Investigation of factors controlling fertility in Japanese quail (*Coturnix Japonica*)

By

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Declaration

The work presented in this thesis is the original work of the author. It has not been submitted for examination at any other university. The experimental design and manuscript preparation were carried out after discussions with my supervisors, Drs Irek Malecki, Johan Greeff and Graeme Martin.

Part of the work on the role of cloacal gland foam was carried out together with Drs Pratap Singh and Paulina Rybnik-Trzaskowska as my study time coincided with their post-doctoral study at UWA.

I carried out the experiments on sperm motility and sperm mobility assay (Chapter 9) together with Mr Dajun Wang, who was working on his honours project and some results presented in this thesis feature in his final report.

I provided care for experimental quails (feeding daily water provision, daily monitoring of birds wellbeing). I also hatched and reared birds for the experiments from eggs produced by the founder flock used in the major experiments, as outlined in Chapter 4. Those birds were supplied by Game Farm Pty. Ltd. (Galston, NSW) that also provided fresh eggs for the study reported in Chapter 3 and commercial quail diets for most of the study.

All experiments and procedures were approved by the Animal Ethics Committee of the University of Western Australia.

Umar Farooq

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Summary

Japanese quail breeding and production has been focused on increasing meat and egg yield but less attention has been paid to improvement of reproductive performance, in particular, fertility. The aims of this study were to determine true fertility of commercial quail over the production span, to estimate the contribution of each sex to egg fertility, and to identify fertility traits that, through selection, could be used to improve egg fertility. The general hypothesis driving this study was that the reproductive performance of Japanese quail could be improved by improving male and female fertility. The work in this thesis comprised four main components: i) investigation of flock fertility and its dependence on age and strain variances; ii) investigation of the contribution of each sex to egg fertility and identification of fertility traits associated with egg fertility; iii) validation of a method for measurement of sperm motility using CASA; and, iv) investigation and assessment of sperm motility and mobility traits that could be used to identify high and low fertility individuals and thus improve egg fertility.

With respect to flock fertility, an in-vivo sperm-egg assay was used and egg fertility was investigated in 5 commercial strains at Weeks 7, 16, 26, 36 and 46 of age. Mean egg fertility peaked at Week 26, while numbers of Sperm_{OPVL} and Holes_{IPVL} peaked at Week 16 and then declined. There was correlation of egg fertility with Sperm_{OPVL} (r = 0.22) and Holes_{IPVL} (r = 0.25), suggesting that Holes_{IPVL} could be used to monitor flock fertility. Most between-strain variations in fertility parameters were observed after the peak of production (Weeks 36 and 46 of age).

To investigate the contribution of each sex to egg fertility and related traits, males and females from five strains were mated individually at Weeks 8, 16, 26 and 36 of age and used in two experiments. In Experiment 1, the effect of age on male and female fertility was studied. Results indicated that egg fertility was higher for older males, while Sperm_{OPVL}, Holes_{IPVL}, sperm storage, and sperm loss rate were the same for 8- and 26-week-old males. Older females had higher body weight, fewer Sperm_{OPVL}, fewer Holes_{IPVL}, shorter sperm storage, and high sperm loss rate than younger females. Strain had significant effect on most of these traits for males. In Experiment 2, males and females were allocated into high (HF) or low (LF) fertility phenotypes based on the mean number of Holes_{IPVL} (± 1 STD), shown in Experiment 1 to be the variable that correlated best with
egg fertility. Subsequently, pair-mating of fertility lines (HF male x HF female, HF male x LF female, LF male x HF female, and LF male x LF female) revealed that egg fertility and numbers of Holes\textsubscript{IPVL} were highest for the HF male x HF female combination and lowest for LF male x LF female combination. On other hand, the HF male x LF female combination produced fewer Holes\textsubscript{IPVL} than the LF male x HF female combination. These observations imply a greater contribution by the female than the male to fertility and Holes\textsubscript{IPVL}, and that these two measures would be good candidate fertility traits in quail.

To analyze sperm motility reliably, the conditions for CASA motility recording were optimized for quail sperm. In the first step, sperm motility was recorded in the presence of 0, 5, 10, 15 or 20 % cloacal gland foam. Addition of foam increased the number of rapid and progressive sperm and enhanced sperm velocity, but only with 5 and 10 % foam concentrations; further increases in foam concentration (i.e. 15 and 20 %) resulted in a decrease in sperm velocity. In the second step, the aim was to classify quail sperm into rapid, medium and slow velocity categories. Quail-specific VCL-cut-off values were determined for the Sperm Class Analyser (Microptic, SL, Spain) using three methods: i) inter-quartile range (or 25 % method); ii) 33 % method; and iii) the SCA chicken setting. The “25 %” setting gave good separation of low- and high-sperm-motility males.

In the next step, the effect of male age (8 to 36 weeks) on ejaculate volume, sperm concentration, total sperm in an ejaculate, live normal and total abnormal sperm, and sperm velocity parameters were measured: percentages of motile, rapid, medium, and progressive sperm; sperm kinematics (i.e., VCL, VSL, VAP, LIN, BCF). Ejaculate volume, and sperm concentration decreased with age, while total abnormal sperm increased. There was an age-related decline (P < 0.05) from Weeks 8 to 26 in the percentages of motile, progressive and rapid sperm, and in most sperm kinematic parameters. These results showed that decline in sperm motility was better indicator of age-related changes in fertility than ejaculate volume or sperm concentration, and that CASA is good tool for detecting age-related changes in sperm motility.

The relationship between sperm motility and egg fertility was also investigated. Semen was collected from males of 5 strains, at ages 8-36 weeks. Ejaculate volume, sperm concentration, and sperm motility were measured, and mating was carried out to obtain egg fertility data. Both ejaculate volume and sperm concentration were negatively correlated
with sperm motility parameters. Medium sperm were positively correlated with $\text{Sperm}_{\text{OPVL}}$ and $\text{Holes}_{\text{IPVL}}$, and the percentage of progressive sperm was positively correlated with $\text{Holes}_{\text{IPVL}}$. The correlation between egg fertility and CASA sperm motility differed between strains. For one strain, sperm kinematic parameters (i.e. VSL, VAP and BCF) were all positively correlated with $\text{Sperm}_{\text{OPVL}}$, whereas, for the other strains, progressive and medium velocity sperm were positively correlated with $\text{Sperm}_{\text{OPVL}}$. Two strains showed no significant correlation between sperm motility and egg fertility traits. These observations suggested that some sperm traits are important to egg fertility and, in this context, male genotype can play an important role.

Subsequently, the relationship between egg fertility and the mobility and motility of sperm was studied. Following development of the sperm mobility assay, 20 males and 20 females were mated individually between weeks 15 to 20 and between weeks 29 to 33 of age, and data were collected for egg fertility, $\text{Sperm}_{\text{OPVL}}$ and $\text{Holes}_{\text{IPVL}}$. At ages 23 to 28 weeks, ejaculates were collected and sperm motility (CASA) and mobility were assessed. Sperm mobility was the highest with 10 % foam extract and an incubating temperature of 37 °C. The sperm mobility assay allowed successful identification of phenotypic classes, but sperm mobility phenotype did not relate to egg fertility under the experimental conditions used in this study.

In conclusion, using objective methods for fertility assessment in quail, factors effecting fertility and traits associated with fertility can be identified and quail reproduction can be improved. The work in this thesis has contributed to our understanding of quail fertility and the findings are applicable to quail breeding and selection programs.
Acknowledgments

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My sincerest thanks are for my Supervisors Associate Professor Irek Malecki, Dr Johan Greeff and Winthrop Professor Graeme Martin. It has been a great experience to work with them. Only due to their guidance and support did I achieve worthwhile results throughout this study.

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I cannot gauge the affection and sweetness of my parents, nor can my words ever begin to describe my love and profound admiration for them. Their spiritual and moral concerns always led my way to the noble and rewarding view of life that I carry in my heart. Having them, I cannot ask more in life from the Almighty.

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<td>Computer assisted semen analysis</td>
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<td>Perivitelline layer</td>
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<td>Rapid</td>
<td>Rapid velocity sperm;</td>
</tr>
<tr>
<td>Medium</td>
<td>Medium velocity sperm;</td>
</tr>
<tr>
<td>Slow</td>
<td>Slow velocity sperm;</td>
</tr>
<tr>
<td>VCL</td>
<td>Curvilinear velocity</td>
</tr>
<tr>
<td>VSL</td>
<td>Straight-line velocity</td>
</tr>
<tr>
<td>VAP</td>
<td>Average path velocity</td>
</tr>
<tr>
<td>STR</td>
<td>Straightness (100 x (VSL/VAP));</td>
</tr>
<tr>
<td>LIN</td>
<td>Linearity (departure of the cell track from the straight line)</td>
</tr>
<tr>
<td>BCF</td>
<td>Beat cross frequency;</td>
</tr>
<tr>
<td>ALH</td>
<td>Lateral head displacement and</td>
</tr>
<tr>
<td>WOB</td>
<td>Wobble</td>
</tr>
<tr>
<td>CP</td>
<td>Crude protein</td>
</tr>
<tr>
<td>ME</td>
<td>Metabolisable energy</td>
</tr>
<tr>
<td>HF</td>
<td>High fertility</td>
</tr>
<tr>
<td>LF</td>
<td>Low fertility</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>TES</td>
<td>N-tris-[hydroxymethyl]-methyl l-2-aminoethane-sulphonic acid</td>
</tr>
<tr>
<td>N/E</td>
<td>Nigrosin-eosin</td>
</tr>
<tr>
<td>VIC</td>
<td>Victoria</td>
</tr>
<tr>
<td>NSW</td>
<td>New south Wales</td>
</tr>
<tr>
<td>SCA</td>
<td>Sperm Class Analyzer</td>
</tr>
<tr>
<td>LSD</td>
<td>Least significant difference</td>
</tr>
<tr>
<td>STD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
</tr>
<tr>
<td>---------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>W.L.H</td>
<td>Width.length.height</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>MOB</td>
<td>Mobility</td>
</tr>
<tr>
<td>LH</td>
<td>LH – luteinizing hormone</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
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</table>
Publications and presentations arising from this thesis

Manuscripts under revision


Manuscript ready to submit to a Journal

Farooq U. and Malecki I.A. (2013) Investigation of sperm curve linear velocity (VCL) cut-off values for the computer aided semen analysis (CASA) to classify motile Japanese quail (Coturnix japonica) spermatozoa into velocity categories. Theriogenology (from Chapter 7).

Published Journal Papers


Refereed Conference Papers


Conference Abstracts


Chapter 1

General introduction

Japanese quail (*Coturnix Japonica*) belongs to the order Galliformes and the family *Phasianidae*, like the chicken (Howard, 1991). Quail are grown for meat and eggs and are attractive for farming due to rapid growth rate (marketable at 5-6 weeks of age), early onset of egg production (6-7 weeks of age) and the nutritive value of their eggs and meat (Santos *et al.*, 2011). Intensive quail production started in the 1920s in Japan and, at present, commercial strains are found worldwide (Asia, Europe, America, Australia), but the focus of production varies: in China, Hong-Kong and Japan, eggs are preferred (9 billion quail produced annually), whereas, in Europe (France, Spain) meat is preferred (around 105 million quail produced annually; Kayang *et al.*, 2004; Minvielle, 2004). In Australia, quail farming began around the early 1970s and, in 2003, the estimated annual production was 6.5 million birds (RIRDC, 2003). However, the number of independent producers has declined over time and the estimated annual production has dropped to 3.5 million quail (Department of Agriculture, Australia, 2010).

Commercial production of Japanese quail developed unequally across the world because of the variation in preference for eggs and meat (Minvielle, 2004). Therefore, modern selection has not been applied equally for all traits in all commercial lines (Minvielle, 1998; Cheng, 2002). Owing to the fact that present-day quail strains originated from a few breeder lines after World Wars I and II, and early quail populations were quite sensitive to inbreeding (Woodard *et al.*, 1973), the choice of unequal selection was suitable for developing lines quickly but, in turn, it resulted in high genetic load in the breeding lines (i.e. loss of alleles in the selection process). In addition, rapid and preferential selection for meat and eggs somewhat overshadowed the genetics of reproduction, as judged from fertility and hatchability (Minvielle, 2004). Consequently, the present day quail flocks (especially the meat type) are characterized by low fertility (45-85 % fertility has been reported for breeder flocks; Marks 1979; El-Filky, 2002; Okenyi *et al.*, 2013), short reproductive span (fertility remains high from 10-19 weeks of age, peaks over weeks
14-16, and then declines; Narahari et al., 1988; Santos et al., 2011) and high fertility losses with small gains (4-5% per generation decline in short-term selection; Okenyi et al., 2013). To counter these problems, quail producers have to maintain large parental stocks, while compromising growth because high selection pressure leads to considerable fertility losses in successive generations (Okenyi et al., 2013). Overall, genetic gains have been slow. Therefore, low general fertility and a decline in fertility with high growth rate are among the biggest constraints to successful commercial production and further development of the quail meat industry.

Growth-related decline in reproductive efficiency in quail is, however, not very surprising, because similar problems have been encountered during the development of other poultry species, specifically chicken and turkey (Berg and Shoffner, 1954; Kondra and Shoffner, 1955). In contrast to these two species, reproduction has been largely ignored in the breeding of quail (Minvielle, 2004) so fertility problems are widespread. The solution is to cull low-fertility individuals and mate those having high fertility merit. In other words, the issue of decline in fertility could have been resolved if quantitative traits linked to high fertility had been identified and incorporated into breeding programs, while those linked to low fertility were eliminated. Unfortunately, no attention has been paid to the causes of poor fertility or to ways to improve fertility by selection. Instead, the focus of quail research has largely been on its use as a model animal for research or for comparative studies (Cheng and Kimura, 1990). However, it can be anticipated that focusing on the selection of males and females with high fertility attributes, while eliminating those factors which are responsible for fertility decline, would improve the reproductive efficiency of the breeder flocks.

Donoghue (1999) reviewed methods of monitoring flock fertility and strategies that had been adopted to improve and maintain high fertility in other poultry species. Certain gender-based strategies have been adopted and successfully used to predict potential fertility of individual birds. In males, traditional measures of semen quality (semen volume, sperm concentration, sperm viability, subjective motility assessment), were proven to be poor predictors of fertility, whereas, objective assessment of sperm motility and sperm mobility were found to be good predictors (Wilson et al., 1979; Wishart and Ross 1985; Wishart, 1995; Froman and McLean, 1996; Robertson et al., 1998; Wishart and Wilson, 1997; Holsberger et al., 1998; McDaniel et al., 1998; Dumpala et al., 2006). On
other hand, hen’s ability to store sperm and supply it to the egg fertilization site (sperm binding and penetration to the ovum) were found to be suitable for predicting female fertility (Brillard and Antoine, 1990; Brillard and Bakst, 1990; Brillard, 1993; Wishart, 1995; Gumulka and Kapkowska (2005).

The data on quail fertility and factors affecting it are insufficient and the available reports are limited to traditional, subjective methods of evaluation. At present male quail, if selected, are selected for physical characteristics such as cloacal gland size and not on the basis of semen properties. We lack basic information on quantitative and qualitative aspects of semen production and its quality, on male-to-male variations, on factors affecting semen quality (e.g., age and genotype), on the relationship between sperm function and egg fertility, and on the possibility of male selection based on sperm characteristics. The focus of female quail selection has largely been on apparent reproductive performance such as age at sexual maturity, egg weight, number of eggs produced per hen and on obtaining higher number of eggs in the early production period (Marks, 1979; El-Fiky, 1994; Nestor et al., 1983; Asasi and Jaafar 2000; Ali et al., 2002; Aboul-Hassan, 2001; Abdel-Azeem, 2005; Abdel-Tawab, 2006). But On other hand, certain crucial factors, having been ignored so far i.e. how much female contribute to egg fertility?, how female fertility is affected by age and strain variations in naturally mated flocks?, what are the female fertility traits and what criteria could be used for selection.

To answer to these questions, the present study was planned and the general objectives were to comprehensively investigating quail fertility and its contributing factors using objective methods of measurement, measuring key fertility traits both in males and females, and then examining the suitability and potential of those traits to counter the fertility problems. The study commenced with an investigation of the reproductive performance of the naturally mated commercial quail breeders using a sperm-egg assay in combination with the appearance of the germinal disc of the egg yolk. The aims were to estimate true fertility, to quantify the roles of age and strain variables on fertility, and to provide baseline data for further experimentation. The focus was then shifted to investigation of fertility in each sex and of variation among strains. The aim was to determine the contribution of each sex to egg fertility traits: percent egg fertility, numbers of sperm (Sperm\textsubscript{OPVL}) and sperm holes (Holes\textsubscript{PVL}) in the perivitelline layer (PVL), sperm storage and sperm loss rate. Studies on male fertility required the development of quail-
specific methods for objective assessment of sperm motility and mobility. In turn, this approach depended on quantification of the effect of cloacal gland foam on sperm motility, and on optimization of computer-assisted sperm analysis (CASA) so we could categorize quail sperm on the basis of sperm velocity (i.e., rapid, medium, or slow). Semen traits, including sperm motility by CASA, were then related to age and strain effects. The utility of CASA measures of sperm motility for identifying sperm traits associated with egg fertility parameters was tested and, subsequently, a sperm mobility assay was developed for quail so we could determine whether poor and good fertility males could be identified using this method. The general hypothesis driving this study was that the reproductive performance of Japanese quail could be improved by improving male and female fertility.
Chapter 2

General literature review

2.1 Quail biology

Japanese quail is the smallest species farmed for meat and egg production. Their physiological, behavioural and managemental aspects have been extensively reviewed over the years (Willson, 1971; Shanaway, 1994; Milles et al., 1997), but certain attributes need to be underscored in brief here. Males and females can be sexed by plumage at about 3 weeks of age – males have an even coloured brown breast with only a few speckles, while females have a grey and black speckled breast. Males also have a cloacal gland, a bulbous structure located at the upper edge of the vent that secretes a white, foamy substance. The adult male weighs about 150-200 g, while the females are slightly heavier and weigh about 160-250 g. On long photoperiods, life span ranges from 3 to 5 years for males and from 2.5 to 3 years for females (Ottinger, 1996).

Male quail begin to secret luteinising hormone (LH) at 3-4 weeks of age, an event that is crucial for initiating testosterone secretion and rapid growth of testes (Denk and Kampenaers, 2005). Males attain sexual maturity at 6 to 7 weeks of age and have relatively large testes size (2.26 % of body mass) but a limited capacity to store spermatozoa for long periods, so their reproductive strategy involves rapid production (14 to 16 days), maturation and transportation (in about 1 day) of spermatozoa through the reproductive tract. The daily output of spermatozoa is high (308 x 10^6 per male). Quail semen is white in colour and the ejaculate ranges from 10 to 35 μl in volume and contains 592 to 812 x 10^6 sperm per ml (Chelmonska et al., 2008). Unlike rooster, turkey or drake semen, quail semen is devoid of seminal plasma (Fujihara et al., 1989). The sperm are vermiform in shape and have an exceptionally long mid-piece, with 67-74 % of the total length covered by a mitochondrial sheath containing large number (>1400) of mitochondria (Korn et al., 2000). Quail are cited as having a non-monogamous mating system involving an intense mate competition with frequent copulations (Birkhead and Moller, 1992).
Female quail begin to reach sexual maturity at 8 weeks of age and differ from other domestic birds by laying eggs in the late afternoon. Based on this behaviour, the daily copulation occurs with an egg inside the uterus, and the egg shell is developed by the early morning. Breeder hens are kept for 8-10 months during which time they lay about 150-200 eggs. At peak production, laying may reach 90 % and may persist at around 80 % for a few months (Murakami and Ariki, 1998). After one year of laying, there is a linear decrease in egg production. Fertility peaks (i.e. 85-90 %) at 14-16 weeks of age and begins to fall after 20 weeks (Narahari et al., 1988). Quail hens can store sperm for 10 to 11 days after the last successful mating, but the probability of egg fertilization drops to 45 % after 5 days (Santos et al., 2013).

2.1.1 Role of cloacal gland foam

Sexually mature males have a functional proctodeal gland, located at the upper dorsal portion of the cloaca that is formed by the aggregation of individual glandular units that become wrapped into a single structure by a connective tissue capsule. The size of the cloacal gland is reported to be positively correlated with androgen levels, testis function and reproductive success (Mohan et al., 2002; Singh et al., 2011a, 2011b; Finseth et al., 2013). The gland size and its secretion (foam), however, differ markedly between wild and the domesticated quail, probably because there is pair bonding in the wild (Wetherbee, 1961; Moreau and Wayre, 1968) but intense competition and lack of pair bonding in domestic (commercial) environments (Schleidt and Shalter, 1972). The exaggerated gland and its secretion is novel to quail and they have no other gland or produce any seminal fluid from seminiferous tubules or epithelia of the testes or ductus deferens (Lake, 1981).

The cloacal gland secretes glycol-muco-proteins that are then whipped into frothy material called “foam” that appears on the surface and when the gland is pinched (Fuji and Tamura, 1967; Seiwert and Adkins-Regan, 1998). The formation of foam is due to the action of bacteria (primarily Escherichia coli and Proteus mirabillia) on the glucose component of the glyco-muco-proteins. Carbon dioxide and hydrogen gasses are produced but the cohesive forces of the muco-proteins prevent them escaping so they appear as froth on the surface. In addition, the hydrogen and carbon dioxide, and perhaps carbonic acid formed by the combination of these two gasses, probably causes the acidic pH (6.3 to 6.5),
with a bicarbonate-carbonic acid equilibrium offering some buffering (McFarland et al., 1968).

The cloacal gland produces foam constantly, regardless of ejaculation of semen or time of the day (Fujihara, 1992). The amount of foam ranges from that of a rice grain to mottled kidney bean. Unlike semen, however, foam is not required for fertilization (Marks and Lepore, 1965) and it is characterized as a non-semeninal copulatory fluid that is produced and stored separately from the semen inside the males. Foam and semen are never found mixed together inside the male, but foam is an inherent component of naturally ejaculated quail semen. Foam production increases rapidly as the male approaches a female (Seiwert and Adkins-Regan, 1998) and, during copulation, the semen is always followed by a large quantity of foam so both are introduced together into the female. The mixture remains in female proctodeum for 3 or more hours and is not eliminated, even by oviposition. Investigations into the role of cloacal gland foam have led to conflicting reports (Perez and Juarez, 1966; Renzoni, 1968; Schleidt and Shalter, 1972; Adkins-Regan, 1999). However, the consensus view is that foam may serve to aerate sperm, to facilitate sperm transportation in the oviduct, and perhaps act as neutralizing agent to protect sperm from the hostile environment of the proctodeum. It might also aid in sperm competition, act as medium for suspending sperm in the female proctodeum to provide an insemination window (Cheng et al., 1989b, Singh et al., 2012), and help sperm reach the sperm storage tubules (SSTs) in sufficient numbers (Adkins-Regan, 1999). In vitro studies have demonstrated that quail sperm form clusters in the absence of foam (Amano and Watanabe, 1966; Kobayashi et al., 1972; Ogawa et al., 1974) whereas, in the presence of foam, sperm are dispersed in the medium, remain motile for longer, and retain high metabolic activity (Fujihara et al., 1989, Cheng et al., 1989b, Biswas et al., 2010; Singh et al., 2011b). On the other hand, it has been reported that foam has a negative impact on sperm morphology (Chelmonska et al., 2006), that only small quantities are required to maintain sperm function in vitro, and that mass motility of sperm either increased or decreased depending upon the concentration of foam added to semen (Biswas et al., 2010). Overall, there is considerable confusion but cloacal foam cannot be ignored in any assessment of male quail fertility.
2.2 Purpose of poultry breeding operations

The ultimate goal of the poultry breeding operations is to produce viable and healthy chicks because they are crucial for the successful continuation of selection and breeding programs, and for genetic gain. However, only a fertile egg can develop into a chick, so egg fertility is the key limiting factor and low fertility or infertility is often the major economic barrier to productivity of breeder flocks.

2.2.1 Definition of fertility

Fertilization, the process of combining two germ cells, egg and sperm, is the consequence of precisely ordered multiple steps, including sperm-egg binding, induction of the acrosome reaction, and fusion of sperm and egg. Thus, fertility is simply the ability to produce young or reproduce (American Heritage Dictionary). In avian species, fertility can be defined as „the percentage of eggs laid that are fertilized” but the concept is complicated because female birds can store sperm and lay multiple fertilized eggs from one insemination. This phenomenon leads us to the concept of a „fertile period”, the period after artificial insemination, or after removal of the male following natural mating, during which the hen continues to lay fertile eggs (Lake, 1975). Therefore, egg fertility percentages can vary with the method of assessment but, in both naturally or artificially inseminated flocks, overall egg fertility also includes the fertile period, unless specified otherwise.

2.3 Factors affecting fertility

Generally, flock fertility is influenced by factors such as strain, age, body condition, health status and mating activity (Wilson et al., 1979). However, as both male and female birds are assumed to contribute half of egg fertility, a low or declining fertility can be caused by factors related to the male, the female, or both.

2.3.1 Male factors affecting fertility in poultry

Factors affecting male fertility can fall into two main categories: i) mating efficiency and ii) semen quality. Mating efficiency reflects libido, aggression, number of copulations and
physical conditions. Low libido might have hormonal causes or be the consequence of physical conditions such as excessive or low body weight, leg problems, or undernutrition (Hocking, 1990; Hocking and Bernard, 2000). Semen factors that are positively correlated with fertility include sperm concentration, insemination dose, ATP levels in sperm (Wishart and Palmer, 1986), sperm motility (Wilson et al., 1979; Wishart and Palmer, 1986), and sperm mobility (Froman and McLean, 1996; Froman et al., 1997; Donoghue et al., 1999).

2.3.2 Decline in male fertility

In broiler breeders and turkeys, declines in fertility are mainly associated with age, low libido, changes in body conformation that inhibit mating, and reductions in sperm quality. Age is associated with reduction in the number of spermatozoa in the ejaculate and also a reduction in semen volume (Sexton et al., 1989).

In quail, male body weight is positively correlated with fertility (Narahari et al., 1988), although some studies have reported decrease in mating efficiency with age (Woodward and Alplanalp, 1967). On the other hand, Cherkin and Eckardt (1977) reported no difference in the latency to mount between 23-week-old and 43-week-old males. Furthermore, mating behavior, plasma testosterone concentration and testis weight did not change from 23-70 weeks of age (Ottinger et al., 1983). Testicular abnormalities and tumors, particularly in the Sertoli cells, are more common in older than younger males, and associated with diminished spermatogenesis whilst maintaining Leydig cell function (Gorham and Ottinger, 1986; Eroschenko et al., 1977). Moreover, aging is associated with testicular regression, sharp decreases in the numbers of LH and FSH receptors, and reductions in sperm production, the percentage of normal sperm, and in egg fertility (Howes, 1968; Chelmonska et al., 2006).

2.3.3 Female factors affecting fertility in poultry

The ability of hens to obtain sperm depends on body weight, health, and ability to mate successfully, and subsequent fertility also depends several aspects of the function of sperm storage tubules (SSTs), especially their ability to store sperm (controlling the rate of sperm loss) and supply it to the egg fertilization site. In addition, females need to ovulate and produce a suitable environment for the fertilization of the egg and development of the
2.3.4 Effect of age on female fertility

The age-related decline in female fertility is partly associated with declines in egg production (Robinson et al., 1991). Usually, fertility starts declining once the age of maximum egg production has passed an age that is specie-specific (Robinson et al., 1991). Older hens are characterized by a continued decrease in fertility and egg hatchability. In naturally mated flocks, a continuous sperm supply is available through mating, but a constant sperm supply for successive egg fertilizations is achieved by the SSTs located in the utero-vaginal junction and infundibular region. Insufficient filling of the SSTs may be responsible for declining fertility in older hens (Etches et al., 1974). An ability of older hens to store sperm in the SSTs may be a cause of decline in fertility (Pierson et al., 1988), as might poor transportation of sperm by the oviduct and reductions in sperm viability inside the SSTs. Changes in the uterine fluid associated with age may be related to declines in spermatozoa viability in the oviduct, but this issue is controversial. Bramwell et al. (1996) reported that the hen-dependent decline in fertility was due to the fact that sperm stored in older hens were viable for a shorter period of time than sperm stored in young hens. However, Brillard (1993) found the same number of sperm residing in sperm storage tubules of young and old hens and, reported that the rate of sperm release from the SSTs was twice as high in old hen than in young hens. This suggested that older hens must be released sperm more often and so need to mate more often to maintain constant sperm supply. Furthermore, a reduction in the number of sperm receptors on the oocyte surface might also be associated with the decline in fertility (Bramwell et al., 1996).

In Japanese quail, there is a similar age-related decline in female reproduction, with older females having fewer ovulations, laying fewer eggs, and also showing a decline in egg fertility and hatchability (Woodard and Alplanalp, 1967). Furthermore, it has also been reported that the age-related decline in fertility arises earlier in female quail that in males and that the number of sperm trapped in the perivitelline layer overlaying the germinal disc falls as a function of female age as well as the time after copulation (Santos et al., 2013).
2.4 Methods of assessing egg fertility

To assess egg fertility, four methods are generally used: i) the appearance of germinal disc (GD) in freshly laid eggs; ii) the appearance of the GD combined with numbers of sperm embedded (Sperm$_{OPVL}$) and sperm-holes present (Holes$_{IPVL}$) in the perivitelline layer (PL) of freshly laid eggs; iii) Egg candling during incubation; and iv) after-hatch break-out of unhatched eggs (clear eggs, early, middle and late embryonic mortalities).

2.4.1 Appearance of GD in freshly laid eggs

The fertility status of an egg can be determined by opening the egg and viewing the GD under low magnification or by light microscopy. Fertile and infertile eggs can be distinguished using method described by Arora and Kosin (1966): fertile eggs contain blastoderm, whereas, infertile eggs do not but instead have numerous vacuoles in the germinal disc. The presence of vacuoles in blastoderm indicates a dead embryo.

2.4.2 In vivo sperm-egg interaction

This process involves quantification of sperm in the outer perivitelline layer (PVL), providing a value for Sperm$_{OPVL}$, and quantification of sperm holes (Holes$_{IPVL}$) in the inner PVL of the egg yolk. These methods are based on two critical steps in the fertilization process: the binding of the sperm to the PVL and the subsequent acrosomal reaction that produces a hydrolysed hole in the inner PVL through which sperm enter the ovum. Spermatozoa that fail to enter the egg cytoplasm get trapped in the outer PVL.

Both, Sperm$_{OPVL}$ and Holes$_{IPVL}$ can be visualized in the perivitelline membrane of laid eggs by the use of specific staining and microscopy techniques (Bramwell et al., 1995; Bramwell and Howarth, 1997). Sperm$_{OPVL}$ are viewed with the aid of a fluorescent, DNA-specific dye that allows sperm nuclei to be viewed as bright comma-shaped structures against a dark background. On the other hand, Holes$_{IPVL}$ are visualized with light microscopy after re-hydrating the same slide used to count Sperm$_{OPVL}$, fixing it with formalin and then staining it with Schiff’s reagent (Bramwell et al., 1995). The number of Sperm$_{OPVL}$ or Holes$_{IPVL}$ can be good predictors of egg fertility. For example, in quail, the presence of 3 Sperm$_{OPVL}$ per mm$^2$ offers a 95 % probability of an egg to be fertilized (Santos et al., 2013). Furthermore, this method uses un-incubated eggs, thus eliminating the errors of fertility assessment that could be caused by undetectable early embryonic mortalities.
mortalities after the incubation, and also provides results promptly (Wishart and Stains, 1999). Furthermore, combining this technique with assessment of the appearance of germinal disc area can give 100% reliable estimates of egg fertility.

Sperm_{OPVL} also indicate mating efficiency in males and the ability of sperm transport and storage in females, whereas, Holes_{IPVL} predict the likely fertilizing ability of the sperm (Wishart, 1995; Robertson et al., 1998). Moreover, it has been suggested that this method can be used to estimate quite accurately the fertility of individual eggs, hens (broiler, turkey) and flocks (Bramwell et al., 1995; Wishart and Staines, 1995), and also to study patterns and changes in egg fertility, such as those caused by aging (Gumulka and Kapkowska, 2005).

2.4.3 Egg candling and after-hatch egg breakout analysis
Egg candling and after-hatch egg-break-out are done on incubated eggs. Candling is done by holding an egg against a light source so that a trained observer (a „grader”) can assess qualitative characteristics of the internal parts of the egg without breaking the shell. Infertile eggs retain the original yolk color and the germinal disc is a distinct white or pale yellow irregular shaped spot (Kosin, 1945). After-hatch breakout analysis involves opening unhatched eggs („dead-in-shell”) to determine at the stage of embryo mortality. Clear eggs with no sign of embryo development are recorded as „infertile”, whereas, eggs with any sign of embryo development are recorded as „fertile”.

However, both of these methods have limitations: candling is not very effective with heavily coloured eggs (e.g., quail) and after-hatch break can be inaccurate in estimating true fertility because the post-incubation interpretation of early developmental pathology is potentially difficult and early embryonic mortalities can be difficult to discriminate from infertility.

2.5 Practical methods of fertility assessment (Male)

2.5.1 Ejaculate volume and sperm concentration
There is a significant positive correlation between number of sperm stored in the SSTs and the number of sperm reaching the PVL, so placing more sperm into the female vagina increases number of sperm reaching the SSTs (Brillard, 1993), thus lengthening the hen’s
fertile period and increasing overall fertility (Wishart, 1987; Brillard and Bakst, 1990). Fertility in quail is higher after insemination with a high volume of semen (15 µl) than with a low volume (2.5 µl; Lepore and Marks, 1966), and by increasing the frequency of inseminations from 1 to 3 per week (Chelmonska et al., 2006). Therefore, the reproductive potential of a male is reflected in his ability to produce a large volume of ejaculate and a high concentration of viable sperm.

Sperm viability depends on keeping the plasma membranes intact, and the ability of semen to fertilize eggs largely depends on the number of morphologically normal sperm in the ejaculate. High proportions of abnormal sperm in a semen sample have been correlated with lower fertility (Steele and Wishart, 1992).

2.5.2 Sperm motility
Sperm motility describes the ability of sperm to move properly towards an egg and reach the fertilization site. In birds, the vaginal portion of the hen’s oviduct regulates sperm entry (Steele and Wishart, 1992; Brillard, 1993) and only motile and morphologically normal sperm are able to traverse the vagina and enter the SSTs (Steel, 1992). Most inseminated sperm are, however, lost and do not reach the SST (Howarth, 1971). Sperm that are more motile are better able to enter and fill the SSTs, and then subsequently fertilize eggs.

Sperm motility becomes critical at the time of fertilization because it allows, or at least facilitates, passage of the sperm through the PVL of the ovum. Non-motile or abnormally motile sperm cannot reach and fertilize the egg, so motility is perhaps the most widely used measure of semen quality (Moce and Graham, 2008). In most species, sperm are classified as non-motile, progressively motile or non-progressively motile. Progressively motile sperm swim forward in an essentially straight line, whereas, a non-progressively motile sperm swim along an abnormal path, as extreme as a tight circle. Percentage of motile sperm is still a reasonable indicator of male fertility (Donoghue, 1999) and, with advent of objective sperm motility assessment (such as CASA), it has become even more useful because motility parameters correlate with fertility (Verstegen et al., 2002).
2.5.2.1 CASA sperm motility

CASA (computer-aided semen analysis) is an automatic or semi-automatic system that performs fast, accurate and reliable sperm analyses. With a minimum investment of time, CASA can objectively assess a variety of sperm characteristics: i.e. percentage motile sperm (PMOT); progressive sperm (PROG); percentage rapid sperm (Rapid); percentage medium sperm (Medium); percentage slow sperm (Slow); velocity curvilinear [VCL (velocity over the normal sperm track)]; velocity straight-line [VSL (straight-line distance between beginning and end of the track/time elapsed)], velocity average path [VAP (velocity/average position of the spermatozoa)]; straightness [STR (100 x (VSL/VAP))]; linearity [LIN (departure of the cell track from the straight line); beat cross frequency (BCF); lateral head displacement (ALH) and wobble (WOB) (Verstegen et al., 2002)

CASA has been used for determining motility parameters in the chicken and turkey (Bakst, 1992; Froman et al., 1999) and many other species. CASA sperm parameters (PMOT, VAP, VSL) have been found to correlate with egg fertility in turkey, chicken and pigeon (Donoghue et al., 1999; Klimowicz et al., 2008), and chicken parental lines have also been reported to vary for CASA sperm motility parameters (Long et al., 2010).

2.5.3 Sperm mobility

The ability of the sperm to reach the egg fertilization site is determined by the extent to which sperm enters and remains sequestrated within the SSTs (Bakst et al., 1994). The vagina has ciliated epithelial walls that move epithelial secretions, and the SSTs also generate a fluid current from the epithelial cell walls that attempt to eject sperm from its tubules (Bakst et al., 1994). Therefore, for sperm to reach the SSTs, they must be able to traverse the vagina and, upon reaching the SSTs, they must also exhibit the necessary motion to remain sequestrated (Froman and Feltmann, 2000; Froman, 2003) and not be ejected from the SSTs.

A sperm mobility assay defines the net movement of a sperm cell population against resistance (such as Accudenz solution) at body temperature. The term "mobility" refers to measurements of migration of sperm from a first volumetric medium to a barrier medium – for example, from a first liquid layer into a second liquid layer of greater density and/or viscosity than the first liquid layer. The second liquid layer provides a physico-chemical barrier to sperm migration that allows entry of only more highly mobile sperm.
Quantitative measurements include photometric measurements of changes in optical density of the barrier medium. The number of sperm that migrate into or through the barrier medium is then used as an index of the mobility of the population of sperm from the test subject. Changes in turbidity or number of sperm adherent to a substrate are then used as an index of mobility of the test sample. In particular, test subjects having highly mobile sperm have been reliably found to be highly fecund.

Sperm mobility is a quantitative trait and has been proven to be a major determinant of fecundity in fowl (Froman and Feltmann, 1998). Thus, male-to-male variation in sperm mobility phenotype has repeatedly shown to be a normally distributed trait in roosters and toms (Froman and McLean, 1996; Froman and Feltmann, 1998; Holsberger et al., 1998). When toms were selected out of a group based on the extreme limits of sperm mobility, and used to provide pooled semen, fertility was related to mobility phenotypes (Donoghue et al., 1999). Moreover, highly mobile sperm reside longer in a female”s SST and henceforth increase the fertilizing efficiency of the ejaculate (Froman et al., 2003).

2.6 Practical methods of fertility assessment (Female)

2.6.1 Sperm supply, storage, sperm loss and fertile period as parameters of fertility

The efficiencies of sperm supply by males and of sperm use by females are two of the most critical factors affecting fertilization rates. However, only a small proportion, probably less than 1% in quail, of the pool of semen deposited in the vagina or cloaca is stored in the SSTs (Brillard and Bakst, 1990; Birkhead and Fletcher, 1994). In avian species in general (Bakst et al. 1994), the capacity of the female oviduct to store sperm determines the duration of sperm storage, and thus the length of the fertile period, defined by Lake (1975) as the interval of time between the last mating or AI and the last fertile egg laid. The fertile period can last from several days to weeks, depending on the species (Birkhead and Moller, 1992). In quail, it lasts for 8-10 days (Birkhead and Fletcher, 1994). Bakst (1993) concluded that the differences between broilers and turkeys in the length of the fertile period were due to the great difference in their numbers of SSTs, with the turkey having a 5-fold more than broilers. Females that can store more sperm in their SSTs are proven to
maintain higher fertility (Schuppin et al., 1984). Importantly the length of the fertile period responds to genetic selection (Pingel, 1990).

The number of viable sperm in the female reproductive tract is directly related to the number of sperm that adhere to the outer PVL (Wishart, 1995) and the numbers of spermatozoa on successive eggs provide a measure of the rate at which spermatozoa are lost from the SSTs (Wishart, 1987; Brillard & Bakst, 1990). The rate of sperm loss can be calculated by regression of the log number of $S_{	ext{OPVL}}$ on eggs in relation to time. The slope of this relationship can be used to indicate the instantaneous sperm loss rate (Birkhead and Fletcher, 1994).

2.7 Selection for effective reproduction

Many studies have been carried out to investigate and improve the reproductive potential of poultry species, especially the chicken and the turkey (Burrows and Titus, 1939; Wilson et al., 1979; Ligon et al., 1990; Wishart and Steele, 1990; Froman et al., 1997; Froman et al., 2003; Pizzari et al., 2004; Brillard, 2004; Bilcik and Estevez, 2005; Wolc et al., 2009). Traditionally, fertility is regarded as a trait that is independent of the male and the female, so most initial efforts to increase reproductive performance of the chicken were concentrated on an individual sex and the selection process was only based on apparent physical characters. In males, those were the comb and wattle, for which a good size and good colour were associated with high fertility whereas, birds with bad legs or that were under-weight were culled (Wilson et al., 1979). The early reproductive selections in females were mainly based on reducing the age at sexual maturity, increasing the number of eggs produced early in the production cycle, and egg size and egg weight.

With advances in reproductive biology and the advent of technology, new methods for predicting male and female fertility have been discovered. For the chicken and turkey, as methods arose for evaluating the male on the basis of semen quality and true fertilizing ability of sperm, it has been realized that physical characteristics are not strongly predictive of male fertility. Instead, the true fertilizing ability of the male seems to depend on semen quality factors (ejaculate volume, sperm concentration, sperm viability, motility, mobility). There are significant differences among ganders in terms of semen quality (Łukaszewicz, 2005; Łukaszewicz and Kruszynski, 2003), and male selection can be based on these parameters (Donoghue, 1999; Froman et al., 2003). However, none of
these factors alone can give an estimate of the true fertilizing ability of a male so males should be evaluated based on as many traits as possible.

In chicken and turkey hens, estimating their ability to store sperm in their SSTs, and the length of their fertile period, makes selection more reliable. Therefore, there has been intensive effort to quantify sperm storage in the reproductive tract (Brillard, 1993; Wishart, 1995). Methods have been developed that use freshly laid eggs to gain insight into sperm numbers in the SSTs (Brillard and Bakst, 1990; Wishart, 1995; Wishart and Staines, 1999). One such assay relies on determining the number of holes created by sperm hydrolysing a passageway through the perivitelline layer of the ovum (Bramwell et al., 1995). This assay can also be used to predict fertility of the egg and provide reliable index of the numbers of spermatozoa stored and their loss rate from the sperm storage tubules (Wishart 1987 & 1988; Brillard & Antoine, 1990).

It is now known that fertility depends both on male and female components, as genetic and non-genetic factors originating from both the male and female affect egg fertilization and embryo development process (Brillard, 2004; Wolc et al., 2009). Moreover, it is known that selection of males and females based on their fertilizing ability and sperm supply and penetration can increase fertility of the breeder flocks significantly.

2.8 Dealing quail fertility problems

Poor fertility in Japanese quail has not yet been solved. This trait appears poorly estimated and there seems to be a tendency to continuously overlook fertility in the selection programs. Additionally, there has been no comprehensive study to quantify and understand this problem, so fertility in Japanese quail remains improperly understood and, consequently, the situation is now drastic. It is important to resolve this problem now so fertility and its related traits should be comprehensively studied. Factors contributing to fertility traits should be thoroughly investigated, key traits contributing to fertility should be highlighted for each gender, and then these traits should be incorporated in the breeding programs to enhance reproduction. To reach these goals, however, variety strategies may be adopted. One approach could be to adopt those methods and strategies which are presently being used, or have been successfully used in the past, to deal with fertility issues in meat-type chickens or turkeys. In this regard, the first and foremost issue in assessment
of quail fertility would be to switch from subjective methods to objective methods, thus allowing the gathering of more information and more data for assessment. As a next step, these methods could be used to investigate the underlying components of fertility on an individual basis, thus helping to eliminate individuals that are responsible for low overall fertility. This possibly would allow selection directly for the key fertility traits and help reduce the fertility problem in quail.

Therefore, the objectives of the present study were:

1. To use objective methods of fertility estimation in quail;
2. To describe contributions from each sex to flock fertility;
3. To identify male and female fertility traits that can be used to select high- and low-fertility phenotypes.
Chapter 3

Effect of age and strain on fertility of commercial Japanese quail breeder flocks as estimated by true egg fertility and \textit{in-vivo} sperm egg assay

3.1 Abstract

The effect of age and strain on egg fertility was studied in Japanese quail meat-type breeder. Three-day egg production was sampled from five strains, five times between 7 to 46 weeks of age (i.e. at Week 7, 16, 26, 36 and 46 of age). True egg fertility (viewing the germinal disc, GD), the number of sperm trapped (Sperm\textsubscript{OPVL}) and sperm-holes (Holes\textsubscript{IPVL}) present in the perivitelline membrane (PL) above and surrounding the GD (sperm-egg assay) were determined. The mean egg fertility increased (P < 0.05) from 79 % at Week 7 reaching a maximum 84 % by Week 26, and then declined to 64 % by Week 46. The mean number of Sperm\textsubscript{OPVL} and Holes\textsubscript{IPVL} were maximum at Week16 and then the numbers declined (Sperm\textsubscript{OPVL}, P<0.05; Holes\textsubscript{IPVL}, P < 0.01) from Week 26 to Week 46 of age. Between-strains, egg fertility (74-86 %), Sperm\textsubscript{OPVL} (13-19), and Holes\textsubscript{IPVL} (32-44) varied significantly (P<0.05). The strain by age interaction was significant (P < 0.001) for egg fertility, Sperm\textsubscript{OPVL} and Holes\textsubscript{IPVL}. However, high between-strain variations in egg fertility were observed at the post peak production (Week 36 and 46 of ages). The age-related changes in the egg fertility correlated more to Holes\textsubscript{IPVL} (r = 0.25; P < 0.05) than to Sperm\textsubscript{OPVL} (r = 0.22; P < 0.05). In conclusion, egg fertility in quail declines with age and this decline is a function of a number of Holes\textsubscript{IPVL} than Sperm\textsubscript{OPVL} present in the PVL. High between-strain variations in fertility parameters are related to post peak production.

3.2 Introduction

The ultimate goal in avian reproduction is to produce large number of viable, healthy offspring, while, egg fertility and embryonic mortality are the limiting factors in achieving this goal. Certain bird and managerial factors, such as genotype, flock age, body
condition, health status, mating efficiency, on farm egg handling, egg storage and incubation conditions may influence the fertility and hatchability of an egg (Kosin and Mun, 1965; Meijerhof, 1992; Brake et al., 1997; Wilson, 1997; Deeming and Middelkoop, 1999; Joseph and Moran, 2005). However, despite all these factors, hatchability largely depends on egg fertility as only fertile eggs can develop into an embryo. In turn, egg fertility changes with the flock age and the natural egg fertility pattern during the production period of a breeder flock is – low at the beginning of the laying period, reaching its peak in the middle, and then declining. For successful commercial operations therefore, it is essential to estimate, describe, and have exact knowledge of reproductive performance of breeder flocks, so that decisions about flock management i.e. flock culling/replacements, strategies to further breed improvement, elimination of farm/hatchery factors which may influence chick output can be exercised and underlying causes of decline in egg fertility and hatchability can be addressed.

To assess egg fertility, generally four methods are used i.e. i) investigation of the appearance of the GD in freshly laid eggs; ii) appearance of the GD combined with in vivo sperm-egg interaction assay (i.e. counting number of Sperm$_{OPVL}$ and Holes$_{IPVL}$ on the PL) of freshly laid eggs; iii) egg candling during egg incubation; and iv) after hatch egg-break-out analysis to differentiate clear eggs from, early, middle and late embryonic mortalities.

The methods of fertility assessment such that egg candling and after hatch egg-break-out analysis are carried out with incubated eggs. However, both of these methods have limitations i.e. candling is more effective for eggs with non-coloured shells and after hatch egg-break out analysis may be inaccurate in estimating true fertility because the post incubation interpretation of early developmental pathology is potentially difficult and early embryonic mortalities can be difficult to discriminate from infertility. On the other hand, the estimation of fertility in freshly laid eggs by viewing the germinal disc area and counting number of Sperm$_{OPVL}$ and Holes$_{IPVL}$ in the PVL is carried out in non-incubated eggs using microscopy, which eliminates the errors in fertility assessment due to early embryonic mortalities, the results can be obtained quickly and the data can be used to identify and interpret patterns and changes in mating activity between flocks or different age groups (Wishart and Stains, 1999; Gumulka and Kapkowska, 2005). This method considers two critical steps in fertilization process, which is binding of the sperm to the perivitelline layer (PL) and then subsequent acrosome reaction followed by entry of sperm
into the ovum. The result is a hydrolysed hole in the inner PVL through which sperm enter the ovum. Spermatozoa that fail to enter the egg cytoplasm become trapped in the outer PL. Both sperm holes and sperm trapped in the PL are identified in the perivitelline membrane of laid eggs through specific staining and microscopy techniques (Wishart, 1987; Bramwell and Howarth, 1992; Bramwell et al., 1995). It has been shown that estimating fertility of an individual egg, hen or flock can be performed quite accurately by combining the information of the appearance of GD area, number of Sperm$_{OPVL}$ and number of Holes$_{IPVL}$ on the PL of freshly laid eggs (Bramwell et al., 1995; Wishart and Staines, 1995 & 1999; Staines et al., 1998).

Fertility estimations in quails have been made through conventional methods i.e. after hatch egg-break-out analysis (Woodard and Alplanalp, 1967; Narahari et al., 1988; Narinc et al., 2013), hence, the true fertility and variations in true flock fertility are unknown. Even though an in-vivo sperm-egg interaction assay has previously been used for quails (Birkhead and Fletcher, 1994; Santos et al., 2011 & 2013), yet the decline in post-peak flock fertility has not been described and underlying causes of fertility decline remained unknown. This study was the first attempt to explore the utility of in-vivo sperm-egg interaction assay method for describing fertility of commercial quail breeder flock.

The aim of this study was to evaluate the effect of age and strain variables on quail fertility, using objective methods i.e. appearance of the GD combined with the count of number of Sperm$_{OPVL}$ and Holes$_{IPVL}$ found in the PVL of freshly laid eggs.

3.3 Material and methods

3.3.1 Housing and management
This study was carried out in commercial setup/farm conditions. Japanese quail breeders belonging to five meat type strains (i.e. A, B, C, D and E; these strains were from different blood lines and had similar 6 week body weight) were studied. The quails were housed in colony cages (11 males and 33 females per cage) and house conditions were maintained at 22-26°C house temperature (Game Farm Pty Ltd; Galston NSW, Australia), 14/10-h light/dark cycle, adequate ventilation and ad-libitum feed and water supply. The quails were fed the quail breeder diet containing 20.0 % CP and 11.5 MJ/kg ME. All the
procedures were carried out in accordance with the protocol approved by the Animal Ethics Committee of the University of Western Australia.

3.3.2 Experimental Design

Egg fertility data were collected at Weeks 7, 16, 26, 36 and 46 of age. 3 cages per strain (i.e. 128 quails per strain) x 5 strains (15 cages, n = 640 quails) approximated 15% of the total flock were marked and the same cages were sampled repeatedly for the entire period of study. At each age eggs were collected for three days and true fertility was estimated by the appearance of the GD – fertilised or not and counting the number of SpermOPVL and HolesIPVL present in the PVL. At each age approximately 250 eggs per strain were analysed.

3.3.3 In-vivo sperm-egg interaction assay

Eggs were opened, the appearance of the GD was captured with the DP-70 digital camera (Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia) and the PVL membranes were collected for SpermOPVL and HolesIPVL counts. The egg fertility status was determined by viewing the PVL around and over the GD area under a low power magnification aided by lateral illumination from a light source before the photograph was taken (Figure 3-A, 3-B). A piece of PVL (i.e. 1.5 x 1.5 cm) was collected around the germinal disc using a paper filter ring (Whatman, Grade 1, Sigma-Aldrich Co., Castle Hill, NSW, Australia). The collected membrane was washed with phosphate buffered saline (PBS; pH 7.4) to remove the adhering yolk and then placed on a microscope glass slide. For counting sperm, the PVL was stained with Hoescht dye 0.01 mM solution (Sigma-Aldrich Co. Castle Hill, NSW, Australia). The sperm nuclei were viewed with the help of a fluorescence microscope (Olympus BX60-FL, Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia) using a „U‟ filter cube with 372 nm excitation and 456 nm emission wavelengths (Figure 3-C). For counting sperm-holes, Schiff’s reagent (Sigma-Aldrich Co. Castle Hill, NSW, Australia) was used for staining the PVL section as described by Bramwell et al., (1995) and the sperm-holes were counted in six fields (5.7 mm² total PVL area) using a 20X objective and bright field optics along the horizontal axis passing through the GD region (Figure 3-D).
The percentage of fertile eggs was estimated by the following formula:
\[
\% \text{ fertile eggs} = \left(\frac{\text{Number of fertile eggs}}{\text{total number of eggs analysed}}\right) \times 100
\]

The Sperm_{OPVL} and Holes_{IPVL} were counted in 5.7 mm\(^2\) area of PVL and reported as Sperm_{OPVL}/PVL and Holes_{IPVL}/PVL area.

The rate of increase or decline in the numbers of Sperm_{OPVL} and Holes_{IPVL} was estimated for each strain between 7 and 16, 16 and 26, 26 and 36, and 36 and 46 weeks of ages. The rate of increase (positive value for the slope) or rate of decrease (negative value of the slope) in sperm or sperm-hole numbers was calculated using linear regression of the log number of Sperm_{OPVL} or Holes_{IPVL} as dependent variables on weeks of age (the back transformed values are presented in results).

3.3.4 Statistical analysis

The frequency distributions for Sperm_{OPVL} and Holes_{IPVL} numbers were positively skewed (i.e. skewness = 2.60 & 2.27; \(P < 0.000\), respectively) and negatively skewed for the percentage of fertile eggs (i.e. skewness = -0.40, \(P < 0.00\)). The egg fertility percentage data were arcsine retransformed, whereas, Sperm_{OPVL} and Holes_{IPVL} data were log transformed before subjecting to analysis (normal values are presented in graphs). The data for the effect of age on egg fertility, Sperm_{OPVL} and Holes_{IPVL} were analysed by analysis of variance, using linear mixed model procedure of the statistics using PASW Statistics 18.0, Release Version 18.0.0 (SPSS©, Inc., 2009, Chicago, IL, www.spss.com). When the significant difference among treatments was found, means were separated using LSD test.

The Linear regression analysis procedure of PASW 18.0 was used to estimate and compare the rate of decline in Sperm_{OPVL} and Holes_{IPVL} across the different ages and strains.

A non-parametric (Kolmogorov-Simirnov test, \(P < 0.01\) for logSperm_{OPVL} or logHoles_{IPVL}) Levene”s test was used to verify the equality of the spread of the eggs (with or without Sperm_{OPVL} or Holes_{IPVL}) between the strains (\(P > 0.05\)).

All data are reported as Mean ± SE and statistical significance was assessed at \(P < 0.05\) unless specified.
Fig 3-A Microscopic view of the GD from a fertile egg. GD characterized by a round, disc like appearance, without any vacuole.

Fig 3-B Microscopic view of the GD from an infertile egg. GD characterized by irregular shape containing numerous vacuoles.

Fig 3-C Sperm on the OPVL membrane. Stained by Hoescht dye 0.01 mM solution and viewed by fluorescent microscope.

Fig 3-D Sperm holes on the IPVL Membrane. Stained by Schiff’s reagent and visualized in bright field using light microscope.
3.4 Results

3.4.1 Effect of flock age, strain, and flock age x strain interaction on egg fertility, Sperm\textsubscript{OPVL} and Holes\textsubscript{IPVL}

There was a significant age effect on egg fertility ($F_{4,4857} = 17.5, P < 0.000$), Sperm\textsubscript{OPVL} ($F_{4,4863} = 52.7, P < 0.000$) and Holes\textsubscript{IPVL} ($F_{4,4863} = 74.1, P < 0.000$). Overall, high egg fertility ($81 \pm 1$ & $84 \pm 1$) and high number of Holes\textsubscript{IPVL} ($61 \pm 1.3$ & $50 \pm 1.6$) were observed at 16 and 26 weeks of ages, respectively, whereas, At week 46 of age, egg fertility ($66 \pm 1$), numbers of Sperm\textsubscript{OPVL} ($8 \pm 1$) and Holes\textsubscript{IPVL} ($18 \pm 2$) were at the lowest (Figure 3-1).

Strain effect was also significant on egg fertility ($F_{4,4857} = 9.6, P < 0.000$), Sperm\textsubscript{OPVL} ($F_{4,4863} = 10.6, P < 0.000$) and Holes\textsubscript{IPVL} ($F_{4,4863} = 7.0, P < 0.000$) although the magnitude was far less compared with the effect of age. Age x strain interaction effect was significant for egg fertility ($F_{4,4857} = 6.7, P < 0.000$), Sperm\textsubscript{OPVL} ($F_{4,4863} = 4.9, P < 0.000$) and Holes\textsubscript{IPVL} ($F_{4,4863} = 3.6, P < 0.000$).

The egg fertility increased for all (except strain A) until Week, 26 and then declined and high between-strain variation was observed mostly in the beginning and after the peak fertility has passed (Figure 3-2). Strain A ($83.3\pm1.4$) and D ($79.8\pm1.7$) showed the highest egg fertility followed by stains E, B and C ($75.2\pm1.5$, $74.8\pm1.3$ and $72.3\pm1.4$, respectively). Numbers of Sperm\textsubscript{OPVL} did not differ between strains A, B and D (mean number for 3 strains was $19.3 \pm 0.7$). Similarly, numbers of Holes\textsubscript{IPVL} did not differ between strains A, B and D (mean number for 3 strains was $43.5 \pm 1.7$). Numbers of Sperm\textsubscript{OPVL} for strains C ($17.0 \pm 0.7$) and E ($12.8 \pm 0.9$) and numbers of Holes\textsubscript{IPVL} for strains C ($39.9 \pm 1.6$) and E ($32.2 \pm 2.1$) were significantly less ($P < 0.05$) compared with all other strains.
3.4.2 Distribution of Sperm\textsubscript{OPVL} and Holes\textsubscript{IPVL} and their correlation with egg fertility

The pattern of logSperm\textsubscript{OPVL} and logHoles\textsubscript{IPVL} distribution suggested that both, Sperm\textsubscript{OPVL} and Holes\textsubscript{IPVL} had binomial distribution although this was more evident in Sperm\textsubscript{OPVL} than Holes\textsubscript{IPVL} with one subpopulation approximated a normal distribution. The population of sperm within normal Sperm\textsubscript{OPVL} and Holes\textsubscript{IPVL} distribution represented eggs with different number of sperm or sperm-holes and the eggs outside of this distribution represented those, which had zero or a few sperm or sperm-holes. The distributions of eggs with sperm or sperm-holes and without sperm or sperm-holes were different between the strains (Figure 3-5). Such that the distribution of eggs (with Sperm\textsubscript{OPVL} or without Sperm\textsubscript{OPVL}) in strain A was different (P < 0.05) from strain B; strain B was different (P < 0.05) from strains C, D and E, while, strain C was different from strain D. Furthermore, distribution of eggs (with Holes\textsubscript{IPVL} or without Holes\textsubscript{IPVL}) in strain A was different (P < 0.05) from strains B and D; strain C was different (P < 0.05) from strain D and strain D was different from strain E (P < 0.05). The correlation of egg fertility with Sperm\textsubscript{OPVL} and Holes\textsubscript{IPVL} was r = 0.22 and r = 0.25 (P < 0.005), respectively (regardless of the age and strain effect).
Fig. 3-2. Age x strain interaction for egg fertility of 5 meat type Japanese quail breeder strains (A, B, C, D and E). The mean fertility values are obtained from 3-day egg (~ 250) production per strain per week-age.

Fig. 3-3. Age x strain interaction for Sperm\textsubscript{OPVL} in 5 meat type Japanese quail breeder strains (A, B, C, D and E). The mean Sperm\textsubscript{OPVL} is obtained from 3-day egg (~ 250) production per strain per week-age.

Fig. 3-4. Age x strain interaction for Holes\textsubscript{IPVL} of 5 meat type Japanese quail breeder strains (A, B, C, D and E). The mean Holes\textsubscript{IPVL} is obtained from 3-day egg (~ 250) production per strain per week-age.
Fig. 3-5. Frequency distribution of Sperm_{OPVL} and Holes_{IPVL} in laid eggs of 5 Japanese quail breeder strains (A, B, C, D and E).
3.5 Discussion

The age and stain related changes in egg fertility correlated well with the changes in Holes$_{IPVL}$ in the PVL. Holes$_{IPVL}$ followed similar patterns of increase or decrease as egg fertility. However, this pattern was more comparable at the start of production and after the peak production had passed. Moreover, the fertility status of the strains and the changes in egg fertility were well explained by numbers of Holes$_{IPVL}$. The high egg fertility strains (e.g. A & D) were characterized by high Holes$_{IPVL}$ numbers and low fertility strains by low Holes$_{IPVL}$ numbers. Furthermore, the distributions of eggs with Sperm$_{OPVL}$ or Holes$_{IPVL}$ and without sperm or Holes$_{IPVL}$ were different for high and low fertility strains. Together, these observations suggested that Holes$_{IPVL}$ can be successfully used to interpret fertility in Japanese quail.

Although egg fertility was well described by Holes$_{IPVL}$ in this study, the correlation between these traits was low whereas, in other species such as in broiler breeders a strong relationship ($r = 0.80$) between egg fertility and the median GD Holes$_{IPVL}$ had been reported (Staines et al., 1998). The poor correlation of Holes$_{IPVL}$ with egg fertility we observed in this study might be due to the fact that we used mean values instead of median. In this study, however, we counted Sperm$_{OPVL}$ and Holes$_{IPVL}$ in 6 fields including both GD and non-GD regions. With these data, both mean and median values were generated for the best presentation (normal distribution) of data, and we used mean values based on these observations. The difference in correlated response might also be due to the use of both GD and non GD areas for counting sperm instead of GD area only, or on other hand these differences might be due to the different strains or species. Furthermore, we observed that age related changes in egg fertility were well represented by Holes$_{IPVL}$, while, the distribution of Holes$_{IPVL}$ was different between strains. These observations are not different from broiler breeders where small age-related changes in flock fertility were associated with relatively large changes in the median Holes$_{IPVL}$ of sampled of eggs (Hazary et al., 2000).

The pattern of fertility we observed in this study agrees in general with poultry species where the age related decline in fertility commences around the middle of the reproduction period and which is associated with the reduced retention of sperm by
females (Bakst et al., 1994; Brillard, 2003). In Japanese quails, age related studies of reproduction indicate high egg fertility (~90 %) between weeks 10 and 19 of age with a peak (96 %) in the 12-14 weeks period (Narahari et al., 1988). In contrast higher fertility beyond 20 weeks of age was observed in our study with a peak at the age of 26 weeks. This could be due to different methods used for estimating flock fertility i.e. methods of true fertility estimation in this study or genotype differences. However, high fertility between Weeks 18 to 21 had also been reported when sperm-egg interaction assay was used to investigate quail fertility in relation to mating ratios (Santos et al., 2011) which suggests that in-vivo sperm egg assay give higher fertility estimates compared with the conventional methods where the ability to differentiate fertile eggs with early embryonic mortalities from the infertile eggs might hamper estimation of true fertility. On the other hand, fertility decline has not been well described in the previous studies. In our study, egg fertility remained close to 80 % in the first 20 weeks of production but in the next 20 weeks it declined sharply (about 20 %). The decline in fertility was, however, rapid (approx. 10 % every 10 weeks). The changes in egg fertility were underpinned by changes in numbers of Sperm_{OPVL} and Holes_{IPVL} but the egg fertility pattern rather corresponded more with the sperm Holes_{IPVL} than with the Sperm_{OPVL}, suggesting that the ability of sperm to fertilize eggs could be lost with age. The underlying causes should be further investigated.

3.6 Conclusion

Numbers of Holes_{IPVL} can be used to express age and strain related changes in Japanese quail fertility. The decline in post-peak egg fertility was due to decline in numbers of sperm entering the ovum.
Chapter 4

Effect of age, sex and strain on fertility as measured by egg fertilization status and numbers of sperm and sperm-holes in the perivitelline layer of laid eggs

4.1 Abstract

Two experiments were carried out with the following objectives i.e. 1) to investigate the effect of age (male and female) and strain on parameters of fertility i.e. the number of sperm (Sperm_{OPVL}) and sperm-holes (Holes_{IPVL}) present in the egg perivitelline layer (PVL), the female sperm storage duration and the sperm loss rate (Experiment I); and 2) to determine if fertility parameters can be used to identify high and low fertility Japanese quail phenotypes (Experiment II).

Males (n = 45) and females (315) of five-meat type Japanese quail strains were studied between weeks 8 to 36 of age. Experiment I comprised of 4 stages and each stage was divided into a mating and non-mating phases. In mating phase of a stage, male (8-week-old in stage I aging to 36 weeks of age in stage IV) were mated separately with four different ages females (8, 16, 26 and 36 weeks of age), eggs were collected after mating and were used to estimate egg fertility, number of Sperm_{OPVL} and Holes_{IPVL}. In a non-mating phase of a stage, eggs were used to estimate female sperm storage duration and the sperm loss rate.

In Experiment I, male body weight and egg fertility increased with age, while, number of Sperm_{OPVL}, Holes_{IPVL}, the sperm storage duration, and the sperm loss rate increased and then decreased with age. The values, however, did not differ for males at week 8 and 36. Female body weight increased with age, wheras, Sperm_{OPVL}, Holes_{IPVL}, the sperm storage duration and the sperm loss rate decreased with age. There was an overall strain effect on male body weight, female body weight, egg fertility, Sperm_{OPVL}, Holes_{IPVL} and the sperm loss rate.
In Experiment II, males and females were selected into high (HF) and low (LF) fertility phenotypes using the mean number of Holes$_{IPVL} \pm 1$ STD; which were found to be the most correlated parameter with egg fertility in Experiment I. The HF male x HF female mating combination produced the highest egg fertility and the highest Holes$_{IPVL}$, while, these values were the lowest for LF male x LF female mating combination.

In conclusion, the Japanese quail female contributes more to the age-related decline in fertility than a male and the Holes$_{IPVL}$ appears to be a good candidate for fertility trait in quails.

4.2 Introduction

In poultry, factors influencing the age-related decline in fertility are still poorly understood. Egg fertility is a combined trait attributed to both, male and female. However, decline in fertility could be associated with factors relating to male (i.e. semen production rate and semen quality), female (i.e. egg quality, hostility of oviduct environment for sperm) or both (i.e. compatibility, physical and physiological). In aging males, reduction in sexual activity and decline in quantitative or qualitative aspects of semen has been observed (Sexton et al., 1989; Cordero & Miller, 1992; Sharpe et al., 2003; Pizzari et al., 2008). On other hand in females, reduction in sperm retention ability in the sperm storage tubules (SSTs) (Fasenko et al., 1992) and decrease in the capability of transporting sperm to the fertilization site (sperm loss rate) has been reported (Bramwell et al., 1996; Gumiłka and Kapkowska, 2005), whereas, a reduction in the number of sperm receptors on the oocyte surface has also been proposed (Bramwell et al., 1996). In broiler breeder however, female has been reported contributing more to the age-related decline in fertility compared with the male.

Japanese quail maintain high egg fertility between weeks 10 and 26 of age with a peak at the age of 20-26 weeks, and then it declines (Santos et al., 2011; Farooq et al., 2012; Chapter 3). So for we know that quail hens can store sperm for up to 10 days and sperm storage is negatively correlated with the sperm loss rate (Birkhead and Fletcher 1994), whereas, number of sperm trapped in the perivitelline layer decrease as function of the age of the female and the time after copulation (Santos et al., 2013). In quails, however, it is not clear whether both male and female contributes equally to the egg
fertility? Furthermore, it is not known what fertility traits should be considered to identify high and low fertility individuals and we don’t know how age and strain related variables effect male and female fertility. This study was planned to answer the aforementioned questions. There were two major objectives i.e. 1) to investigate the effect of age and strain variables on fertility traits i.e. egg fertility, Sperm_{OPVL}, Hole_{IPVL}, sperm storage duration and the sperm loss rate in commercial meat-type breeder quails and to determine which sex contributes more to the decline in fertility and 2) to investigate whether any particular measure of fertility could be used for identifying fertility phenotypes.

Two hypotheses were tested: 1) Male and the female contribute equally to egg fertility; and 2) Sperm_{OPVL} and Hole_{IPVL} are correlated with egg fertility and can be used to identify high and low fertility phenotypes.

### 4.3 Material and methods

#### 4.3.1 Housing and management

Japanese quail males (9 male x 5 strains; n = 45; all males were 8 weeks old to start with) and females (63 females x 5 strains; n = 315, comprising 4 ages; weeks 8, 16, 26 and 36) sampled randomly from 5 meat-type quail breeder strains (A, B, C, D and E, these strains were from different blood lines and had similar 6 week body weight), reared at the Game Farm (Pty Ltd. Galston NSW, Australia), and transferred to the Native Animal Facility of the University of Western Australia. The males and females were housed individually in commercial quail cages (male pen size = 19 x 40 x 23 cm WLH, Cimuka, Turkey; female pen size = 19 x 54 x 30 cm WLH, Venturi Valter, Predappio, Italy). The house environment was maintained at 22-26°C, 14/10-h light/dark cycle (lights on at 6:00 am and off at 8:00 pm), adequate ventilation and ad-libitum feed and water supply. The quails were fed the quail breeder diet containing 20.0 % CP and 11.5 MJ/kg ME. Cage enrichment was achieved by the provision of sand bath. All the procedures were carried out in accordance with the protocol approved by the Animal Ethics Committee of the University of Western Australia.
4.3.2 Experimental design

Two experiments were carried out

Experiment I

This experiment was carried out in four age-stages (i.e. stage I to IV; Figure 4-1) to test the hypothesis that the male and female contribute equally to the egg fertility.

In stage I, within a strain, each male (week 8 of age) was mated individually with four females, each of different age (8, 16, 26 and 36 weeks). Rotational mating was carried out for 16 days in which for the first 2 days a male stayed with each female for 4 hours and then move to next female, and for the next 14 days (mating phase) a male stayed with each female for 12 hours/day. During mating phase eggs were collected from each female and evaluated for fertilisation status, numbers of Sperm$_{OPVL}$, and Holes$_{IPVL}$.

At the end of the mating phase, the males and females were mated frequently (rotational mating; 2 hours per female) for 2 days to make the female fertile status similar because of their interval between the last mating and the end of the mating phase differed. Then the females were separated from males, eggs were collected for 14 days and numbers of Sperm$_{OPVL}$, Holes$_{IPVL}$, sperm storage duration, and the sperm loss rate were estimated.

In stage II, III and IV, the males were older (i.e. 16, 26 and 36 weeks) respectively, and the rest of procedures i.e. mating phase, non-mating phase and data collection remained the same as in stage I.

After stage I, every next stage started 10 weeks after a previous stage. All females moved to the older age groups, while, the females that started the stage I at 36 weeks of age left the experiment at the completion of this stage and were replaced by the new 8-week-old females for the next stage. Females removed from experiment after stage I were culled but, those removed after stage II and III were retained until the end of experiment for the final selection and potential allocation to Experiment II (Figure 4-1).
Fig. 4-1 Schematic diagram for experiment I showing male and female ages (Weeks; 8, 16, 26, 36) at the time of mating and data collection (egg fertility, $\text{Sperm}_{\text{OPVL}}$, $\text{Holes}_{\text{IPVL}}$, sperm storage and sperm loss rate at stages I, II, III and IV) and II (selection of high fertility male and females and data collection for egg fertility, $\text{Sperm}_{\text{OPVL}}$ and $\text{Holes}_{\text{IPVL}}$).
**Experiment II**

This experiment was carried out to test the hypothesis that $\text{Sperm}_{\text{OPVL}}$ and $\text{Holes}_{\text{IPVL}}$ are correlated with egg fertility and can be used to identify high and low fertility phenotypes.

The egg fertility, $\text{Sperm}_{\text{OPVL}}$ and $\text{Holes}_{\text{IPVL}}$, the sperm storage duration and sperm loss rate data of males and females that had more than one age-stage record were used in analysis and selection to Experiment II. Analysis of fertility parameters showed correlations between egg fertility, $\text{Sperm}_{\text{OPVL}}$, $\text{Holes}_{\text{IPVL}}$, sperm storage duration, and the sperm loss rate with $\text{Holes}_{\text{IPVL}}$ correlating more strongly with all other measured parameters than any other single parameter (Table 4-11 in results section).

Predicted values of egg fertility $\text{Sperm}_{\text{OPVL}}$, $\text{Holes}_{\text{IPVL}}$, sperm storage duration, and the sperm loss rate were calculated and assigned to each male and female. The high and low fertility males and females were identified using mean $\text{Holes}_{\text{IPVL}} \pm 1 \text{ Std.}$

20 x 20 high x low fertility males and females ($n = 40$) were selected and mating was carried out as shown in Figure 4-2. Five high fertility males (HF-male) were paired with 5 high fertility females (HF-female) and the other 5 high fertility males (HF-male) were paired with 5 low fertility females (LF-female). Similarly, 5 low fertility males (LF-male) were paired with 5 high fertility females (HF-female) and the other 5 low fertility males (LF-male) were paired with 5 low fertility females (LF-female).

All birds were kept individually and were joined for mating once a day for 12 hours. Individual mating were carried out for 7 consecutive days. The eggs were collected and fertility of those paired mating estimated by determining egg fertility status from GD and counting the numbers of $\text{Sperm}_{\text{OPVL}}$ and $\text{Holes}_{\text{IPVL}}$ present in the PL. Similar housing and management conditions were maintained as described for Experiment I.
4.3.3 In vivo sperm-egg interaction assay

Eggs were opened, the appearance of the GD was captured with the DP-70 digital camera (Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia), and the PVL was collected for Sperm_{OPVL} and Holes_{IPVL} counts. The fertilisation status (fertilised or not) of eggs was determined by viewing the GD under a low power magnification aided by lateral illumination from a light source before the photograph was taken. A piece (1.5 x 1.5 cm) of PVL was collected around the germinal disc using a paper filter ring (Whatman, Grade 1, Sigma-Aldrich Co., Castle Hill, NSW, Australia). The collected membrane was washed with phosphate buffered saline (PBS; pH 7.4) to remove the adhering yolk and then placed on a microscope glass slide. For counting Sperm_{OPVL}, the membrane was stained with Hoescht dye 0.01 mM solution (Sigma-Aldrich Co. Castle Hill, NSW, Australia). The sperm nuclei were visualized with a fluorescence microscope (Olympus BX60-FL, Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia) using a „U“ filter cube with 372 nm excitation and 456 nm emission wavelengths. For counting Holes_{IPVL}, Schiff’s reagent (Sigma-Aldrich Co. Castle Hill, NSW, Australia) was used for staining the PVL section as described by Bramwell et al., (1995). Numbers of Sperm_{OPVL} and Holes_{IPVL} were counted in six microscope fields (5.7 mm² total area) defined by 20x objective along the horizontal axis passing through the GD region.

1. The percentage of fertile eggs (egg fertility) was estimated by the following formula.

\[
\text{(Number fertile eggs produced by a female in a mating phase/total number of eggs produced by female in this phase)} \times 100
\]
2. The number of Sperm$_{OPVL}$ and Holes$_{IPVL}$ were counted in 5.7 mm$^2$ area of PVL and presented as Sperm$_{OPVL}$ and Holes$_{IPVL}$ per 5.7 mm$^2$ of PVL area.

4.3.4 Estimation of the sperm storage duration

Eggs collected during the non-mating phases were used. The female sperm storage duration was defined as determined from the duration of sperm storage and defined as the number of days the eggs containing Sperm$_{OPVL}$ or Holes$_{IPVL}$ were laid after the last mating.

4.3.5 Estimation of sperm loss rate

The number of spermatozoa in the outer perivitelline layer of laid eggs provides a reliable index of the numbers of spermatozoa in the sperm storage tubules, and the numbers of spermatozoa on successive eggs provide a measure of the rate at which spermatozoa are lost from the sperm storage tubules (Wishart, 1987; Brillard & Bakst, 1990). To calculate sperm loss rate the method described by Birkhead and Fletcher 1994 was used, except that the numbers of Sperm$_{OPVL}$ plus Holes$_{IPVL}$ were combined because in some eggs Holes$_{IPVL}$ were present while there was no Sperm$_{OPVL}$ or vice versa. The sperm loss rate was calculated for each female at each age-stage using the linear regression and log number of Sperm$_{OPVL}$ plus Holes$_{IPVL}$ counted in eggs laid between the last mating and the last egg containing sperm or sperm-holes. The slope of this relationship was taken for the instantaneous sperm loss rate.

4.3.6 Statistical Analysis

All percentage values were arcsine transformed and Sperm$_{OPVL}$ and Holes$_{IPVL}$ values were log transformed before analysis (normal values are presented in tables). The data were analysed as 3 x 3 factorial design (age of male x age of female x strain) with 9 replicates in each category using linear mixed model procedure for repeated measures of PASW Statistics 18.0, Release Version 18.0.0 (SPSS©, Inc., 2009, Chicago, IL, www.spss.com). The Linear regression analysis procedure of PASW 18.0 was used to estimate and compare male and female Sperm$_{OPVL}$ and Holes$_{IPVL}$ rate of decline with the age. Statistical significance was assessed at $P < 0.05$ unless specified. Pearson correlation procedure of PASW 18.0 was used to estimate correlation between different fertility traits.
4.4 Results

Experiment I

4.4.1 Effect of strain, male age and female age on body weight

There was a significant (P < 0.01) age effect on male and female body weight. Both, male and females body weight were lowest at Week 8 and highest at Week 36 of ages (Figure 4-3). The male body weight was positively (P < 0.05) correlated with male age and the percentage of fertile eggs whereas, the female body weight was positively (P < 0.01) correlated with female age and negatively (P < 0.01) correlated with $\text{Holes}_{\text{IPVL}}$, sperm storage duration, and with the sperm loss rate (Table 4-1).

There was a significant strain effect on male and female body weights (P < 0.01), moreover, strain x male age interaction was significant (P < 0.01), whereas, strain x female body weight interaction was not significant (P > 0.05). The male body weight ranged from 263-277 g being the highest (P < 0.05) for strain D and lowest for strain A, while, female body weight ranged from 311-338 g being the highest (P < 0.05) for strain E and lowest for strain C (Table 4-2). Between-strains, male and female body weight ranged from 261-278 g and 282-307 g at Week 8 and 265-303 g & 317-354 g at Week 36 respectively. Within-strains, the old males and females (Week 36) had higher body weight than the young male and females (Week 8) (Table 4-3).
Fig. 4-3 Effect of age on male (■) and female (□) body weight. Within sex, means containing different superscripts differ (P<0.05).

Table 4-1 Pearson correlation between male body weight and female body weight with male age, female age, percentage of fertile eggs, Sperm$_{OPVL}$, Holes$_{IPVL}$, sperm storage duration and sperm loss rate.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Female body weight (g)</th>
<th>Female age (weeks)</th>
<th>Male age (weeks)</th>
<th>Fertile eggs (%)</th>
<th>Sperm$_{OPVL}$</th>
<th>Holes$_{IPVL}$</th>
<th>Sperm storage duration (days)</th>
<th>Sperm loss rate (sperm/per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male body weight (g)</td>
<td>-0.47</td>
<td>-0.004</td>
<td>0.30**</td>
<td>0.08*</td>
<td>0.05</td>
<td>0.06</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>Female body weight (g)</td>
<td>0.36**</td>
<td>0.11**</td>
<td>-0.01</td>
<td>-0.06</td>
<td>-0.11**</td>
<td>-0.10**</td>
<td>-0.12**</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05 ** P < 0.01
Table 4-2 Mean (± SEM) effect of strain on egg fertility, Sperm_{OPVL}, Holes_{IPVL}, sperm storage duration and sperm loss rate

<table>
<thead>
<tr>
<th>Strain</th>
<th>Male body weight (g)</th>
<th>Female body weight (g)</th>
<th>Fertile eggs (%)</th>
<th>Sperm_{OPVL}</th>
<th>Holes_{IPVL}</th>
<th>Sperm storage duration (days)</th>
<th>Sperm loss rate (sperm/per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>263.3^a</td>
<td>325.7^a</td>
<td>69.6^a</td>
<td>6.9^a</td>
<td>20.7^a</td>
<td>6.8^a</td>
<td>-0.140^a</td>
</tr>
<tr>
<td>B</td>
<td>270.0^b</td>
<td>327.5^a</td>
<td>76.2^b</td>
<td>10.4^c</td>
<td>23.0^b</td>
<td>6.9^a</td>
<td>-0.157^b</td>
</tr>
<tr>
<td>C</td>
<td>274.5^bc</td>
<td>311.1^b</td>
<td>83.0^c</td>
<td>13.4^b</td>
<td>32.2^c</td>
<td>6.9^a</td>
<td>-0.159^b</td>
</tr>
<tr>
<td>D</td>
<td>277.2^c</td>
<td>328.2^a</td>
<td>71.1^ab</td>
<td>12.3^bc</td>
<td>26.6^b</td>
<td>7.0^a</td>
<td>-0.166^b</td>
</tr>
<tr>
<td>E</td>
<td>270.4^b</td>
<td>338.5^c</td>
<td>75.2^b</td>
<td>11.0^c</td>
<td>24.9^b</td>
<td>6.6^a</td>
<td>-0.160^b</td>
</tr>
<tr>
<td>SEM</td>
<td>2.1</td>
<td>2.3</td>
<td>1.9</td>
<td>0.7</td>
<td>1.6</td>
<td>0.2</td>
<td>0.007</td>
</tr>
</tbody>
</table>

^a-c Within a column, means without a common superscript differ (P < 0.05)

Table 4-3 Mean effect of strain x male age and strain x female age interactions on body weight (SEM = 4.4)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Male age (weeks)</th>
<th>Female age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>A</td>
<td>261.9^aA</td>
<td>258.9^aA</td>
</tr>
<tr>
<td>B</td>
<td>264.6^aA</td>
<td>264.0^aA</td>
</tr>
<tr>
<td>C</td>
<td>276.8^aB</td>
<td>261.4^aB</td>
</tr>
<tr>
<td>D</td>
<td>278.0^aB</td>
<td>259.4^aB</td>
</tr>
<tr>
<td>E</td>
<td>246.4^aC</td>
<td>268.6^bA</td>
</tr>
</tbody>
</table>

^a-c Within a row and sex, means without a common superscript differ (P < 0.05)

^A-C Within a column, means without a common superscript differ (P < 0.05)
4.4.2 Effect of strain, male age and female age on egg fertility percentage

There was observed a significant effect of male age (P < 0.01) but no significant (P > 0.05) effect of female age on the percentage of fertile eggs. The mean for the male ranged from 70-79 % being the lowest at Week 8 and the highest at Week 36 (P < 0.05). The mean female egg fertility percentage ranged from 73-76 % being the highest at Week 16 and 26 and the lowest at Week 36 (Table 4-4).

The male age x female age interaction was significant (P < 0.001). The percentage of fertile eggs was higher when same age male and females were mated or when old males (Week 26 or 36) were mated to young females (Week 8 or 16) (Table 4-10).

There was significant strain effect on the percentage of fertile eggs (P < 0.001) (Table 4-2).

The strain x male age (P < 0.001) and strain x female age (P < 0.001) interactions were also significant. Between-strains male and female egg fertility percentage ranged from 45-84 & 66-82 % at Week 8 and between 69-87 & 58-83 % at Week 36, respectively. Within-strains, the old males (Week 36) had higher egg fertility then the young ones (Week 8) from strain A (59 vs. 77 %), C (84 vs. 88 %) and D (45 vs. 86 %) while opposite was observed for strain B (82 vs. 76 %) and E (77 vs. 73 %) whereas, the old females (Week 36) had higher egg fertility than the young females (Week 8) from strain C (78 vs. 83 %), D (64, 74 %) and E (66 vs. 74 %) and opposite was observed for strain A (75 vs. 54 %) and B (80 vs. 68 %) (Table 4-5).

The strain x male age x female age interaction was not significant for egg fertility (P>0.05).
Table 4-4 Mean effect of age on male and female percentage of fertile eggs, $S_{\text{OPVL}}$, $H_{\text{IPVL}}$, sperm storage duration and sperm loss rate.

<table>
<thead>
<tr>
<th>Age</th>
<th>Fertile eggs (%)</th>
<th>$S_{\text{OPVL}}$</th>
<th>$H_{\text{IPVL}}$</th>
<th>Sperm storage (days)</th>
<th>Sperm loss rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>70.6$^a$</td>
<td>10.9$^c$</td>
<td>28.2$^a$</td>
<td>6.8$^b$</td>
<td>-0.15$^b$</td>
</tr>
<tr>
<td>16</td>
<td>76.6$^{bc}$</td>
<td>8.9$^b$</td>
<td>23.6$^b$</td>
<td>7.2$^a$</td>
<td>-0.16$^b$</td>
</tr>
<tr>
<td>26</td>
<td>73.3$^{ac}$</td>
<td>13.7$a$</td>
<td>25.2$^{ab}$</td>
<td>6.5$b$</td>
<td>-0.14$a$</td>
</tr>
<tr>
<td>36</td>
<td>79.6$^b$</td>
<td>9.4b$^b$</td>
<td>24.1$^{ab}$</td>
<td>6.7$b$</td>
<td>-0.17$b$</td>
</tr>
<tr>
<td>SEM</td>
<td>1.7</td>
<td>0.7</td>
<td>1.4</td>
<td>0.1</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age</th>
<th>Fertile eggs (%)</th>
<th>$S_{\text{OPVL}}$</th>
<th>$H_{\text{IPVL}}$</th>
<th>Sperm storage (days)</th>
<th>Sperm loss rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>74.5$^a$</td>
<td>12.2$^a$</td>
<td>29.1$^a$</td>
<td>7.5$^a$</td>
<td>-0.16$^a$</td>
</tr>
<tr>
<td>16</td>
<td>76.2$^a$</td>
<td>11.7$^a$</td>
<td>27.9$^a$</td>
<td>7.1$^b$</td>
<td>-0.16$^a$</td>
</tr>
<tr>
<td>26</td>
<td>76.2$^a$</td>
<td>10.4$^{ab}$</td>
<td>23.8$^b$</td>
<td>6.7$c$</td>
<td>-0.16$a$</td>
</tr>
<tr>
<td>36</td>
<td>73.3$^a$</td>
<td>8.8$^b$</td>
<td>21.0$^b$</td>
<td>6.0$d$</td>
<td>-0.14$b$</td>
</tr>
<tr>
<td>SEM</td>
<td>1.7</td>
<td>0.7</td>
<td>1.4</td>
<td>0.1</td>
<td>0.01</td>
</tr>
</tbody>
</table>

$^a-d$ Within a row and sex, means without a common superscript differ (P < 0.05)

Table 4-5 Mean effect of strain x male age and strain x female age interaction on percentage fertile eggs (SEM = 1.7).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Male age</th>
<th>Female age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Male age</th>
<th>Female age</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>61.2$^{AB}$</td>
<td>71.2$^{BA}$</td>
</tr>
<tr>
<td>B</td>
<td>82.9$^{AB}$</td>
<td>74.6$^{BA}$</td>
</tr>
<tr>
<td>C</td>
<td>84.0$^{AB}$</td>
<td>77.7$^{AB}$</td>
</tr>
<tr>
<td>D</td>
<td>45.5$^{AC}$</td>
<td>85.2$^{AB}$</td>
</tr>
<tr>
<td>E</td>
<td>79.6$^{AB}$</td>
<td>74.1$^{A}$</td>
</tr>
</tbody>
</table>

$^{ac}$ Within a row and sex, means without a common superscript differ (P < 0.05)

$^{A-C}$ Within a column, means without a common superscript differ (P < 0.05)
4.4.3 Effect of male age, female age and strain on Sperm\textsubscript{OPVL} numbers (Exp I)

The male age (P < 0.001) and female age (P < 0.01) effects were significant on Sperm\textsubscript{OPVL} numbers. The mean number of male Sperm\textsubscript{OPVL} ranged from 8-13 being the lowest at Week 16 and highest at Week 26 while, for the females the mean number of Sperm\textsubscript{OPVL} ranged from 8-12 being the lowest at Week 36 and highest at Week 8 (Table 4-4).

The male age x female age interaction effect was significant (P < 0.05) for Sperm\textsubscript{OPVL}. The high number of Sperm\textsubscript{OPVL} was observed when young males were mated to young females (Week 8 males x Week 8 females) or when old males (Week 26 or 36) were mated to young females (Week 8, 16 or 26; Table 4-10).

There was significant strain effect on Sperm\textsubscript{OPVL} numbers (P < 0.001). The strain x male age interaction was also significant (P < 0.001) while, the strain x female age interaction was not significant (P > 0.05). An older male (36 weeks old) from strains A, B, D and E had lower Sperm\textsubscript{OPVL} than a younger male (8 weeks old), and opposite was observed for strain C. On other hand 36 weeks old females from strain A, B, C and D had lower Sperm\textsubscript{OPVL} than 8 weeks old females and opposite was observed for strain E. Decline in Sperm\textsubscript{OPVL} between 8 and 36 weeks old females was significant (P < 0.05) for strains C and D only (Table 4-6). The strain x male age x female interaction effect was not significant for Sperm\textsubscript{OPVL} (P > 0.05)

4.4.4 Effect of male age, female age and strain on Holes\textsubscript{IPVL} numbers (Exp I)

There was significant (P < 0.001) female age effect on Holes\textsubscript{IPVL} numbers while, male age effect was not significant (P > 0.05). For females the numbers of Holes\textsubscript{IPVL} ranged from 21-29 being the highest at Week 8 and the lowest at Week 36 (Table 4-4)

The male age x female age interaction was significant (P < 0.05) for number of Holes\textsubscript{IPVL}. The high numbers of Holes\textsubscript{IPVL} were observed when 8-week-old males were mated with 8 and 16-week-old females or when older males were mated to younger females (Table 4-10).

There was significant strain effect on numbers of Holes\textsubscript{IPVL} (P < 0.01). Number of Holes\textsubscript{IPVL} ranged from 21-32 being the lowest (P < 0.05) for strain A and the highest for strain C (Table 4-2). Young males (8 Weeks old) from strain A had lowest (P < 0.05)
Holes$_{IPVL}$, while, young males from all other strains were similar. On other hand old males (36 Weeks old) from strain C and D had highest (P < 0.05) Holes$_{IPVL}$, wheras, old males from strain A, B and E were similar and had lower number of Holes$_{IPVL}$. Young females (8 week old) form strain C and D had highest (P < 0.05) Holes$_{IPVL}$, however, young females were similar from all other strains. Old females (36 Weeks) did not differ significantly between the strains for Holes$_{IPVL}$ (4-7)

The strain x male age and the strain x female age interaction were not significant (P > 0.05) for numbers of Holes$_{IPVL}$ (Table 4-7). Moreover, the strain x male age x female age interaction effect was also not significant for egg fertility (P > 0.05).
### Table 4-6 Mean effect of strain x male age and strain x female age interaction on SpermOPVL (SEM = 0.7)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Male age (weeks)</th>
<th>Female age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>A</td>
<td>7.3&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>6.0&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>12.8&lt;sup&gt;bB&lt;/sup&gt;</td>
<td>9.2&lt;sup&gt;bABC&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>13.4&lt;sup&gt;abB&lt;/sup&gt;</td>
<td>10.3&lt;sup&gt;abBC&lt;/sup&gt;</td>
</tr>
<tr>
<td>D</td>
<td>12.2&lt;sup&gt;abB&lt;/sup&gt;</td>
<td>12.6&lt;sup&gt;aC&lt;/sup&gt;</td>
</tr>
<tr>
<td>E</td>
<td>9.2&lt;sup&gt;aAB&lt;/sup&gt;</td>
<td>6.8&lt;sup&gt;bB&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-c</sup> Means in rows, within sex and strain, not having a common superscript differ (P < 0.05)

<sup>A-C</sup> Means in columns not having a common superscript differ between strains (P < 0.05)

### Table 4-7 Mean effect of strain x male age and strain x female age interaction on HoleSPVL (SEM = 1.4)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Male age (weeks)</th>
<th>Female age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>A</td>
<td>20.7&lt;sup&gt;aA&lt;/sup&gt;</td>
<td>17.9&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td>B</td>
<td>30.1&lt;sup&gt;bB&lt;/sup&gt;</td>
<td>20.2&lt;sup&gt;bAB&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
<td>35.1&lt;sup&gt;aB&lt;/sup&gt;</td>
<td>28.2&lt;sup&gt;aB&lt;/sup&gt;</td>
</tr>
<tr>
<td>D</td>
<td>28.7&lt;sup&gt;aAB&lt;/sup&gt;</td>
<td>28.8&lt;sup&gt;aB&lt;/sup&gt;</td>
</tr>
<tr>
<td>E</td>
<td>26.6&lt;sup&gt;abAB&lt;/sup&gt;</td>
<td>23.3&lt;sup&gt;abAB&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-c</sup> Means in rows, within sex and strain, not having a common superscript differ (P < 0.05)

<sup>A-C</sup> Means in columns not having a common superscript differ between strains (P < 0.05)
4.4.5 Effect of strain, male age and female age on female sperm storage duration (Exp I)
The effect of male (P < 0.05) and female age (P < 0.001) on the female sperm storage duration was significant. The mean male sperm storage duration ranged from 6.5-7.2 days being the lowest at Week 26 and the highest at Week 16 while, for the females, it ranged from 6.0-7.5 days, being the lowest at Week 36 and highest at Week 8 (Table 4-4).

The male age x female age interaction was not significant (P > 0.05) for female sperm storage duration. Similarly, the effect of strain, strain x male age, strain x female age and strain x male age x female age interaction were also not significant (P > 0.05) (Table 4-8).

4.4.6 Effect of strain, male age and female age on sperm loss rate
The effect of male and female age on sperm loss rate was significant (P < 0.001). The mean sperm loss rate for males ranged from -0.17 to -0.14 (sperm/day) being the lowest at Week 36 and the highest at Week 26 (Table 4-3). For the female, the sperm loss rate ranged from -0.16 to -0.14 sperm/day being the highest at Week 36 compared with all other ages. The male x female age interaction effect was not significant (P > 0.05) for sperm loss rate.

The effect of strain on sperm loss rate was significant (P < 0.01). The effect of strain x male age interaction was significant (P < 0.001) for sperm loss rate, while the effect of strain x female age interaction was not significant (P > 0.05). Old females had higher sperm-loss-rate for all the strains than the young females (Table 4-9). Males from strain A and C had highest sperm loss rate at Week 8 of age, while, at Week 36 males from strain B had highest sperm loss rate but all other strains were same. The effect of strain x male age x female age interaction was not significant for the sperm loss rate (P > 0.05).
Table 4-8 Mean effect of strain x male age and strain x female age interaction on days of sperm storage (SEM = 0.15).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Male age (weeks)</th>
<th>Female age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>A</td>
<td>6.7\textsuperscript{aAC}</td>
<td>7.7\textsuperscript{bA}</td>
</tr>
<tr>
<td>B</td>
<td>6.2\textsuperscript{aA}</td>
<td>7.7\textsuperscript{bA}</td>
</tr>
<tr>
<td>C</td>
<td>6.8\textsuperscript{aAC}</td>
<td>7.3\textsuperscript{aA}</td>
</tr>
<tr>
<td>D</td>
<td>7.2\textsuperscript{abBC}</td>
<td>7.5\textsuperscript{aA}</td>
</tr>
<tr>
<td>E</td>
<td>7.2\textsuperscript{abBC}</td>
<td>6.2\textsuperscript{bB}</td>
</tr>
</tbody>
</table>

\textsuperscript{ac} Within a row and sex, means without a common superscript differ (P < 0.05)
\textsuperscript{A-C} Within a column, means without a common superscript differ (P < 0.05)

Table 4-9 Mean effect of strain x male age and strain x female age interaction on sperm loss rate (sperm/per day) (SEM = 0.004).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Male age (weeks)</th>
<th>Female age (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>A</td>
<td>-0.125\textsuperscript{aA}</td>
<td>-0.127\textsuperscript{aA}</td>
</tr>
<tr>
<td>B</td>
<td>-0.178\textsuperscript{bB}</td>
<td>-0.167\textsuperscript{abB}</td>
</tr>
<tr>
<td>C</td>
<td>-0.133\textsuperscript{aA}</td>
<td>-0.176\textsuperscript{bBC}</td>
</tr>
<tr>
<td>D</td>
<td>-0.162\textsuperscript{bB}</td>
<td>-0.194\textsuperscript{bc}</td>
</tr>
<tr>
<td>E</td>
<td>-0.183\textsuperscript{bB}</td>
<td>-0.133\textsuperscript{hA}</td>
</tr>
</tbody>
</table>

\textsuperscript{ac} Within a row and sex, means without a common superscript differ (P < 0.05)
\textsuperscript{A-C} Within a column, means without a common superscript differ (P < 0.05)
Table 4-10 Mean effect of male and female age interaction on the percentage of fertile eggs, Sperm$_{OPVL}$ and Holes$_{IPVL}$.

<table>
<thead>
<tr>
<th>Male age (weeks)</th>
<th>Female age (weeks)</th>
<th>Egg fertility (%)</th>
<th>Sperm$_{OPVL}$</th>
<th>Holes$_{IPVL}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>8</td>
<td>76.6$^{aAB}$</td>
<td>15.0$^{aA}$</td>
<td>38.5$^{aA}$</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>70.0$^{aA}$</td>
<td>11.2$^{bAB}$</td>
<td>30.3$^{bA}$</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>73.3$^{aA}$</td>
<td>9.6$^{bA}$</td>
<td>22.4$^{bAB}$</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>69.4$^{aA}$</td>
<td>8.7$^{bA}$</td>
<td>23.1$^{bA}$</td>
</tr>
<tr>
<td>16</td>
<td>8</td>
<td>83.1$^{aA}$</td>
<td>10.7$^{aA}$</td>
<td>30.0$^{bB}$</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>85.1$^{bB}$</td>
<td>9.9$^{aA}$</td>
<td>26.0$^{aAB}$</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>73.9$^{bA}$</td>
<td>7.5$^{aB}$</td>
<td>19.9$^{bB}$</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>72.6$^{bA}$</td>
<td>8.6$^{aA}$</td>
<td>21.6$^{bA}$</td>
</tr>
<tr>
<td>26</td>
<td>8</td>
<td>70.1$^{bB}$</td>
<td>13.0$^{abA}$</td>
<td>25.0$^{abB}$</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>76.7$^{aA}$</td>
<td>16.8$^{abB}$</td>
<td>31.5$^{aB}$</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>85.9$^{bB}$</td>
<td>16.8$^{abB}$</td>
<td>29.1$^{aA}$</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>72.0$^{aA}$</td>
<td>10.2$^{bA}$</td>
<td>19.1$^{bA}$</td>
</tr>
<tr>
<td>36</td>
<td>8</td>
<td>81.2$^{aA}$</td>
<td>11.5$^{aA}$</td>
<td>26.8$^{abB}$</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>85.9$^{abB}$</td>
<td>10.8$^{aA}$</td>
<td>28.7$^{aA}$</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>81.5$^{abB}$</td>
<td>9.0$^{aA}$</td>
<td>25.9$^{abB}$</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>87.4$^{abB}$</td>
<td>8.1$^{aA}$</td>
<td>21.4$^{bA}$</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>3.8</td>
<td>1.5</td>
<td>2.9</td>
</tr>
</tbody>
</table>

$^{a,b}$Means for female age within same male age not having a common superscript differ (P < 0.05)

$^{A,B}$Means for same age females mated to different age males not having a common superscript differ (P < 0.05)

4.4.7 Selection of males and females to high and low fertility phenotypes (Experiment II, Part I)

There was significant correlation between the percentage of fertile eggs, Sperm$_{OPVL}$, Holes$_{IPVL}$, female sperm storage duration, and the sperm loss rate. The trait of “number of Holes$_{IPVL}$” showed the strongest relationship with most of the observed traits i.e. Holes$_{IPVL}$ with egg fertility ($r = 0.45$), Sperm$_{OPVL}$ ($r = 0.82$), sperm storage duration ($r = 0.23$), and sperm loss rate ($r = 0.31$) (Table 4-11). The mean number of Holes$_{IPVL}$ was 18.2. One standard deviation from the mean Holes$_{IPVL}$ separated the males and females into the low
and high fertility phenotypes having significant differences for fertility variables (i.e. percentage of fertile eggs, Sperm\textsubscript{OPVL}, Holes\textsubscript{IPVL}, female sperm storage duration, and sperm loss rate) (Table 4-12).

4.4.8 Mating of male and female fertility phenotypes (Experiment II, Part II)

The percentage of fertile eggs (100 vs. 55 %), number of Sperm\textsubscript{OPVL} (49.6 vs. 4.1), and number of Holes\textsubscript{IPVL} (60 vs. 14) were significantly (P < 0.05) higher for HF male x HF female compared to LF male x LF female combination. On other hand, mating HF male with LF female produced almost half (P < 0.05) the Holes\textsubscript{IPVL} (27 vs 48) compared with LF male x HF female combination, whereas, the percentage of fertile eggs between the two combinations did not differ significantly (P > 0.05) (Table 4-13).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fertile eggs (%)</th>
<th>Sperm\textsubscript{OPVL}</th>
<th>Holes\textsubscript{IPVL}</th>
<th>Sperm storage duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm\textsubscript{OPVL}</td>
<td>0.35**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holes\textsubscript{IPVL}</td>
<td>0.45**</td>
<td>0.82**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm storage duration (days)</td>
<td>0.23**</td>
<td>0.17**</td>
<td>0.23**</td>
<td></td>
</tr>
<tr>
<td>Sperm loss rate (per day)</td>
<td>0.34**</td>
<td>0.30**</td>
<td>0.31**</td>
<td>0.09*</td>
</tr>
</tbody>
</table>

* (P < 0.05), ** (P < 0.01)
Table 4-12 Mean (± SEM) effect of high fertility (HF) and low fertility (LF) phenotypes on the percentage of fertile eggs, Sperm\textsubscript{OPVL}, Holes\textsubscript{IPVL}, sperm storage duration and the sperm loss rate. High and low fertility males were selected based on numbers of Holes\textsubscript{IPVL}.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Female</th>
<th></th>
<th></th>
<th>Male</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fertile eggs (%)</td>
<td>Sperm\textsubscript{OPVL}</td>
<td>Holes\textsubscript{IPVL}</td>
<td>Sperm storage (days)</td>
<td>Sperm loss rate (sperm/day)</td>
<td>Fertile eggs (%)</td>
<td>Sperm\textsubscript{OPVL}</td>
<td>Holes\textsubscript{IPVL}</td>
<td>Sperm storage (days)</td>
</tr>
<tr>
<td>HF</td>
<td>70.1\textsuperscript{a}±0.2</td>
<td>9.6\textsuperscript{a}±0.5</td>
<td>26.1\textsuperscript{a}± 0.8</td>
<td>7.1\textsuperscript{a}±0.2</td>
<td>-0.16\textsuperscript{a}±0.0</td>
<td>77.1\textsuperscript{a}±1.6</td>
<td>9.3\textsuperscript{a}±0.5</td>
<td>22.3\textsuperscript{a}± 0.8</td>
<td>6.7\textsuperscript{a}±0.2</td>
</tr>
<tr>
<td>LF</td>
<td>65.1\textsuperscript{b}± 5.4</td>
<td>5.4\textsuperscript{b}±0.6</td>
<td>14.4\textsuperscript{b}±2.8</td>
<td>6.6\textsuperscript{b}± 0.2</td>
<td>-0.14\textsuperscript{b}±0.0</td>
<td>64.4\textsuperscript{b}± 2.3</td>
<td>5.5\textsuperscript{b}±0.4</td>
<td>14.5\textsuperscript{b}± 0.7</td>
<td>6.5\textsuperscript{b}±0.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a-b} Within a column and sex, means without a common superscript differ (P < 0.05)

Table 4-13 Mean (± SEM) effect of mating combinations (HF male x HF female, HF male x LF female, LF male x HF female, LF male x LF female) on the percentage of fertile eggs, Sperm\textsubscript{OPVL} and Holes\textsubscript{IPVL}.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HF male x HF female</th>
<th>HF male x LF female</th>
<th>LF male x HF female</th>
<th>LF male x LF female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of fertile eggs</td>
<td>100\textsuperscript{a}±5</td>
<td>92.1\textsuperscript{b}±6</td>
<td>91.1\textsuperscript{b}±6</td>
<td>55.6\textsuperscript{a}±7</td>
</tr>
<tr>
<td>Sperm\textsubscript{OPVL}</td>
<td>49.6\textsuperscript{c}±8</td>
<td>9.6\textsuperscript{b}±8</td>
<td>22.8\textsuperscript{c}±8</td>
<td>4.1\textsuperscript{d}±10</td>
</tr>
<tr>
<td>Holes\textsubscript{IPVL}</td>
<td>60.5\textsuperscript{b}±9</td>
<td>27.1\textsuperscript{b}±9</td>
<td>48.1\textsuperscript{c}±9</td>
<td>14.1\textsuperscript{d}±11</td>
</tr>
</tbody>
</table>

\textsuperscript{a-b} Within a row, means without a common superscript differ (P < 0.05)
4.5 Discussion

The findings of the study did not support the hypothesis that the male and the female contribute equally to egg fertility. The parameters of fertility differed between male and female as they aged. This was evident from decline in Spem$_{OPVL}$, Hole$_{IPVL}$, sperm storage duration and sperm loss rate for females but not for males.

The hypothesis that a parameter that has a strong correlation with egg fertility can be used to identify high and low fertility phenotypes has been accepted. The Hole$_{IPVL}$, a parameter mostly associated with the egg fertility, could be used to select males and females into fertility phenotypes. Mating within high fertility phenotype resulted with higher fertility than mating within a low fertility phenotype or between low and high phenotype.

Males and females maintained high fertility until Week 36 in this study but data suggest trend in age-related decline in fertility. Older females had less Spem$_{OPVL}$ and Hole$_{IPVL}$ compared with the young females. These observations suggest that there is a general tendency for females to drop their reproductive efficiency irrespective of male age or strain. These observations are in agreement with general perception of age-related decline in female reproduction i.e. the reduction in the ability of the female to retain sperm in the SSTs (Fasenko et al., 1992) and decrease in the capability of transporting them to the egg fertilization site.

Moreover, we observed that sperm storage duration decreased while the rate of sperm loss increased with female age. The pattern of sperm loss was similar to that described for domestic fowl, the zebra finch and for quails previously (Wishart, 1987; Birkhead et al., 1993; Birkhead and Fletcher, 1994). On other hand, males maintained high sperm storage duration and low sperm loss rate at older age, which suggest that the decline in female sperm storage duration was associated with high sperm loss rate rather than what was inseminated from male side which is in agreement with reports by Birkhead and Fletcher, (1994) and Santos et al., (2013) for quails and by Malecki and Martin, (2002) for emus.

Egg fertility decline, previously observed in a farm flock after 26 weeks of age (Farooq et al., 2012; Chapter 3) was not confirmed in this study. Possibly holding animals individually and pair-mating account for this difference as normal farm practice for this
species uses colony mating, characterised by intense mate competition. However, in a similar study in broiler breeder, no age-related decline in egg fertility was observed (Hocking and Bernard, 2010) indicating that high egg fertility can be maintained longer with frequent individual mating. In our study, however, we also observed feather loss condition among all males between stage III and stage IV (i.e. 26 to 36 Weeks of age). We are not sure about this, but this might be due to the self-molting of the males which then allow them to perform better at 36 weeks of age. But on other hand this condition was not observed in the females, while, still they maintained high egg fertility, though data presented in chapter 3 and in this chapter, clearly showed an age related decline in female ability to store and transporting them to the egg fertilization site. There are two possibilities for this i.e. 1) male molted themselves and intern their sperm production increased and due to continuous supply by pair mating, masked the effect of age related decline in female egg fertility or 2) pair mating made sure of continuous sperm supply and female SSTs remained filled and due to this reason egg fertility did not decline. However, both of these possibilities need to be further analysed to clarify these effects on age related decline in egg fertility.

In experiment II, numbers of Holes_{IPVL} was a parameter of egg fertility and the relationship was stronger than for any other single parameter. Allocation to high and low fertility phenotypes based on Holes_{IPVL} resulted in difference in most fertility parameters between high and low fertility phenotypes. Moreover, when selected males and females were mated, the egg fertility, the Sperm_{OPVL} and Holes_{IPVL} were improved confirming that numbers of Holes_{IPVL} are associated with the egg fertility and fertility phenotype of the quails. Holes_{IPVL} numbers have been indicator of fertility throughout this study. They were found to be useful in analysing farm flock fertility (Chapter 3), and in selection of high fertility males and females that could maintain or improve fertility in mating high fertility phenotypes. Previously, it has also been proposed that breeding efficiency (broiler breeder and turkey) is directly quantifiable as the number of spermatozoa that become associated with the egg perivitelline layer at the time of egg fertilization (Bramwell et al., 1996; Wishart, 1997). Moreover, it has been reported that, the median number of points of hydrolysis produced by spermatozoa in the inner perivitelline layer over the germinal disc (sperm-holes) was related to sample and flock fertility (Staines et al., 1998; Hazary et al., 2000), and in broilers sperm-holes changed with age (Hazary et al., 2000). This suggests
that expression of sperm-holes in egg fertility in quails is similar to the other species and is potentially a quantitative fertility trait that could be used in selection to maintain or improve fertility.

The fact that male age did not affect numbers of \( \text{Holes}_{\text{IPVL}} \) (Experiment I) and mating of HF male with LF female produced half the \( \text{Holes}_{\text{IPVL}} \) compared with LF male and HF female cross (Experiment II), indicates that this parameter is controlled by female. However, given that the cross between LF male and HF female produced half the number of \( \text{Holes}_{\text{IPVL}} \) compared with cross between HF male and HF females which suggests that the male phenotype also has some effect on number of \( \text{Holes}_{\text{IPVL}} \). The high number of \( \text{Holes}_{\text{IPVL}} \) produced by HF males might refer to their ability to produce better sperm compared with the low fertility males or this effect might be due to different genotypes. However, to answer this question that what really led HF male to give twice the \( \text{Holes}_{\text{IPVL}} \) than LF male produced when crossed with the HF female needs further investigation.

Furthermore, male and female body weight increased with age. The egg fertility was positively affected by male body weight while, female body weight was negatively associated with most egg fertility traits. This suggests that in Japanese quail male and female body weight is an important factor in fertility and it should be controlled where high male body weight seems desirable. This is not surprising, as in other species such as broiler breeder, special consideration is given to male and female body weight not only for proper reproductive development but also due to the fact that control of male body weight helps preventing some of the fertility losses which might be observed in over weight-old males due to increase in muscular-skeletal disorders (Hocking and Duff, 1989; Hocking, 1990; Attia et al., 1993). Moreover, significant male age and male by strain interactions for most of the egg fertility traits, and no strain by female age interaction for most of the observed traits suggested that more within and between-strain variation exists for males compared with the females and these variations should be accounted for, to maintain high male and flock fertility. The body weight dimorphism favouring females and selection for high body weight might be responsible for poor fertility due to mating incompatibility (greater female aggression/problems to achieve cloacal contact during mounting and copulation).
4.6 Conclusion

The fertility in Japanese quail, as evident from the numbers of Hole$_{IPVL}$ present in PL, is mainly controlled by the female. Age-related decline in fertility is dependent on female age and is independent of the male age and strain. Number of Hole$_{IPVL}$ can be successfully sued to identify high and low fertility phenotypes in quails and selection for high fertility in the male is also important to flock fertility.
Chapter 5

The velocity of Japanese quail (*Coturnix japonica*) spermatozoon and proportions of different categories of motile sperm change with concentration of the cloacal gland foam

5.1 Abstract

The motility of Japanese quail sperm has so far been subjectively assessed. Using the same traditional methods many efforts have been carried out to explain the role of proctodeal gland foam on quail sperm motility, however, the results have been conflicting till now. We used computer assisted semen analysis (CASA) to objectively describe quail sperm motility in response to addition of different foam concentrations. We found that addition of foam to semen significantly increased \( P < 0.05 \) the number of rapid and progressive motile sperm and also boosted \( P < 0.05 \) the sperm velocity parameters (i.e. VCL, VAP and VSL). However, the number of rapid and progressive motile sperm and sperm velocities increased only with 5 and 10 % foam concentrations, while, further increased in foam concentration (i.e. 15 and 20 %) resulted in decline \( P < 0.05 \) of the sperm velocities. In conclusion, in *vitro*, quail sperm motility increases with up to 10 % foam supplementation, while, addition of more than 10 % foam cause decline in sperm motility.

5.2 Introduction

Sexually mature quail male possess a functional proctodeal gland, which is located, immediately dorsal to the cloacal opening (McFarland *et al.*, 1968, Klemm *et al.*, 1973, Siopes and Wilson 1975). Proctodeal gland secretes a viscous muco-protein that is then whipped into foam by the action of the sphincter cloaca (Fuji and Tamura, 1967, Seiwert and Adkins-Regan 1998). Foam production increases rapidly as male approaches a female (Seiwert and Adkins-Regan 1998), and during copulation both semen and foam are
introduced together to the female. Unlike semen, foam is not required for fertilization (Marks and Lepore, 1965) and it is characterized as non-semen copulatory fluid, which is produced and stored separately from the semen. In quails the size of cloacal gland is directly related to plasma testosterone level (Balthazart et al., 1979). Males with well-developed proctodeal gland are characterized by high fertilization success (Mohan et al., 2002) and are preferred by the females in natural matings (Shit et al., 2010). This suggests that proctodeal gland size is important for quail reproduction; therefore, its functions need to be understood to comprehend male strategies in achieving fertilization success and to improve productivity of commercial flocks. However, with the available data and in the presence of many conflicting reports it is not clear yet as to why proctodeal gland secretions (i.e. foam) should accompany semen at copulation time.

Many hypotheses have been proposed and rejected with regards to explaining the role of cloacal gland foam in reproductive strategy of quails. The foam was thought to act as a post-copulatory sperm leakage plug from the female tract (Perez and Juarez 1966), a lubricant for the male's phallus (Renzoni 1968), a territorial marker (Schleidt and Shalter 1972), or as a source to reduce the rival male chance to fertilize a female (Adkins-Regan, 1999). However, no conclusive evidence supported these ideas. While, the popular views are that foam may serve to aerate sperm to facilitate sperm transportation in the oviduct, or may act as neutralizing agent to protect sperm from the hostile environment of the proctodeum, or that it aids in sperm competition, or it can act as medium for suspending sperm in the female proctodeum to provide insemination window (Cheng et al., 1989b, Singh et al., 2012) and may help them to reach the sperm storage tubules (SSTs) in sufficient numbers (Adkins-Regan, 1999). In support to these views males with intact proctodeal gland were found to give higher egg fertility compared with those having no foam or whose proctodeal glands were removed surgically (Ogawa et al., 1974; Cheng et al., 1989a; Adkins–Regan, 1999). Similarly, significantly higher fertilization success was achieved when semen was inseminated mixed with foam compared with no foam (Chelmonska et al., 2006). However, contrary to these findings no difference in the fertility was observed when semen was inseminated with or without foam (Lepore and Marks, 1966 and Kobayashi et al., 1972). Similarly, in naturally mated quails, no difference in egg fertility was observed when the males with intact proctodeal gland were mated to the
females in comparison to those males whose glands were removed surgically (Singh et al., 2008).

Although it is not certain as to how foam and sperm interact in vivo because no direct study has demonstrated it, yet it is evident that foam enhances sperm ability to reach the egg fertilization site (Cheng et al., 1989a; Adkins–Regan, 1999; Singh et al., 2012). However, it is not known as yet that what exactly happens to a sperm when it comes in contact with foam. In vitro studies (based on conventional methods of sperm motility evaluation) have shown that quail sperm form clusters when they are not supplemented with foam (Amano and Watanabe, 1966; Kobayashi et al., 1972 and Ogawa et al., 1974), however, when supplemented with foam sperm are found disintegrated in the medium, remained motile for an extended period of time (Fujihara et al., 1989, Cheng et al., 1989b, Biswas et al., 2010) and also show higher metabolic activity (Singh et al., 2011b). Contrary to these, a negative effect of foam supplementation was observed on normal sperm morphology compared with no foam (Chelmonska et al., 2006). However, recently it has been reported (Biswas et al., 2010) that only small quantity of foam is required to maintain sperm functions in vitro and mass motility of sperm either increased or decreased depending upon the concentration of foam added to semen. No such concentration effect was considered in the any of the previous (in vitro or in vivo) studies and it is unsure whether the available results are truly representative of real time foam-sperm relationship.

Discrepancies in the available data might partly be due to the fact that until now, the subjective (conventional) methods of quail sperm motility assessment have been used. Conventional method of sperm motility assessment in general are associated with large variation (as using traditional methods 30 to 60 % variation in sperm motility assessment for the same ejaculate in humans and animals has been reported), which makes comparison between studies difficult (Jorgensen et al., 1997; Verstegen et al., 2002) and are incapable of describing subtle changes in the sperm motion due to small changes in the treatments. The effect of foam on sperm motility has never been objectively assessed and even after the most recent report of foam concentrations effect (Biswas et al., 2010), it remains unknown whether changes in sperm motility score were due to changing numbers of motile sperm or changes in sperm velocities.

Contrary to conventional methods of semen evaluation new techniques of in vitro sperm motility assessment such as computer assisted sperm analysis (CASA) provide an
objective and sophisticated motility assessment and have the advantage of increasing the accuracy and reproducibility of measurements (Moce and Graham 2008). Furthermore, CASA permits determination of various sperm movement characteristics (kinematics) and the detection of subtle changes in sperm motion that cannot be identified by conventional manual sperm motility analysis. The CASA technology has been widely used to assess sperm movement characteristics from range of species such as rats (Moore and Akhondi, 1996), rabbits (Farrell et al., 1993), boars (Holt et al., 1997), bulls (Amann, 1989), turkeys (Bakst and Cecil, 1992), and humans (MacLeod and Irvine, 1995) and significant correlations of CASA motility measurements with fertility have been reported. Therefore, we used CASA system to provide an objective assessment of foam-sperm interaction in quails

The aims of this study were to devise a workable protocol for quail sperm CASA analysis and to evaluate quail sperm motility objectively in relation to different foam concentrations using CASA.

5.3 Materials and methods

5.3.1 Housing and Management
Japanese quail males (n = 65) were randomly selected from quail breeder flock and housed in commercial quail cages (pen size = 30 x 54 x 25 cm W.L.H., Cimuka, Turkey). The house environment was adjusted to 22 to 26 ºC temperature, 14/10 h light/dark cycle, adequate ventilation and ad-libitum feed and water supply. The males were fed a quail breeder diet containing 20.0 % CP and 11.5 MJ/kg ME. Cage enrichment was achieved by the provision of sand bath. Before start of semen collection, three weeks acclimatization period was given, during which time males were accustomed to handling and produce semen after getting stimulated by a teaser female. All the procedures were carried out in accordance with the protocol approved by the Animal Ethics Committee of the University of Western Australia.
5.3.2 Experimental design

1. To study the effect of different foam concentrations on sperm motility, proctodeal gland foam was collected from 20 males (age = 16 weeks) up to 2 days and then pooled together. Foam extract was prepared and used to set up 5, 10, 15 and 20 % foam treatments. Motility buffer (150 mM NaCl and 20 mM $\text{N}$-tris-[hydroxymethyl]-methyl-2-aminoethane-sulphonic acid, 35mM glucose and 0.1 % bovine serum albumin adjusted to pH 8.00 at 25 °C) without foam was used as control (0 % foam). Two ejaculates (one ejaculate per week) per male were collected and CASA sperm motility was recorded against the treatments.

2. The effect of “age” on foam-sperm interaction was assessed at Weeks 8, 16 and 26 of age. Proctodeal gland foam was collected from 45 males (for two days at each age) and pooled for particular “age”. Foam extract was prepared and diluted to give 10 % foam concentration. Motility buffer without foam was used as control or as 0 % foam. At each “age”, two ejaculates (one ejaculate per week) per male were analyzed and CASA sperm motility was recorded with 0 and 10 % foam treatments.

5.3.3 Foam collection and preparation of foam extract

The proctodeal gland foam was collected by gently squeezing either side of the cloacal gland with fingers and thumb (Mohan et al., 2002). The collected foam was pooled and to prepare foam extract, 1 part of foam was mixed (w/v) with 2 parts of normal saline (0.89 % w/v NaCl) in a centrifuged tube. The mixture was thoroughly vortexed for 20 to 30 minutes and then centrifuged at 10,000g for 30 minutes. The supernatant was collected and centrifuged again at 10,000g for 30 minutes. The final supernatant obtained was calculated as 33.3 % foam extract. The foam extract was transferred into small eppendorf tubes (i.e. 100 $\mu$l tubes) and stored at -20 °C.

5.3.4 Semen collection and preparation

Teaser method was used to stimulate male for semen collection (Chelmonska et al., 2008). Briefly, males were conditioned for stimulation by a teaser female and produce semen after receiving a gentle massage on the lumber region, followed by gentle squeezing of cloaca. While collecting semen to avoid contamination of semen with any debris
materials, feathers around the vent were clipped off and before each collection foam was used to be carefully removed from the proctodeal gland each time.

Immediately after collection semen was diluted 1 is to 2 (semen: buffer) with NaCl-TES (v/v) “collection buffer” (150 mM NaCl, 20 mM N-tris-[hydroxymethyl]-methyl 1-2-aminoethane-sulphonic acid, with pH adjusted to 7.40 at 25 ºC). After collection diluted samples were stored in water bath at 30 ºC for 10 minutes during which time sperm concentration was measured and foam treatments were prepared. To measure sperm concentration 1.5 μl of neat semen was diluted with 598.5 μl of formalin buffer (2 % formalin PBS buffer) in a 1 ml vial, mixed well and transferred to polystyrene cuvette and sperm concentration was immediately determined by recording the absorbance at 560nm in a spectrophotometer (Metertech UV/VIS SP8001, Taipei, Taiwan). The unknown concentration was then calculated from already established standard quail sperm concentration curve with the recorded absorbance.

The foam extract (33.3 %) was diluted with motility buffer (foam : motility buffer) in 1:15, 1:30, 1:45 and 1:60 ratios i.e. to prepare 5, 10, 15 and 20 % foam treatments respectively. Motility buffer without any foam addition was used as control and also taken as 0 % foam treatment.

5.3.5 CASA equipment, capturing properties and analysis
Sperm motility in sperm preparations (0, 5, 10, 15 and 20 % foam) was assessed using the Motility module of the Sperm Class Analyzer® (SCA) version 5.2 (Microptic S.L., Barcelona, Spain). Capturing data were collected using a Basler A602fc digital camera (Microptic S.L.) that was mounted on Olympus BX53 microscope (Olympus Optical Co., Tokyo, Japan) equipped with phase contrast optics and motorized heated stage (Prior Optiscan II, Prior Scientific Intl.).

The capturing properties of the SCA® system were set as follows: images per sec = 25, Frame rate = 60Hz, optics = Ph- (negative phase contrast), particle size 10 to 130 micron (particle area was determined by visually evaluating captured fields and selecting the particle area size range that resulted in detecting only the sperm head for motility analysis, thereby excluding any other cells or debris), progressivity = 70 % STR, light filter = green filter (IF 550, Olympus, Japan) and slides = Leja 10 (standard count four-chamber slide, Leja products B.V. Nieuw-Vennep, Netherlands). The SCA “Chicken”
setting with sperm curvilinear velocity (VCL, µm/s) intervals (i.e. 10 < slow sperm < 50 < medium sperm < 100 < rapid sperm) was used and sperm were categorized based on these cut-off values into slow, medium and rapid sperm.

For motility analysis we used NaCl-TES diluent (Holm and Wishart, 1998) with the supplementation of 35 mM glucose and 0.1% BSA (we also tested different other available diluents or semen extenders i.e. Lake’s, Ringer’s, BPSE, Normal saline, NaCl-TES, Tyrod’s and Quail diluent (Shit et al., 2010) with their original formulation and also with manipulation of different glucose and bovine serum albumen (BSA) concentrations, but we found NaCl-TES a simple and suitable diluent for quail sperm motility assessment, For motility recording a 2 to 3 µl of diluted semen was loaded on to the pre-warmed slide placed on a heated stage at 39 °C temperature and 45 seconds settle down time was given before starting motility recording. Ten to fifteen fields were captured until a total of 200 motile spermatozoa were collected (Mortimer, 1994; WHO, 1999). Fields were captured randomly to eliminate biasness toward higher sperm concentration or motility but fields that included debris or clumps of sperm were excluded. All captured sperm tracks were verified visually to delete incorrectly recorded tracks, e.g. due to colliding spermatozoa.

5.3.6 Statistical Analysis
All percentage values were subjected to arcsine transformation and all velocity values were square root transformed before subjecting to statistical analysis. The effect of different foam concentrations and age on sperm motility was analyzed using linear mixed model procedure of PASW Statistics 18.0, (SPSS©, Inc., 2009, Chicago, IL, USA). Males within treatments were used as an error term to test treatment effects and least square significant (LSD) test was used to separate the significantly different means (P < 0.05). Regression analysis procedure of simple linear regression (PASW 18.0) was used to estimate the decrease or increase in sperm motility per unit increased in foam concentration.

To investigate the pattern of sperm clustering in different foam concentrations, 2-step cluster analysis procedure was carried out in which “factor scores: were used to rank sperm in different categories.. The “factor scores” were obtained by PCA (principle component analysis) factorization of following sperm kinematic parameters i.e. (velocity curvilinear (VCL), velocity straight line (VSL), velocity average path (VAP), straightness (STR), linearity (LIN), lateral head displacement (ALH) and beat cross frequency (BCF).
5.4. Results

5.4.1 Effect of foam on sperm motility (Foam vs. no foam)

The mean number of motile, rapid and progressive sperm was significantly (P < 0.05) higher, while, medium sperm was significantly (P < 0.05) lower in 5, 10, 15 and 20% foam treatments compared with 0 % foam (Table 5-1). Similarly, Sperm velocity parameters (VCL, VAP and VSL) and progression ratios (LIN, STR, and BCF) were significantly (P < 0.05) higher in foam treatments compared with 0 % foam (Table 5-1).

The number of slow sperm did not differ between 0, 5, 15 and 20 % foam treatments (Table 5-1). Maximum VSL and VAP and Average VSL and average VAP for top 10 % sperm (within rapid sperm category) were same for 0 and 20 % foam treatments, while, maximum VCL and average VCL for top 10 % sperm were higher (P < 0.05) for 20 % foam treatment compared with 0 % foam. For 20 % foam treatment, ALH was significantly (P < 0.05) less compared with 0 % foam.
Table 5-1 Mean (±SEM) percentage of sperm in different sperm categories and values for sperm kinematic parameters (VCL, VAP, VSL, LIN, STR, BCF and ALH) as affected by different concentrations (0, 5, 10, 15 and 20 %) of foam estimated with CASA. Maximum VCL, VSL and VAP represent single maximum value obtained within a particular treatment. Avg. VCL, VSL and VAP top 10 % sperm represent mean (±SEM) values from top 10 % sperm within rapid sperm categories.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Foam (%)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>PROG (%)</td>
<td></td>
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<tr>
<td></td>
<td>22.4±1.7</td>
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<tr>
<td>Rapid (%)</td>
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<td></td>
<td>25.1±2.0</td>
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<tr>
<td>Medium (%)</td>
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<tr>
<td></td>
<td>29.4±0.9</td>
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<tr>
<td>Slow (%)</td>
<td></td>
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<tr>
<td></td>
<td>45.4±1.2</td>
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<tr>
<td>Avg. VAP (µm/s)</td>
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<tr>
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<td>47.2±2.4</td>
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<tr>
<td>Avg. VSL (µm/s)</td>
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<tr>
<td></td>
<td>35.1±2.1</td>
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<tr>
<td>Avg. VCL (µm/s)</td>
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<tr>
<td></td>
<td>70.8±2.8</td>
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<tr>
<td>Maximum VCL</td>
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<tr>
<td></td>
<td>256.5±7.9</td>
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<tr>
<td>Maximum VSL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>241.6±7.5</td>
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<tr>
<td>Maximum VAP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>246.1±7.4</td>
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<tr>
<td>Avg. VCL top 10% sperm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>196.7±0.3</td>
</tr>
<tr>
<td>Avg. VSL top 10% sperm</td>
<td></td>
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<tr>
<td></td>
<td>130.9±0.9</td>
</tr>
<tr>
<td>Avg. VAP top 10% sperm</td>
<td></td>
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<tr>
<td></td>
<td>156.1±0.6</td>
</tr>
<tr>
<td>STR (%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72.6±8.1</td>
</tr>
<tr>
<td>LIN (%)</td>
<td></td>
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<tr>
<td></td>
<td>48.1±1.1</td>
</tr>
<tr>
<td>BCF (Hz)</td>
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<tr>
<td></td>
<td>7.4±0.1</td>
</tr>
<tr>
<td>ALH (µm)</td>
<td></td>
</tr>
<tr>
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<td>4.1±0.05</td>
</tr>
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</table>

Means without a common superscript within a row differ (P < 0.05)

5.4.2 Effect of different concentrations of foam on sperm motility

The mean number of motile sperm, rapid and progressive sperm increased (P < 0.05) with the increase in foam concentration from 0 to 10 % and then declined (P < 0.05) (Figure 5-1 and 5-2). The percentage of medium sperm was significantly (P < 0.05) lower in 10 % foam compared with 15 and 20 %, while, the percentage of slow sperm remained significantly lower in 10 % concentration compared with all other foam treatments. The regression analysis showed that for every one percent increase in foam concentration from
0 to 10 %, the number of rapid sperm and progressive sperm increased by 1.43 and 1.02 % respectively, while, these parameters decreased at rate of 0.72 and 0.33 % between 10 to 20 % foam concentrations, respectively.

The mean sperm velocity (VCL, VAP and VSL) increased (P < 0.05) as the foam concentration increased from 0 to 10 % and then declined. On other hand although maximum values for VCL, VSL and VAP were significantly lower in 10 % foam concentration, yet the mean from top 10 % sperm for these parameters were significantly in 10 % foam higher treatments compared with 0, 15 and 20 % foam concentrations. The values for STR, LIN and BCF were same for all foam treatments.

![Graph](image)

**Fig. 5-1** Percent motile and non-motile sperm in different foam concentrations (0, 5, 10, 15 and 20 %).
5.4.3 Clustering within rapid sperm populations obtained in different foam concentrations
The population of rapid sperm obtained within each foam concentration was further classified using 2-step cluster analysis. The principle component analysis (PCA) of eight sperm motility factors (VCL, VSL, VAP, STR, LIN, WOB, ALH and BCF) reduced this data into two factors, which accounted for 86 % variability in the data. Sperm clustering based on these two factor scores gave three different sperm populations for each 0, 5, 10 and 15 % foam concentrations, and 6 populations for 20 % foam concentration (Table 5-2). The clustering results showed that 10 % foam had the highest proportion of top ranking sperm as compared to all other foam treatments.

5.4.4 Foam sperm relationship in response to age
Sperm motility parameters were significantly higher (P<0.05) for 10 % foam concentration as compared to no foam for all ages and the increased in sperm motility with the addition of foam was independent of the age effect (Table 5-3).
Table 5-2 Further categorization of sperm based on high, average and low sperm kinematics within rapid sperm population obtained in each treatment (0, 5, 10, 15 and 20 %). Cluster ranked from high to low in ascending order in each treatment. The percentage from original population represents proportion of sperm ranked high or low from the total rapid sperm obtained in each treatment. VCL (curvilinear velocity), VSL (straight line velocity), VAP (average path velocity), LIN (linearity), STR (straightness), WOB (Wobble), ALH (Lateral head displacement) and BCF (beat cross frequency)

<table>
<thead>
<tr>
<th>Foam %</th>
<th>Cluster no</th>
<th>VCL (µm/sec)</th>
<th>VSL (µm/sec)</th>
<th>VAP (µm/sec)</th>
<th>LIN (%)</th>
<th>STR (%)</th>
<th>WOB (%)</th>
<th>ALH (µm)</th>
<th>BCF (Hz)</th>
<th>Sperm no</th>
<th>Percentage from original population</th>
<th>Cluster ranking</th>
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Table 5-3 Percentage of motile (PMOT), percent rapid (Rapid), progressive motile (PROG) sperm and sperm kinematics; curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), Lateral head displacement (ALH) and beat cross frequency (BCF) recorded at Weeks 8, 16 and 26 of age against 0 and 10 % foam concentrations

<table>
<thead>
<tr>
<th>Age (Wks)</th>
<th>Foam (%)</th>
<th>PMOT (%)</th>
<th>Rapid (%)</th>
<th>PROG (%)</th>
<th>VCL (µm/sec)</th>
<th>VSL (µm/sec)</th>
<th>VAP (µm/sec)</th>
<th>LIN (%)</th>
<th>STR (%)</th>
<th>ALH (µm)</th>
<th>BCF (Hz)</th>
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<tr>
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<td>93.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>104.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>73.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>88.6&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>117.5&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>10</td>
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<td>104.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.5&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>0.6</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a-b</sup> Means without a common superscript in columns (within a specific age) differ (P<0.05)
5.5 Discussion

The first objective of this study was to devise a workable protocol for quail sperm CASA analysis. Quail produces a very minute quantity of semen (< 25 µl) per ejaculation, while, its semen is highly viscous and concentrated (3-4 billion sperm per ml). Undiluted quail semen is prone to agglutinate and form clusters in most commonly used diluents or extenders like Lake’s, Ringer’s and Tyrod’s (Amano and Watanabe, 1966; Fujihara et al., 1989; Chelmonska et al., 2006) therefore; it was necessary to add proctodeal gland foam to a medium or buffer for proper dispersion of sperm (Meda, 2002). However, to test, foam as a treatment, a medium without foam was needed in which sperm could move freely and disperse properly without forming clumps and also they would not be affected by high dilution rate required for CASA sperm motility assessment. For this purpose we used NaCl-TES as a collection buffer and semen was diluted with this buffer immediately after collection (Holm and Wishart, 1998). For CASA sperm motility assessment, however, we supplemented NaCl-TES buffer with 35 mg of glucose and BSA, and sperm motility was recorded at pH = 8.50 (which is close to the pH of female oviduct). This pH was chosen due to the fact that quail sperm motility decreases slightly between pH range of 8 to 9, but on other hand sperm velocity increases at this pH range (Holm and Wishart, 1998). Quail body temperature ranges from 41-42 °C however, we analysed sperm motility at 39 °C because in the given conditions, at 41 to 42 °C temperature, sperm were very highly motile and the duration for which the sperm were highly motile was very short and it was not possible to make unbiased motility recordings. To record CASA sperm motility we used standard CASA procedures as provided for sperm CASA analysis (see material and methods) and we found detectable difference among the treatments. Addition of foam to semen increased sperm motility, number of rapid sperm, progressively motile sperm and sperm velocities. The maximum numbers of motile sperm and the highest sperm kinematic parameters were recorded with 10 % foam and number of rapid and progressive motile sperm were nearly twice and sperm velocities were 30 to 40 percent higher compared with no foam. Moreover, these observations were consistent when foam effect was studied in response to age. Together, these results suggested that foam increases sperm motility when sperm come in contact with foam and the increase in sperm motility is due to increase in number of fast moving sperm and also with the increase in their speed. However, we found
that maximum motility is reached with 10 % foam at 39 °C, while in contrast, maximum velocity was reported with 5 % foam extract at room temperature (30 to 35 °C) where higher concentrations (>5 % foam) were found suppressing motility (Biswas et al., 2010). The differences in the obtained results through this study might be due to objective assessment of sperm motility we used or it can be due to different method of foam extract preparations.

On the other hand, in natural mating, full foam is inserted into the female proctodeum and it was found improving number of sperm reaching the egg fertilization site (Cheng et al., 1989a; Singh et al., 2012). A recent study (Finseth et al., 2013) has suggested that foam might be performing two functions, it increases the probability of fertilization and it might be decreasing the relative fertilization of rival males. The results of our study support both of these ideas as acceleration in sperm motility with the addition of foam (10 %) could thus improve number of sperm reaching the sperm storage glands and increase the rate of fertilization through high sperm velocity (Wishart and Palmer, 1986; Froman and McLean, 1996), while, on other hand high concentrations of foam may lead to suppression of motility of sperm from subsequent mating.

In addition to that, the decrease in sperm motility with higher foam concentration may also support the idea that foam holds the sperm in the proctodeum away from the path of the hard shelled egg coming down the tube, and then gradually release sperm to help them to reach the SSTs (Cheng et al., 1989 b). High foam concentration would be similar to the situation when the foam was freshly deposited in the ptoctodeum. At this time the function of the foam would be to hold the sperm in place and suppressing sperm motility to conserve energy and then later on when the disintegration of the foam mass starting from the outside edge at this time the function of the foam would be to gradually disperse the sperm and help them move up the oviduct as lower foam concentration has also been reported to stimulate sperm motility (Singh et al., 2011a). However, the foam concentrations i.e. 5, 10, 15, 20 % we used in this study were equal to 16.5, 33, 49.5 and 66 mg of foam. In natural quail produces 15-35 mg foam/bird and sometimes more. In this case, 5 to 10 % foam we used in our study represented the amount of foam which is deposited during natural copulations; therefore, our study is not in agreement with Cheng et al., 1989b) hypothesis unless 49-66 mg of foam is produced. However, demonstrations
of acceleration or cessation of sperm movements in vivo under different foam concentrations are still required to fully adopt or reject different hypothesis.

5.6 Conclusion

The proctodeal gland foam enhances sperm motility in-vitro, in concentrations up to 10%, whereas higher foam concentrations lead to suppression of sperm motility.
Chapter 6

Effect of age and strain on fertility on meat-type Japanese quail males

6.1 Abstract
Semen traits are associated with egg fertility and therefore, often used to evaluate male reproductive ability. In Japanese quail very little information is available on semen quality. Male evaluation based on sperm parameters is lacking, while, no quantitative information on factors that affects semen quality and sperm parameters is available. We used semen parameters to evaluate changes in male reproduction in relation to age and genotype. Four ejaculates (n = 16) per male (9 male x 5 strain; n = 45) were collected at each of Week 8, 16, 26, and 36 of age. The following parameters were estimated: volume of an ejaculate, sperm concentration, total number of sperm in an ejaculate, percentage of live normal sperm, and percentage of total abnormal sperm. The sperm motility was recorded using CASA system and the percentage of motile sperm (PMOT); percentage of Rapid, Medium and Slow sperm; percentage of progressive sperm (PROG) and other sperm kinematic parameters i.e. velocity curvilinear (VCL), velocity straight line (VSL), velocity average path (VAP); straightness (STR); linearity (LIN); wobble (WOB); beat cross frequency (BCF); and lateral head displacement (ALH) were determined.

The volume of an ejaculate and total sperm in an ejaculate remained high at Week 26 of age, whereas, sperm concentration started declining after Week 16 of age. A gradual increase (P < 0.05) in numbers of abnormal sperm was observed between Weeks 8 to 36 of age. On the other hand significant (P < 0.05) age-related decline in PMOT, PROG, VCL, VSL, VAP and ALH, was observed between Weeks 8 and 26 of age. The parameters like, LIN, STR and WOB decreased continuously (P < 0.05) between Week 8 and 36 of age.

The volume of an ejaculate, sperm concentration and total number of sperm in ejaculate, the percentage of live normal sperm and abnormal sperm varied significantly (P < 0.05) between the strains. Out of five strains, two strains (i.e. A & E) were characterized by high ejaculate volume and sperm concentration, while, two other (i.e. C & D) were
categorised as low for these parameters. High strain variations in the percentage of live normal sperm occurred at Week 8 and Week 36 of age, while, for percentage of abnormal sperm strain variations were high at Week 36 of age.

The strains varied (P < 0.05) in most CASA sperm parameters i.e. PMOT, Rapid, Medium, VCL, VSL, VAP, LIN, BCH, and STR. Strain A was low for most of the sperm kinematics, while, on the other hand rate of decline in sperm kinematics was highest for strain E after Week 16 of age.

It is concluded that age affects ejaculate of Japanese quail quantitatively and qualitatively, where the qualitative parameters decline earlier than quantitative parameters. Moreover, genetic strains differ in their response to age-related decline in sperm motility parameters.

6.2 Introduction

In avian species fertilization of an egg depends on production of quality ova and ability of the female to supply sperm to the egg fertilization site, while, on other hand production of quality sperm by male and their transfer to the female oviduct are important male factors. Both male and female produce good quality gametes in the early part of their production cycle, but, after peak production there is general tendency of decline in gamete quality. Post peak decline in egg fertility might have certain reasons such that, aging male quails are reported to show a sharp decline in LH and FSH receptors numbers, lowered sperm production and also reduction in sexual behaviour (Ottinger et al., 1983; Gorham and Ottinger, 1986; Howes, 1968). Similarly, aging female quails are reported to have lowered number of sperm trapped in the perivitelline layer overlaying the germinal disc, a decline in ability to store sperm in the sperm storage tubeless and increased in sperm loss rate (Santos et al., 2013; Chapter 3 & 4). In commercial quail flocks, the age-related decline in female reproduction is seen from decline in egg production (Ottinger et al., 1983) or deterioration of egg quality, while, the contribution of male to the decline in egg fertility remain unclear unless male specific traits are investigated.

In poultry, the most commonly used methods of assessing male reproduction are semen volume, color, concentration, viability, morphology, sperm motility and mobility (Donoghue and Wishart, 2000). However, in quail, quantitative and qualitative aspects of
semen evaluation are only limited to visual examination, estimation of ejaculate volume, and measurement of sperm concentration or subjective assessment of sperm motility (Chelmonska et al., 2008; Biswas et al., 2010; Singh et al., 2011).

This study was planned to provide an insight that how age affects male reproduction in present day meat type quails. Both, quantitative (ejaculate volume and sperm concentration) and qualitative (sperm motility) aspects of semen were studied through the production cycle of males. The age-related decline in sperm motility was given special consideration in this chapter as the quality of the sperm to be motile is important to male fertility and also to egg fertilization. We used CASA for sperm motility assessment as it can provide repeatable measurements and can detect subtle changes in sperm movement. CASA has also been successfully used to assess age-related changes in sperm motility characteristics in human (Sloter et al., 2006).

The aim of this study was to determine how ejaculate characteristics and sperm motility are affected by male age and genotype.

6.3 Material and methods

6.3.1 Housing and Management

The male Japanese quail (9 males x 5 strains, n= 45) were randomly selected from five meat type breeder strains (A, B, C, D and E; these strains were from different blood lines and had similar 6 week body weight) hatched and reared in commercial setup (Game Farm Pty Ltd; Galston NSW, Australia) to Week 6 of age and then transferred to lab facility. Males were housed individually in special quail cages (pen size = 19 width x 40 depth x 23 height cm, Cimuka quail cages, Turkey). The house environment was maintained at 22-26 °C, with 14/10-h light/dark cycle, adequate ventilation and ad-libitum feed and water supply. Males were fed on quail breeder diet containing 20.0 % CP and 11.5 MJ/kg ME. Cage enrichment was achieved by the provision of sand bath in each individual pen and sand was provided daily. Before start of semen collection two weeks acclimatization period was given during which males were accustomed to handling and semen collection using a teaser female. All the procedures and protocols were approved by the Animal Ethics Committee, The University of Western Australia.
6.3.2 Experimental design
The study was carried out at Week 8, 16, 26 and 36 of male age. At each age 4 ejaculates per male (2 ejaculate per week) were collected and duplicate samples were analysed. Ejaculate volume, sperm concentration (SC), total number of sperm per ejaculate, the percentage of live normal and abnormal sperm were estimated. Sperm motility was analysed using CASA. The following CASA motility parameters were studied and analysed; PMOT, Rapid, Medium, Slow, PROG, VSL, VCL, STR, LIN, WOB, BCF and ALH. Before start of semen collection at each age, males were mated to four females individually for two weeks (as described in Chapter 4, “mating phase”).

6.3.3 Semen collection and preparation
Teaser method was used for semen collection (Chelmonksa et al., 2008). The male quails were trained to stimulate by a teaser female and produce semen after given gentle massage on the lumber region followed by gentle squeezing of cloaca. To avoid any possible contamination of semen during collection, the feathers around the vent were clipped off and before collection of an ejaculate foam was carefully removed from the proctodeal gland.

Immediately after collection semen was diluted 1:2 with Nacl-TES buffer (collection buffer) (150 mM NaCl and 20 mM N-tris-[hydroxymethyl]-methyl 1-2-aminoethane-sulphonic acid), and adjusted to pH 7.40 at 25 °C (Holm and Wishart, 2000). Diluted samples were stored (~10 min) at 30°C for further use.

Ejaculate volume was measured using micro-pipette up to 0.1-0.2 µl precision. To measure sperm concentration 1.5 µl of neat semen was diluted with 598.5 µl of formalin buffer (2 % formalin PBS buffer) in a 1 ml vial, mixed well and transferred to polystyrene cuvette and sperm concentration was immediately determined by recording the absorbance at 560nm in a spectrophotometer (Metertech UV/VIS SP8001, Taipei, Taiwan). The unknown concentration was then calculated from already established standard curve for Japanese quail semen.
6.3.4 Foam collection and preparation of foam extract

To record sperm motility by CASA, semen samples were diluted in a medium comprised of motility buffer (150 mM NaCl and 20 mM N-tris-[hydroxymethyl]-methyl-2-aminoethane-sulphonic acid, 35mM glucose and 0.1 % bovine serum albumin adjusted to pH 8.00 at 25 °C) supplemented with 10 % proctodeal gland foam extract. The 10 % foam was used in this study as we established previously that for maximum in-vitro motility quail sperm required this much amount of foam foam (Chapter 5). To prepare foam extract, proctodeal gland foam was collected from individual males by gently squeezing either side of the cloacal gland with fingers and thumb (Mohan et al., 2002). Foam was pooled for particular strain and freezed at -20°C in airtight bottles. Foam extract was prepared by mixing the foam in normal saline (0.89 % w/v NaCl) in 1:2 ratios, followed by homogenization for 20-30 minutes (Biswas et al., 2010). The mixture was centrifuged at 10000g for 30 min. The supernatant was collected and centrifuged again at 10000g for 30 min. Supernatant was collected again and considered as 33.33 % of foam extract. The 10 % foam extract was prepared diluting 1 part of foam extract (33 %) with 30 parts of motility buffer (1: 30; 33 % foam: motility diluent). For each particular age fresh foam was collected and fresh foam extract was prepared.

6.3.5 Sperm viability and morphology

The percentage of viable morphologically normal sperm and total abnormal sperm were estimated by staining aliquots of sperm suspension with eosin-nigrosin (nigrosin 5 % w/v; eosin 1 % w/v; diluted in buffer for N/E stain; Sigma-Aldrich Co., Castle Hill, NSW, Australia) prepared as described by Bakst and Cecil (1992). Staining was done by mixing an aliquot of 3 µL (already diluted 1:2; semen: collection buffer) sperm suspension in 6 µL eosin-nigrosin solution and the mixture was left for two minutes at room temperature. A smear was made, dried and observed under 100 x oil objective on an Olympus BX60 microscope (Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia) mounted with digital camera (Olympus DP 70, Olympus Australia Pty. Ltd.) and connected to a computer screen. Sperm viability and morphology were evaluated on the same slide (Living stone International Pty. Ltd., Rosebery, NSW Australia) by the same person and categorised as follows: percent live sperm, and total abnormal sperm. A total of 350 sperm
were counted on each slide and duplicate samples were analysed for each male at each age.

6.3.6 CASA equipment, capturing properties and analysis

Sperm motility in semen and sperm preparations was assessed using the Motility/Concentration module of the Sperm Class Analyzer ® (SCA) version 5.2 (Microptic S.L., Barcelona, Spain). Sperm motility video data were collected using a Basler A602fc digital camera (Microptic S.L.) that was mounted on Olympus BHS microscope (Olympus Optical Co., Tokyo, Japan) equipped with phase contrast optics and a heated stage (Tokai hit thermo plate, Japan).

The capturing properties of the SCA ® system were set as follows: images per sec = 25; Frame rate = 60Hz; optics = Ph- (negative phase contrast, particle size = 10 micron, maximum particle size 130 micron (particle area was determined by visually evaluating captured fields and selecting the particle area size range that resulted in detecting only the sperm head for motility analysis, thereby excluding any other cells or debris); progressivity at 70 % STR; light filter = green filter and slides = Leja 10 (standard count four-chamber slide, Leja products B.V. Nieuw-Vennep, Netherlands). Chicken sperm curvilinear velocity (VCL, µm/s) intervals (i.e. 10 < slow sperm < 50 < medium sperm < 100 < rapid sperm) were selected as a default setting and sperm were categorized based on these cut-off values into slow, medium and rapid sperm. For motility recording A 2-3 µl of diluted semen was loaded on to the pre-warmed slide placed on a heated stage at 39 °C temperature and then 45 seconds settle down time was given before recordings were carried out. Ten to fifteen fields were captured until a total of 200 motile spermatozoa were collected (WHO, 1999). Fields were captured randomly to eliminate biasness toward higher sperm concentration or motility but fields that included debris or clumps of sperm were excluded. All captured sperm tracks were verified visually to delete incorrectly recorded tracks, e.g., due to colliding spermatozoa.

6.3.7 Statistical analysis

The CASA Motility parameters approximated the normal distribution and normal values were used for ejaculate volume and sperm concentration in analysis. Linear regression analysis procedure of PASW 18.0 (Release Version 18.0.0 (SPSS, Inc., 2009, Chicago, IL
www.spss.com) was used to investigate the age-related changes in each observed parameters. General linear model procedure of the PASW 18.0 was used to analyze the effect of strain and age on sperm motility traits and mean with significant differences were separated by least significant difference test (LSD). All significant values are reported at P < 0.05 level unless specified.

6.4 Results

6.4.1 Effect of age, strain and their interaction on ejaculate volume and sperm concentration and total sperm in ejaculate

There was significant age effect on ejaculate volume (F = 35.9, P < 0.000), sperm concentration (F = 10.1, P < 0.000) and total sperm per ejaculates (F = 24.9, P < 0.000) (Table 6-1). The effect of strain on ejaculate volume (F = 74.4, P < 0.001), sperm concentration (F = 10.6, P < 0.001) and total sperm per ejaculate (F = 50.9, P < 0.001) was also significant (Table 6-2). The strain E had the highest ejaculate volume, sperm concentration and total sperm per ejaculate followed by strain A, B, C and D (Table 6-2).

The age x strain interaction for ejaculate volume (F = 3.6, P < 0.001) and total sperm per ejaculate (F = 2.9, P < 0.001) was significant (Figure 6-1 & 6-2) while, it was not significant for sperm concentration (P > 0.05). Ejaculate volume increased between Week 8 to 26 of age for all the strains, however at Week 36 it decreased for strains A and D but remain similar to Week 26 for all other strains. For ejaculate volume and total sperm per ejaculate strains C and D were similar and the lowest, strains A and E were the highest and strain B was the mid strain (Fig 6-1 & 6-2).
Table 6-1 Mean (± STD) age values for ejaculate volume, sperm concentration and total sperm in an ejaculate. Four ejaculates in each age stage was collected form every male (n = 45)

<table>
<thead>
<tr>
<th>Male age</th>
<th>Ejaculate volume (µl)</th>
<th>Sperm concentration (x 10^6/µl)</th>
<th>Total sperm in an ejaculate (x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>7.3^a±4.2</td>
<td>1.40^a±0.44</td>
<td>11.3^a±8.6</td>
</tr>
<tr>
<td>16</td>
<td>10.5^b±5.1</td>
<td>1.70^b±0.53</td>
<td>19.2^b±12.1</td>
</tr>
<tr>
<td>26</td>
<td>11.3^b±4.7</td>
<td>1.50^c±0.45</td>
<td>18.3^bc±0.2</td>
</tr>
<tr>
<td>36</td>
<td>10.6^b±4.4</td>
<td>1.50^c±0.50</td>
<td>17.2^c±11.3</td>
</tr>
</tbody>
</table>

^a-c Means in column, without a common superscript differ (P < 0.05)

Table 6-2 Mean (± STD) strain (A, B, C, D and E) values for ejaculate volume, sperm concentration and total sperm in an ejaculate. 4 ejaculates per male were evaluated and 9 males per strain was analysed.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ejaculate volume (µl)</th>
<th>Sperm Concentration (x 10^6/µl)</th>
<th>Total sperm in an ejaculate (x 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12.4^a±0.3</td>
<td>1.50^a±0.1</td>
<td>20.5^a±0.7</td>
</tr>
<tr>
<td>B</td>
<td>9.6^b±0.3</td>
<td>1.50^a±0.1</td>
<td>16.1^b±0.7</td>
</tr>
<tr>
<td>C</td>
<td>7.4^c±0.3</td>
<td>1.50^a±0.1</td>
<td>11.6^c±0.7</td>
</tr>
<tr>
<td>D</td>
<td>7.4^c±0.3</td>
<td>1.30^b±0.1</td>
<td>10.4^c±0.7</td>
</tr>
<tr>
<td>E</td>
<td>13.3^d±0.3</td>
<td>1.70^a±0.1</td>
<td>23.8^d±0.8</td>
</tr>
</tbody>
</table>

^a-d Means with different superscript in columns for a particular parameter differ significantly (P < 0.05)
Fig. 6-1 Effect of age by strain interaction on ejaculate volume. 4 ejaculates per male and 9 males per strain (A, B, C, D and E) were analysed at each age stage.

Fig. 6-2 Effect of age x strain interaction on total sperm in ejaculate. 4 ejaculates per male and 9 males per strain were analysed at each age stage.
6.4.2 Effect of age, strain and their interaction on live normal and abnormal sperm numbers

The effect of age on the percentage of live normal (F = 20.4, P < 0.001) and abnormal sperm (F = 13.3, P < 0.001) was significant. The effect of strain was also significant for the percentage of live normal (F = 5.9, P < 0.001) and abnormal sperm (F = 2.5, P < 0.027). Overall, strain A had the lowest number of live normal sperm (69.0 ± 1.1 %) and the highest number of abnormal sperm (8.6 ± 0.6), whereas, strain D had the highest number of live normal (76.3 ± 1.1 %) and the lowest abnormal sperm (6.0 ± 0.7).

The strain by age interaction was significant for live normal (F = 7.5, P < 0.001) and abnormal sperm (F = 1.9, P < 0.05). The live normal sperm declined significantly for strain A and B between Weeks 8 and 16, and for strain B and D between Weeks 26 and 36 of ages. The age related changes in live normal sperm were not significant for strain C and E (Figure 6-3).

The percentages of abnormal sperm were similar for all the strains between Weeks 8 to 26 of age (except strain E where abnormal sperm increased significantly between Weeks 16 to 26). Total abnormal sperm increased significantly for strains A, B and D between Weeks 26 to 36 while, remained similar to Week 26 for strains C and E (Figure 6-4).
Fig. 6-3 Strain by age interaction on live normal sperm. 4 ejaculates from 9 males per strain (A, B, C, D and E) were collected at each age i.e. 8, 16, 26 and 36 weeks. Duplicate slides were prepared from each ejaculate were analysed.

Fig. 6-4 Strain by age interaction on the percentage of abnormal sperm. 4 ejaculates per male and 9 males per strain were analysed at each age i.e. 8, 16, 26 and 36 weeks. Duplicate slides were prepared for morphology from each ejaculate. Total abnormal sperm represents both live and abnormal sperm.
6.4.3 Effect of age, strain and their interaction on sperm kinematics parameters

The effect of age was significant for PMOT, PROG, Rapid, Medium, Slow sperm, VCL, VSL, VAP, LIN, STR, ALH and BCF parameters (Table 6-3, 6-4). The effect of strain was also significant for all above mentioned parameters except the percentage of Medium velocity sperm.

There was a significant age-related decline in PROG (0.48 % per week; P < 0.05) and percentage rapid sperm (0.50 % per week; P < 0.000) between Weeks 8 and 26 of age. However, PROG and percentage rapid sperm increased (0.77, 0.56 and 0.64 % per week; P < 0.001) between Weeks 26 and 36 of age. Number of medium sperm decreased (0.06 % per week; P < 0.05) between Weeks 8 and 16 and then increased (0.61 % per week; P < 0.05) between Weeks 16 to 36 of age (Table 6-3).

The effect of age by strain interaction was significant for PMOT, VCL, VSL, VAP, LIN, STR and BCF parameters. Out of five strains, three strains (i.e. B, D and E) showed a gradual decline (P < 0.05) in PMOT between Week 8 and 26 of age and then an increase between Week 26 & 36 of age. However, for strain A and C there was no specific age related trend for PMOT.

The sperm velocity parameters i.e., VCL, VSL and VAP decreased (P < 0.05) between Weeks 8 to16 of age for all the strains and then increased significantly (P < 0.05) between Weeks 26 to 36 (Figure 6-9 to 6-11).

The LIN, STR, BCF remained same between Weeks 8 and 16 of age, decreased reaching Week 26 of age for all the strains (except STR for strain C). However, at Week 36; LIN, STR and BCF values increased (P < 0.05 ) for strain A, C and D, while a further decrease was observed for strain B and E as compared to Week 26 of age (Figure 6-12 to 6-14).
Table 6-3 Mean (± STD) effect of age on PMOT, PROG, rapid and medium sperm percentage. 4 ejaculate per male per age were collected and sperm motility parameters was analysed by CASA. PMOT (percent motile sperm), Rapid (percent rapid sperm), PROG (progressive motile sperm), Medium (percent medium sperm)

<table>
<thead>
<tr>
<th>Male age</th>
<th>PMOT (%)</th>
<th>Rapid (%)</th>
<th>PROG (%)</th>
<th>Medium (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>67.4\textsuperscript{a}±14.1</td>
<td>23.2\textsuperscript{a}±10.2</td>
<td>43.1\textsuperscript{a}±12.4</td>
<td>35.1\textsuperscript{a}±10.3</td>
</tr>
<tr>
<td>16</td>
<td>63.9\textsuperscript{a}±15.5</td>
<td>19.8\textsuperscript{b}±12.7</td>
<td>35.9\textsuperscript{b}±14.1</td>
<td>27.3\textsuperscript{c}±10.4</td>
</tr>
<tr>
<td>26</td>
<td>59.4\textsuperscript{b}±18.2</td>
<td>14.0\textsuperscript{c}±9.8</td>
<td>34.0\textsuperscript{b}±14.0</td>
<td>33.5\textsuperscript{ab}±13.7</td>
</tr>
<tr>
<td>36</td>
<td>67.1\textsuperscript{a}±17.4</td>
<td>19.8\textsuperscript{b}±12.3</td>
<td>40.7\textsuperscript{a}±13.6</td>
<td>37.4\textsuperscript{b}±13.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a,b} Means with different superscript in columns for a particular parameter differ significantly (P < 0.05)
Table 6-4 Mean ± Std. Effect of age on sperm kinematics. 4 ejaculates per male per age were collected and sperm motility was analysed by CASA: VCL (curvilinear velocity), VSL (straight line velocity), VAP (average path velocity), LIN (linearity), STR (straightness), WOB (Wobble), ALH (Lateral head displacement) and BCF (beat cross frequency).

<table>
<thead>
<tr>
<th>Male age</th>
<th>VCL (µm/sec)</th>
<th>VSL (µm/sec)</th>
<th>VAP (µm/sec)</th>
<th>LIN (%)</th>
<th>STR (%)</th>
<th>BCF (Hz)</th>
<th>ALH (µm)</th>
<th>WOB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>115.3±19.2</td>
<td>82.0±19.3</td>
<td>93.7±19.2</td>
<td>70.5±7.3</td>
<td>87.0±4.6</td>
<td>9.3±0.9</td>
<td>3.9±0.5</td>
<td>80.7±4.7</td>
</tr>
<tr>
<td>16</td>
<td>100.6±23.5</td>
<td>73.3±22.5</td>
<td>82.6±23.1</td>
<td>71.5±7.8</td>
<td>87.7±4.6</td>
<td>9.4±1.1</td>
<td>3.7±0.4</td>
<td>81.1±5.4</td>
</tr>
<tr>
<td>26</td>
<td>100.5±19.2</td>
<td>69.8±19.0</td>
<td>80.8±18.9</td>
<td>67.3±9.4</td>
<td>85.5±5.5</td>
<td>8.8±1.2</td>
<td>3.6±0.5</td>
<td>78.3±6.8</td>
</tr>
<tr>
<td>36</td>
<td>106.2±23.0</td>
<td>72.2±21.8</td>
<td>84.4±21.8</td>
<td>66.9±9.3</td>
<td>84.5±6.3</td>
<td>9.0±1.3</td>
<td>3.8±0.5</td>
<td>78.5±5.7</td>
</tr>
</tbody>
</table>

Means with different superscript in columns for a particular parameter differ significantly (P < 0.05)
Fig. 6-5 to 6-8 Effect of strain (A, B, C, D and E) by age (Week 8, 16, 26 and 36) interaction on PMOT, PROG, rapid and medium sperm. PMOT (percent motile sperm), PROG (progressive motile sperm)
Effect of strain (A, B, C, D and E) by age (Week 8, 16, 26 & 36) interaction on VCL, VSL, VAP, LIN, BCF and STR.

VCL (curvilinear velocity), VSL (straight line velocity), VAP (average path velocity), LIN (linearity), STR (straightness) and BCF (beat cross frequency)
6.5 Discussion

The age-related changes in semen quantity and quality were investigated in this study. The ejaculate volume, sperm concentration, live normal sperm and most parameters of sperm motility declined with age. The values of some parameters were lower already at Week 16 than Week 8 of age, while some were declined after 16 weeks of age. However, some parameters returned to high levels at 26 or 36 weeks of age. All strains showed age-related changes in sperm velocity parameters, and therefore, these parameters appeared to be genotype dependent.

No significant age-related decline in ejaculate volume was observed in this study. Drop in sperm concentration was observed after Week 16 of age, but males seemed to maintain high sperm production with a further increased in age (Weeks 26 and 36). These observations suggested that the studied age duration did not have any strong effect on semen quantity. Previous work with quail reproduction has described no change in the mating behavior, plasma testosterone level and testes weight for Weeks 23 to 70 old males (Ottinger et al., 1983). Similarly, no change in the latency of mount was observed between Week 23 to 243 of male age (Cherkin and Eckardt, 1977) which suggested that quail males maintain high reproduction for a longer period of time which was evident with the ejaculate volumes in this study. Together, these observations implies that that subjective method of semen assessment such as ejaculate volume and sperm concentrations might not be used to study age-related decline in quail breeders, as breeder are culled around Week 40 of age and these parameters do not give much information about age related changes among individuals.

We observed increase in abnormal sperm and decline in live normal sperm with the advance in male age. Previously, high testicular abnormalities have been documented with quail age, (Eroschenko et al., 1977) which, presumably contributed to lower number of viable sperm and high number of sperm with abnormalities. While, to counter the fact that older males maintained high testosterone level (which is related to testis functions) it has been argued that abnormalities on spermatogenesis may be possible without significant changes in the testosterone level (Ottinger et al., 1983).

CASA sperm motility assessments have shown a quantitative evidence of age-related decline (Weeks 8-26) in proportion of motile sperm and their motion characteristics
in quails in this study while, similar age related changes have been reported in human (Sloter *et al.*, 2006). However, improvement in PMOT and all other sperm kinematics parameters were observed at later age (Week 36) which suggested that the age-related decline in semen quality parameter was reversible. The major effect of advancing male age (Week 8-26) was observed on PMOT, PROG, Rapid sperm and the VCL, VAP, VSL, LIN and BCF which suggested that older males produce less proportion of motile sperm with lower forward movement which might be associated with the reduced number of sperm present at PVL. This might be true as certain sperm kinematics parameters have been found associated with fertility such as VSL, LIN and BCF in turkeys were positively correlated with sperm mobility in freshly collected semen (King *et al.*, 2000); whereas, VSL, LIN and VAP were found to be associated with fertilization in rates (Hirano *et al.*, 2001), and PMOT, Rapid and PROG found to be associated with the low pregnancy in old men (Abramsson, 1988). Therefore, decline in sperm kinematics in quails might be one of the causes of lower post peak egg fertility.

Furthermore, the age related decline in LIN and BCF parameters suggest that sperm from older males (Week 26) follow less linear path and their frequency at which sperm cross the average path declined. Therefore, age-related decline in number of sperm reaching the egg PVL might be due to less PMOT produced by old males on the one hand and with reduced LIN and BCF motion of the remaining sperm on the other. However, no change in the STR and ALH parameters were observed with the age. ALH has been associated with the ability of sperm to penetrate cervical mucus in humans and fuse with oocyte, if same is assumed for the quail sperm motion (in hen’s oviduct), it might be suggested that sperm ability to fuse with ovum did not decrease within the studies ages.

Increase in proportion of motile sperm and sperm motility parameters between Week 26 to 36 was observed in this study. We don’t know the actual cause of it, but we did observe feather lost and re-growth from males between Week 26-36 of age which might be due to the molting and this condition would have reconditioned the males and increased the overall sperm quality as has been reported previously that molting may affect the morpho-physiological parameters in quails including reproductive organs (Faitarone *et al.*, 2008; Kashmiri and Vatsalya, 2011). Moreover, molting has also been found associated with increased in semen output from turkeys (Woodard *et al.*, 1975). In our study ejaculate volume decreased arithmetically between Week 26 to 36 of age and sperm concentration
remained same although sperm concentration was declined significantly between Week 16 to 26 of age. In a previous study (Chapter 4), a positive correlation of male body weight with age, ejaculate volume and sperm concentration was observed. Similar response was observed in this study however, male body weight and age was negatively correlated with most of the sperm motility parameters (data not presented). This suggests that an increase in these parameters was not due to male body weight. On other hand, improved semen quantity and quality could be due to molting, however, this was noted and further studies would need to confirm that.

In general, quail breeders are selected for the genetic traits that they will pass on to their offspring, e.g. growth rate or feed conversion efficiency, while, their reproductive performance, if assessed, is measured by judging physical traits such as cloacal gland area on the basis that physical appearance is determined by testosterone production by the testes. However, it is possible for a male to have normal testosterone production, but low fertility, and so these birds may produce large volume with good sperm concentration whereas, sperm quality may be compromised. The application of CASA to pick age-related changes in sperm motility characteristics may help to identify these males.

6.6 Conclusion

The quantity of ejaculate produced and sperm motility parameters motility Japanese depend on male age and strain of Japanese quail. Sperm motility parameters appear to decline earlier than ejaculate volume and sperm concentration.
Chapter 7

Investigation of sperm curve linear velocity (VCL) cut-off values for the computer aided semen analysis (CASA) to classify motile Japanese quail (*Coturnix japonica*) spermatozoa into velocity categories

7.1 Abstract

Using the Sperm Class Analyzer (SCA), we captured quail sperm motility (after suspending sperm in a motility buffer containing 10% proctodeal gland foam extract) and analysed the video files of 22,300 sperm tracks. Since, SCA uses curvilinear velocity (VCL) cut-off values to categorize sperm into different velocity categories (i.e. slow, medium and rapid sperm), to correctly categorize quail sperm into different categories, we determined quail sperm specific VCL cut-off values. Based on 22,300 sperm tracks, the median and mean VCL values were 84.6 µm/s and 97.3±67.9 (SD) µm/s, respectively. When the SCA default setting (i.e. slow sperm < 50 < medium sperm < 100 < rapid sperm) termed „chicken“ was applied to categorise quail sperm into three subpopulations (i.e. Rapid, medium and slow sperm), nearly 50 % of sperm were classified as „rapid“. Therefore, we sought new cut-off values using two approaches i.e. 1) we defined the upper and lower VCL cut-off values using mean and median VCL values i.e. median VCL ± 25 %, median VCL ± 50 %, median VCL ± 75 %; first and third quartile values (25 % cut-off setting); and 33 % and 66 % (33 % cut-off setting) of the VCL data; 2) we calculated heteroscedasticity in the original sperm data (i.e. n = 22,300 sperm tracks) using PCA and 2-step clustering analysis method.

The VCL cut-off values obtained either by median VCL ± 25 %, median VCL ± 50 %, or median VCL ± 75 % were similar to the values obtained from 25 % or 33 %, or the default (SCA) cut-off setting, therefore, we continued only with the three i.e. „default”, ”25 %” and ”33 %” settings. Amongst these three settings, when 25 % setting (slow < 36 <
medium < 154 < rapid) was used to categories sperm, and the males were clustered based on sperm categories and their corresponding sperm kinematics, the most distinct clusters (K-mean clustering based on factor score obtained by PCA of CASA sperm motility parameters) were obtained as compared to default or 33 % methods. On other hand when the 2nd approach was used to determine VCL cut-off values the mean of the high (164.6 µm/s) and low (51 µm/s) clusters were nearest to the upper and lower VCL cut-off values obtained by 25 % cut-off setting. Therefore, we concluded that the first and third quartile of VCL data or 2-step cluster analysis (after PCA factorization of sperm kinematics) can be to categorise quail sperm into rapid, medium and slow velocity categories.

7.2 Introduction

Sperm motility is often used to describe and predict fertility of an individual or a population as naturally only motile sperm can move through the barriers of the female reproductive tract and fertilize an egg. Thus sperm motility has become an essential criterion of most semen evaluations (Mocé and Graham, 2008). Traditionally, sperm motility is subjectively assessed and therefore, only motile and non-motile sperm are marked.

However, a normal sperm population is heterogeneous and consists of combinations of spermatozoa that can be grouped into subpopulations according to differences in measureable characteristics possibly because each sperm subpopulation represents spermatozoa in different physiological states (Abaigar et al., 2001) and each of these respond differently to changes in environment (natural or treatments). Thus to study these population, an objective assessment such as the computer assisted semen analysis (CASA) is carried out now-a-days. The CASA system details several parameters for both the wholesome sperm population and individual spermatozoa and can separate motile sperm into three categories i.e. rapid, medium and slow sperm. This categorization is based on the velocity of individual sperm, thus, velocity cut-off points (or values) are used to categorise sperm into rapid, medium and slow velocity groups. Given that each species sperm differ in motility characteristics it needs to be assumed that the VCL cut-off values
are unique to specie and they should be defined before the analysis is carried out (Maree and Horst, 2012).

Japanese quail farming has a worldwide presence but a common problem is variable fertility in the breeder flocks. Control of fertility is still little understood in quail reproduction, and our knowledge of sperm motility is based on conventional (subjective) methods of motility assessment, which in general are associated with large variation and potentially unable to give useful information. The computer-aided semen analysis (CASA) has been used to assess sperm movement characteristics of many species, for example of rat (Moore and Akhondi, 1996), rabbit (Farrell et al., 1993), boar (Holt et al., 1997), bull (Amann, 1989), turkey (Bakst and Cecil, 1992), chicken (McDaniel et al., 1998; Long et al., 2010), pigeon (Klimowicz et al., 2008) or a human (Barratt et al., 1993; MacLeod and Irvine, 1995). A significant correlation between CASA motility parameters and fertility has been found in most studies. In Japanese quail, CASA has not been used to describe sperm motility, and before this system can be used to categorise sperm into different velocity categories, the VCL cut off values specific for Japanese quail sperm should be determined.

This study was carried out to investigate different approaches to determine VCL cut-off values for Japanese quail sperm that CASA could use to classify sperm into different velocity categories.

7.3 Materials and methods

7.3.1 Housing and Management

Japanese quail males (n = 65, randomly selected from meat type breeders) were housed in commercial quail cages (pen size = 30 x 54 x 25 cm W.L.H., Cimuka, Turkey). The house environment was maintained at 22 to 26 ºC temperature, 14/10 h light/dark cycle, adequate ventilation and ad-libitum feed and water supply. The males were fed the quail breeder diet containing 20.0 % CP and 11.5 MJ/kg ME. Cage enrichment was achieved by the provision of sand bath. Before, start of semen collection three weeks acclimatization period was given during which males were accustomed to manhandling and produce semen after stimulated by a teaser female. All the procedures were carried out in accordance with the protocol approved by the Animal Ethics Committee of the University of Western Australia.
7.3.2 Experimental design

Semen was collected from individual males and sperm motility was recorded. Duplicate samples were analysed and each male was replicated twice.

Different methods based on the mean and median VCL values were used to define new VCL cut-off points for quail sperm. Sperm were re-categorised into rapid, medium and slow classes based on these new values. The data were interpreted to find the most suitable cut-off values which can give best ranking of males based on their sperm motility.

7.3.3 Semen collection and preparation

Teaser method was used for semen collection (Chelmonska et al., 2008). Briefly, males were trained to become stimulated by a teaser female and produce semen after being gently massaged on the lumber region followed by gentle squeezing of the cloaca. Immediately after collection semen was diluted 1:2 (v/v; semen/buffer) with Nacl-TES “collection buffer” (150 mM NaCl, 20 mM N-tris-[hydroxymethyl]-methyl-1-2-aminoethane-sulphonic acid, 35mM glucose with pH adjusted to 7.40 at 25 °C (Holm and Wishart, 1998). Diluted samples were stored at 30°C in a water bath for 10 min. To measure sperm concentration, 1.5 μl of neat semen was diluted with 598.5 μl of formalin buffer (2 % formalin PBS buffer) in a 1 ml vial, mixed well and transferred to polystyrene cuvette and sperm concentration was immediately determined by recording the absorbance at 560nm in a spectrophotometer (Metertech UV/VIS SP8001, Taipei, Taiwan). The unknown concentration was then calculated from already established Japanese quail sperm concentration standard curve with the recorded absorbance.

The proctodeal gland foam was collected from each male by gently squeezing the either side of the cloacal gland with fingers and a thumb (Mohan et al., 2002). The foam extract was prepared by mixing the foam (pooled foam) in normal saline (0.89 % w/v NaCl) in 1:2 ratio (foam: normal saline) followed by thorough mixing for 20-30 minutes (Biswas et al., 2010). The mixture was centrifuged at 10000g for 30 minutes. The supernatant was collected and centrifuged again at 10000g for 30 minutes. The final supernatant obtained was estimated as 33.3 % foam extract. A foam extract was diluted with a specific volume of the “motility buffer” (150 mM NaCl and 20 mM N-tris-[hydroxymethyl]-methyl-2-aminoethane-sulphonic acid, 35mM glucose and 0.1 % bovine
serum albumin adjusted to pH 8.00 at 25 °C) so that the buffer contained 10 % foam (the amount of foam that gives the maximum sperm velocity). Semen samples were diluted with this buffer and after equilibration for approximately 30 sec sperm motility was recorded.

7.3.4 CASA equipment, capturing properties and analysis
Sperm motility was recorded using the Motility module of the Sperm Class Analyzer® (SCA) version 5.2 (Microptic S.L., Barcelona, Spain). Capturing was achieved using a Basler A602fc digital camera (Microptic S.L.) that was mounted on the Olympus BX53 microscope (Olympus Optical Co., Tokyo, Japan) equipped with a phase contrast optics and motorized heated stage (Prior Optiscan II, Prior Scientific Intl.).

The capturing properties of the SCA® system were set as follows: images per sec = 25, Frame rate = 60Hz, optics = Ph- (negative phase contrast, particle size = 10 micron, maximum particle size 130 micron; particle area was determined by visually evaluating captured fields and selecting the particle area size range that resulted in detecting only the sperm head, thereby excluding any other cells or debris), progressivity at 70 % STR. The sperm VCL intervals (i.e. 10 µm/s < slow sperm < 50 µm/s < medium sperm < 100 µm/s < rapid sperm) were selected from a default SCA setting termed “Chicken” and sperm were categorized based on these cut-off values into slow, medium and rapid sperm. For motility recording a 2-3 µl of diluted semen was loaded onto a pre-warmed slide chamber (10µm-4-chamber slide, Leja products B.V. Nieuw-Vennep, Netherlands) placed on a heated stage at 39°C temperature and 45 seconds settling down time was given before recording. Ten to fifteen fields were captured until at least 200 tracks of motile spermatozoa were collected (Mortimer, 1994; WHO, 1999). Fields were captured randomly to eliminate biasness toward higher sperm concentration or motility but fields that included debris or clumps of sperm were excluded. All captured sperm tracks were verified visually to delete incorrectly recorded tracks, e.g., due to colliding spermatozoa.
7.3.5 Interpretation of sperm categories (i.e. rapid, medium and slow) for Japanese quail sperm

For classifying sperm into the „rapid“, „medium“ and „slow“ categories the SCA provided a default setting termed „chicken“ (i.e. 10 < slow < 50 < medium < 100 < rapid). After data collection (n = 22,300 sperm tracks) and preliminary evaluation, the median and mean sperm values were 84.6 µm/s and 97.3±67.9 (±SD) µm/s, respectively, (min. 10.0 to max. 303.7 µm/s). With the SCA default setting, the 100 µm/s cut-off value classified nearly 50 % of Japanese quail sperm into „rapid“ category, which could not be in agreement with the data distribution (Fig. 1). To determine the cut-off values that would better reflect distribution of sperm velocity in Japanese quail we used two approaches. First, we compared the SCA default setting with the other arbitrarily chosen mean and median VCL values. Using VCL (Table 1) we calculated several different range of values as follows: i.e. (1) using the natural VCL distribution (Fig. 1), the upper (rapid) and lower (slow) cut-off values were defined by subtracting and adding 25 %, 50 % and 75 % deviation from the median VCL value (i.e. median±25 %, median±50 %, median±75 %); (2) the inter-quartile ranges were calculated and H-spread was defined for lower, middle and upper quartiles. The upper-cut-off value was then taken as the third quartile (75 %) and the first quartile (25 %) was taken as lower cut-off value; (3) the upper cut-off point was taken at 66% and lower cut-off value was taken at 33 % of the VCL. Alternately (i.e. through the 2nd method), we determined the cut-off values using 2-step cluster analysis after factorization of sperm kinematic parameters (VCL, VSL, VAP, STR, LIN, ALH, WOB, and BCF using the principle component analysis (PCA).

7.3.6 Statistical Analysis

The mean and median values and distribution of the data were calculated using SPSS 18.0 (PASW Statistics 18, Release Version 18.0.0 (=SPSS, Inc., 2009, Chicago, IL, www.spss.com) and H-spreads approximating the categories of sperm in different cut-off settings were defined. The natural VLC distribution was positively skewed (skewness; 0.450, Kolmogorov-Smirnov test, P < 0.000, Fig.1) and the mean was 97.3 µm/s and the median 84.6 µm/s, while, in case of square root transformed data amen and median values were 84.2 µm/s and 84.6 µm/s respectively. All recorded data were categorized to new values of „rapid“, „medium“ and „slow“ sperm using excel “if” function (based on cut-off
points) and results for different settings were compared (P<0.05) using t-test (paired two samples for mean). Pearson correlation procedure was used to estimate correlation between the category of sperm obtained by each cut-off setting and their corresponding kinematic values. Alternatively, we investigated the pattern of clustering in the original data. We obtained two PCA components by performing PCA analysis of this data and brought all kinematic parameters (VCL, VSL, VAP, ATR, LIN, ALH and BCF) down to two components (factor score) and then used these two components for clustering (2-step cluster analysis) using factors score as continuous variables and setting VCL as an evaluation field.

To determine as to which cut-off setting (25 %, 33 % or default setting) would give better interpretation of studied male population, parameters obtained from CASA for the sperm populations of individual males were tested. Principle component analysis was applied to the covariance matrix of the 9 semen parameters i.e. percentage of rapid sperm, VCL, VSL, VAP, ATR, LIN, WOB, ALH and BCF and variables were weighted against their eigenvectors. Two components were drawn for each cut-off setting (25 %, 33 % and default setting), which accounted for more than 87 % variability in the data on an average basis (the first principle component accounted for 69 % variability and the 2\textsuperscript{nd} component accounted for 19 % variability in the data). For all cut-off settings, the first principle component was termed „Score 1” and was more related to sperm movement characteristics whereas, the 2\textsuperscript{nd} component was termed „Score 2” and was more related to number of rapid sperm (Table 2). K-means cluster analysis for each cut-off setting was carried out where „Score 1” and „Score 2” were used as variables and the male IDs as labelling variable with a fixed 4-cluster model to categorise males into high-high, high-low, low-low and low-high ranking.
7.4 Results

7.4.1 Proportions of sperm and sperm kinematics in different sperm categories as defined by cut-off setting

By reducing the VCL intervals, increasing or decreasing the upper and lower VCL cut-off values, the proportions of rapid and slow sperm altered and the mean velocities in those categories increased and decreased respectively (Table 7-1 and 7-2). The value of upper VCL cut-off for “median±25 % cut-off setting” was close to upper VCL cut-off value of default setting; “median±50 % cut-off setting” was close to the “33 % setting” values, while upper cut-off values for “median±75 % setting” was close to the “25 % setting” value. On other hand lower cut-off value for “median±75 % setting” gave very few slow sperm (slow sperm < 21 µm/s VCL) which was not in agreement with the distribution of original data (Fig. 1) where skewness was due to more slow sperm. However, H-spread
obtained (Fig. 1) form the “25 % setting” and “33 % setting” showed more realistic presentation of the sperm categories (i.e. rapid, medium and slow sperm) as compared to the “default setting” or any other setting.

7.4.2 Correlated response between proportion of sperm in velocity categories and sperm kinematics according to different cut-off setting

To estimate, in which cut-off setting (i.e. 25 %, 33 % and default setting), the number of rapid, medium or slow sperm had stronger relationship with their corresponding velocity and other movement parameters; we run correlations (Table 7-3). However, all the cut-off settings showed similar relation between a category of sperm (i.e. rapid, medium or slow sperm) and their corresponding kinematic characteristics. For all the settings percentage of rapid sperm was positively correlated (P < 0.01), while, percentage of slow sperm was negatively correlated (P < 0.01) with sperm velocity parameters (i.e. VCL, VAP and VSL). On other hand, LIN, STR and BCF had significant (P < 0.01; P < 0.05) negative correlations with the number of medium sperm obtained by each cut-off setting.
Table 7-1 Mean (± SEM) proportion of percent rapid sperm (Rapid), percent medium sperm (Medium) and percent slow sperm (Slow) separated according to different settings.

<table>
<thead>
<tr>
<th>Velocity category</th>
<th>Setting</th>
<th>Default</th>
<th>25%</th>
<th>33%</th>
<th>Median ± 25%</th>
<th>Median ± 50%</th>
<th>Median ±75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>41.3±2</td>
<td>23.8±1.4</td>
<td>32.2±1.6</td>
<td>39.4±2.2</td>
<td>32.1±1.6</td>
</tr>
<tr>
<td>Medium (%)</td>
<td></td>
<td></td>
<td>21.9±0.7</td>
<td>48.5±1.3</td>
<td>31.3±0.9</td>
<td>18.2±0.7</td>
<td>34.4±0.9</td>
</tr>
<tr>
<td>Slow (%)</td>
<td></td>
<td></td>
<td>36.8±0.9</td>
<td>27.7±1.8</td>
<td>36.5±1.9</td>
<td>42.4±0.9</td>
<td>33.3±2.0</td>
</tr>
</tbody>
</table>

Means without a common superscript within a row differ (P<0.05)
Means without a common superscript within column (for specific cut-off method) differ (P<0.05)

Table 7-2 Mean± SEM Comparison for kinematics characteristics of rapid, medium and slow sperm obtained in different cut-off settings (25% and “33%).

Rapid (percent rapid sperm), Medium (percent medium sperm), Slow (percent slow sperm), VCL (curvilinear velocity), VSL (straight line velocity), VAP (average path velocity), LIN (linearity), STR (straightness), WOB (Wobble), ALH (Lateral head displacement) and BCF (beat cross frequency)

<table>
<thead>
<tr>
<th>Category</th>
<th>VCL (µm/sec)</th>
<th>VSL (µm/sec)</th>
<th>VAP (µm/sec)</th>
<th>LIN (%)</th>
<th>STR (%)</th>
<th>WOB (µm)</th>
<th>ALH (µm)</th>
<th>BCF (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid (25%)</td>
<td>192.5±0.3</td>
<td>140.8±0.7</td>
<td>162.1±0.5</td>
<td>72.6±0.03</td>
<td>84.0±0.04</td>
<td>83.8±0.01</td>
<td>5.3±0.01</td>
<td>9.2±0.01</td>
</tr>
<tr>
<td>Rapid (33%)</td>
<td>178.6±0.4</td>
<td>124.7±0.7</td>
<td>146.5±0.5</td>
<td>68.4±0.03</td>
<td>81.0±0.04</td>
<td>81.1±0.01</td>
<td>5.3±0.01</td>
<td>8.6±0.01</td>
</tr>
<tr>
<td>Default</td>
<td>163.7±0.3</td>
<td>109.2±0.6</td>
<td>131.0±0.5</td>
<td>64.3±0.03</td>
<td>78.0±0.04</td>
<td>78.5±0.01</td>
<td>5.1±0.01</td>
<td>8.0±0.01</td>
</tr>
<tr>
<td>Medium (25%)</td>
<td>88.2±0.3</td>
<td>43.2±0.3</td>
<td>60.5±0.3</td>
<td>46.4±0.03</td>
<td>64.0±0.04</td>
<td>67.0±0.01</td>
<td>3.8±0.01</td>
<td>5.4±0.01</td>
</tr>
<tr>
<td>Medium (33%)</td>
<td>85.1±0.3</td>
<td>39.8±0.3</td>
<td>57.1±0.5</td>
<td>45.9±0.03</td>
<td>64.0±0.04</td>
<td>66.6±0.01</td>
<td>3.8±0.01</td>
<td>5.3±0.01</td>
</tr>
<tr>
<td>Default</td>
<td>73.5±0.2</td>
<td>32.8±0.3</td>
<td>48.3±0.5</td>
<td>44.1±0.03</td>
<td>62.4±0.04</td>
<td>65.4±0.01</td>
<td>3.4±0.01</td>
<td>5.0±0.01</td>
</tr>
<tr>
<td>Slow (25%)</td>
<td>21.1±0.1</td>
<td>5.9±0.1</td>
<td>11.5±0.1</td>
<td>26.5±0.01</td>
<td>43.1±0.03</td>
<td>53.4±0.01</td>
<td>1.4±0.01</td>
<td>1.9±0.01</td>
</tr>
<tr>
<td>Slow (33%)</td>
<td>26.3±0.1</td>
<td>8.3±0.1</td>
<td>15.0±0.1</td>
<td>29.0±0.02</td>
<td>45.8±0.03</td>
<td>55.3±0.01</td>
<td>1.6±0.01</td>
<td>2.6±0.01</td>
</tr>
<tr>
<td>Default</td>
<td>26.7±0.1</td>
<td>8.5±0.1</td>
<td>15.2±0.1</td>
<td>29.3±0.02</td>
<td>46.1±0.04</td>
<td>55.4±0.01</td>
<td>1.6±0.01</td>
<td>2.6±0.01</td>
</tr>
</tbody>
</table>

Means without a common superscript within column (same category of sperm) differ (P<0.05)
Table 7-3 Correlation between the number of rapid, medium and slow sperm obtained by each setting and their corresponding sperm kinematic parameters.
VCL (curvilinear velocity), VSL (straight line velocity), VAP (average path velocity), LIN (linearity), STR (straightness), ALH (Lateral head displacement) and BCF (beat cross frequency)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Rapid 25%</th>
<th>Rapid 33%</th>
<th>Rapid default</th>
<th>Medium 25%</th>
<th>Medium 33%</th>
<th>Medium default</th>
<th>Slow 25%</th>
<th>Slow 33%</th>
<th>Slow default</th>
</tr>
</thead>
<tbody>
<tr>
<td>VCL (µm/sec)</td>
<td>0.809**</td>
<td>0.818**</td>
<td>0.804**</td>
<td>0.183</td>
<td>0.288</td>
<td>-0.031</td>
<td>-0.759**</td>
<td>-0.797**</td>
<td>-0.787**</td>
</tr>
<tr>
<td>VAP (µm/sec)</td>
<td>0.825**</td>
<td>0.818**</td>
<td>0.789**</td>
<td>0.114</td>
<td>0.209</td>
<td>-0.116</td>
<td>-0.723**</td>
<td>-0.760**</td>
<td>-0.746**</td>
</tr>
<tr>
<td>VSL (µm/sec)</td>
<td>0.800**</td>
<td>0.770**</td>
<td>0.716**</td>
<td>0.001</td>
<td>0.069</td>
<td>-0.225</td>
<td>-0.624**</td>
<td>-0.654**</td>
<td>-0.640**</td>
</tr>
<tr>
<td>STR (%)</td>
<td>0.011</td>
<td>-0.095</td>
<td>-0.204</td>
<td>-0.480**</td>
<td>-0.581**</td>
<td>-0.493**</td>
<td>0.329*</td>
<td>0.352*</td>
<td>0.355*</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>0.247</td>
<td>0.159</td>
<td>0.064</td>
<td>-0.359*</td>
<td>-0.420**</td>
<td>-0.493**</td>
<td>0.060</td>
<td>0.071</td>
<td>0.089</td>
</tr>
<tr>
<td>ALH (µm)</td>
<td>0.156</td>
<td>0.167</td>
<td>0.124</td>
<td>0.007</td>
<td>0.023</td>
<td>0.096</td>
<td>-0.126</td>
<td>-0.146</td>
<td>-0.152</td>
</tr>
<tr>
<td>BCF (Hz)</td>
<td>0.181</td>
<td>0.120</td>
<td>0.032</td>
<td>-0.342*</td>
<td>-0.400*</td>
<td>-0.303</td>
<td>0.099</td>
<td>0.092</td>
<td>0.062</td>
</tr>
</tbody>
</table>

* P < 0.05; ** P < 0.01
7.4.3 Application of the sperm characteristic obtained by each cut-off setting to evaluate Japanese quail males according to their sperm motility characteristics

Pair-wise correlation revealed strong relationship between the number of rapid sperm and their corresponding sperm kinematic values for each cut-off setting. Therefore, a principle component analysis was applied to minimize the set (i.e. percentage of rapid sperm, VCL, VSL, VAP, ATR, LIN, WOB, ALH and BCF) and summarize the variation in these 9 sperm traits. Two components were derived for each cut-off setting. The two components derived for each of 25 %, 33 %, and default cut-off settings (Table 7-4) accounted for 88.7 % (first component 69.2 and 2nd component 19.4), 86.6 % (first component 68.1 and 2nd component 18.5), and 87.7 % (first component 69.4, 2nd component 18.2) variability in above mentioned data, respectively. However, in 25 % cut-off setting, the 2nd principle component accounted for 19.4 % variability, out of the 19.7%, which was not accounted for by the 1st component while, in 33 % cut-off setting and default setting, the 2nd principle component accounted for 18.5 and 18.2 % variability, out of the 21.9 and 32.3 % variability respectively, which was not accounted by the 1st component. This showed that the maximum variability in the 1st and 2nd component was accounted for in 25 % cut-off setting as compared to other two settings.

PCA scores were derived after factorizing 9 sperm traits (as mentioned above) and these scores were used by clustering procedure to rank male with in each cut-off setting. The average values (average for a cluster) for number of rapid sperm, sperm kinematic parameters, corresponding score obtained by PCA and number of male categorised into each cluster in each cut-off setting is shown in Table 7-5. The four clusters obtained by each cut-off setting represented differences in allocation of males to the clusters (Table 7-6). In 25 % cut-off setting, however, each cluster was represented by a similar number of males, whereas, in the default and 33 % cut-off settings this pattern was not observed. Secondly, only 1 out of 4 males allocated to the „top” cluster by 25 % cut-off settings was present in „top” cluster of 33 %, and 2 out of 4 males in default setting.

7.4.4 Identifying VCL cut-off values based on clustering procedure and PCA factorization (2nd method)

PCA analysis of VCL data (22,300 sperm) revealed two components (Table 2, “overall data”), which accounted for more than 86 % variability in the variance. 2-step cluster
analysis based on two PCA components revealed 4 clusters. Cluster 1 and 2 regarded as high with the mean VCL of 174.0 and 155.3 \( \mu \text{m/s} \), whereas, cluster 3 and 4 regarded as low, with mean VCL of 48.0 \( \mu \text{m/s} \) and 54.0 \( \mu \text{m/s} \). Together, mean VCL of clusters 1 & 2 was 164.6 \( \mu \text{m/s} \) while, mean VCL of cluster 3 and 4 was 51\( \mu \text{m/s} \). Taking these two values as upper and lower cut-off values the following SCA setting could be suggested for quail sperm categorization into rapid, medium and slow sperm; “slow < 51 < medium <165< rapid”. For this setting, the upper cut-off value (i.e. 165 \( \mu \text{m/s} \)) was close to that of the upper cut-off setting of25 % setting while, lower cut-off value (51 \( \mu \text{m/s} \)) was close to that of the lower cut-off setting of SCA default setting (Table 7-7).
Table 7-4 PCA components (equations) for sperm kinematic parameters of rapid sperm as categorized by different settings.

<table>
<thead>
<tr>
<th>Setting</th>
<th>Score</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rapid (percent rapid sperm), VCL (curvilinear velocity), VSL (straight line velocity), VAP (average path velocity), LIN (linearity), STR (straightness), WOB (Wobble), ALH (Lateral head displacement) and BCF (beat cross frequency)</td>
</tr>
<tr>
<td>25%</td>
<td>1</td>
<td>(VCL x .11) + (VSL x 0.98) + (VAP x 0.92) + (STR x 0.98) + (WOB x 0.97) + (LIN x 0.96) + (BCF x 0.84) + (ALH x 0.89) + (number of rapid sperm x -0.21)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>(VCL x 0.92) + (VSL x 0.13) + (VAP x 0.34) + (STR x -0.14) + (WOB x -0.14) + (LIN x -0.21) + (BCF x -0.03) + (ALH x 0.22) + (Number of rapid sperm x 0.80)</td>
</tr>
<tr>
<td>33%</td>
<td>1</td>
<td>(VCL x 0.33) + (VSL x 0.92) + (VAP x 0.79) + (STR x 0.98) + (WOB x 0.97) + (LIN x 0.99) + (BCF x 0.68) + (ALH x -0.86) + (Number of rapid sperm x -0.34)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>(VCL x 0.88) + (VSL x 0.37) + (VAP x 0.58) + (STR x 0.07) + (WOB x 0.05) + (LIN x 0.02) + (BCF x 0.46) + (ALH x 0.26) + (Number of rapid sperm x 0.65)</td>
</tr>
<tr>
<td>Default</td>
<td>1</td>
<td>(VCL x -0.91) + (VSL x 0.91) + (VAP x 0.96) + (STR x 0.75) + (WOB x 0.71) + (LIN x 0.72) + (BCF x 0.83) + (ALH x -0.21) + (Number of rapid sperm x 0.11)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>(VCL x -0.26) + (VSL x 0.40) + (VAP x 0.18) + (STR x 0.63) + (WOB x 0.66) + (LIN x 0.68) + (BCF x 0.19) + (ALH x -0.84) + (Number of rapid sperm x -0.77)</td>
</tr>
<tr>
<td>Overall</td>
<td>1</td>
<td>(VSL x 0.78) + (VAP x 0.66) + (STR x 0.89) + (WOB x 0.89) + (LIN x 0.97) + (BCF x 0.60) + (ALH x -0.04)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>(VSL x 0.53) + (VAP x 0.66) + (STR x 0.08) + (WOB x 0.12) + (LIN x 0.11) + (BCF x 0.54) + (ALH x 0.92)</td>
</tr>
</tbody>
</table>
Table 7-5 Male clustering based on PCA derived scores within each setting and their sperm kinematic properties.
Rapid (percent rapid sperm), VCL (curvilinear velocity), VSL (straight line velocity), VAP (average path velocity), LIN (linearity), STR (straightness), WOB (Wobble), ALH (Lateral head displacement) and BCF (beat cross frequency)

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Rapid (%)</th>
<th>VCL (µm/sec)</th>
<th>VSL (µm/sec)</th>
<th>VAP (µm/sec)</th>
<th>LIN (%)</th>
<th>STR (%)</th>
<th>WOB (%)</th>
<th>ALH (µm)</th>
<th>BCF (Hz)</th>
<th>Score 1</th>
<th>Score 2</th>
<th>N*</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% setting</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>29.8</td>
<td>194.9</td>
<td>150.8</td>
<td>169.2</td>
<td>75.0</td>
<td>86.6</td>
<td>86.1</td>
<td>5.0</td>
<td>9.5</td>
<td>0.47</td>
<td>1.06</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>24.1</td>
<td>189.9</td>
<td>155.8</td>
<td>168.6</td>
<td>81.4</td>
<td>90.6</td>
<td>88.4</td>
<td>4.5</td>
<td>9.9</td>
<td>0.91</td>
<td>0.05</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>28.2</td>
<td>191.7</td>
<td>122.9</td>
<td>150.0</td>
<td>63.1</td>
<td>77.4</td>
<td>77.7</td>
<td>6.0</td>
<td>7.9</td>
<td>-1.22</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>16.9</td>
<td>183.3</td>
<td>138.0</td>
<td>156.1</td>
<td>74.8</td>
<td>85.4</td>
<td>84.9</td>
<td>5.1</td>
<td>9.1</td>
<td>-0.05</td>
<td>-1.18</td>
<td>6</td>
</tr>
<tr>
<td>33% setting</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>35.2</td>
<td>193.3</td>
<td>157.0</td>
<td>170.3</td>
<td>80.4</td>
<td>90.4</td>
<td>87.5</td>
<td>4.8</td>
<td>10.9</td>
<td>1.20</td>
<td>1.58</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>37.5</td>
<td>184.6</td>
<td>126.1</td>
<td>150.8</td>
<td>66.9</td>
<td>79.7</td>
<td>80.7</td>
<td>5.4</td>
<td>8.3</td>
<td>-0.55</td>
<td>0.91</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>31.3</td>
<td>170.2</td>
<td>111.7</td>
<td>134.6</td>
<td>64.2</td>
<td>77.8</td>
<td>78.2</td>
<td>5.4</td>
<td>8.2</td>
<td>-0.87</td>
<td>-0.47</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>28.7</td>
<td>175.4</td>
<td>137.8</td>
<td>152.3</td>
<td>77.4</td>
<td>87.7</td>
<td>86.1</td>
<td>4.6</td>
<td>9.0</td>
<td>0.83</td>
<td>-0.50</td>
<td>7</td>
</tr>
<tr>
<td>SCA default setting</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>43.6</td>
<td>175.7</td>
<td>125.4</td>
<td>144.9</td>
<td>69.3</td>
<td>82.4</td>
<td>81.4</td>
<td>5.1</td>
<td>9.1</td>
<td>1.09</td>
<td>-0.73</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>38.7</td>
<td>165.8</td>
<td>127.0</td>
<td>141.5</td>
<td>74.2</td>
<td>85.8</td>
<td>83.8</td>
<td>4.6</td>
<td>8.8</td>
<td>0.56</td>
<td>0.69</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>45.3</td>
<td>156.1</td>
<td>98.0</td>
<td>121.5</td>
<td>60.4</td>
<td>74.7</td>
<td>76.3</td>
<td>5.2</td>
<td>7.5</td>
<td>-0.88</td>
<td>-0.53</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>23.9</td>
<td>149.5</td>
<td>111.7</td>
<td>126.5</td>
<td>73.0</td>
<td>84.2</td>
<td>83.6</td>
<td>4.3</td>
<td>7.4</td>
<td>-1.18</td>
<td>2.36</td>
<td>1</td>
</tr>
</tbody>
</table>

*N* No of males per cluster
Table 7-6 Male ranking (allotment) to specify the usefulness of a SCA cut-off setting (i.e. 25 %, 33% and default) to identify high and low fertility males based on their clustering characteristics (i.e. number of rapid sperm and their velocity parameters).

<table>
<thead>
<tr>
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<th>Ranking</th>
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<td>1</td>
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</tr>
<tr>
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<td>Top</td>
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<tr>
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</tr>
<tr>
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<td>4</td>
<td>1,2,3,13,14,19,22</td>
<td>Low % rapid sperm +average velocities</td>
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<td>SCA default</td>
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<td>Top</td>
</tr>
<tr>
<td>2</td>
<td>1,2,3,13,14,15,19</td>
<td>Average % rapid sperm + high velocities</td>
<td>--</td>
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<td>4</td>
<td>22</td>
<td>Low % rapid sperm + low velocities</td>
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Table 7-7 CASA settings for estimating proportions of rapid, medium and slow sperm using different methods.

<table>
<thead>
<tr>
<th>Method for obtaining VCL cut-off values</th>
<th>Cut-off values used for classifying sperm into different velocity category</th>
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</thead>
<tbody>
<tr>
<td>Median ± 25 %</td>
<td>Slow &lt; 63 &lt; Medium &lt; 106 &lt; Rapid</td>
</tr>
<tr>
<td>Median ± 50 %</td>
<td>Slow &lt; 42 &lt; Medium &lt; 126 &lt; Rapid</td>
</tr>
<tr>
<td>Median ± 75 %</td>
<td>Slow &lt; 21 &lt; Medium &lt; 150 &lt; Rapid</td>
</tr>
<tr>
<td>First and third quartile (25 %)</td>
<td>Slow &lt; 36 &lt; Medium &lt; 154 &lt; Rapid</td>
</tr>
<tr>
<td>At 33 and 66 % of the data</td>
<td>Slow &lt; 49 &lt; Medium &lt; 127 &lt; Rapid</td>
</tr>
<tr>
<td>Default setting</td>
<td>Slow &lt; 50 &lt; Medium &lt; 100 &lt; Rapid</td>
</tr>
</tbody>
</table>
We used two approaches, i.e. arbitrarily chosen VCL values and PCA analysis followed by cluster analysis, to investigate how the proportions of rapid, medium or slow velocity sperm changes and how the changes in these sperm categories and their corresponding kinematic values influence male ranking. The first approach was to define different cut-off values using the mean and median VCL. Adjusting VCL cut-off values to 25 % setting (based on mean values), produced more reliable results, as there was more pronounced difference in the number of rapid, medium and slow sperm, while, on the same time the distribution of rapid, medium and slow sperm obtained by this setting was more realistic according to obtained data structure where there were much more medium and slow sperm as compared to rapid sperm and moreover, there was good separation of males in cluster ranking with this setting. On other hand, 25 % setting did classify those males to the top ranking, which were close to the top ranking in the other two cut-off settings, however, masked by other males having a lot more rapid sperm at lower cut-off values. The reason why 33 % and default setting did not give the same male ranking, as that by 25 %, might be that in these two settings (33 % and SCA default) the number of rapid sperm and movement characteristics were not evaluated at the threshold levels where most variability in the males can be observed.

Using the second approach, clustering of data on the basis of factors obtained by PCA analysis showed mean value of high and low VCL clusters close to the values obtained by 25 % setting. It indicates that this setting splits different subpopulations closer to the original distribution of data as compared to 33 % or the SCA default setting.

We used the VCL parameter to define different categories of sperm, as VCL was reported to be species specific and found effective to categorize rapid, medium and slow sperm in different species (Maree and Horst, 2012). However, Maree and horst 2012, adjusted VCL cut-off values manually to include 80 % rapid, 15 % medium and 5 % slow swimming sperm in their study while, based on our data, such approach (80 % of total motile sperm as rapid) appears artificial and does not reflect the natural distribution of sperm velocity in quails, and this, may also apply to sperm in other species (Abaigar et al., 1999; Holt et al., 2007). The frequency distributions of quail sperm were skewed as
were reported for other species (Holt *et al.*, 2007) where there were more slow sperm, more medium and less rapid sperm. We derived different VCL cut-off values using statistical interpretations and compared the suitability of these values defining H-spread on other hand we used clustering procedure, which is an effective method to identify different sperm subpopulations (Quintero-Moreno *et al.*, 2003 & 2007) and also to estimate the cut-off values for the data, and compared results from both methods. These procedures gave us more realistic VCL cut-off values that define quail sperm subpopulations in the samples.

It appears that simply choosing first and third quartile (i.e. 25 % setting) intervals for the CASA setting may be adequate to describe and compare quail sperm subpopulations and ranking of different males. However, while so obtained VCL cut-off values might be useful for male ranking, when it comes to relate, male ranking to female fertility, the female factors need to be taken into account (Bakst *et al.*, 1994; Bakst, 1998; Brillard, 2003). Further studies are needed to relate sperm subpopulations defined by different cut-off settings to in vivo fertility. It is clear though that each species sperm population need to be investigated under the optimal in vitro conditions before cut-off values are applied in CASA setting.

7.6 Conclusion

In Japanese quail, the first and third quartile of VCL data or 2-step cluster analysis (after PCA factorization of sperm kinematics) appears adequate to categorise sperm into rapid, medium and slow velocity categories.
Chapter 8

Parameters of sperm motility are important to egg fertility in Japanese quail

8.1 Abstract

This study was carried out to investigate the relationship between sperm motility and egg fertility in Japanese quail. Four ejaculates (4 ejaculates x 4 ages; n= 16), per male (9 male x 5 strains; n = 45) were collected at successive age intervals of 8, 16, 26 and 36 weeks. The parameters recorded thereby included the volume of an ejaculate, sperm concentration, total sperm in an ejaculate and CASA sperm motility parameters (i.e. the percentages of motile (PMOT), percentage of rapid sperm (Rapid), percentage of medium sperm (Medium), percentage of slow sperm (Slow) and percentage of progressive motile sperm (PROG), the curvilinear velocity (VLC), straight line velocity (VSL) and average path velocity (VAP), straightness (STR), linearity (LIN), lateral head displacement (ALH), beat cross frequency (BCF) and wobble (WOB). At each age (i.e. 8, 16, 26 and 36 weeks), prior to semen collection each male was mated to four females (n = 180) for a two-week period. During mating eggs were collected and numbers of sperm and sperm-holes present in the egg perivitelline layer were recorded and true egg fertility was determined by the appearance of germinal disc area. The volume of an ejaculate positively correlated with sperm concentration (r = 0.35; P < 0.01), while sperm concentration positively correlated with egg fertility (r = 0.08; P < 0.05). However, ejaculate volume and sperm concentration were negatively correlated with CASA sperm motility parameters.

The PMOT was positively correlated (P < 0.01) with Rapid, PROG and Medium sperm and sperm velocities i.e. VCL, VSL and VAP. The Rapid and PROG were positively (P < 0.01) correlated with sperm velocities.

Medium sperm were positively correlated with SpermOPVL (r = 0.08; P < 0.05) and HolesIPVL (r = 0.08; P < 0.05), while, PROG were positively correlated with
Holes$_{IPVL}$ ($r = 0.1; P < 0.05$). No other sperm motility parameter showed any significant relationship with the egg fertility traits.

Strains differ with their response to correlations between CASA sperm motility parameters and egg fertility. For one strain (i.e. strain A) VSL, VAP and BCF were positively correlated with Sperm$_{OPVL}$ ($r =0.18; 0.18$ and $0.23$, respectively, $P < 0.05$), while, for others i.e. for strain B, PROG ($r = 0.17$, $P < 0.05$) and for strain C, Medium sperm ($r = 0.18; P < 0.05$) sperm were positively correlated with Sperm$_{OPVL}$, respectively.

At week 8 of age, those males which were characterized by poor fertility (based on their sperm motility records ($n = 4$ ejaculates) were compared with those males, which were ranked poor based on all records ($n= 16$ ejaculates). Only one third of those males which were categorized as poor at Week 8 were ranked poor after lifetime performance, while, two third of the males were ranked average or high.

It may, therefore, be suggested to conclude that sperm concentration, Medium and PROG, VAP and VSL parameters are important (however, not very powerful) male traits in relation to egg fertility, however, these expressions are strain specific in quails.

**8.2 Introduction**

Japanese quail, given its substantial natural resources, offers huge commercial prospects to cater for the ever growing consumptive needs worldwide. However, the genetic selection for meat and egg production carried out, so far, demonstrate certain anomalies, especially with regards to traits such as egg fertility and hatchability which are often overlooked (Minvielle, 2004). Not surprisingly, therefore, a wide range of egg fertility rates has been reported for quail flocks ranging from 45 to 85 % (Marks, 1979; El-Filky, 2002). Moreover, 4-5 percent per generation decline in fertility has been reported after short-term selection for high egg production (Okenyi et al., 2013), which is alarmingly high in quails compared with less than 0.5 percent per generation decline in broiler breeders (Reddy and Sajadi, 1990). In quails, fertility has remained a serious issue with a threat to genetic gains and future development of the industry, however, till date no intensified efforts has been carried out to deal with this issue.

Fertility is a mutual trait denoting the genetic and non-genetic factors originating from both the male and female that affect egg fertilization and embryo development.
(Brillard, 2003). However, in domestic poultry one male serves many females during his life time, contributing to fertility of many eggs and having fifty percent probability for genetic share, therefore, his fertility is important and may become one of the first limiting factors to achieve the highest egg fertility possible. Accordingly, males are made responsible for decline in flock fertility (Bramwell et al., 1995) and the remedies to manage fertility related issues are also supposed to rely on manipulating male options. On other hand, based on the same reason, that a male has served many females and therefore, sired many offspring, the genetic gains or improvement in the fertility is assumed to be quick and easy with male options compared with females.

It has been suggested, however, that if fertility is to be managed or optimized from the male side, assessment of sperm quality and culling of poor males need to be incorporated in management procedures and individuals must be evaluated based on sperm parameters (Donoghue, 1999). Certain semen parameters such as sperm metabolism, sperm concentration, sperm motility, and percentage of abnormal or dead sperm (Wilson et al., 1979) can be important in relation to individual male fertility., However, as all these parameters are interlinked and contribute to make sperm swim through the barriers of female oviduct and reach the egg fertilization site in sufficient numbers (Holt and Van look, 2004), thus a good sperm motility reflects the characteristics of good quality of sperm Therefore, the ability of sperm to move, be motile or mobile makes it an important component of semen analysis (Froman and Mclean, 1996; Froman et al., 1997; Froman and Feltman, 1998; Froman, 2003; Moce and Graham, 2008).

Sperm motility is traditionally assessed by subjective method (optical microscopic evaluation), which is not highly repeatable or reliable (Moce and Graham, 2008) and has been reported to give 30 to 60% variation for the same ejaculate in humans and animals (see review; Verstegen et al., 2002). However, technologies such as CASA can provide sophisticated assessment of these parameters and have the advantage of increasing the accuracy and reproducibility of measurements (Moce and Graham, 2008). The CASA technology has been widely used to assess sperm movement characteristics for a range of species i.e. rat, rabbit, pig, bull, broiler chicken, pigeon, turkey or human. Furthermore,
significant correlations of CASA motility parameters with fertility have also been reported, lately (King et al., 2000; Verstegen et al., 2002).

In broiler and turkey industries selection for high fertility males on the basis of their sperm movement characteristics (motility and mobility) has become a common practice and this has proved an effective tool to maintain fertility (see review; Donoghue, 1999). Moreover, genetic strains have been reported to differ based on their sperm motility parameters (Long et al., 2010). However, in Japanese quail no attempt has been made so far to assess male sperm motility traits in relation to selection or culling of high or poor fertility males, thus the crucial link between sperm motility and true egg fertility had also remained undiscovered. However, in quail sperm motility has unique physiology, because male possess functional cloacal gland which produces a meringue like fluid, called “foam”, to affect sperm functions. The foam always accompanies male semen and increases not just in-vitro sperm motility but also the number of sperm reaching the egg fertilization site (Biswas et al., 2010; Singh et al., 2011a & b; Chapter 5).

Four outlined objectives for this study were: 1) to investigate quail sperm motility objectively using CASA; 2) to study how semen and sperm motility traits are linked to each other; 3) To identify male sperm motility traits which are linked to egg fertility; and 4) to investigate the utility of sperm motility assessment to separate poor and good fertility males.

8.3 Material and methods

8.3.1 Housing and Management

The Japanese quails used in this study (9 males x 5 strains; n= 45 & 36 female x 5 meat-type strains; n = 180) were hatched and reared till Week 6 of age in the farm conditions (Game Farm Pty. Ltd., Galston, NSW) and then transferred to a research facility at the University of Western Australia. These five strains (i.e. A, B, C, D and E) were from different blood lines and had similar 6 week body weight. The males and females were housed individually in commercial quail cages (pen size: Male =19 width x 40 depth x 23 high cm (Cimuka quail cages, Turkey); Female = 54 length x 30 width x 25 height cm
(Venturi Valter quail breeding cages, Italy). The house environment was adjusted to 22-26°C temperature, with 14/10-h light/dark cycle, adequate ventilation and ad-libitum feed and water supply. Male and females were fed on the same diet, containing 20.0% CP and 11.5 MJ/kg ME. Cage enrichment was achieved by the provision of sand bath in each individual pen and sand was provided daily. Before start of semen collection three weeks acclimatization period was given during which males got accustomed to manhandling and semen collection using a teaser female. All the procedures and protocols were approved by the Animal Ethics Committee, The University of Western Australia.

8.3.2 Experimental design
The study was carried out at Week 8, 16, 26 and 36 of age and at each age, four ejaculates (n = 16) per male (2 ejaculate per week) were collected and samples were analysed for CASA sperm motility assessment for individual male. Duplicate motility recordings were made for each ejaculate.

At each age before start of semen collection, males were allowed to mate individually with four females for two weeks (mating phase, Chapter 4), eggs were collected and true egg fertility was estimated by assessing the fertilisation status of the germinal disc and by in vivo sperm-egg assay i.e. counting the number of sperm (Sperm\textsubscript{OPVL}) and Sperm-holes (Holes\textsubscript{IPVL}) present at egg perivitelline layer (PVL) around GD.

The following sperm motility parameters were analyzed: PMOT, Rapid, Medium, Slow, ROG sperm, VSL, VCL, VAP, STR, LIN, ALH, BCF, and WOB.

8.3.3 Sperm-egg assay
Eggs were opened, appearance of the GD was captured with the DP-70 digital camera (Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia), and the PVL membranes were collected for sperm-hole counts. The fertility status of eggs was determined by viewing the GD under a low power magnification aided by lateral illumination from a light source before the photograph was taken. A piece (1.5 x 1.5 cm) of PVL was collected around the germinal disc using a paper filter ring (Whatman, Grade 1, Sigma-Aldrich Co., Castle Hill, NSW, Australia). The collected membrane was washed with
phosphate buffered saline (PBS; pH 7.4) to remove the adhering yolk and then placed on a microscope glass slide. For sperm counting, the membrane was stained with Hoescht dye 0.01 mM solution (Sigma-Aldrich Co. Castle Hill, NSW, Australia). The sperm nuclei were visualized with a fluorescence microscope (Olympus BX60-FL, Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia) using a „U“ filter cube with 372 nm excitation and 456 nm emission wavelengths. For counting HolesIPVL, Schiff’s reagent (Sigma-Aldrich Co. Castle Hill, NSW, Australia) was used, staining the PVL section as described by Bramwell et al., (1995). Counting was carried out in six fields (5.7 mm² total PVL area) along the horizontal axis passing through the GD region. The percentage of egg fertility was estimated by the following formula:

“Number of fertile eggs produced by a female in mating phase/total number of eggs produced in that phase) x 100.“ The number of SpermOPVL and HolesIPVL were counted in 5.7 mm² area of PVL and values presented as SpermOPVL/PVL area and HolesIPVL/PVL area.

8.3.4 Foam collection and preparation of foam extract

To record sperm motility by CASA, semen samples were diluted in a medium, comprising motility buffer (150 mM NaCl and 20 mM N-tris-[hydroxymethyl]-methyl-2-aminoethane-sulphonic acid, 35 mM glucose and 0.1 % bovine serum albumin adjusted to pH 8.00 at 25 °C) supplemented with 10 % proctodeal gland foam extract. The foam was used in this study (like we already established in Chapter, 5) that to make reliable in-vitro analysis possible, quail sperm requires 10 % foam to gain maximum motility at 39 °C (Chapter 5). To prepare foam extract, proctodeal gland foam was collected from individual male (9 male x 5 strains; n = 45) (by gently squeezing the either side of the cloacal gland with fingers and thumb (Mohan et al., 2002), pooled for particular strain and frozen at -20 °C in airtight bottles. Foam extract was then prepared by mixing the foam in normal saline (0.89 % NaCl) in 1:2 ratio, followed by homogenization for 30 minutes (Biswas et al., 2010). The mixture was centrifuged at 10,000g for 30 min. The supernatant was collected and centrifuged again at 10,000g for 30 min. Supernatant was collected again and considered as 33 % of foam extract. The 10 % foam extract was then prepared diluting 1 part of foam extract (33 %) with 30 parts of motility buffer (1:30;
foam extract: motility diluent). To record sperm motility, at each particular age (i.e. Weeks 8, 16, 26 or 36), fresh foam was collected and foam extract was prepared consequently.

### 8.3.5 Semen collection and preparation

Teaser method was used for semen collection (Chelmonska et al., 2008). The males were trained to get stimulated by a teaser female and then produce semen after receiving gentle massage on the lumber region and gentle squeezing of cloaca. To avoid any possible semen contamination feathers around the vent of each male were clipped off and each time before semen collection, cloacal gland foam used to be carefully removed from the gland.

Immediately after collection semen was diluted 1:2 with Nacl-TES buffer (collection buffer) (150 mM NaCl and 20 mM N-tris-[hydroxymethyl]-methy l-1-2-aminoethane-sulphonic acid), 35mM glucose and adjusted to pH 7.40 at 25 °C (Holm and Wishart, 2000). Diluted samples were stored (~10 min) at 30°C for later use.

To measure sperm concentration 1.5 μl of neat semen was diluted with 598.5 μl of formalin buffer (2% formalin PBS buffer) in a 1 ml vial, mixed well and transferred to polystyrene cuvette and sperm concentration was immediately determined by recording the absorbance at 560nm in a spectrophotometer (Metertech UV/VIS SP8001, Taipei, Taiwan). The unknown concentration was then calculated from already established Japanese quail sperm concentration standard curve.

### 8.3.6 CASA equipment, capturing properties and analysis

Sperm motility was assessed using the Motility/Concentration module of the Sperm Class Analyzer ® (SCA) version 5.2 (Microptic S.L., Barcelona, Spain). Capturing data were collected using a Basler A602fc digital camera (Microptic S.L.) that was mounted on Olympus BHS microscope (Olympus Optical Co., Tokyo, Japan) equipped with phase contrast optics and a heated stage (Tokai hit thermo plate, Japan).

The capturing properties of the SCA ® system were as follows: images per sec = 25, Frame rate = 60Hz, optics = Ph- (negative phase contrast, particle size = 10 micron, maximum particle size 130 micron (particle area was determined by visually evaluating
captured fields and selecting the particle area size range that resulted in detecting only
the sperm head for motility analysis, thereby excluding any other cells or debris),
progressivity = 70% STR, light filter = green filter (IF 550, Olympus, Japan) and slides =
Leja 10 (standard count four-chamber slide, Leja products B.V. Nieuw-Vennep,
Netherlands). Chicken sperm curvilinear velocity (VCL, µm/s) intervals (10 < Slow
sperm < 50 < Medium sperm < 100 < Rapid sperm) were selected as a default setting
and sperm were categorized based on these cut-off values into Slow, Medium and Rapid
sperm. For motility recording, 2-3 µl of diluted semen was loaded onto a pre-warmed
slide placed on a heated stage at 39°C temperature and then 45 seconds settle down time
was given before recording. Ten to fifteen fields were captured until a total of 200 motile
spermatozoa were collected (WHO, 1999). Fields were captured randomly to eliminate
biasness toward higher sperm concentration or motility but fields that included debris or
clumps of sperm were excluded. All captured sperm tracks were verified visually to
delete incorrectly recorded tracks, e.g., due to colliding spermatozoa.

8.3.7 Analysis VCL cut-off values to define rapid, medium and slow sperm velocity
categories
Initially, the sperm curvilinear velocity (VCL, µm/s) intervals (i.e. 10 < Slow sperm < 50
< Medium sperm < 100 < Rapid sperm) were selected from a default SCA setting termed
“Chicken” and sperm were categorized based on these cut-off values into slow, medium
and rapid. The mean VCL for Japanese quail in this study was 106 µm/s. If default
CASA setting (slow sperm < 50 < medium sperm < 100 < rapid sperm) were used, nearly
50 % of Japanese quail would fall into Rapid category. Two quail VCL cut-off ranges
defined previously (Chapter 6) were used i.e. 1) 25 % or quartile method; the inter-
quartile ranges where the upper VCL cut-off point was taken as the third quartile (75 %)
and the first quartile (25 %) was taken as lower cut-off point resulting in Slow < 36 µm/s
< Medium < 154 µm/s < Rapid setting for CASA; and 2) 33 % method; the upper cut-off
point was taken at 66 % and lower cut-off point was taken at 33 % of the VCL data
resulting in Slow < 49 µm/s < Medium < 127 µm/s < Rapid setting for CASA. The sperm
motility video recordings were re-analysed for each chosen quail setting and outputs were
used for further analysis.
8.3.8 Statistical Analysis

All percentage values were arcsine transformed sperm velocity values were square root transformed, and Sperm_{OPV} and Holes_{IPVL} were log-transformed before analysis was carried out. Pearson correlation procedure of the SPAW 18.0 was used to estimate relationship between sperm motility and egg fertility parameters. Male predicted values for sperm motility parameters were estimated using linear mixed model procedure of the GenStatat 12th edition. To rank male into high and low fertility categories PCA analysis procedure of the PASW 18.0 was used where sperm motility i.e. percentage of rapid sperm, progressive motile sperm, VCL, VSL, VAP, ATR, LIN, WOB, ALH and BCF and egg fertility parameters i.e. Sperm_{PVL}, Holes_{PVL} percentage fertile eggs were weighted against their eigenvectors. PCA-components scores were obtained and K-means cluster analysis with fixed 4-cluster model was used to categorise males into high-high, high-low, low-low and low-high ranking and the values for statistical significance were compared using T-test procedure of PASW 18.0

8.4 Results

8.4.1 Choosing VCL cut-off values to define categories of rapid, medium and slow sperm (using predicted values calculated from life-time sperm motility and egg fertility data).

The correlation between egg fertility and sperm kinematics obtained by either of the two methods of sperm categorization i.e. 25 % and 33% cut-off methods were of the same magnitude. To decide which cut-off method should be used for further data analysis, we used cluster analysis. Separate principle component analysis was used to factorize sperm motility traits i.e. PMOT, Rapid, Medium, Slow, PROG, VCL, VSL, VAP, LIN, ALH, BCF and WOB obtained either by 25 % or 33 % cut-off methods to give factor scores. Three factor scores were obtained for 25 % cut off setting which accounted for 85 % variability in the data. Based on factor score males were clustered to give 4 clusters (K-mean clustering). Clustering for 25 % cut-off setting ranked seven males in the lowest cluster (70.7 % egg fertility; Table 8-1). Similarly, factorization of 33 % cut-off method values gave two factor scores, which accounted for 84.9 % variability in the data. Males were clustered into four groups based on these factor scores. For 33 % cut-off method
clustering ranked four males in the lowest fertility (72.0 % egg fertility; Table 8-1). These results indicated that 25 % cut-off method was more invasive (which identify 7 low fertility males at 70 % egg fertility compared with 4 males at 72 % egg fertility obtained by 33% cut-off method) to detect lower fertility males compared with 33 % cut-off method. So we continue with 25 % cut-off method for further data interpretation. The obtained mean values for sperm motility traits through 25 % cut-off method are given in (Table 8-2).

8.4.2 Correlations between ejaculate volume, sperm concentration, sperm kinematics and egg fertility parameters (overall data without adjustment for age and strain effects).
There was positive correlation between ejaculate volume and sperm concentration (r = 0.35; P < 0.01), while, sperm concentration was positively correlated with percent fertile eggs (r = 0.08; P < 0.05: Table 8-3). However, both, the ejaculate volume and sperm concentration were negatively correlated with sperm kinematics.
Table 8-1 Male ranking based on sperm motility and true egg fertility parameters. Males were ranked based on their egg fertility data and sperm motility parameters obtained by either 25 % or 33 % cut-off methods using n=720 ejaculates) without adjusting for age and strain.

Rapid (rapid sperm), Medium (medium sperm), PROG (progressive motile sperm), VCL (curvilinear velocity), VSL (straight line velocity), VAP (average path velocity), Sperm\textsubscript{OPVL} (sperm present at outer perivitelline layer of egg), Holes\textsubscript{IPVL} (sperm-holes present at inner perivitelline layer of egg).

<table>
<thead>
<tr>
<th>Cluster number</th>
<th>Rapid (%)</th>
<th>Medium (%)</th>
<th>PROG (%)</th>
<th>VCL (µm/s)</th>
<th>VSL (µm/s)</th>
<th>VAP (µm/s)</th>
<th>Sperm\textsubscript{OPVL}</th>
<th>Holes\textsubscript{IPVL}</th>
<th>Fertile eggs (%)</th>
<th>N</th>
<th>Male rank</th>
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</tr>
<tr>
<td>1</td>
<td>14.6\textsuperscript{a}</td>
<td>30.9\textsuperscript{a}</td>
<td>32.4\textsuperscript{a}</td>
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<td>9.8\textsuperscript{a}</td>
<td>23.5\textsuperscript{a}</td>
<td>70.7\textsuperscript{a}</td>
<td>7</td>
<td>Poor</td>
</tr>
<tr>
<td>2</td>
<td>20.4\textsuperscript{b}</td>
<td>36.8\textsuperscript{b}</td>
<td>40.2\textsuperscript{b}</td>
<td>106.9\textsuperscript{b}</td>
<td>73.0\textsuperscript{b}</td>
<td>85.0\textsuperscript{b}</td>
<td>10.8\textsuperscript{b}</td>
<td>27.5\textsuperscript{b}</td>
<td>76.0\textsuperscript{b}</td>
<td>10</td>
<td>High</td>
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<td><strong>33% cut-off setting</strong></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>21.3\textsuperscript{a}</td>
<td>21.8\textsuperscript{a}</td>
<td>32.6\textsuperscript{a}</td>
<td>96.3\textsuperscript{a}</td>
<td>65.7\textsuperscript{a}</td>
<td>76.0\textsuperscript{a}</td>
<td>10.0\textsuperscript{a}</td>
<td>23.8\textsuperscript{a}</td>
<td>72.0\textsuperscript{a}</td>
<td>4</td>
<td>Poor</td>
</tr>
<tr>
<td>2</td>
<td>29.0\textsuperscript{b}</td>
<td>23.9\textsuperscript{b}</td>
<td>39.2\textsuperscript{b}</td>
<td>107.4\textsuperscript{b}</td>
<td>73.5\textsuperscript{b}</td>
<td>85.6\textsuperscript{b}</td>
<td>11.3\textsuperscript{b}</td>
<td>27.5\textsuperscript{b}</td>
<td>76.6\textsuperscript{b}</td>
<td>7</td>
<td>High</td>
</tr>
</tbody>
</table>

\textsuperscript{a,b} Mean without common superscripts with in column for a specified cut-off setting differ significantly (P < 0.05)
Table 8-2 Mean (±STD) values for Japanese quail semen volume, sperm concentration, and sperm motility parameters (predicted values from data collected at Week 8, 16, 26 and 36 of age). Allocation of sperm into rapid and medium velocity categories was based on 25% cut-off method.

PMOT (percent motile sperm), Rapid (rapid sperm), PROG (progressive motile sperm), VCL (curvilinear velocity), VSL (straight line velocity), VAP (average path velocity), LIN (linearity), STR (straightness of trajectory); WOB (Wobble), ALH (amplitude of lateral head), BCF (beat cross frequency).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean ± STD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMOT (%)</td>
<td>64.5±16.8</td>
</tr>
<tr>
<td>Rapid (%)</td>
<td>19.2±1.9</td>
</tr>
<tr>
<td>PROG (%)</td>
<td>38.3±14.2</td>
</tr>
<tr>
<td>VCL (µm/s)</td>
<td>106.3±22.1</td>
</tr>
<tr>
<td>VSL (µm/s)</td>
<td>74.5±21.2</td>
</tr>
<tr>
<td>VAP (µm/s)</td>
<td>85.5±21.4</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>69.1±8.7</td>
</tr>
<tr>
<td>STR (%)</td>
<td>86.3±5.4</td>
</tr>
<tr>
<td>WOB (%)</td>
<td>79.8±5.8</td>
</tr>
<tr>
<td>ALH (µm)</td>
<td>3.9±3.1</td>
</tr>
<tr>
<td>BCF (Hz)</td>
<td>9.1±1.2</td>
</tr>
<tr>
<td>Ejaculate volume (µl)</td>
<td>9.9±4.9</td>
</tr>
<tr>
<td>Sperm concentration (x 10^6/µl)</td>
<td>1.5±0.5</td>
</tr>
</tbody>
</table>
Table 8-3 Correlations of ejaculate volume and sperm concentration with sperm motility parameters, sperm and sperm-holes present at PVL and percent fertile eggs. Over all data (n = 1152 motility recordings) were used with out adjusting age and strain effects. Rapid (rapid sperm), Medium (percent medium sperm), PROG (progressive motile sperm), VCL (curvilinear velocity), VSL (straight line velocity), VAP (average path velocity), Sperm_{OPVL} (sperm present at outer perivitelline layer of egg), Holes_{IPVL} (sperm-holes present at inner perivitelline layer of egg).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sperm conc.</th>
<th>Rapid (%)</th>
<th>Medium (%)</th>
<th>PROG (%)</th>
<th>VCL (µm/s)</th>
<th>VSL (µm/s)</th>
<th>VAP (µm/s)</th>
<th>Sperm_{OPVL}</th>
<th>Holes_{IPVL}</th>
<th>Fertile eggs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejaculate volume (µl)</td>
<td>0.35**</td>
<td>-0.18**</td>
<td>-0.07</td>
<td>-0.21**</td>
<td>-0.18**</td>
<td>-0.21**</td>
<td>-0.20**</td>
<td>-0.07</td>
<td>-0.10**</td>
<td>-0.03</td>
</tr>
<tr>
<td>Sperm concentration (x 10^6/µl)</td>
<td>-0.05</td>
<td>-0.07</td>
<td>-0.06</td>
<td>-0.11*</td>
<td>-0.08*</td>
<td>-0.09*</td>
<td>-0.01</td>
<td>0.04</td>
<td>0.08*</td>
<td></td>
</tr>
</tbody>
</table>

*(P < 0.05); ** (P < 0.01)

Table 8-4 Correlation among different CASA sperm parameters without adjusting for age and strain effects (n = 720 ejaculates). PMOT (percent motile sperm), Rapid (rapid sperm), PROG (progressive motile sperm), VCL (curvilinear velocity), VSL (straight line velocity), VAP (average path velocity), LIN (linearity), STR (straightness of trajectory); WOB (Wobble), ALH (amplitude of lateral head), BCF (beat cross frequency).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>PMOT (%)</th>
<th>Rapid (%)</th>
<th>PROG (%)</th>
<th>Medium (%)</th>
<th>Slow (%)</th>
<th>VCL (µm/s)</th>
<th>VSL (µm/s)</th>
<th>VAP (µm/s)</th>
<th>LIN (%)</th>
<th>STR (%)</th>
<th>WOB (%)</th>
<th>ALH (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid (%)</td>
<td>0.50**</td>
<td>0.79**</td>
<td>0.55**</td>
<td>0.31**</td>
<td>-0.23**</td>
<td>-0.13*</td>
<td>0.01</td>
<td>-0.53*</td>
<td>0.43**</td>
<td>0.31**</td>
<td>0.51**</td>
<td>0.08*</td>
</tr>
<tr>
<td>PROG (%)</td>
<td>0.64**</td>
<td>0.85**</td>
<td>0.72**</td>
<td>0.09*</td>
<td>0.06</td>
<td>-0.20**</td>
<td>0.31**</td>
<td>0.43**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
</tr>
<tr>
<td>Medium (%)</td>
<td>0.56**</td>
<td>0.08*</td>
<td>0.08*</td>
<td>0.09*</td>
<td>-0.23**</td>
<td>-0.13*</td>
<td>0.15</td>
<td>0.92**</td>
<td>0.78**</td>
<td>0.69**</td>
<td>0.78**</td>
<td>0.69**</td>
</tr>
<tr>
<td>Slow (%)</td>
<td>0.06</td>
<td>-0.20**</td>
<td>-0.23**</td>
<td>-0.13*</td>
<td>0.01</td>
<td>-0.46*</td>
<td>0.92**</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>VCL (µm/s)</td>
<td>0.32**</td>
<td>0.72**</td>
<td>0.09*</td>
<td>-0.31*</td>
<td>0.01</td>
<td>-0.10</td>
<td>0.43**</td>
<td>0.69**</td>
<td>0.52**</td>
<td>0.52**</td>
<td>0.52**</td>
<td>0.52**</td>
</tr>
<tr>
<td>VSL (µm/s)</td>
<td>0.20**</td>
<td>0.43**</td>
<td>0.39**</td>
<td>-0.31*</td>
<td>0.10</td>
<td>-0.10</td>
<td>0.43**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
</tr>
<tr>
<td>VAP (µm/s)</td>
<td>0.27**</td>
<td>0.31**</td>
<td>0.27**</td>
<td>-0.39*</td>
<td>-0.10</td>
<td>0.43**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>-0.05</td>
<td>0.43**</td>
<td>0.39**</td>
<td>-0.31*</td>
<td>-0.10</td>
<td>0.43**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
</tr>
<tr>
<td>STR (%)</td>
<td>-0.15</td>
<td>0.31**</td>
<td>0.27**</td>
<td>-0.39*</td>
<td>-0.10</td>
<td>0.43**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
</tr>
<tr>
<td>WOB (%)</td>
<td>0.05</td>
<td>0.50**</td>
<td>0.48**</td>
<td>-0.20*</td>
<td>-0.09*</td>
<td>0.55**</td>
<td>0.72**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
<td>0.69**</td>
</tr>
<tr>
<td>ALH (µm)</td>
<td>0.01</td>
<td>0.08*</td>
<td>0.03</td>
<td>-0.04</td>
<td>0.02</td>
<td>0.04</td>
<td>0.11**</td>
<td>0.05</td>
<td>0.09**</td>
<td>0.09**</td>
<td>0.09**</td>
<td>0.09**</td>
</tr>
<tr>
<td>BCF (Hz)</td>
<td>0.01</td>
<td>0.51**</td>
<td>0.31**</td>
<td>-0.31*</td>
<td>-0.10</td>
<td>0.58**</td>
<td>0.68**</td>
<td>0.63**</td>
<td>0.63**</td>
<td>0.63**</td>
<td>0.63**</td>
<td>0.63**</td>
</tr>
</tbody>
</table>

*P < 0.05, ** P < 0.01.
8.4.3 Correlation between sperm motility and egg fertility parameters (overall data-without adjustment for age and strain effects)

The correlations between sperm motility parameters are given in Table 8-4. PMOT was significantly (P < 0.01) correlated with rapid PROG, medium sperm and sperm velocities i.e. VCL, VSL and VAP. Similarly, Rapid and PROG sperm were positively (P < 0.01) correlated with sperm velocities (VCL, VAP and VSL), velocity ratios (LIN, STR, WOB), and BCF.

The correlations between sperm motility and egg fertility parameters are given in Table 8-5. There was significant positive correlation of Medium sperm with SpermOPVL (r = 0.08; P < 0.05) and HolesIPVL (r = 0.08; P < 0.05), while, PROG sperm were positively correlated with HolesIPVL (r = 0.1; P < 0.05).

8.4.4 Correlation between sperm motility and egg fertility parameters within strain (predicted values from life time record 8-36 week of age)

The correlations between sperm motility and egg fertility parameters differed between the strains (Table 8-6). There was significant positive correlation of sperm velocity parameters i.e. VSL, VAP and BCF with SpermOPVL (r = 0.18; 0.18 and 0.23, respectively, P < 0.05) for strain A. Likewise, in the case of strain B, there was significant positive correlation of PROG sperm with SpermOPVL (r = 0.17, P < 0.05), while, for strain C, Medium sperm were correlated with SpermOPVL (r = 0.18; P < 0.05). However, for strain D and E no significant correlations were obtained neither for any of the sperm motility nor for egg fertility traits.

8.4.5 Correlation between sperm motility and egg fertility parameters adjusted for strain and age effects

The correlations between predicted sperm motility values and predicted egg fertility values (predicted from lifetime records of males) were similar to overall data described in section 8.4.3, the only difference being in terms of the stronger correlations between the sperm and egg motility parameters. Medium sperm were positively correlated with HolesIPVL and percentage of fertile eggs (0.36 and 0.31; P < 0.05 respectively) (Figures 8-1 & 8-2), while, PROG sperm were significantly correlated with SpermOPVL and
Holes<sub>IPVL</sub> (r = 0.30 and r = 0.30; P < 0.05, respectively) (Figures 8-3 & 8-4). Whereas, ejaculate volume was negatively correlated with Sperm<sub>OPVL</sub> and Holes<sub>IPVL</sub> (r = -0.36 and r = -0.31; P < 0.05, respectively).

**Table 8-5** Correlation between sperm motility parameters and Sperm<sub>OPVL</sub> and Holes<sub>IPVL</sub> and percentage of fertile eggs without adjusting for age and strain effects (n = 720 ejaculates).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sperm&lt;sub&gt;OPVL&lt;/sub&gt;</th>
<th>Holes&lt;sub&gt;IPVL&lt;/sub&gt;</th>
<th>Fertile eggs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMOT</td>
<td>-0.01</td>
<td>0.05</td>
<td>-0.05</td>
</tr>
<tr>
<td>PROG</td>
<td>0.06</td>
<td>0.10*</td>
<td>0.02</td>
</tr>
<tr>
<td>Rapid %</td>
<td>0.01</td>
<td>0.05</td>
<td>-0.04</td>
</tr>
<tr>
<td>Medium %</td>
<td>0.08*</td>
<td>0.08*</td>
<td>0.04</td>
</tr>
<tr>
<td>Slow %</td>
<td>-0.05</td>
<td>-0.01</td>
<td>0.04</td>
</tr>
<tr>
<td>VCL (µm/s)</td>
<td>0.04</td>
<td>0.05</td>
<td>-0.04</td>
</tr>
<tr>
<td>VSL (µm/s)</td>
<td>0.03</td>
<td>0.05</td>
<td>-0.01</td>
</tr>
<tr>
<td>VAP (µm/s)</td>
<td>0.03</td>
<td>0.05</td>
<td>-0.02</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>0.01</td>
<td>0.05</td>
<td>0.06</td>
</tr>
<tr>
<td>STR (%)</td>
<td>0.02</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>WOB (%)</td>
<td>0.01</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>ALH (µm)</td>
<td>-0.03</td>
<td>-0.03</td>
<td>-0.05</td>
</tr>
<tr>
<td>BCF (Hz)</td>
<td>0.03</td>
<td>0.04</td>
<td>0.02</td>
</tr>
</tbody>
</table>

*P < 0.05
Table 8-6 Correlation between sperm motility parameters recorded for each strain (A, B, C, D and E) with SpermOPVL and percentage of fertile eggs without adjusting for age effect (n = 144 ejaculates per strain).

PMOT, percentage motile sperm; Rapid, rapid sperm; Medium, medium sperm; PROG, progressive sperm; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness of trajectory; BCF, bear cross frequency

<table>
<thead>
<tr>
<th>Strain</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SpermOPVL</td>
<td>Fertile eggs (%)</td>
<td>SpermOPVL</td>
<td>Fertile eggs (%)</td>
<td>SpermOPVL</td>
</tr>
<tr>
<td>PMOT (%)</td>
<td>-0.23**</td>
<td>-0.18*</td>
<td>0.11</td>
<td>0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>Rapid (%)</td>
<td>0.10</td>
<td>0.10</td>
<td>0.09</td>
<td>0.01</td>
<td>-0.08</td>
</tr>
<tr>
<td>Medium (%)</td>
<td>-0.05</td>
<td>0.08</td>
<td>0.12</td>
<td>-0.01</td>
<td>0.18*</td>
</tr>
<tr>
<td>PROG (%)</td>
<td>0.02</td>
<td>0.13</td>
<td>0.17*</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>VCL (um/s)</td>
<td>0.16</td>
<td>0.06</td>
<td>0.13</td>
<td>-0.04</td>
<td>-0.04</td>
</tr>
<tr>
<td>VSL (um/s)</td>
<td>0.18*</td>
<td>0.07</td>
<td>0.13</td>
<td>-0.03</td>
<td>-0.06</td>
</tr>
<tr>
<td>VAP (um/s)</td>
<td>0.18*</td>
<td>0.08</td>
<td>0.13</td>
<td>-0.03</td>
<td>-0.05</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>0.15</td>
<td>0.08</td>
<td>0.06</td>
<td>-0.15</td>
<td>-0.05</td>
</tr>
<tr>
<td>STR (%)</td>
<td>0.10</td>
<td>0.00</td>
<td>0.04</td>
<td>-0.03</td>
<td>-0.05</td>
</tr>
<tr>
<td>BCF (Hz)</td>
<td>0.23*</td>
<td>0.09</td>
<td>0.05</td>
<td>-0.06</td>
<td>-0.12</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01.
Fig. 8-1 Correlation between Medium velocity sperm and Holes\_IPVL (predicted values of sperm motility and egg fertility parameters after adjusting for strain and age effect (n = 45 males).

Fig. 8-2 Correlation between Medium velocity sperm and the percentage of fertile eggs (predicted values of sperm motility and egg fertility parameters after adjusting for strain and age effect (n = 45 males).

Fig. 8-3 Correlation between the percentage of progressive (PROG) sperm and Sperm\_OPVL (predicted values of sperm motility and egg fertility parameters after adjusting for strain and age effect (n = 45 males).

Fig. 8-4 Correlation between the percentage of progressive (PROG) and Holes\_IPVL (predicted values of sperm motility and egg fertility parameters after adjusting for strain and age effect (n = 45 males).
8.4.6 Identifying poor fertility males at younger age using sperm motility or egg fertility parameters and comparison with poor fertility males identified after their life-time performance.

Males were clustered based on sperm motility and egg fertility parameters at 8 weeks of age using predicted values. PCA analysis of sperm motility parameters i.e. PMOT, Rapid, Medium, PROG, VCL, VAP, VSL, LIN, STR and BCF obtained at Week 8 reduced this data to 3 factor-scores which accounted for 90.0 % variability thereof. These scores were used to rank males into four clusters i.e. high-high, high-low, low- low and high-low.

Similarly, Sperm\text{OPVL} and Holes\text{IPVL} and the percentage of fertile eggs data obtained at age of 8 weeks were factorized into one factor using PCA analysis, which accounted for 67.8% variability in the data. This score was used to rank males into four clusters i.e. high-high, high-low, low- low and high-low.

The males assigned to the lowest fertility clusters from sperm motility data and egg fertility data at 8 weeks of age were compared with males having life-time poor fertility records. Only 30% of males identified as low fertility at week 8 of male age by sperm motility parameters were confirmed as low fertility males after life-time performance (Figure 8-5), whereas, their ratio at the same age level with egg fertility parameters was found to be only 47 % (Figure 8-5), the remaining percentages in both cases being of high fertility males.
Fig 8-5 Comparison between the number of low fertility male identified at 8 week of age and after lifelong records using sperm motility parameters as selection criterion. 13 males were identified as low fertility at 8 weeks of age, while, 7 males were identified as low fertility after lifelong record. Only 4 males (i.e. male number 1, 2, 6, 8) which were of low fertility at week 8 of age were of lower fertility after lifelong record.

Fig. 8-6 Comparison between the number of low fertility male identified at 8 week of age and after lifelong record using egg fertility parameters as selection criterion. 21 males were identified as low fertility at 8 weeks of age, while, 15 males were identified as low fertility after lifelong record. 10 males (i.e. male number 1, 2, 6, 12, 17, 20, 22, 24, 31, 33) which were of low fertility at week 8 of age were of lower fertility after lifelong record.

8.5 Discussion

To understand the relationship between sperm motility and egg fertility parameters, data were analysed in three different ways i.e. i) overall data without adjusting for age and strain effects, ii) within-strain correlation of sperm motility with egg fertility parameters and, iii) using predicted values where data were adjusted for strain and age effects. Overall values and the predicted values showed positive correlation between sperm concentration
and the percentage of fertile eggs, medium sperm and \( \text{Sperm}_{OPVL} \) and \( \text{Holes}_{IPVL} \), and the percentage of progressive motile sperm and \( \text{Holes}_{IPVL} \). Within-strain, different sperm motility parameters were correlating with egg fertility parameters i.e. medium sperm, PROG, VSL, VAP and BCF. This suggested that in Japanese quail relationship between sperm motility and egg fertility is influenced by genotype and on overall bases Rapid, Medium and the PROG sperm, sperm VSL, VAP and BCF could be important parameters for egg fertility. However, poor correlation between sperm motility and egg fertility parameters suggested strong influence of female environment (oviduct) on sperm reaching the egg fertilization site.

Sperm concentration and the PMOT are often linked to fertility in different species (Froman and Mclean, 1996; Froman et al., 1997; Froman and Feltman, 1998; Froman, 2003; Donoghue et al., 1999; Verstegen et al., 2000). In Japanese quail positive correlation of these parameters with egg fertility emphasized that these parameters were also desirable in quail fertility assessment. However, in relation to sperm concentration in this study, the whole population of motile sperm was not found correlated; instead the category of medium sperm was correlated, with the percentage of fertile eggs. This observation seems similar to observations in roosters where motile sperm with VSL values, only above 30 \( \mu \text{m/s} \) were found penetrating the Accudenz solution (Froman, 2003), which suggests that in Japanese quail motile sperm population above some critical velocity would be important. In this study, this population was represented by the medium sperm category which comprised of sperm with VCL values between 36 and 154 \( \mu \text{m/sec} \), however, this is not clear yet if 36 \( \mu \text{m/sec} \) is the requisite velocity for quail sperm to penetrate Accudenz solution.

Ejaculate volume was negatively correlated with most of the observed sperm motility and egg fertility parameters. This is consistent with previous studies where negative or no correlation between semen volume and sperm fertilising ability has been reported (Wilson et al., 1979; Wishart, 1986) However, the question as to why high ejaculate volume in quails is negatively correlated with sperm motility parameters requires further investigation.

Our results for overall data or predicted values did not show any significant relationship between sperm VCL, VSL, VAP, LIN, BCF, STR and egg fertility parameters.
In contrast, studies in broilers and turkeys showed differences in VSL, LIN, BCF, and VAP between high and low sperm mobility males (King et al., 2000). VSL is also reported to be associated with fertility in rats and boars (Moore and Akhondi, 1996; Holt et al., 1997) while, in humans reports have shown both positive as well as negative association of these parameters with fertility (see review; Verstegen et al., 2000). We observed differences in strains for their relation between sperm motility and egg fertility parameters. One strain showed significant correlations between VSL, VAP and the egg fertility parameters, while, the other showed none at all. This was in line with previous studies with roosters where genetic lines were found differing in sperm velocity and linearity parameters (Long et al., 2010) and with boars in which genetic lines differ in the relationship between VCL and farrowing rate and in their relationship between VAP and total number of newborn piglets (Broekhuijse et al., 2012). In Japanese quail a relationship between sperm motility and egg fertility parameters appears to be strain specific. Further studies should factorize genetic line differences, when relating these parameters to egg fertility.

There was a weak relationship between sperm motility and egg fertility parameters, which resulted only in partial success in identifying low/high fertility males at early age. On other hand, when egg fertility parameters were used to predict good or poor fertility males, relatively better results were obtained. It appeared that in Japanese quail female oviduct environment had considerable influence on numbers of sperm reaching the fertilization site and fertilizing the egg. However, this did not exclude the possibility of male selection on the basis of sperm motion parameters in quails. The reason for poor correlation between sperm motility and male ability to fertilize an egg might be the mean CASA values which were used in this study while, only small portion of total sperm were able to fertilize an oocyte (Holt and Van look, 2004).

At the moment nothing is known about the sperm-subpopulations in quail ejaculates, while, it has been suggested that the sperm that fertilize the oocytes in vivo may be subpopulation that is small and highly selected, but not representative of the average sperm evaluated in the ejaculate. Moreover, in quails cloacal gland foam is known to affect sperm motility (Biswas et al., 2010; Chapter 5) but nothing is known about foam composition and relationship with genotype. We used 10% foam sperm motility diluent...
because this concentration produces the highest motility in vitro (Chapter 5). The effect of foam concentration is not yet clear in vivo as well, though it has been reported to affect numbers of sperm reaching the egg fertilization site and to improve fertility (Singh et al., 2012). Thus sperm motility and egg fertility relationship remain to be explored for better understanding of the relationship between sperm motility, genotype and foam composition, as well as the relationship between different sperm sub populations and egg fertility.

8.6 Conclusion

The motility of quail spermatozoa and their relationship to egg fertility is strain specific. Sperm concentration, numbers of Medium and PROG, VAP and VSL are male parameters relevant to egg fertility. The female oviduct seems to have high influence on numbers of sperm reaching the ova.
Chapter 9

Mobility of Japanese quail spermatozoa and its relationship to egg fertility

9.1 Abstract

The relationship between sperm mobility and CASA (computer assisted sperm analysis) sperm motility recoded at 37 °C assay temperature, and egg fertility was analyzed. The effect of cloacal gland foam on sperm mobility was assessed at 37 °C and 41°C following the development of the sperm mobility assay.

Study was carried out in three stages. Males and females (20 x 20; n = 40) were mated individually between weeks 15 to 20 (Stage I), and weeks 29 to 33 (Stage III) of age and egg fertility, Sperm_{OPVL} and Holes_{IPVL} data were collected. In Stage II (weeks 23-28 of age), ejaculates were collected (n = 20 males) and sperm mobility was measured.

Sperm mobility, progressively motile sperm (PROG), curvilinear velocity (VCL), straight-line velocity (VSL) and average-path velocity (VAP) were negatively affected by Accudenz concentration \((P < 0.05)\). Sperm mobility was the highest with 10 % foam extract concentration and an incubating temperature of 37°C. Sperm motility and mobility did not relate to egg fertility under the present conditions of the assay.

Sperm mobility assay identified different mobility phenotypes amongst males and categorized them accordingly but the poor relationship between sperm mobility phenotype and egg fertility stresses the need for further studies in which the potentially crucial role of cloacal gland foam shall be determined.

9.2 Introduction

Sperm motility is defined by the cell”s ability to express any movement in the flagella or sperm-head of its own accord (Lake, 1989; Malo et al., 2006). However, any sperm
movement does not guarantee that a sperm has sufficient mobility to traverse the oviduct. All mobile sperm exhibit motility, whereas, not all motile sperm have the capacity to be mobile. To define the net movement of a sperm cell population against the oviduct secretory flow and cilia movement at body temperature, a sperm mobility assay has been developed (broiler breeder: Froman and McLean, 1996; turkey: Donoghue et al., 1998).

Sperm mobility has been shown to be a quantitative trait and has proved to be one of the determinants of fecundity in fowl (Froman and Feltmann, 1998), and male-to-male variation in sperm mobility phenotype has repeatedly been shown to be a normally distributed trait in roosters and toms (Froman and McLean, 1996; Froman et al., 1997; Froman and Feltmann, 1998; Holsberger et al., 1998). Furthermore, when toms were selected on the basis of extreme levels of sperm mobility, fertility was directly related to the mobility phenotype (Donoghue, 1999).

In Japanese quail, male reproduction is still poorly understood compared to most other commercially important species. The assessment or selection of males based on sperm traits has been slow in quails because the available methods of sperm assessment have not been applied and sperm traits linked to high and low fertility phenotypes has not been identified. The relationship between sperm motility and egg fertility is poor in quail (Chapter 8), but sperm mobility has not been investigated. This study therefore, aimed to test whether sperm mobility is related to egg fertility in quail. However, to develop an assay for sperm mobility for this species, cloacal gland foam needed consideration (Cheng et al., 1989). The foam is not required for fertilization (Marks and Lepore, 1965) so it is characterized as non-semen copulatory fluid but, during mating, it accompanies the semen and it is thought to play a fundamental role in the success of natural mating and fertilization (Biswa et al., 2010; Singh et al., 2011; Singh et al., 2012). We have observed a significant increase in the motility of quail sperm after adding cloacal gland foam (Chapters 5 and 8), so it was clear that any sperm mobility assay developed for quail sperm had to include the foam.
The aims of the study were to investigate the relationship between CASA-based measures of sperm motility and sperm mobility, and to the relationships between sperm motility, sperm mobility, and egg fertility.

9.3 Materials and methods

9.3.1 Experimental birds, housing and management

20 male x 20 female offspring (n = 40) were produced from high fertility parents (n = 40; previously selected from a pool of 45 males x 350 females after assessment of life-long performance) and were housed at the Native Animal Facility of The University of Western Australia. Upon reaching sexual maturity, males and females were moved to individual breeding cages, using Cimuka quail cages (Ankara, Turkey) for males (pen size; 19 cm wide x 40 cm deep x 23 cm high) and Venturi Valter quail breeding cages (Predappio FC, Italy) for the females (pen size; 54 cm long x 30 cm wide x 25 cm high; 1190 cm² floor space per bird). The housing environment was tuned to 22-26 ºC temperature with a 14:10 h light/dark cycle, adequate ventilation, and ad-libitum access to feed and water. The males and females were fed the same quail breeder diet containing 20.0% CP and 11.5 MJ/kg ME. Cages were enriched with a sand bath. There was a 3-week acclimatization period before semen collection during which males were accustomed to handling and to semen collection using a teaser female. All the procedures were carried out in accordance with the protocol approved by the Animal Ethics Committee of the University of Western Australia.

9.3.2 Experimental design

The study comprised of three main stages.

Stage I (mating), 15-20 weeks of age; with two further divisions:

i.e. Stage 1a – individual mating; each male randomly allotted to a female for 7 days, eggs were collected and used to estimate egg fertility by viewing the germinal disc area and counting SpermOPVL and HolesIPVL.

Stage 1b – males were randomly swapped to another female; individual mating were carried out for 7 days, and data were collected as described in Stage 1a.
Stage II (sperm mobility study), 23-28 weeks of age; ejaculates were collected from males; sperm motility was recorded by CASA, and sperm mobility was measured using the Accudenz technique (see below).

Stage III (mating), 29-33 weeks of age; individual mating were carried out for 14 days (pairs were swapped after 7 days), and similar mating and data collection procedures were followed as were described for Stages 1a and 1b.

9.3.3 Preparation of sperm mobility assay

Stock and working Accudenz solutions

A 30% (w/v) Accudenz stock solution (Accurate Chemical and Scientific Corporation, NY) was prepared in 3 mM KCl containing 5mM N-tris-[hydroxymethyl] methyl-2-amino-ethanesulfonic acid (TES), in distilled water pH adjusted to 7.9. The working Accudenz solution was prepared by diluting the stock solution with the Accudenz dilution buffer (111 mM NaCl and 25 mM glucose in 50 mM TES in distilled water, pH adjusted to 7.9). Additionally, 0.1% BSA was added into the final volume of the Accudenz working solution and pH was verified as 7.9.

9.3.4 Estimating sperm concentration

An aliquot of 1.5 μl neat semen was diluted with 598.5 μl formalin buffer (2% formalin PBS buffer) in a 1 ml vial, mixed well and transferred to polystyrene cuvette and sperm concentration was immediately determined by recording the absorbance at 560 nm in a spectrophotometer (Metertech UV/VIS SP8001, Taipei, Taiwan). The unknown concentration was then calculated from the recorded absorbance using a previously established quail sperm concentration standard curve.
Sperm suspension (200 x 10\(^6\) sperm/ml) was prepared by diluting semen with the sperm motility buffer (150 mM NaCl and 20 mM N-tris-[hydroxymethyl]-methyl-2-aminoethanesulphonic acid, 35 mM glucose and 0.1 % bovine serum albumin adjusted to pH 8.00 at 25°C). The sperm suspension was overlaid on 750 μl of pre-warmed Accudenz working solution in a cuvette (SARSTEDT Polystyrene Cuvettes, Germany; 10 x 4 x 45 mm) and initial absorbance was recorded immediately. Samples were then incubated in a water bath for the required time and at the desired temperature, and absorbance readings were taken as per protocol (described below).

**Effect of increasing sperm numbers on absorbance (Experiment 1)**

Semen was collected in the absence of cloacal gland foam from 3 males, the sperm concentration was determined, and the semen was then diluted with motility buffer to get 200 x 10\(^6\) sperm/ml. This sperm suspension was then divided into three different treatment volumes, each replicated: 20 μl (4 x 10\(^6\) sperm), 40 μl (8 x 10\(^6\) sperm) and 60 μl (12 x 10\(^6\) sperm). The treatments were then overlaid on 750 μl pre-warmed 6% (w/v) Accudenz working solution at 41°C in separate cuvettes. Absorbance was immediately recorded at 560 nm and the cuvettes were then returned to the water bath for further incubation at 41°C. Subsequent recordings of each cuvette in each treatment were conducted at 4-min intervals for 20 min.

**Effect of Accudenz concentration on sperm motility and mobility (Experiment 2)**

Two concentrations of the Accudenz working solution i.e. 2% and 6% were prepared from stock solution (30%) and incubated at 41°C in a water bath. Fresh semen was collected from 6 males in the absence of cloacal gland foam. Sperm suspensions (200x 10\(^6\)

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1 The sperm mobility assay procedures described below were carried out in collaboration with Mr Dajun Wang who was developing the mobility assay for his Honors with my assistance and Dr I. Malecki, Wang’s and my supervisor. They are outlined here as described in Mr Dajun Wang final report. Statistical evaluation of experimental results are given in 9.3.10
sperm/ml) were prepared in motility buffer, and 60 µl of the sperm suspension was overlaid upon both Accudenz pre-warmed (41°C) treatment cuvettes. Absorbance was immediately recorded at 560 nm. The cuvettes were returned to the water bath for further incubation (at 41°C), and subsequent absorbance were recorded at 2-min intervals upto 10 minutes time.

Parallel to sperm mobility absorbance readings, CASA sperm motility was also recorded for each of the treatments (i.e. 2% and 6% Accudenz). The sperm motility was recorded when the mobility assay was initiated and then again after 4 min of incubation time. For sperm motility recordings, aliquots containing 3 µl semen (sampled from the 200 x 10⁶ sperm/ml sperm suspension) were mixed with 37.5 µl of 2% or 6% Accudenz working solution to give final concentration of 20 x 10⁶ sperm/ml. The suspensions were mixed well and incubated at 41°C. Duplicate samples were analyzed for motility using CASA.

Effect of foam extract and incubation temperature on sperm mobility (Experiment 3)
Cloacal gland foam was pooled from 3 males and foam extract was prepared (see section 9.3.7). Sperm suspensions (200 x 10⁶ sperm) were prepared in motility buffer containing 10 %, 5 % or 0 % foam. Immediately after preparation, 60 µl sperm suspension from each of the three treatments were overlaid on pre-warmed (41°C) duplicate cuvettes containing 750 µl of 2% Accudenz solution (w/v). Assay temperature was maintained at 41°C and absorbance was recorded at 0 min and then at 2-min intervals for 10 min. Parallel to sperm mobility readings, sperm motility was also recorded (20 x 10⁶/ml sperm concentration) for each sample at 2 minutes intervals.

This experiment was repeated at 37 °C of assay temperature, using ejaculates from the same males

Intra- and inter-male variation in sperm motility and mobility (Experiment 4)
Cloacal gland foam was collected from twenty (27-week-old) males over a period of 10 days, but not pooled. Ejaculates were collected from individual males and were diluted to obtain 200 x 10⁶ sperm/ml motility buffer containing 10% foam from the same male that provided the ejaculate. Duplicate samples of 60 µl sperm suspension were overlaid on 750
μl of 2% Accudenz solution, pre-warmed (37°C) in polystyrene cuvettes. Absorbance was recorded immediately and then at 1-min, 2-min and 4-min intervals through the course of the experiment. In parallel with this mobility assay, sperm motility was measured by CASA in duplicate aliquots containing 3 μl of the diluted semen sample (containing 10% foam) and 37.5 μl of 2% Accudenz solution (final sperm concentration: 20 x 10⁶/ml), incubated at 37 °C. The intra-assay CV was 12.9% for Day 1 and 5.6% for Day 2, and the inter-assay CV was 9.2% for both days.
9.3.6 Estimating egg fertility by appearance of GD and in vivo sperm-egg assay

The eggs were opened, the appearance of the GD was captured with the DP70 digital camera (Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia), and the PVL was collected for sperm-hole counts. The egg fertility status was determined by viewing the GD under a low power magnification aided by lateral illumination from a light source before the photograph was taken. A piece (1.5 x 1.5 cm) of the PVL was collected around the germinal disc using a filter paper ring (Whatman, Grade 1, Sigma- Aldrich Co., Castle Hill, NSW, Australia), washed with phosphate-buffered saline (PBS; pH 7.4) to remove the adhering yolk, and then placed on a glass microscope slide. To count sperm, the membranes were stained with 0.01 mM Hoescht dye (Sigma-Aldrich Co. Castle Hill, NSW, Australia) so the sperm nuclei (Sperm_{OPVL}) could be seen with a fluorescence microscope (Olympus BX60-FL, Olympus Australia Pty. Ltd., Mt Waverley, VIC, Australia) using a „U” filter cube with 372 nm excitation and 456 nm emission wavelengths. For counting sperm-holes on the inner perivitelline layer (Holes_{IPVL}), membranes were stained with Schiff’s reagent (Sigma-Aldrich Co. Castle Hill, NSW, Australia) as described by Bramwell et al., (1995). Counting of Sperm_{OPVL} and Holes_{IPVL} were carried out in six fields (5.7 mm² total area) along the horizontal axis passing through the GD region. The percentage of fertile eggs was estimated by the following formula:

\[
\text{Number fertile eggs produced by a female in a mating phase/total number of eggs produced by female in mating phase} \times 100
\]

Sperm_{OPVL} and Holes_{IPVL} were counted in 5.7 mm² total PVL area and presented as Sperm_{OPVL}/PVL and Holes_{IPVL}/PVL

9.3.7 Foam collection and preparation of foam extract

The proctodeal gland foam was collected by gently squeezing either side of the cloacal gland with fingers and thumb (Mohan et al., 1991). The foam extract was prepared by mixing 1 part foam with 2 parts normal saline (0.89 % w/v NaCl), followed by thorough vortexing for 20-30 min. The mixture was centrifuged at 10,000 g for 30 min. The supernatant was separated and centrifuged again at 10,000 g for 30 min. The final supernatant, being foam extract containing 33.3 % foam, was stored at -20 °C until further use. To prepare 5 and 10 % foam treatments, foam extract (33.3 % foam) was diluted to
1:15 and 1:30 with motility buffer. Motility buffer without any foam was used as control treatment.

9.3.8 Semen collection and preparation
The teaser method was used for semen collection (Chelmonska et al., 2008). In short, males were trained to be stimulated by a teaser female and to produce semen after receiving a gentle massage on the lumber region followed by gentle squeezing of cloaca. To avoid any contamination of the semen samples, feathers around the vent were clipped off and, before each collection, foam was carefully removed from the proctodeal gland.

Immediately after collection, semen was diluted 2-fold with NaCl-TES “collection buffer” (150 mM NaCl, 20 mM N-tris-[hydroxymethyl]-methyl l-2-aminoethane-sulphonic acid, 35 mM glucose; pH adjusted to 7.40 at 25 °C; Holm and Wishart, 1998). To record sperm motility, samples were prepared as per treatment requirement and motility was recoded in duplicate.

9.3.9 CASA equipment, capturing properties and analysis
Sperm motility was assessed using the Motility module of the Sperm Class Analyzer® (SCA) version 5.2 (Microptic S.L., Barcelona, Spain). Captured data were collected using a Basler A602fc digital camera (Microptic S.L.) that was mounted on an Olympus BX53 microscope (Olympus Optical Co., Tokyo, Japan) equipped with phase contrast optics and a motorized heated stage (Prior Optiscan II, Prior Scientific Intl.). The capturing properties were set as follows: 25 images per sec; frame rate = 60Hz; optics Ph- (negative phase contrast); particle size = 10 to 130 micron (particle area was determined by visually evaluating captured fields and selecting the particle area size range that resulted in detecting only the sperm head for motility analysis, thereby, excluding any other cells or debris); progressivity = 70% STR; green filter (IF 550, Olympus, Japan); and Leja 10 slides (standard count four-chamber slide; Leja Products B.V. Nieuw-Vennep, Netherlands). The “quail” SCA setting, with curvilinear velocity (VCL, µm/s) and intervals (10 < slow sperm < 36 < medium sperm < 154 < rapid sperm), was used and sperm were categorized based on these cut-off values into slow, medium and rapid sperm. For motility recording 2-3 µl diluted semen was loaded onto a pre-warmed slide placed on
a heated stage at 39 °C and allowed to settle for 45 seconds before recording. Ten to fifteen fields were captured until a total of 200 motile spermatozoa were recorded (Mortimer, 1994; WHO, 1999). Fields were captured randomly to eliminate biasness towards higher sperm concentration or motility, but fields that included debris or sperm clumps were excluded. All captured sperm tracks were verified visually to allow for the deletion of incorrectly recorded tracks (e.g., due to colliding spermatozoa).

9.3.10 Statistical analysis
For each experiment, the consistency between duplicates was assessed from the intra-assay coefficient of variation (CV) which is calculated by dividing the observed standard deviation by the mean absorbance and then multiplying the proportion by 100. Additionally, in Experiment 4, the inter-assay CV was also calculated to assess male-to-male consistency between Day 1 and Day 2. It is reported as the mean intra-assay CV in Day 1 and Day 2. Moreover, correlations (Experiment 4) were drawn between the sperm mobility absorbance and CASA sperm motility properties recorded at the 4-min interval. In Experiment 1, a single-factor ANOVA was used to determine if differences in recorded absorbance were significant between the treatments. Single-factor ANOVA was used in Experiment 2 to determine the effect of concentration of the Accudenz working solution on recorded absorbance. Likewise, differences between Accudenz treatments in CASA motility properties were analyzed with ANOVA. For Experiment 3, Tukey’s HSD (honest significant difference) test was used to determine whether the concentration of foam and the change in temperature significantly affected absorbance recorded in the mobility assay and the sperm motility properties recorded by CASA. For Experiment 4, the Shapiro-Wilks test for goodness of fit was used to determine whether observed frequencies approximated a normal distribution. Both original and square-root transformed distributions were tested. Males were then ranked by their recorded sperm mobility absorbance. The males with absorbance near average were categorized as average and males with absorbance greater or less than one standard deviation were categorized as high or low mobility, respectively. Subsequently, recorded absorbance was analyzed by split-plot design to detect significant differences between categories and between males within categories.
Alternately, to rank males based on their sperm motility and mobility traits in relation to egg fertility (percentage of fertile eggs, sperm and holes), principle component analysis was applied to the covariance matrix of the 7 variables (mobility absorbance, percentage of rapid sperm, PROG, VCL, VSL, VAP and STR) and variables were weighted against their eigenvectors. One component was drawn (Score) which accounted for 80% variability in the data. K-means cluster analysis was carried out and the „factor Score“ obtained by principle component analysis was used to categorise males into high-high, high-low, low-low and low-high fertility ranking.

9.4 Results

9.4.1 Assay parameters

Effect of increasing sperm numbers on absorbance units (Experiment 1)
The Absorbance recorded at the 4-minute interval differed ($P < 0.05$) with each of the three treatments. The 12-million sperm treatment had higher absorbance than 8- and 4-million ($P < 0.05$) at 4-, 8-, 12- and 16-min intervals, while the 4 million sperm treatment had lower absorbance ($P < 0.05$) than 8-million and 12-million at the 4- and 8-min intervals. The 4-min interval was defined as the plateau of recorded absorbance, as it was found to be within the standard errors of subsequent recordings (i.e. 8-, 12-, 16- and 20-min).

Effect of Accudenz concentration on sperm mobility and motility (Experiment 2)
Absorbance was higher ($P < 0.001$) in the 2% Accudenz treatment than in the 6% treatment. A plateau was reached after 2 min in both treatments, i.e. for 2 and 6% Accudenz concentrations. Sperm suspended in 2% Accudenz showed a higher percentage of progressively motile sperm (PROG), curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) than 6% Accudenz (Table 9-1). The percentage of motile sperm (PMOT) did not differ between Accudenz concentrations. In both treatments, absorbance measurements were correlated with PMOT ($r = 0.76; P < 0.001$) and PROG ($r = 0.90; P < 0.001$).
Table 9-1. Percentage of motile sperm (PMOT), percentage of progressive sperm (PROG), curve linear velocity (VCL), straight line velocity (VSL) and average path velocity (VAP) between 2% and 6% Accudenz treatments. Measurements were made using ejaculates collected from 6 males in each treatment. Values are mean (± STD).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PMOT</th>
<th>PROG</th>
<th>VCL</th>
<th>VSL</th>
<th>VAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2%</td>
<td>58.6±19.6</td>
<td>37.9±12.4</td>
<td>121±24.5</td>
<td>87.5±28.4</td>
<td>97.2±25.5</td>
</tr>
<tr>
<td>6%</td>
<td>53.8±15.4</td>
<td>27.7±11.8</td>
<td>88.8±15.7</td>
<td>59.1±16.7</td>
<td>67.1±16.2</td>
</tr>
</tbody>
</table>

*ab* Mean without common superscripts with in column differ significantly (P < 0.05)

**Effects of foam extract concentration and incubation temperature on sperm mobility (Experiment 3)**

Absorbance was higher (P < 0.001) in the 10% foam extract than in the 5% or the control, and higher (P < 0.05) in the 5% foam extract than the control at 37 °C. On the other hand, with 41°C incubating temperature, sperm mobility absorbance readings in the 10% foam extract were lower (P < 0.05) than the 5% foam extract and control. The 5% foam extract treatment did not differ from the control (Figure 9-1).

For the mobility assay conducted at 37 °C, PMOT and PROG were both found to be higher (P < 0.05) in 10% foam extract than in the control treatment. And Sperm mobility absorbance correlated (P < 0.05) with PMOT and PROG in all treatments at both incubating temperatures.

**Intra-and inter-male variation in sperm motility and mobility (Experiment 4)**

At 37 °C, in the presence of 10% foam extract sperm mobility absorbance reached a plateau at the 4-min interval, though no significant difference was found among values recorded at 2, 4 and 6 min (Figure 9-1). The absorbance measured at 4 min did not differ between Days 1 and 2 (P > 0.05), and neither did CASA motility parameters (i.e. PMOT, PROG, VCL, VSL and VAP), while, sperm mobility (absorbance) and sperm motility analyzed from the same samples (Experiment 4) were positively correlated (Table 9-2).
Fig 9-1. The effect of foam concentration, 10% foam extract (□) and 5% foam extract (■), and control (i.e. no foam extract added) (○), on absorbance recording when sperm suspension was overlaid upon 2% pre-warmed Accudenz solution. Initial recording was conducted immediately after overlaying the sperm suspension and subsequent recordings were conducted at 2, 4, 6, 8, 10 minutes after incubating at 37°C (solid lines) and then assay was repeated at 41°C (dotted lines)
Table 9-2 Correlation between CASA sperm motility parameters and egg fertility traits. Sperm collected from 20 quail and motility and mobility were recorded in parallel at 0 and 4 min. The correlations presented are between 0 min sperm motility and 4 min mobility absorbance values. PMOT, percentage motile sperm; Rapid, rapid sperm; PROG, progressive motile sperm; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; LIN, linearity; STR, straightness of trajectory; ALH, amplitude of lateral head; BCF, beat cross frequency.

<table>
<thead>
<tr>
<th>Mobility (absorbance)</th>
<th>PMOT (%)</th>
<th>Rapid (%)</th>
<th>Medium (%)</th>
<th>PROG (%)</th>
<th>VCL (µm/s)</th>
<th>VSL (µm/s)</th>
<th>VAP (µm/s)</th>
<th>LIN (%)</th>
<th>STR (%)</th>
<th>ALH (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMOT (%)</td>
<td>0.80**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rapid (%)</td>
<td>0.46**</td>
<td>0.66**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium (%)</td>
<td>0.77**</td>
<td>0.92**</td>
<td>0.37*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PROG (%)</td>
<td>0.78**</td>
<td>0.95**</td>
<td>0.72**</td>
<td>0.89**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VCL (µm/s)</td>
<td>0.73**</td>
<td>0.84**</td>
<td>0.79**</td>
<td>0.75**</td>
<td>0.91**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VSL (µm/s)</td>
<td>0.62**</td>
<td>0.72**</td>
<td>0.86**</td>
<td>0.56**</td>
<td>0.84**</td>
<td>0.95**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VAP (µm/s)</td>
<td>0.65**</td>
<td>0.76**</td>
<td>0.85**</td>
<td>0.61**</td>
<td>0.87**</td>
<td>0.96**</td>
<td>0.90**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIN (%)</td>
<td>0.38*</td>
<td>0.46**</td>
<td>0.70**</td>
<td>0.30*</td>
<td>0.62**</td>
<td>0.75**</td>
<td>0.90**</td>
<td>0.88**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STR (%)</td>
<td>0.43**</td>
<td>0.48**</td>
<td>0.64**</td>
<td>0.37*</td>
<td>0.64**</td>
<td>0.78**</td>
<td>0.88**</td>
<td>0.86**</td>
<td>0.96**</td>
<td></td>
</tr>
<tr>
<td>ALH (µm)</td>
<td>0.40**</td>
<td>0.39*</td>
<td>0.12</td>
<td>0.41**</td>
<td>0.31*</td>
<td>0.35*</td>
<td>0.16</td>
<td>0.19</td>
<td>-0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>BCF (Hz)</td>
<td>0.41**</td>
<td>0.44**</td>
<td>0.61**</td>
<td>0.28*</td>
<td>0.52**</td>
<td>0.70**</td>
<td>0.77**</td>
<td>0.75**</td>
<td>0.76**</td>
<td>0.78**</td>
</tr>
</tbody>
</table>

*P < 0.05; ** P < 0.01
9.4.2 Male ranking based on sperm mobility

The frequency analysis of square root transformed data approximated a normal distribution (Kolmogorov-Smirnov test: P = 0.08 for original values, P = 0.82 for square root transformed data; Mean Absorbance = 0.26 ± 0.10 STD). Based on frequency analysis and separation by ± 1STD, three mobility categories were identified: low mobility (n = 3), average mobility (n = 14) and high mobility (n = 3).

9.4.3 The correlation between sperm mobility and egg fertility parameters

The correlation between sperm motility, sperm mobility and egg fertility (Sperm_{OPVL}; Holes_{IPVL}; percentage of fertile eggs) is given in Table 9-3. The values for PROG, VCL, VSL, VAP and STR were more strongly correlated with the measures of egg fertility than PMOT, Medium sperm, BCF and LIN, but none of the correlations were significant. The correlations between sperm mobility and egg fertility parameters were non-significantly negative.

Table 9-3 Correlations between sperm motility, sperm mobility and measures of egg fertility for 20 males (Experiment 4). Rapid, rapid sperm; PROG, progressive motile sperm; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity; STR, straightness of trajectory PMOT, percentage motile sperm; Medium, medium sperm; BCF, beat cross frequency; LIN, linearity; ALH, amplitude of lateral head.

<table>
<thead>
<tr>
<th></th>
<th>Sperm_{OPVL}</th>
<th>Holes_{IPVL}</th>
<th>Egg fertility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid (%)</td>
<td>0.21</td>
<td>0.21</td>
<td>0.15</td>
</tr>
<tr>
<td>PROG (%)</td>
<td>0.16</td>
<td>0.21</td>
<td>0.11</td>
</tr>
<tr>
<td>VCL (µm/s)</td>
<td>0.12</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>VSL (µm/s)</td>
<td>0.12</td>
<td>0.17</td>
<td>0.16</td>
</tr>
<tr>
<td>VAP (µm/s)</td>
<td>0.11</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>STR (%)</td>
<td>0.13</td>
<td>0.15</td>
<td>0.23</td>
</tr>
<tr>
<td>PMOT (%)</td>
<td>0.06</td>
<td>0.11</td>
<td>0.09</td>
</tr>
<tr>
<td>Medium (%)</td>
<td>0.06</td>
<td>0.13</td>
<td>0.04</td>
</tr>
<tr>
<td>BCF (Hz)</td>
<td>0.09</td>
<td>0.06</td>
<td>0.16</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>0.05</td>
<td>0.08</td>
<td>0.15</td>
</tr>
<tr>
<td>Mobility (absorbance)</td>
<td>-0.12</td>
<td>-0.21</td>
<td>0.03</td>
</tr>
<tr>
<td>Maximum VCL (µm/s)</td>
<td>-0.11</td>
<td>-0.01</td>
<td>0.21</td>
</tr>
<tr>
<td>ALH (µm)</td>
<td>-0.07</td>
<td>-0.08</td>
<td>-0.05</td>
</tr>
</tbody>
</table>
9.4.4 Male ranking based on sperm motility and mobility information

The male clustering based on sperm motility parameters (Rapid, PROG, VCL, VSL, VAP and STR) and sperm mobility data (given strong correlation among these parameters) assign 5 males (Cluster 4) into the „poor fertility” category (Table 9-4). Clusters 1, 2 and 3 had higher percentages of fertile eggs. Males from Clusters 1 and 2 also had high MOB, high sperm kinematics and egg fertility values, whereas the poor males (Cluster 4) were characterized by low values of MOB, PMOT, Rapid, Medium and PROG. Moreover, the values for VCL, VSL, VAP, SpermOPVL and the percentage of fertile eggs were also low for Cluster 4 compared with Clusters 1 and 2 (high and average sperm mobility clusters).
Table 9-4 Male clustering by factorizing data for cluster 20 males in Experiment 4: MOB, mobility; PMOT, percentage motile sperm; Rapid, rapid sperm; Medium, medium sperm; PROG, progressive sperm; VCL, curvilinear velocity; VSL, straight line velocity; VAP, average path velocity.

<table>
<thead>
<tr>
<th>Cluster No.</th>
<th>MOB (absorbance)</th>
<th>PMOT (%)</th>
<th>Rapid (%)</th>
<th>Medium (%)</th>
<th>PROG (%)</th>
<th>VCL (µm/s)</th>
<th>VSL (µm/s)</th>
<th>VAP (µm/s)</th>
<th>SpermOPVL (no/PL)</th>
<th>HolesIPVL (no/PL)</th>
<th>Egg fertility (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.12^a</td>
<td>49.3^a</td>
<td>5.0^a</td>
<td>26.1^a</td>
<td>21.6^a</td>
<td>72.9^a</td>
<td>42.6^a</td>
<td>50.4^a</td>
<td>19.0^a</td>
<td>49.8^a^b</td>
<td>94.4^ab</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>0.07^b</td>
<td>62.8^b</td>
<td>9.5^b</td>
<td>35.7^b</td>
<td>32.9^b</td>
<td>54.8^b</td>
<td>54.5^b</td>
<td>62.8^b</td>
<td>19.9^a</td>
<td>51.7^a^a</td>
<td>90.7^a</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>0.04^b</td>
<td>41.1^c</td>
<td>1.1^c</td>
<td>18.3^c</td>
<td>11.9^c</td>
<td>52.0^b</td>
<td>24.9^c</td>
<td>31.8^c</td>
<td>23.4^b</td>
<td>45.7^b^b</td>
<td>100.0^b</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>0.04^b</td>
<td>29.1^d</td>
<td>0.4^c</td>
<td>9.2^d</td>
<td>5.4^d</td>
<td>39.0^c</td>
<td>16.8^d</td>
<td>23.0^d</td>
<td>14.8^a</td>
<td>46.3^b^b</td>
<td>81.2^c</td>
<td>5^*</td>
</tr>
</tbody>
</table>

* low fertility males
9.5 Discussion

Sperm mobility and CASA sperm motility values were highly correlated suggesting that the absorbance recorded in the sperm mobility assay was a function of PMOT, PROG and VCL in Japanese quail. Sperm mobility was found to be higher with 10% foam extract than with 5% or 0% at 37°C. Likewise, PMOT and PROG sperm were highest with 10% foam extract and they were also correlated with sperm mobility. Therefore, the suggestion that foam extract might improve sperm transport through the oviduct (Ikeda and Taji, 1954; Cheng et al., 1989b) appears supported by this study. On the other hand, at an incubation temperature of 41°C, sperm mobility did not improve with increasing concentrations of foam extract – instead, with 10% foam sperm mobility was lower than with 0% foam. In our previous study, we observed a decline in sperm motility with more than 10% foam at 39 °C (Chapter, 5), while, Biswas et al., (2010) reported suppressed motility with 25% foam at room temperature (18 °C). It is not clear yet as to how interactions between foam concentration and temperature affect motility and mobility, so this aspect of quail reproductive biology needs further investigation.

In the present study, sperm mobility under the described assay conditions (i.e. 37 °C, 10% foam extract, 2% Accudenz and 4 min incubation time), was not found to be correlated with SpermOPVL and HoleSIPVL, and with percentage of fertile eggs, these observations do not agree with the claim that, in domestic fowl, sperm mobility is a primary determinant of male fertility (Froman et al., 1999). However, in quail, correlations between sperm motility and sperm mobility were highly significant and those males which were categorized into the lowest mobility category had lower values for sperm motility and egg fertility than all other male clusters. On other hand, in our own previous study (Chapter 8), we had observed a positive correlation between medium sperm and PROG with egg fertility parameters (i.e. SpermOPVL, HoleSIPVL and percentage of fertile eggs), relationships could not be evidently established in the present study. There are three possible explanations: 1) Only 20 males were used in the present study, so the sample size for the assay might has been too small; 2) All males were from highly fertile parents (F0), thus there was less than normal variation in egg fertility traits, as it is evident also by the similar values for SpermOPVL and HoleSIPVL in the high mobility and low mobility clusters; 3) We used pair mating (individual mating), in contrast with commercially practiced
method where a male is grouped with many females (colony mating), i.e., a situation which presents greater variation in fertility rate than our resorted strategy.

Reportedly, during natural mating foam is fully inserted into the female proctodeum, increasing the chances of egg fertility (Cheng et al., 1989a; Adkins-Regan., 1999). However, differences in sperm motility at 37 °C and 41 °C with the same amount of foam have been quite intriguing to rationalize. It may be that in vitro environment, in which sperm motility and mobility are measured, does not reflect seminal or female oviduct environment. Hence, the role of cloacal gland foam in Japanese quail reproduction remains to be elucidated and male traits that can be linked to egg fertility are yet to be determined.

9.6 Conclusion

The sperm mobility assay can be used to identify sperm mobility phenotypes in quail, however, the poor relationship between sperm mobility and egg fertility stresses the need for further studies, particularly with respect to the role of cloacal gland foam.
Chapter 10

General Discussion

The general hypothesis driving this research was that the fertility of Japanese quail could be improved by selecting high fertility males and females. To accomplish this, objective methods of fertility estimation were used to study 5 meat-type breeder quail strains and to determine the contribution of each sex to the egg fertility. Furthermore, traits linked to egg fertility were identified for each sex.

The number of sperm holes in the egg perivitelline layer was quantitatively related to fertility phenotype, with the strongest contribution coming from the female. Among the male traits, the number of medium and progressive velocity sperm, as well as body weight, were linked to egg fertility, and cloacal gland foam had a major effect in vitro semen assessment and interpretation of in vivo fertility outcomes. The results support the hypothesis that quail production can be improved by selecting high-fertility individuals. There is also evidence that fertility is affected by age, strain, and cloacal gland foam.

These findings are a significant advance in our understanding of quail reproduction, and in our ability to identify the male and female fertility traits which are related to egg fertility.

10.1 Flock fertility

To date, estimation of fertility in quail has relied on conventional methods, such as after-hatch egg-break-out analysis (Woodard and Alplanalp, 1967; Narahari et al., 1988, Narinc et al., 2013), which indicate high egg fertility between ages 10-19 weeks with a peak at 14-16 weeks. After-hatch egg-break out analysis has its disadvantages, particularly the fact that early embryonic mortality cannot be distinguished from infertility with naked eye. Estimation of fertility in freshly laid eggs by viewing the germinal disc status and counting the Sperm$_{OPVL}$ and Holes$_{IPVL}$ eliminates such errors and can be used to monitor flock fertility and to show how flock fertility is affected by age and strain (Wishart and Stains,
With the work in this thesis, we have validated the use of the germinal disc assessment and in vivo sperm-egg assay for quail.

In this study, flock fertility peaked at 26 weeks of age, far later than any other reports. The apparent delay might be due to the strain or to our use of an objective method for fertility estimation. High fertility up to 21 weeks has been reported when Holes\textsubscript{IPVL} assay was used to investigate quail fertility in relation to mating ratio (Santos \textit{et al.}, 2011), supporting the view that the objective methods (germinal disc assessment and \textit{in vivo} sperm-egg assay) have a major advantage over the after-hatch egg break-out analysis. Age and strain-specific egg fertility appear to correspond more with Holes\textsubscript{IPVL} than with Sperm\textsubscript{OPVL}, suggesting that egg fertility in quail is a function of the number of sperm that can penetrate the inner PVL.

\textit{10.1.1 Effect of age, sex and strain on egg fertility}

Egg fertility, a combined trait attributed to both the male and the female, should be affected by male factors (semen production rate, semen quality) and female factors (egg quality, hostility of oviduct environment for sperm), as well as gender interaction (physical and physiological compatibility of male and female), and a change in fertility in either sex can thus lead to a decline or increase in egg fertility. Detailed knowledge of the contributions of each sex to fertility is therefore important for management of breeder flocks and for flock improvement. We expected that both males and females would contribute equally to age-related fertility changes. Egg fertility, Sperm\textsubscript{OPVL} and Holes\textsubscript{IPVL}, the duration of sperm storage and sperm loss rate were therefore estimated, from 8 to 36 weeks of age, using GD appearance and the \textit{in-vivo} sperm-egg assay. Fertility measures differed for the two sexes, with age-related decline being greater in females than in males, an observation that was consistent within and between the strains.

Male age and body weight was positively correlated with egg fertility. Overall, values for Sperm\textsubscript{OPVL} and Holes\textsubscript{IPVL}, duration of sperm storage, and for sperm loss rate did not differ between young and old males, but this observation did not apply not to all strains. Strain differences might be due to the fact that male body weight and age were positively correlated with egg fertility, two parameters for which there was between-strain variation, or to the different genotypes. Taking all observations into account, it seems likely that males maintain fertility longer than females.
Female body weight and age were negatively correlated with $\text{Holes}_{\text{IPVL}}$, duration of sperm storage and sperm loss rate. Older females had fewer $\text{Holes}_{\text{IPVL}}$ than younger females, even when mated to same-age males, suggesting that a reduction in $\text{Holes}_{\text{IPVL}}$ was independent of male age, as previously reported for quail (Santos et al., 2013). Similarly, older females showed a shorter duration of sperm storage and higher sperm loss rate compared to young females, whereas older males showed higher duration of sperm storage and lower sperm loss rate than young males, suggesting that a decline in duration of sperm storage was due to reduction in the ability of the female to store sperm for extended periods, as seen on other species (Birkhead and Fletcher, 1994; Santos et al., 2013, in quail; Malacki and Martin, 2002, in emus). The findings of the present study provide further insight into quail reproduction and will help breeders to develop strategies to counter age-related declines in egg fertility.

### 10.1.2 Selection for egg fertility traits and its impact on fertility

In first part of this study, the number of $\text{Holes}_{\text{IPVL}}$ was found to be related to egg fertility in quail, as had been shown for broiler breeders and turkeys (Bramwell et al., 1996; Wishart, 1997). This relationship was stronger than for any other single variable. Allocation to high and low fertility phenotypes on the basis of $\text{Holes}_{\text{IPVL}}$ resulted in differences between the high and low fertility phenotypes for most fertility parameters. Moreover, when selected males and females were mated, egg fertility and the numbers of $\text{Sperm}_{\text{OPVL}}$ and $\text{Holes}_{\text{IPVL}}$ were all improved, confirming the importance of $\text{Holes}_{\text{IPVL}}$ for egg fertility. Furthermore, the number of $\text{Holes}_{\text{IPVL}}$ was not affected by male age (Experiment I) but was halved when HF males were mated with LF females, compared mating LF males with HF females (Experiment II). The observations suggest that the number of $\text{Holes}_{\text{IPVL}}$ is determined by the female and could be used as a female fertility trait in selection. This finding will help the management of flock fertility and will be useful for breeding programmes.

### 10.2 Development and optimization of procedures for CASA sperm motility assessment

The males used in all of the semen evaluation studies were kept under similar conditions (cages, housing) and the same methods and procedures for semen collection were used every time. Before the start of each experiment, the males were trained for two weeks.
Sometimes, a male was not already in the same cage, so an extra week was added before the start of training for semen collection (hence the mention of “2 or 3 weeks” in some of the methodologies). Importantly, this was not extra week of training – each experiment was started when the males started showing similar responses to the teaser females.

10.2.1 Optimizing foam concentration for CASA sperm motility assessment

It has been reported previously that 5% cloacal gland foam is required to maintain mass motility \textit{in vitro} (Biswas \textit{et al.}, 2010). However, this result was obtained using subjective motility assessment, a method that suffers from 30-60\% variation and cannot detect small changes in sperm motion (Jorgensen \textit{et al.}, 1997; Verstegen \textit{et al.}, 2002). Moreover, it was not clear from the reports by Biswas \textit{et al.}, (2010) and others whether changes in sperm motility score were due to changing numbers of motile sperm or changes in sperm velocity. We therefore, decided to re-test the optimum foam concentration using assessment of sperm motility by CASA. We observed maximum sperm motility with 10\% foam, with highest values for numbers of both rapid and progressive sperm and for sperm velocities parameters (VCL, VAP and VSL).

We now have a more valid optimum foam concentration for the assessment of quail sperm motility and we have shown that CASA can be used to assess treatment effects in a wide range of experiments.

10.2.2 Estimation of quail specific VCL cut-off values to categorise motile sperm into rapid, medium and slow velocity categories

For classifying sperm into the „rapid”, „medium” and „slow” categories, the Sperm Class Analyser (SCA) allows the entry of specific VCL values. One of the default settings on the SCA was suitable for chicken sperm (slow < 50 < medium < 100 < rapid), but this setting classified nearly 50\% of Japanese quail sperm into the „rapid” category, a poor reflection of the real data distribution for quail. As reported elsewhere, species differ in their sperm motility characteristics (Maree and Horst, 2012), so the VCL cut-off values will be unique to a species and should be defined before analysis and before correlation with egg fertility.

The studies in this thesis together generated huge amounts of sperm data, placing us in an ideal position for defining new cut-off settings. We used several statistical methods to derive suitable cut-off values for categorizing quail sperm. The best outcome was obtained with lower cut-off values of 25 \% or 33 \% of the data and upper cut-off
values of 66 % or 75 % of the data. Males were ranked based on categories defined by these two methods, and the 25% cut-off identified the lowest motility sperm more effectively than the 33% cut-off. The results suggested following classes for quail sperm: slow < 36 < medium < 154 < rapid. This finding will improve the interpretation of data for quail sperm motility as measured by CASA and will help in comparison of results from different studies. However, the wide range of values for medium velocity suggests that further investigation is needed.

The accumulation of a huge dataset also allowed us to re-analyse data for all experiments. When we did so, no differences in any treatment effects were revealed, only changes in the categories of rapid, medium and slow sperm.

10.3. Male assessment based on semen analysis

10.3.1 Effect of age and strain on semen characteristics

Aging male quail are reported to have more gonadal abnormalities and tumours, particularly Sertoli cell tumours, a sharp decrease in numbers of LH and FSH receptors, lower sperm production and reduced sexual behaviour (Howes, 1968; Ottinger et al, 1983; Gorham and Ottinger, 1986). It was therefore expected that age would affect semen quantity (ejaculate volume, sperm concentration) and quality (sperm morphology, sperm motility).

We observed no significant age-related decline in ejaculate volume and, at old age, males seemed to maintain high numbers of sperm per ejaculate. Aging might not have affected semen quantity because our males were maintained good body condition and their endocrine system remained relatively active (Ottinger et al., 1983). However, age-related increases in the numbers of abnormal sperm and decreases in the number of live normal sperm might be caused by increases in the frequency of testicular abnormalities (Eroschenko et al., 1977).

Advancing age, from 8 to 26 weeks, was seen to reduce PMOT, PROG, Rapid sperm and the VCL, VAP, VSL, LIN and BCF, but, at 36 weeks of age, there was an increase in motility parameters. The initial decline suggests that aging males produce a smaller proportion of motile sperm with less forward movement. Furthermore, the age-related decline in LIN and BCF suggests that sperm from older males (Week 26) follow paths that are less linear and have a lower beat frequency, suggesting that age-related
declines in the number of sperm reaching the PVL might be caused by a loss of PMOT and reduced LIN and BCF. The lack of age-related changes in STR and ALH suggest that the ability of sperm to fuse with the ovum did not decrease with age (Sloter et al., 2006). The reason for an increase in percentage of motile sperm and sperm motility from Week 26 to Week 36 is not clear. Males gaining body weight and body condition can maintain reproductive function, but the environmental conditions were controlled in all of our studies. Self-molting could be an explanation because feather loss was observed in a few males, and molting in turkeys has been linked to increases in semen output (Woodard et al., 1975). However, there has been no study of this issue for quail.

The findings of the present study suggest that measurement of ejaculate volume and sperm concentration might not be useful for assessing age-related decline in male reproductive function because many males maintained a good ejaculate volume up to the age when present-day breeder flocks are normally culled (36-40 weeks). Clearly, objective assessment of male semen needs to be implemented.

10.3.2 Relationship between sperm motility and egg fertility

Only motile sperm can swim through the barriers of female oviduct and reach the egg fertilization site, so sperm motility is considered an essential part of semen evaluation. This is supported by the significant correlation of CASA sperm motility parameters with fertility (Verstegen et al., 2002). Sperm motility has not been giving due weight in selection of male quail, perhaps because specific information on the links between sperm motility traits and egg fertility are not available for this species. We addressed this issue by using CASA to study quail sperm motility with the aim of identifying sperm motility traits that could be used to improve fertility. We observed significant correlations between egg fertility and the numbers of medium velocity sperm and progressive sperm. In contrast to previous observations, the whole motile sperm population was not correlated with egg fertility, so it is suggested that quail sperm with a specific velocity have the best chance of reaching the egg. Indeed, in the chicken, only sperm with VSL values above 30 um/sec can penetrate Accudenz solution (Froman et al., 2003).

Overall, there were no significant relationships between measures of egg fertility and sperm velocity parameters (i.e. VCL, VSL, VAP, LIN, BCF, and STR). However, among the strains, some velocity parameters (VSL, VPA, BCF) were correlated with egg fertility traits, suggesting that, in quail, CASA motility parameters and their association
with the egg fertility could be strain specific, as reported for broiler chickens (Long et al., 2010) and for boars (Broekhuijse et al., 2012).

On the other hand, the generally poor correlations between sperm motility parameters and egg fertility traits support the notion that egg fertility is primarily controlled by females, as described in Chapter 4. However, this does not exclude the use of sperm motility traits for male fertility assessment. Considering that only a small proportion of sperm reside in the SSTs and reach the egg fertilization site, it is feasible that, in a sperm population, only sperm with specific characteristics are useful. Identification of hypothetical small subpopulations that are involved in egg fertilization would greatly improve our understanding of the relationship between sperm motility to egg fertility.

10.3.3 Development and quantification of the sperm mobility assay

The ability of sperm to reach the egg fertilization site is determined by the extent to which they enter and remain sequestrated within the SSTs (Bakst et al., 1994). Any sperm can be motile, but motility alone does not ensure an ability to move through the female oviduct. Sperm mobility, a measure of the net movement of a sperm population against resistance, is recognized as a quantitative trait that determines fertility in the chicken and turkey (Froman and McLean, 1996; Froman and Feltmann, 1998). We expected a similar situation in quail.

The mobility assay could distinguish mobility phenotypes and categorize males accordingly. When males were clustered on the basis of sperm motility and mobility values, the lowest mobility category also showed the lowest values for sperm motility and egg fertility, suggesting that, as in other poultry species (Froman and McLean, 1996), the sperm mobility assay can be used to identify poor and high fertility males quail. Furthermore, measures of sperm velocity (PMOT, PROG, VCL, VSL, VAP) were correlated with mobility values, suggested that sperm mobility in quail is inherent to a subpopulation (PROG, PMOT) and to individual sperm (VCL, VSL, VAP).

Sperm mobility was higher in 10% foam than in 5% foam or control at 37°C, but was lower at 41°C. We observed a reduction in sperm motility with more than 10% foam at 39°C (Chapter 5), whereas Biswas et al., (2010) reported a reduction with 25% foam at room temperature (18°C), suggesting that, at quail body temperature, foam might be performing other functions in addition to increasing motility. This hypothesis needs to be
tested, and we also need a deeper understanding of how interactions between foam extract and temperature affect motility and mobility.

The negative correlation between sperm mobility and both $\text{Sperm}_{\text{OPVL}}$ and $\text{Holes}_{\text{IPVL}}$, and the absence of a correlation with percentage fertile eggs observed in the present study do not agree with previous work in other species (e.g., Froman and McLean, 1996). The reason might be found in unexplained roles of cloacal gland foam in sperm mobility at body temperature (41°C). On the other hand, the low sample size and similar fertility phenotypes in the present study suggest a need for caution.

At the commencement of this thesis (September, 2009), the protocols for assessing quail fertility were in preliminary stages of development and some of them needed to be optimized. The complex nature of the study, involving production and supply of different age birds, and the length of the experiments with intensive data collection and time constraints, did allow analysis of foam and seminal plasma data from GC-MSD analysis, investigation of the lack of relationship between sperm mobility and egg fertility in quail, or exploration of the reasons for foam acting differently at quail body temperature and room temperature. These avenues of research remain open. Nevertheless, the research described in this thesis will help breeders to identify and eliminate poor fertility males from their flocks and has provided important insights into the foam-sperm relationship.

10.4 General Conclusions

The research in this thesis has demonstrated that age- and strain-related changes in quail fertility can be quantified and that fertility could be improved by selecting for high fertility males and females using $\text{Holes}_{\text{IPVL}}$ as a criterion. The detailed findings are:

1. The age- and strain-related changes in egg fertility are a function of $\text{Holes}_{\text{IPVL}}$;
2. The age-related decline in fertility is earlier in females than in males;
3. The age-related decline in female fertility is independent of male age;
4. The age-related decline in duration of sperm storage depends on sperm loss rate from the oviduct rather than the amount of sperm supplied by males;
5. The number of $\text{Holes}_{\text{IPVL}}$ is related to egg fertility and this trait is controlled by the female;
6. Flock fertility can be improved by selecting males and females for high numbers of $\text{Holes}_{\text{IPVL}}$;
7. 10% cloacal gland foam in the motility buffer produces maximum sperm motility *in vitro* whereas more than 10% decreases sperm motility;
8. There are quail-specific CASA settings for VCL cut-off values for categorizing sperm velocity as rapid, medium and slow;
9. Sperm categorization based on the upper and lower inter-quartile range better identifies low and high sperm motility males, compared with 33% cut-offs;
10. The age-related decline in male fertility can be assessed by studying the proportions of motile sperm and their kinematics;
11. Sperm from old males have a lower proportion of motile sperm, less linearity and less forward movement, compared with sperm from young males;
12. Sperm concentration, the numbers of medium and progressive sperm, the average path velocity (VAP) and straight line velocity (VSL) are all important male traits in relation to female fertility;
13. Sperm motility and sperm mobility are strongly correlated;
14. Sperm motility and mobility are poorly correlated with egg fertility;
15. Cloacal gland foam appears to play a decisive role in sperm mobility and its effect on egg fertility. An inhibitory effect of foam on sperm mobility cannot be ruled out.
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