T. 2002. Postcopulatory sexual selection. *Nature* 3: 262-273.

Birkhead, T.R. *et al*. 1999. Sperm mobility determines the outcome of sperm competition in the domestic fowl. *Proc. R. Soc. B*. 266: 1759-1764.

Bonduriansky, R. & Chenoweth, S.F. 2009. Intralocus sexual conflict. *T.Ecol.Evol.* 24:280-288.

Braisted, J.C. *et al.* 2008. The APEX quantitative proteomics tool: generating protein quantitation estimates from LC-MS/MS proteomics results. *BMC Bioinformatics*. 9: 529.

Bresnick, A.R., Weber, D.J. & Zimmer, D.B. 2015. S100 proteins in cancer. *Nat. Rev. Cancer* 15: 96-109.

Bretman A,. Fricke, C. & Chapman, T. 2009. Plastic responses of male *Drosophila melanogaster* to the level of sperm competition increase male reproductive fitness. *Proc. R. Soc. B*. 276: 1705-1711.

Bretman, A., Newcombe, D. & Tregenza, T. 2009. Promiscuous females avoid inbreeding by controlling sperm storage. *Mol. Ecol.* 18: 3340-3345.

Brillard, J. 1993. Sperm storage and transport following natural mating and artificial insemination. *Poult. Sci*. 72: 923-928.

Burke, T. *et al.* 1989. Parental care and mating behaviour of polyandrous dunnocks *Prunella modularis* related to paternity by DNA fingerprinting. *Nature* 338: 249-251.

Burrows, W.H. & Quinn, J.P. 1935. A method of obtaining spermatozoa from the domestic fowl. *Poult. Sci*. 14, 251–254.

Butts, I. A. E. *et al*. 2011. Seminal plasma biochemistry and spermatozoa characteristics of Atlantic cod (*Gadus morhua* L.) of wild and cultivated origin. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* 159: 16-24.

Callaway, E. 2014. Chicken project gets off the ground. *Nature*. 509: 546.

Calvete, J.J. *et al.* 1995. Spermadhesins: sperm-coating proteins involved in capacitation and zona pellucida binding. *Fertilitat*. 11: 35-40.

Cameron, E., Day, T. & Rowe, L. 2007. Sperm competition and the evolution of ejaculate composition. *Am*. *Nat*. 169: E158-172.

Carazo, P., Molina-Vila, P. & Font, E. 2011. Male reproductive senescence as a potential source of sexual conflict in a beetle. *Behav. Ecol.* 22: 192-198.

Carvalho, G. B. *et al*. 2006. Allocrine modulation of feeding behavior by the sex peptide of *Drosophila*. *Curr. Biol*. 16: 692–696.

Chandler, C.H., Ofria, C. & Dworkin, I. 2012. Runaway sexual selection leads to good genes. *Evolution* 67: 110-119.

Chantepie, S. *et al.* 2015. Quantitative genetics of the aging of reproductive traits in the houbara bustard. *PLoS One.* 10: e0133140.

Chapman, T. 2001. Seminal fluid-mediated fitness traits in *Drosophila*. *Heredity* 87: 511-521.

Chapman, T. 2006. Evolutionary conflicts of interest between males and females. *Curr*. *Biol.* 16: 744-754.

Chapman, T. 2008. The soup in my fly: evolution, form and function of seminal fluid proteins. *PLoS B.* 6: e179.

Chapman, T. & Davies, S.J. 2004. Functions and analysis of the seminal fluid proteins of male *Drosophila melanogaster* fruit flies. *Peptides*. 25: 1477-1490.

Chapman, T., Hutchings, J. & Partridge, L. 1993. No reduction in the cost of mating for *Drosophila melanogaster* females mating with spermless males. *Proc. R. Soc. B*. 253: 211-217.

Chapman, T. *et al.* 1995. Cost of mating in *Drosophila melanogaster* females is mediated by accessory gland products. *Nature* 373: 241-244.

Chapman, T. *et al*. 2000. The role of male accessory gland protein Acp36DE in sperm competition in *Drosophila melanogaster*. *Proc. R. Soc. B*. 267: 1097-1105.

Chapman, T. *et al.* 2003. The sex peptide of *Drosophila melanogaster*: Female post-mating responses analyzed by using RNA interference. *Proc. Natl. Acad. Sci. U.S.A.* 100: 9923-9928.

Chauvin, T. *et al.* 2012. A Systematic analysis of a deep mouse epididymal sperm proteome. *Biol. Reprod.* 87: 141.

Chen, P.S. & Balmer, J. 1989. Secretory proteins and sex peptides of the male accessory gland in *Drosophila sechellia*. *J. Ins. Physiol.* 35: 759-764.

Chin, D. & Means, A. R. 2000. Calmodulin: a prototypical calcium sensor. *Trends Cell. Biol.* 10: 322-328.

Clutton-Brock, T.H. & Parker, G.A. 1992. Potential reproductive rates and the operation of sexual selection. *Q. Rev. Biol.* 67: 437-456.

Clutton-Brock, T.H. & Vincent, A.C.J. 1991. Sexual selection and the potential reproductive rates of males and females. *Nature* 351: 58-60.

Collet, J. 2010. The operation of sexual selection in the red junglefowl. DPhil dissertation. University of Oxford.

Collias, N.E. & Collias, E.C. 1996. Social organization of a red junglefowl, *Gallus gallus*, population related to evolution theory. *Anim. Behav*. 51: 1337-1354.

Collias, N.E, Collias, E.C. & Jennrich, R.I. 1994. Dominant red junglefowl (*Gallus gallus*) hens in an unconfined flock rear the most young over their lifetime. *Auk* 111: 863-872.

Connallon, T. & Clark, A.G. 2011. The resolution of sexual antagonism by gene duplication. *Genetics* 187: 919-937.

Cook, B.J. & Wagner, R.M. 1992. Some pharmacological properties of the oviduct muscularis of the stable fly *Stomoxys calcitrans*. *Comp. Biochem. Physiol. C* 102: 273–280.

Cornwallis, C.K. 2004. The reproductive tactics of male and female fowl, *Gallus gallus*. PhD thesis. University of Sheffield.

Cornwallis, C.K. & Birkhead, T.R. 2006. Social status and availability of females determine patterns of sperm allocation in the fowl. *Evolution.* 60: 1486-1493.

Cornwallis, C.K. & Birkhead, T.R. 2007. Changes in sperm quality and numbers in response to experimental manipulation of male social status and female attractiveness. *Am. Nat.* 170: 758-770.

Cornwallis, C.K. & Birkhead, T.R. 2008. Plasticity in reproductive phenotypes reveals status-specific correlations between behavioral, morphological, and physiological sexual traits. *Evolution*. 62: 1149-1161.

Cornwallis, C.K. & O'Connor, E.A. 2009. Sperm: seminal fluid interactions and the adjustment of sperm quality in relation to female attractiveness. *Proc. R. Soc. B*. 276: 3467-3475.

Cornwallis, C.K., Dean, R. & Pizzari, T. 2014. Sex-specific patterns of aging in sexual ornaments and gametes. *Am. Nat*. 184: E66-E78.

Corrigan, L. *et al*. 2014. BMP-regulated exosomes from *Drosophila* male reproductive glands reprogram female behavior. *J. Cell. Biol*. 206, 671-688.

Craig, J.V., Ortman, L.L. & Guhl, A.M. 1965. Genetic selection for social dominance ability in chicks. *Anim. Behav*. 13: 114-131.

Craig, R. & Beavis, R.C. 2004. TANDEM: Matching proteins with tandem mass spectra. *Bioinformatics*. 20: 1466-1467.

Crawley, M.J. 2005. *Statistics. An introduction using R.* Chichester: Wiley.

Darwin, C. 1871. *The descent of man, and selection in relation to sex*. London: John Murray.

Davies, N.B., Krebs, J.R. & West, S.A. 2012. *An introduction to behavioural ecology*. Oxford: Oxford University Press.

Dawkins, R. & Krebs, J.R. 1979. Arms races between and within species. *Proc. R. Soc. B*. 205: 489-511.

Dean, M. D. 2013. Genetic disruption of the copulatory plug in mice leads to severely reduced fertility. *PLoS Genet*. 9, e1003185.

Dean, M. D. *et al*. 2011. Identification of ejaculated proteins in the house mouse (*Mus domesticus*) via isotopic labelling. *BMC Genom*. 12.

Dean, R., Nakagawa, S. & Pizzari, T. 2011. The risk and intensity of sperm ejection in female birds. *Am. Nat.* 178: 343-354.

Dean, R. *et al.* 2010. Male reproductive senescence causes potential for sexual conflict over mating. *Curr. Biol*. 20: 1192-1196.

Decuypere, E. *et al*. 2010. Ethical, genetic and physiological perspectives, and suggestions for solutions. *Br. Poult. Sci.* 51: 569-579.

del Barco-Trillo, J. & Ferkin, M.H. 2004. Male mammals respond to a risk of sperm competition conveyed by odours of conspecific males. *Nature*. 431: 446-449.

Delacour, J. 1951. *The pheasants of the world*. London: Country Life Limited.

den Boer, S.P.A., Baer, B. & Boomsma, J.J. 2010. Seminal fluid mediates ejaculate competition in social insects. *Science* 327: 1506-1509.

Deutsch, E. W. *et al*. 2010. A guided tour of the Trans-Proteomic Pipeline. *Proteomics* 10, 1150-1159.

Dewsbury, D. A. 1981. Effects of novelty on copulatory behavior: the Coolidge effect and related phenomena. *Psychol. Bull.* 89: 464-482.

Ding, Z. *et al*. 2007. Identification of sperm forward motility-related proteins in human seminal plasma. *Mol. Reprod. Dev.* 74: 1124–1131.

Domanitskaya, E.V. *et al.* 2007. The hydroxyproline motif of male sex peptide elicits the innate immune response in *Drosophila* females. *FEBS J.* 274: 5659-5668.

Donato, R. *et al*. 2013. Functions of S100 proteins. *Curr. Mol. Med*. 13: 24-57.

Dong, J. *et al*. 2014. MicroRNA-26a inhibits cell proliferation and invasion of cervical cancer cells by targeting protein tyrosine phosphatase type IVA 1. Molecular Medicine Reports. 10: 1426-1432.

Dorus, S., Skerget, S. & Karr, T.L. 2012. Proteomic discovery of diverse immunity molecules in mammalian spermatozoa. *Sys. Biol. Repr. Med.* 58: 218-228.

Dorus, S. *et al.* 2004. Rate of molecular evolution of the seminal protein gene SEMG2 correlates with levels of female promiscuity. *Nat. Genet.* 36: 1326-1329.

Eberhard, W.G. 1996. *Female control: sexual selection by cryptic female choice.* Princeton: Princeton University Press.

Egger, B. *et al*. 2011. A sensory bias has triggered the evolution of egg-spots in Cichlid fishes. *PLoS One* 6: e25601.

Emlen, S.T. & Oring, L.W. 1977. Ecology, sexual selection, and the evolution of mating systems. *Science* 197: 215-223.

Eng, J. K., Jahan, T. A. & Hoopmann, M. R. 2013. Comet: an open-source MS/MS sequence database search tool. *Proteomics* 13: 22-24.

Eriksson, J. *et al*. 2008. Identification of the *yellow skin* gene reveals a hybrid origin of the domestic chicken. *PLoS G.* 4: e1000010.

Esponda, P. & Bedford, J.M. 1985. Surface of the rooster spermatozoa changes in passing through the Wolffian duct. *J. Exp. Zool.* 234: 441–449.

Etches, R.J. 1996. *Reproduction in poultry*. Oxon: CAB International.

Fardilha, M. *et al*. 2011. Protein phosphatase 1 complexes modulate sperm motility and present novel targets for male infertility. *Mol. Hum. Reprod.* 17: 466-477.

Farrah, T. *et al.* 2011. A high-confidence human plasma proteome reference set with estimated concentrations in PeptideAtlas. *Mol. Cell. Proteomics* 10: M110 006353.

Fedorka, K.M. Winterhalter, W.E. & Ware, B. 2011. Perceived sperm competition intensity influences seminal fluid protein production prior to courtship and mating. *Evolution*. 65: 584-590.

Fincke, O.M. 1984. Sperm competition in the damselfly *Enallagma hageni* Walsh (Odonata: Coenagrionidae): benefits of multiple matings to males and females. *Behav*. *Ecol*. *Sociob.* 14: 235-240.

Findlay, G.D. *et al.* 2008. Proteomics reveals novel *Drosophila* seminal fluid proteins transferred at mating. *PLoS B*. 6: e178.

Finseth, F.R., Bondra, E. & Harrison, R.G. 2014. Selective constraint dominates the evolution of genes expressed in a novel reproductive gland. *Mol. Biol. Evol.* 31: 3266-3281.

Fisher, R.A. 1930. *The genetical theory of natural selection*. Oxford: The Clarendon Press.

Flink, L.G. *et al.* 2014. Establishing the validity of domestication genes using DNA from ancient chickens. *Proc. Natl. Acad. Sci. U.S.A.* 111: 6184-6189.

Forsberg, G. *et al.* 1990. β-Endorphin acts on the reproductive tract of female rats to suppress sexual receptivity. *Neurosci. Lett.* 115: 92-96.

Fricke, C., Bretman, A. & Chapman, T. 2010. Sexual conflict. In: Westneat, D.F. & Fox, C.W. *Evolutionary Behavioral Ecology* (pp. 400-415). Oxford: Oxford University Press.

Fricke, C. *et al*. 2009. The benefits of male ejaculate sex peptide transfer in *Drosophila melanogaster*. *J. Evol. Biol*. 22: 275-286.

Friedländer, M., Jeshtadi, A. & Reynolds, S.E. 2001. The structural mechanism of trypsin-induced intrinsic motility in *Manduca sexta* spermatozoa in vitro. *J. Insect Physiol*. 47: 245-255.

Froman, D.P. 1995. Biology of semen production and ejaculation. In: Bakst, M.R. & Wishart, G.J. *Proc. First. Int. Symp. Art. Insem. Poult*. (pp.21-38). Poultry Science Association.

Froman, D.P. 2003. Deduction of a model for sperm storage in the oviduct of the domestic fowl (*Gallus domesticus*). *Biol. Reprod*. 69: 248-253.

Froman, D. P. & Feltmann, A.J. 1998. Sperm mobility: A quantitative trait of the domestic fowl (*Gallus domesticus*). *Biol. Reprod.* 58: 379–384.

Froman, D. P. & Feltmann, A. J. 2005. Fowl (Gallus domesticus) sperm motility depends upon mitochondrial calcium cycling driven by extracellular sodium. *Biol. Reprod.* 72: 97-101.

Froman, D.P. & Kirby, J.D. 2005. Sperm mobility: Phenotype in roosters (*Gallus domesticus*) determined by mitochondrial function. *Biol. Reprod*. 72: 562-567.

Froman, D.P., Wardell, J.C. & Feltmann, A.J. 2006. Sperm mobility: Deduction of a model explaining phenotypic variation in roosters (*Gallus domesticus*). *Biol. Reprod*. 74: 487-491.

Froman, D. P. *et al*. 1999. Sperm mobility: A primary determinant of fertility in the domestic fowl (*Gallus domesticus*). *Biol. Reprod.* 61: 400-405.

Froman, D. P. *et al*. 2002. Sperm mobility: mechanisms of fertilising efficiency, genetic variation and phenotypic relationship with male social status in the fowl, *Gallus g. domesticus*. *Proc. R. Soc. B*. 269: 607-612.

Froman, D.P. *et al.* 2011. Physiology and endocrinology symposium: A proteome-based model for sperm mobility phenotype. *J. Anim. Sci.* 89: 1330-1337.

Fry, C.L. & Wilkinson, G.S. 2004. Sperm survival in female stalk-eyed flies depends on seminal fluid and meiotic drive. *Evolution* 58: 1622-1624.

Fujihara, N. 1992. Accessory reproductive fluids and organs in male domestic birds. *World Poultry Sci. J.* 48: 39-56.

Fujihara, N. & Howarth Jr., B. 1978. Lipid peroxidation in fowl spermatozoa. *Poultry Sci.* 57: 1766-1768.

Fulceri, R. *et al.* 1997. Fatty acyl-CoA-acyl-CoA-binding protein complexes activate the Ca2+ release channel of skeletal muscle sarcoplasmic reticulum. *Biochem. J*. 325: 423-428.

Fumihito, A. *et al.* 1996. Monophyletic origin and unique dispersal patterns of domestic fowls. *Proc. Natl. Acad. Sci. U.S.A.* 93: 6792-6795.

Gage, M.J.G. 1991. Risk of sperm competition directly affects ejaculate size in the Mediterranean fruit fly. *Anim. Behav*. 42: 1036-1037.

Gage, M.J.G. 2003. Scramble for the eggs. *Nature*. 426: 22-23.

Gasparini, C. & Pilastro, A. 2011. Cryptic female preference for genetically unrelated males is mediated by the ovarian fluid in the guppy. *Proc. R. Soc. B*. 278: 2495-2501.

Gavrilets, S. 2000. Rapid evolution of reproductive barriers driven by sexual conflict. *Nature* 403: 886-889.

Gavrilets, S. & Hayashi, T.I. 2005. Speciation and sexual conflict. *Evol. Ecol.* 19: 167-198.

Gentil, B.J. *et al*. 2001. The giant protein AHNAK is a specific target for the calcium-and zinc-binding S100B protein: potential implications for Ca2+ homeostasis regulation by S100B. *J. Biol. Chem*. 276: 23253-23261.

Gershman, S.N. & Sakaluk, S.K. 2009. No Coolidge effect in decorated crickets. *Ethology* 115: 774-780.

Gilbert, L.E. 1976. Postmating female odor in *Heliconius* butterflies: A male-contributed antiaphrodisiac? *Science* 193: 419-420.

Gilchrist, A.S. & Partridge, L. 1995. Male identity and sperm displacement in *Drosophila melanogaster*. *J. Ins. Phys*. 41: 1087-1092.

Gillingham, M. 2007. MHC and sexual selection in the fowl, *Gallus gallus*. DPhil dissertation. University of Oxford.

Gioti, A. *et al.* 2012. Sex peptide of *Drosophila melanogaster* males is a global regulator of reproductive processes in females. *Proc. R. Soc. B*. 279: 4423-4432.

Girouard, J., Frenette, G. & Sullivan, R. 2009. Compartmentalization of proteins in epididymosomes coordinates the association of epididymal proteins with the different functional structures of bovine spermatozoa. *Biol. Reprod*. 80: 965-972.

Goeritz, F. *et al.* 2003. Seasonal timing of sperm production in roe deer: interrelationship among changes in ejaculate parameters, morphology and function of testis and accessory glands. *Theriogenology*. 59: 1487-1502.

Gutsche, S. *et al.* 2003. Seminal plasma induces mRNA expression of IL-1β, IL-6 and LIF in endometrial epithelial cells in vitro. *Mol. Hum. Reprod*. 9: 785-791.

Gwathmey, T.M. *et al*. 2006. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30 kDa share functional roles in storing sperm in the oviduct. *Biol. Reprod.* 75: 501–507.

Haase, B. *et al.* 2005. Evolution of the spermadhesin gene family. *Gene*. 352: 20-29.

Hadjisavas, M., Laurenz, J.C. & Bazer, F.W. 1994. Seminal Plasma (SPL): a potential mediator of inflammation in the uterus following mating in the pig. *Biol. Reprod*. 50 (Suppl. I): 73-78.

Hajirezaee, S., Amiri, B.M. & Mirvaghefi, A.R. 2010. Changes in sperm production, sperm motility, and composition of seminal fluid in Caspian brown trout, *Salmo trutta caspius*, over the course of a spawning season. *J. Appl. Aquacult.* 22: 157-170.

Halliday, T.R. 1983. The study of mate choice. In: Bateson, P. *Mate choice* (pp. 3-32). Cambridge, UK: Cambridge University Press.

Hamilton, W.D. 1964. The genetical evolution of social behaviour. I. *J. Theoret. Biol*. 7: 1-16.

Hartmann, R. & Loher, W. 1999. Post-matting effects in the grasshopper, *Gomphocerus rufus* L. mediated by the spermatheca. *J. Comp. Physiol. A.* 184: 325-332.

Haubruge, E. *et al*. 1999. Fertilization by proxy: rival sperm removal and translocation in a beetle. *Proc. R. Soc. B*. 266: 1183-1187.

Heifetz, Y. *et al.* 2005. Two cleavage products of the *Drosophila* accessory gland protein ovulin can independently induce ovulation. *Proc. Natl. Acad. Sci. U.S.A.* 102: 743-748.

Hernandez-Lopez, L. *et al.* 2012. Aging-related reproductive decline in the male spider monkey (*Ateles geoffroyi*). *J. Med. Primatol.* 41: 115-121.

Herndon, L.A. & Wolfner, M.F. 1995. A *Drosophila* seminal fluid protein, Acp26Aa, stimulates egg laying in females for 1 day after mating. *Proc. Natl. Acad. Sci. U.S.A.* 92: 10114-10118.

Hill, R.W., Wyse, G.A. & Anderson, M. 2004. *Animal physiology*. Massachusetts: Sinauer Associates, Inc.

Hocking, P. *et al*. 2001. Welfare assessment of modified rearing programmes for broiler breeders. *Brit. Poultry Sci.* 42: 424-432.

Hodgson, D.J. & Hosken, D.J. 2006. Sperm competition promotes the exploitation of rival ejaculates. *J. Theoret. Biol.* 243: 230-234.

Holland, B. & Rice, W.R. 1998. Perspective: Chase-away sexual selection: Antagonistic seduction versus resistance. *Evolution* 52: 1-7.

Holman, L. 2009. *Drosophila melanogaster* seminal fluid can protect the sperm of other males. *Funct. Ecol*. 23: 180-186.

Howard, R.D. 1978. The evolution of mating strategies in bullfrogs, *Rana catesbeiana. Evolution* 32: 850-871.

Howarth Jr., B. 1983. Fertilizing ability of cock spermatozoa from the testis, epididymis and vas deferens following intramagnal insemination. *Biol. Reprod*. 28: 586–590.

Huang da, W., Sherman, B. T. & Lempicki, R. A. 2009a. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res*. 37: 1-13.

Huang da, W., Sherman, B. T. & Lempicki, R. A. 2009b. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4: 44-57.

Husby, A., Ekblom, R. & Qvarnstrom, A. 2011. Let's talk turkey: immune competence in domestic and wild fowl. *Heredity* 107: 103-104.

Ivanova-Kicheva, M. & Dimov, G. 2011. Influence of selected seminal plasma proteins on mitochondrial integrity and speed parameters of ram sperm stored at low temperature. *Biotechnol. Biotec. Eq.* 25: 2591-2596.

Janicke, T. *et al*. 2016. Darwinian sex roles confirmed across the animal kingdom. *Sci. Adv.* 2: e1500983.

Janssen, S.J. *et al*. 2000. Identification of epididymal stones in diverse rooster populations. *Poultry Sci.* 79: 568-574.

Jennions, M.D. & Kokko, H. 2010. Sexual selection. In: Westneat, D.F. & Fox, C.W. *Evolutionary Behavioral Ecology* (pp. 343-364). Oxford: Oxford University Press.

Ji, Y. *et al*. 2014. S100 family signaling network and related proteins in pancreatic cancer (review). *Int. J. Mol. Med.* 33: 769-776.

Jin, S. *et al*. 2014. Oncogenic function and prognostic significance of protein tyrosine phosphatase PRL-1 in hepatocellular carcinoma. *Oncotarget* 5: 3685-3696.

Jones, A.G. & Ratterman, N. L. 2009. Mate choice and sexual selection: What have we learned since Darwin? *Proc. Natl. Acad. Sci. USA.* 106: 10001-10008.

Jones, O.R. *et al*. 2014. Diversity of ageing across the tree of life. *Nature*. 505: 169-173.

Kalb, J.M., Dibenedetto, A.J. & Wolfner, M.F. 1993. *Probing the function of Drosophila melanogaster* accessory glands by directed cell ablation. *Proc. Natl. Acad. Sci. U.S.A.* 90: 8093-8097.

Keerthikumar, S. *et al*. 2016. ExoCarta: A Web-Based Compendium of Exosomal Cargo. *J. Mol. Biol.* 428: 688-692.

Kelly, C.D. & Jennions, M.D. 2011. Sexual selection and sperm quantity: meta-analyses of strategic ejaculation. *Biol. Rev.* 86: 863-864.

Kempenaers, B., Verheyen, G.R. & Dhondt, A.A. 1997. Extrapair paternity in the blue tit (*Parus caeruleus*): female choice, male characteristics, and offspring quality. *Behav. Ecol.* 8: 481-492.

Khan, R.U. 2011. Antioxidants and poultry semen quality. *World Poultry Sci. J.* 67: 297-308.

Kirby, J.D. *et al.* 1990. Decreased spermatozoal survivability associated with aberrant morphology of the ductuli efferentes proximales of the chicken (*Gallus domesticus*). *Biol. Reprod*. 42: 383–389.

Kirkpatrick, M. & Barton, N.H. 1997. The strength of indirect selection on female mating preferences. *Proc. Natl. Acad. Sci. USA.* 94: 1282-1286.

Kirkwood, T.B.L. 2001. Sex and ageing. *Exp. Gerontol.* 36: 413–418.

Kirkwood, T.B.L. & Austad, S.N. 2000. Why do we age? *Nature*. 408: 233–238.

Klowden, M.J. 2001. Sexual receptivity in *Anopheles gambiae* mosquitoes: Absence of control by male accessory gland substances. *J. Insect Physiol*. 47: 661-666.

Klowden, M.J. 2006. Switchover to the mated state by spermathecal activation in female *Anopheles gambiae* mosquitoes. *J. Insect Physiol*. 52: 679-684.

Koene, J.M. & Ter Maat, A. 2007. Coolidge effect in pond snails: male motivation in a simultaneous hermaphrodite. *BMC Evol. Biol.* 7: 212.

Kokko, H., Jennions, M.D. & Brooks, R. 2006. Unifying and testing models of sexual selection. *Annu. Rev. Ecol. Evol. Syst.* 37: 43-66.

Krzanowski, J. 1979. Between-groups comparison of principal components. *J Am Stat Assoc* 74: 703-707.

Labas, V. *et al.* 2015. Qualitative and quantitative peptidomic and proteomic approaches to phenotyping chicken semen. *J. Proteomics.* 112: 313-335.

Lahnsteiner, F. *et al*. 1996. Motility of spermatozoa of *Alburnus alburnus* (Cyprinidae) and its relationship to seminal plasma composition and sperm metabolism. *Fish Physiol. Biochem.* 15: 167-179.

Lahnsteiner, F. *et al*. 1998. Determination of semen quality of the rainbow trout, *Oncorhynchus mykiss*, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. *Aquaculture*. 163: 163-181.

Lake, P.E. 1956. A retarding factor in the problem of fowl semen storage. *Proceedings of 3rd International Congress on Animal Reproduction, Cambridge, UK*, vol. 3 (pp. 104-106). London: Printed by Brown Knight & Truscott.

Lake, P.E. 1966. Physiology and biochemistry of poultry semen. In: McLaren, A. *Advances in reproductive physiology* (pp. 93-123). London: Logos Press.

Lake, P.E. 1971. The male in reproduction. In: Bell, D.J. & Freeman, B.M. *Physiology and Biochemistry* of *the Domestic Fowl, vol.3* (pp. 1411-1447). London: Academic Press.

Lake, P.E. 1981. Male genital organs. In: King, A.S. & McLelland, J. *Form and Function in Birds, Vol. 2 (pp. 1-61).* London: Academic Press.

Lake, P.E. 1984. The male in reproduction. In: Freeman, B.M. *Physiology and biochemistry of the domestic fowl*, *vol. 5* (pp. 381-405). London: Academic Press.

Lake, P.E. & Ravie, O. 1987. Effect on fertility of low numbers of fowl spermatozoa inseminated in aqueous diluent or semen components of the fowl and turkey. *Br. Poult. Sci.* 28: 75-80.

Lake, P.E. & Wishart, G.J. 1984. Comparative physiology of turkey and fowl semen. In: Cunningham F.J., Lake, P.E. & Hewitt, D. *Reproductive Biology of Poultry* (pp. 151-160). Harlow: Longman Group.

Lange, A.B. 2009a. Neural mechanisms coordinating the female reproductive system in the locust. *Front. Biosci.* 14: 4401–4415.

Lange, A.B. 2009b. The female reproductive system and control of oviposition in Locusta migratoria migratorioides. *Can. J. Zool.* 87: 649-661.

Laughlin, K. 2009. Breeder management: how did we get here?. In: P.M. Hocking. *Biology of Breeding Poultry, Poultry Science Symposium Series, vol.3* (pp. 10-26). Wallingford: CAB International.

Liu, H. & Kubli, E. 2003. Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 100: 9929-9933.

Liu, X. *et al*. 2014. Proteome profiling of the sperm maturation milieu in the rhesus monkey (Macaca mulatta) epididymis. *Reprod. Fert. Develop*. 28: 732-741.

Locatello, L., Poli, F. & Rasotto, M.B. 2013. Tactic-specific differences in seminal fluid influence sperm performance. *Proc. R. Soc. B*. 280: 1755.

Løvlie, H., Cornwallis, C.K. & Pizzari, T. 2005. Male mounting alone reduces female promiscuity in the fowl. *Curr. Biol.* 15: 1222-1227.

Løvlie, H. *et al*. 2013. Cryptic female choice favours sperm from major histocompatibility complex-dissimilar males. *Proc. R. Soc. B*. 280: 20131296.

Lu, J.W. *et al*. 2012. Increased expression of PRL-1 protein correlates with shortened patient survival in human hepatocellular carcinoma. *Clin. Transl. Oncol.* 14: 287-293.

Lung, O., Kuo, L. & Wolfner, M.F. 2001. *Drosophila* males transfer antibacterial proteins from their accessory gland and ejaculatory duct to their mates. *J. Insect Physiol*. 47: 617-622.

Ma, Y. *et al*. 2014. Towards an animal model of ovarian cancer: Cataloging chicken blood proteins using combinatorial peptide ligand libraries coupled with shotgun proteomic analysis for translational research. *OMICS A J. Integr. Biol.* 18: 280-297.

Mann, T. 1964. *The biochemistry of semen and of the male reproductive tract*. London: Methuen.

Martin, O.Y. & Hosken, D.J. 2003. The evolution of reproductive isolation through sexual conflict. *Nature*. 423: 979-982.

Maynard-Smith, J. 1956. Fertility, mating behaviour and sexual selection in *Drosophila suboscura*. *J. Genet.* 54: 261-279.

McDonald, G. C. & Pizzari, T. 2016. Why patterns of assortative mating are key to study sexual selection and how to measure them. *Behav Ecol Sociob.* 70: 209-220.

McGraw, L. A., Suarez, S. S. & Wolfner, M. F. 2015. On a matter of seminal importance: Insights and perspectives. *BioEssays*. 37: 142-147.

McGraw, L.A. *et al.* 2004. Genes regulated by mating, sperm, or seminal proteins in mated female *Drosophila melanogaster*. *Curr. Biol.* 14: 1509-1514.

Melloni, E. *et al.* 2000. Acyl-coA-binding protein is a potent m-calpain activator. *J. Biol. Chem.* 275: 82-86.

Middleton, C.A. *et al*. 2006. Neuromuscular organization and aminergic modulation of contractions in the *Drosophila* ovary. *BMC Biol.* 4:17.

Miyata, H. *et al*. 2012. Protease activation and the signal transduction pathway regulating motility in sperm from the water strider *Aquarius remigis*. *Cytoskeleton*. 69: 207-220.

Møller, A.P. 1998. Sperm competition and sexual selection. In: Birkhead, T.R. & Møller, A.P. *Sperm competition and sexual selection*. London: Academic Press.

Møller, A.P. *et al*. 2009. Senescence sperm performance in old male birds. *J. Evol. Biol.* 22: 334-344.

Morimoto, K. & Harrington, W.F. 1974. Evidence for structural changes in vertebrate thick filaments induced by calcium. *J. Mol. Biol*. 88: 693-709.

Morris, S.A. *et al.* 1987. Specificity of sperm-binding Wolffian duct proteins in the rooster and their persistence on spermatozoa in the female host glands. *J. Exp. Zool.* 242: 189–198.

Mueller, J.L., Page, J.L. & Wolfner, M.F. 2007. An ectopic expression screen reveals the protective and toxic effects of Drosophila seminal fluid proteins. *Genetics*. 175: 777-783.

Mulder, M.B. & Rauch, K.L. 2009. Sexual conflict in humans: variations and solutions. *Evol. Anthropol.* 18: 201-214.

Munro, S.S. 1935. Motility and fertilizing capacities of fowl sperm in the excretory ducts. *Proc. Soc. Exp. Biol. Med.* 33: 255–257.

Munro, S.S. 1938. Functional changes in fowl sperm during their passage through the excurrent ducts of the male. *J. Exp. Zool.* 79: 71–92.

Neubaum, D.M. & Wolfner, M.F. 1999. Mated *Drosophila melanogaster* females require a seminal fluid protein, Acp36DE, to store sperm efficiently. *Genetics*. 153: 845-857.

Nguyen, T. M. *et al*. 2016a. Ca2+/Calmodulin-dependent protein kinase kinases (CaMKKs) effects on AMP-Activated Protein Kinase (AMPK) Regulation of Chicken Sperm Functions. *PLoS One* 11: e0147559.

Nguyen, T. M. *et al*. 2016b. Calcium channels in chicken sperm regulate motility and the acrosome reaction. *FEBS J* : 283: 1902-1920.

Nishiyama, H. 1955. Studies on the accessory reproductive organs in the cock. *J. Fac. Agric. Kyushu Univ*. 10: 277-305.

Nishiyama, H., Ogawa, K. & Nakanishi, Y. 1971. Studies on the artificial insemination in the domestic fowl. III. Sperm concentration of semen at collection and sperm-quality of the semen. *Mem. Fac. Agri. Kagoshima Univ.* 8: 355-366.

Noguera, J.C. *et al*. 2012. Age-specific oxidative status and the expression of pre- and postcopulatory sexually selected traits in male red junglefowl, *Gallus gallus*. *Ecol. Evol*. 2: 2155-2167.

Nussey, D.H. *et al*. 2013. Senescence in natural populations of animals: widespread evidence and its implications for bio-gerontology. *Ageing Res. Rev*. 12: 214-225.

Ohashi, Y.Y., Haino-Fukushima, K. & Fuyama, Y. 1991. Purification and characterization of an ovulation stimulation substance from the male accessory glands of *Drosophila suzukii*. *Ins. Biochem*. 21: 413-419.

Otti, O. *et al.* 2009. Bacteriolytic activity in the ejaculate of an insect. *Am. Nat.* 174: 292-295.

Paliy, O. & Shankar, V. 2016. Application of multivariate statistical techniques in microbial ecology. *Mol. Ecol.* 25: 1032-1057.

Park, Y.-J. *et al*. 2013. Proteomic revolution to improve tools for evaluating male fertility in animals. *J. Proteome. Res.* 12: 4738-4747.

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in the insects. *Biol. Rev.* 45: 525-567.

Parker, G.A. 1984. Sperm competition and the evolution of animal mating strategies. In: Smith, R.L. *Sperm competition and the evolution of animal mating systems* (pp. 1-60). London: Academic Press.

Parker, G.A. 1998. Sexual conflict and speciation. *Philos. T. Roy. Soc. B*. 353: 261-274.

Parker, G.A. 2006. Sexual conflict over mating and fertilization: an overview. *Philos. T. Roy. Soc. B.* 361: 235-259.

Parker, G.A. 2014. The sexual cascade and the rise of pre-ejaculatory (Darwinian) sexual selection, sex roles, and sexual conflict. *Cold Spring Harb. Perspect. Biol.* 6: a017509.

Parker, G.A. & Birkhead, T.R. 2013. Polyandry: the history of a revolution. *Philos. T. Roy. Soc. B.* 368: 20120335.

Parker, G.A. & Pizzari, T. 2010. Sperm competition and ejaculate economics. *Biol. Rev.* 85: 897-934.

Parker, G.A., Baker, R.R. & Smith, V.G.F. 1972. The origin and evolution of gamete dimorphism and the male-female phenomenon. *J. Theor. Biol.* 36: 529-533.

Parker, G.A. *et al*. 1996. Sperm competition games: individual assessment of sperm competition intensity by group spawners. *Proc. R. Soc. B*. 263: 1291-1297.

Partridge, L. & Gems, D. 2002. The evolution of longevity. *Curr. Biol.* 12: R544–R546.

Partridge, L. & Harvey, P.H. 1988. The ecological context of life history evolution. *Science*, 241: 1449-1455.

Peng, J., Zipperlen, P. & Kubli, E. 2005. *Drosophila* sex-peptide stimulates female innate immune system after mating via the toll and Imd pathways. *Curr. Biol*. 15: 1690-1694.

Peng, Y. *et al*. 2016. Seminal fluid of honeybees contains multiple mechanisms to combat infections of the sexually transmitted pathogen *Nosema apis*. *Proc. R. Soc. B*. 283: 20151785.

Perez, L. *et al.* 2003. Ionic composition and physio-chemical parameters of the European eel (*Anguilla anguilla*) seminal plasma. *Fish Physiol. Biochem.* 28: 221-222.

Perry, J.C., Sirot, L. & Wigby, S. 2013. The seminal symphony: how to compose an ejaculate. *T.Ecol.Evol.* 28: 414-422.

Pilastro, A. Scaggiante, M. & Rasotto, M.B. 2002. Individual adjustment of sperm expenditure accords with sperm competition theory. *Proc. Natl. Acad. Sci. U.S.A.* 99: 9913-9915.

Pilch, B. & Mann, M. 2006. Large-scale and high-confidence proteomic analysis of human seminal plasma. *Genome Biol.* 7: R40.

Pitnick, S. & Hosken, D.J. 2010. Postcopulatory sexual selection. In: Westneat, D.F. & Fox, C.W. *Evolutionary Behavioral Ecology* (pp. 379-399). Oxford: Oxford University Press.

Pitnick, S., Wolfner, M.F. & Suarez, S.S. 2009. Ejaculate-female and sperm-female interactions. In: Birkhead, T.R., Hosken, D.J. & Pitnick, S. *Sperm biology: an evolutionary perspective* (pp. 247-304). Elsevier.

Pizzari, T. 1999. Reproductive Strategies of Feral Fowl. *Gallus gallus*. PhD dissertation, University of Sheffield.

Pizzari, T. 2002. Sperm allocation, the Coolidge effect and female polyandry. *T.Ecol.Evol.* 17: 456.

Pizzari, T. 2003. Food, vigilance, and sperm: the role of male direct benefits in the evolution of female preference in a polygamous bird. *Behav. Ecol.* 14: 593-601.

Pizzari, T. & Birkhead, T.R. 2000. Female feral fowl eject sperm of subdominant males. *Nature* 405: 787-789.

Pizzari, T. & Birkhead, T. R. 2002. The sexually-selected sperm hypothesis: sex-biased inheritance and sexual antagonism. *Biol. Rev. Camb. Philos. Soc.* 77: 183-209.

Pizzari, T. & Parker, G.A. 2009. Sperm competition and sperm phenotype. In: Birkhead, T.R., Hosken, D.J. & Pitnick, S. *Sperm biology: an evolutionary perspective* (pp. 207-245). Elsevier.

Pizzari, T. & Snook, R.R. 2003. Perspective: Sexual conflict and sexual selection: Chasing away paradigm shifts. *Evolution* 57: 1223-1236.

Pizzari, T. & Snook, R.R. 2004. Sexual conflict and sexual selection: measuring antagonistic coevolution. *Evolution* 58: 1389-1393.

Pizzari, T., Cornwallis, C.K. & Froman, D.P. 2007. Social competitiveness associated with rapid fluctuations in sperm quality in male fowl. *Proc. R. Soc. B*. 274: 853-860.

Pizzari, T., Froman, D.P. & Birkhead, T.R. 2002. Pre- and post- insemination episodes of sexual selection in the fowl, *Gallus g. domesticus*. *Heredity* 88: 112-116.

Pizzari, T., Løvlie, H. & Cornwallis, C.K. 2004. Sex-specific, counteracting responses to inbreeding in a bird. *Proc. R. Soc. B*. 271: 2115-2121.

Pizzari, T. *et al*. 2003. Sophisticated sperm allocation in male fowl. *Nature* 426: 70-74.

Pizzari, T. *et al*. 2008a. The evolutionary ecology of pre- and post- meiotic sperm senescence. *T.Ecol.Evol.* 23: 131-140.

Pizzari, T. *et al.* 2008b. Sperm competition dynamics: Ejaculate fertilising efficiency changes differentially with time. *BMC Evol. Biol.* 8: 332.

Poiani, A. 2006. Complexity of seminal fluid: a review. *Behav. Ecol. Sociob*. 60: 289-310.

Pound, N. & Gage, M.J.G. 2004. Prudent sperm allocation in Norway rats, Rattus norvegicus: A mammalian model of adaptive ejaculate adjustment. *Anim. Behav*. 68: 819-823.

Portus, B.J., Reilas, T. & Katila, T. 2005. Effect of seminal plasma on uterine inflammation, contractility and pregnancy rates in mares. *Equine Vet. J.* 37: 515-519.

Preston, B.T. & Stockley, P. 2006. The prospect of sexual competition stimulates premature and repeated ejaculation in a mammal. *Curr. Biol*. 16: R239-R241.

Price, C.S.C. 1997. Conspecific sperm precedence in *Drosophila*. *Nature* 388: 663-666.

Price, C.S.C., Dyer, K.A. & Coyne, J.A. 1999. Sperm competition between *Drosophila* males involves both displacement and incapacitation. *Nature* 400: 449-452.

Price, C.S.C. *et al.* 2000. Mechanisms of conspecific sperm precedence in *Drosophila*. *Evolution* 54: 2028-2037.

Queller, D.C. 1997. Why do females care more than males? *Proc. R. Soc. B*. 264: 1555-1557.

Radwan, J., Michalczyk, L. & Prokop. Z. 2005. Age dependence of male mating ability and sperm competition success in the bulb mite. *Anim. Behav.* 69: 1101-1105.

Raina, A.K., Kingan, T.G. & Giebultowicz, J.M. 1994. Mating-induced loss of sex pheromone and sexual receptivity in insects with emphasis on *Helicoverpa zea* and *Lymantria dispar*. *Arch. Insect. Biochem. Physiol.* 25: 317-327.

Ramm, S.A. & Stockley, P. 2007. Ejaculate allocation under varying sperm competition risk in the house mouse, *Mus musculus domesticus*. *Behav. Ecol*. 18: 491-495.

Ramm, S.A. & Stockley, P. 2009a. Adaptive plasticity of mammalian sperm production in response to social experience. *Proc. R. Soc. B*. 276: 745-751.

Ramm, S.A. & Stockley, P. 2009b. Male house mice do not adjust sperm allocation in response to odours from related or unrelated rivals. *Anim. Behav.* 78: 685-690.

Ramm, S.A. *et al.* 2008. Sexual selection and the adaptive evolution of mammalian ejaculate proteins. *Mol. Biol. Evol*. 25: 207-219.

Ramm, S.A. *et al.* 2009. Comparative proteomics reveals evidence for evolutionary diversification of rodent seminal fluid and its functional significance in sperm competition. *Mol. Biol. Evol*. 26: 189-198.

Ramm, S.A. *et al*. 2015. Sperm competition risk drives plasticity in seminal fluid composition. *BMC Biol.* 13:87.

Reinhold, K. *et al*. 2015. Male birch catkin bugs vary copula duration to invest more in matings with novel females. *Anim. Behav*. 109: 161-166.

Rice, W.R. 1984. Sex chromosomes and the evolution of sexual dimorphism. *Evolution* 38: 735-742.

Rodd, *et al*. 2002. A possible non-sexual origin of mate preference: are male guppies mimicking fruit? *Proc. R. Soc. B*. 269: 475-481.

Rodrigues, M.A.M. *et al*. 2013. Seminal plasma proteins and their relationship with sperm motility in Santa Ines rams. *Small Ruminant Res.* 109: 94-100.

Rodriguez-Martinez, H. *et al.* 2010. Spermadhesin PSP-I/PSP-II heterodimer induces migration of polymorphonuclear neutrophils into the uterine cavity of the sow. *J. Reprod. Immunol*. 84: 57-65.

Rodriguez-Valentin, R. *et al*. 2006. Oviduct contraction in *Drosophila* is modulated by a neural network that is both, octopaminergic and glutamatergic. *J. Cell. Physiol.* 209: 183-198.

Rose, T.M., Schultz, E.R. & Todaro, G.J. 1992. Molecular cloning of the gene for the yeast homolog (ACB) of diazepam binding inhibitor/endozepine/acyl-CoA-binding protein. *Proc. Natl. Acad. Sci. USA.* 89: 11287-11291.

Rowe, M. & McGraw, K.J. 2008. Carotenoids in the seminal fluid of wild birds: Interspecific variation in fairy-wrens. *Condor.* 110: 694-700.

Rowe, M., *et al*. 2011. Sexual ornamentation reflects antibacterial activity of ejaculates in mallards. *Biol. Lett.* 7: 740-742.

Rowe, M., *et al.* 2013. Lysozyme-associated bactericidal activity in the ejaculate of a wild passerine. *Biol. J. Linn. Soc.* 109: 92-100.

Rubin, C.J. *et al.* 2010. Whole-genome resequencing reveals loci under selection during chicken domestication. *Nature*. 464: 587-591.

Rubinstein, C.D. & Wolfner, M.F. 2013. *Drosophila* seminal protein ovulin mediates ovulation through female octopamine neuronal signaling. *Proc. Natl. Acad. Sci. U.S.A.* 110: 17420-17425.

Rubinstein, C.D. & Wolfner, M.F. 2014. Reproductive hacking: A male seminal protein acts through intact reproductive pathways in female *Drosophila*. *Fly*: 8: 2.

Rudolfsen, G., Serrano, J.V. & Folstad, I. 2015. Own, but not foreign seminal fluid inhibits sperm activation in a vertebrate with external fertilization. *Front. Ecol. Evol*. 3: 92.

Samakovlis, C. *et al*. 1991. The Andropin gene and its product, a male-specific antibacterial peptide in *Drosophila melanogaster*. *EMBO J*. 10: 163-169.

Sarsaifi, K. *et al*. 2015. Two-dimensional polyacrylamide gel electrophoresis of Balibull (*Bos javanicus*) seminal plasma proteins and their relationship with semen quality. *Theriogenology*. 84: 956-968.

Schulz *et al.* 2008. An antiaphrodisiac in *Heliconius melpomene* butterflies. *J. Chem. Ecol.* 34: 82-93.

Sefiani, M. 1987. Regulation of egg laying and *in vitro* oviductal contractions in *Gryllus bimaculatus*. *J. Insect Physiol.* 33: 215-222.

Sharma, R. *et al*. 2004. Antioxidant role of Glutathione *S*-Transferases: protection against oxidant toxicity and regulation of stress-mediated apoptosis. *Antioxid. Redox. Sign*. 6: 289-300.

Simmons, L.W. 2001. *Sperm competition and its evolutionary consequences in insects*. Princeton: Princeton University Press.

Simmons, L.W. & Beveridge, M. 2011. Seminal fluid affects sperm viability in a Cricket. *PLoS One* 6: e17975.

Simmons, L.W., Parker, G.A. & Stockley, P. 1999. Sperm displacement in the yellow dung fly, *Scatophaga stercoraria*: an investigation on male and female processes. *Am. Nat.* 153: 302-314.

Simmons, L.W. *et al*. 2007. Male crickets adjust ejaculate quality with both risk and intensity of sperm competition. *Biol. Lett*. 3: 520-522.

Singhal, S. *et al*. 2015. Antioxidant role of glutathione S-transferases: 4-Hydroxynonenal, a key molecule in stress-mediated signalling. *Toxicol. Appl. Pharm*. 289: 361-370.

Sirot, L.K., Wolfner, M.F. & Wigby, S. 2011. Protein-specific manipulation of ejaculate composition in response to female mating status in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 108: 9922-9926.

Sirot, L. K. *et al*. 2011. Towards a semen proteome of the dengue vector mosquito: protein identification and potential functions. *PLoS Negl. Trop. Dis.* 5, e989.

Skerget, S. *et al*. 2015. Sperm proteome maturation in the mouse epididymis. *PLoS One* 10**:** e0140650.

Snook, R.R. 2005. Sperm in competition: not playing by the numbers. *T. Ecol. Evol.* 20: 46-53.

Souza, C.E.A. *et al*. 2008. Binding patterns of bovine seminal plasma proteins A1/A2, 30 kDa and osteopontin on ejaculated sperm before and after incubation with isthmic and ampullary oviductal fluid. *Anim. Reprod. Sci.* 105: 72–89.

Spence, R., Reichard, M. & Smith, C. 2013. Strategic sperm allocation and a Coolidge effect in an externally fertilizing species. *Behav. Ecol.* 24: 82-88.

Steiger, S. *et al*. 2008. The Coolidge effect, individual recognition and selection for distinctive cuticular signatures in a burying beetle. *Proc. R. Soc. B*. 275: 1831-1838.

Storey, B.T. 2008. Mammalian sperm metabolism: oxygen and sugar, friend and foe. *Int. J. Dev. Biol.* 52: 427–37.

Stürup, M. *et al*. 2013. When every sperm counts: factors affecting male fertility in the honeybee *Apis mellifera*. *Behav. Ecol.* 24: 1192-1198.

Svoboda, P. *et al*. 2004. Biochemistry of transmembrane signaling mediated by trimeric G proteins. *Physiol. Res*. 53 (Sup.1), S141-S152.

Swanson, W.J. & Vacquier, V.D. 2002. The rapid evolution of reproductive proteins. *Nat. Rev. Genet*. 3: 137-144.

Szczesna, D. *et al*. 2001. Familial hypertrophic cardiomyopathy mutations in the regulatory light chains of myosin affect their structure, Ca2+ binding, and phosphorylation. *J. Biol. Chem*. 276: 7086-7092.

Tan, C.K.W. 2012. The effect of relatedness on sexual dynamics. Studies of red junglefowl and fruit flies. DPhil dissertation. University of Oxford.

Tan, C.K.W. *et al*. 2013. Sex-specific responses to sexual familiarity, and the role of olfaction. *Proc. R. Soc. B.* 280: 20131691.

Teixeira, D.I.A. *et al*. 2002. Isolation and partial characterisation of a protein from buck seminal plasma (*Capra hircus*), homologous to spermadhesins. *Protein Pept. Lett.* 9: 331-335.

Teixeira, D.I.A. *et al*. 2006. Ion-exchange chromatography used to isolate a spermadhesin-related protein from domestic goat (*Capra hircus*) seminal plasma. *Gen. Mol. Res.* 5: 79–87.

Thomas, M.L. & Simmons, L.W. 2015. No Coolidge effect in the Australian field cricket *Teleogryllus oceanicus* (Orthoptera: Gryllidae). *Austral Entomol.* 54: 433-437.

Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly Harpobittacus nigriceps. *Am. Nat*. 122: 765-788.

Thornhill, R. & Alcock, J. 1983. *The evolution of insect mating systems*. Cambridge, MA: Harvard University Press.

Töpfer-Petersen, E. *et al*. 1998. Spermadhesins: A new protein family. Facts, hypotheses and perspectives. *Andrologia*. 30: 217-224.

Tram, U. & Wolfner, M.F. 1999. Male seminal fluid proteins are essential for sperm storage in *Drosophila melanogaster*. *Genetics* 153: 837-844.

Tregenza, T. & Wedell, N. 2000. Genetic compatibility, mate choice and patterns of parentage: Invited review. *Mol. Ecol.* 9: 1013-1027.

Tregenza, T. & Wedell, N. 2002. Polyandrous females avoid costs of inbreeding. *Nature* 415: 71-73.

Trivers, R.L. 1972. Parental investment and sexual selection. In: Campbell, B. *Sexual selection and the descent of man 1871-1971* (pp. 136-179). London: Heinemann.

Vanha-Pettula, T., Ronkko, S. & Lahtinen, R. 1990. Hydrolases from bovine seminal vesicle, prostate and Cowper's gland. *Andrologia*. 22 (Suppl.1): 10-24.

Waage, J.K. 1979. Dual function of the damselfly penis: Sperm removal and transfer. *Science* 203: 916-918.

Wang, G. *et al.* 2013. In-depth proteomic analysis of the human sperm reveals complex protein compositions. *J Proteomics* 79: 114-122.

Watanasit, S. 1997. Sperm displacement in the damselfly, *Xanthagrion erythroneurum* (Zygoptera: Coenagrionidae) –variance in female sperm count and genital morphology. *J. Sci. Soc. Thail.* 23: 115-122.

Watson, E.D. *et al*. 1999. Oxitocin in the semen and gonads of the stallion. *Theriogenology*. 51: 855-865.

Waxham, M.N. 1999. Neurotransmitter receptors. In: Zigmond M.J. *et al*. *Fundamental Neuroscience*. (pp. 235-267). New York: Academic Press.

Wedell, N. & Cook, P.A. 1999a. *Strategic sperm allocation in the Small White butterfly Pieris rapae* (Lepidoptera: Pieridae). *Funct. Ecol*. 13: 85-93.

Wedell, N. & Cook, P.A. 1999b. Butterflies tailor their ejaculate in response to sperm competition risk and intensity. *Proc. R. Soc. B*. 266: 1033-1039.

Wedell, N., Gage, M.J.G. & Parker, G.A. 2002. Sperm competition, male prudence and sperm-limited females. *T.Ecol.Evol.* 17: 313-320.

Westneat, D. & Rambo, T. 2000. Copulation exposes female Red-winged Blackbirds to bacteria in male semen. *J. Avian Biol.* 31: 1-7.

White, J. *et al*. 2010. Sexually transmitted bacteria affect female cloacal assemblages in a wild bird. *Ecol. Lett*. 13: 1515-1524.

Wigby, S. & Chapman, T. 2005. Sex peptide causes mating costs in female *Drosophila melanogaster*. *Curr. Biol*. 15: 316-321.

Wigby, S. *et al*. 2009. Seminal fluid protein allocation and male reproductive success. *Curr. Biol.* 19: 751-757.

Wilson, J.R., Kuehn, R.E. & Beach, F.A. 1963. Modification in the sexual behavior of male rats produced by changing the stimulus female. *J. Comp. Physiol. Psychol.* 56: 636-644.

Wishart, G. J. 1987. Regulation of the length of the fertile period in the domestic fowl by numbers of oviducal spermatozoa, as reflected by those trapped in laid eggs. *J. Reprod. Fert.* 80: 493-498.

Wishart, G.J. 2009. Semen quality and semen storage. In: P.M. Hocking. *Biology of Breeding Poultry, Poultry Science Symposium Series, vol.3* (pp. 151-178). Wallingford: CAB International.

Wishart, G. J. & Palmer, F. H. 1986. Correlation of the fertilising ability of semen from individual male fowls with sperm motility and ATP content. *Br. Poult. Sci.* 27: 97-102.

Wolfner, M. 1997. Tokens of love: functions and regulation of *Drosophila* male accessory gland products. *Ins. Bioch. Molec. Biol.* 27: 179-192.

Xue, L. & Noll, M. 2000. *Drosophila* female sexual behavior induced by sterile males showing copulation complementation. *Proc. Natl. Acad. Sci. U.S.A.* 97: 3272-3275.

Zackular, J.P., Chazin, W.J. & Skaar, E.P. 2015. Nutritional immunity: S100 proteins at the host-pathogen interface. *J. Biol. Chem.* 290: 18991-18998.

Zahavi, A. 1975. Mate selection: A selection for a handicap. *J. Theor. Biol.* 53: 205-214.

Zeh, J.A. & Zeh, D.W. 1996. The evolution of polyandry I: intragenomic conflict and genetic incompatibility. *Proc. R. Soc. B*. 263: 1711-1717.

Zeh, J.A. & Zeh, D.W. 1997. The evolution of polyandry II: post-copulatory defences against genetic incompatibility. *Proc. R. Soc. B*. 264: 69-75.

Zhou, L. *et al*. 2014. Enhanced expression of IMPDH2 promotes metastasis and advanced tumor progression in patients with prostate cancer. *Clin. Transl. Oncol.* 16: 906-913.

Zou, J. *et al*. 2015. Elevated expression of IMPDH2 is associated with progression of kidney and bladder cancer. *Med. Oncol*. 32: 373.

Zuk, M. *et al*. 1990. Mechanisms of Female Choice in Red Jungle Fowl. *Evolution* 44: 477-485.