Unraveling the Physiology of Sperm Maintenance in the Vas Deferens of Japanese Quail (Coturnix japonica)

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(ウズラの輸精管における精子維持に関する生理学的研究)

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Chapter I: General Introduction

1.1 Sperm production process in avian species

Sperm are produced within the seminiferous tubules of testis through the process of spermatogenesis. Compare to mammals, spermatogenesis occurs rapidly by about 14.5-15.8 days in Japanese quail (Clulow and Jones, 1982). After spermatogenesis, sperm are suspended in the fluid of seminiferous tubules (Vizcarra et al., 2014). The volume of suspensory fluid and its composition are changed prior to sperm transportation from seminiferous tubules to epididymal region (Esponda and Bedford, 1985), and sperm concentration changes from low concentration in testis to high concentration in vas deferens and ejaculated semen.

1.2 Sperm maturation and transportation process in avian species

Clulow and Jones (1982) investigated the process of sperm production, transportation, storage and survival in male reproductive tract of Japanese quail and found a significantly high percentage of motile sperm in the vas deferens compare to testis and epididymis. This result shows sperm become mature or capable of movement during their transportation in the reproductive tract. Probably, sperm are transported through peristaltic movement in the male reproductive tract. To prevent back flow of semen, contraction of lumen happens at the end of the epididymis. Thus, sperm are continuously produced and transported through the reproductive tract. In male Japanese quail, the process of sperm transportation is accomplished by about 1 day (Clulow and Jones, 1982). The sperm production, and transportation process in Japanese quail is shown in Fig. 1-1.

1.3 Reproductive fluid in avian species

Most components of seminal plasma are derived from primary sex glands (seminal vesicle, prostate gland and bulbourethral gland) in mammals (Mann and Lutwak-Mann, 1951; Maňásková et al., 2002; Duncan and Thompson, 2007), and insects (Happ, 1984; Gillott, 1996). The reproductive tract of avian species generally differs from that of most mammals in two main aspects. First, the internal testis and second, the accessory reproductive organs, namely seminal vesicles, prostate and bulbourethral gland. Unlike mammals and insects, avian species lack the prostate gland, seminal vesicle, and bulbourethral gland. Some avian species produces lymph-like fluid and/or foam at the time of ejaculation (Fujihara, 1992). Male quail produces large quantities of cloacal foam at the time of ejaculation (Seiwert and Adkins-Regan, 1998). Thus, in avian species, the fluid adds with the sperm mainly derived from testis, epididymis and cloacal region.

1.4 Sperm storage *in vivo* condition

In physiological condition, the capacity of sperm to store in the female reproductive tract is relatively commonplace in reptiles, fishes, birds and amphibians (Holt, 2011). In mammals, some species of bat can also store sperm in the female reproductive tract for several months (Holt and Lloyd, 2010). The female in these species possesses a specialized reproductive structure for sperm storage *in vivo* condition (Suarez, 2010; Wolfner, 2011; Kuehnel and Kupfer, 2012). Depending on the species, the capacity of sperm storage period and fertilizing ability varies from several hours to months. In female bird, there is a junction between uterus and vagina, known as utero-vaginal junction (UVJ) where sperm storage tubules (SSTs) are located. In avian species, ejaculated sperm can be stored in the lumen of SSTs and may retaining fertilizing ability up to 15 weeks at body temperature 41 °C (Sasanami et al., 2013).

Though sperm storage in female reproductive tract is considerably higher than male reproductive tract, sperm stores in the vas deferens for a considerable period of time before ejaculation. In avian species, sperm are highly concentrated in the vas deferens and ejaculated semen (Donoghue and Wishart, 2000). Sperm production and storage occurs at body temperature 41 °C (Beaupré et al., 1997). pH of seminal plasma seems to be neutral to slightly alkaline (Siudzińska and Łukaszewicz, 2008; Ondho, 2014; Getachew, 2016) and, isotonic condition (Siudzińska and Łukaszewicz, 2008; Dietrich et al., 2010).

1.5 Role of seminal plasma protein

Seminal plasma (SP) is a complex fluid (Poiani, 2006), varies among species and plays an essential role for sperm functioning in both male and female reproductive tract (Juyena and Stelletta, 2012). The roles of SP on sperm viability, motility and fertilizing ability are well studied in mammalian species (Thaler, 1989; Killian et al., 1993; Manjunath and Thérien, 2002; Moura et al., 2006; Manjunath et al., 2007; Koppers et al., 2011; Juyena and Stelletta, 2012; Crawford et al., 2015; Viana et al., 2018), and insects (Bertram et al., 1996; Lung et al., 2001; Bloch Qazi and Wolfner, 2003; Holman, 2009). The role of SP on sperm viability, motility and, fertilizing ability in avian species are limited and are shown in table 1-1. Probably, the first proteomic analysis of SP was investigated in rooster semen (Marzoni et al., 2013). SP contains several components and specific SP components are associated with sperm maintenance (Santiago-Moreno et al., 2019). Identification of SP proteins and the application of SP proteins on sperm viability and motility study may give clearer scenario about the role of SP in avian species.

1.6 Molecular mechanism of sperm maintenance

The molecular mechanisms of sperm maintenance in the male reproductive tract are still

not fully understood. It is believed that the fluid added with sperm in the male reproductive tract contributes important role for sperm maintenance. The extensive study on reproductive fluid can help to unravel the physiology of sperm maintenance in the male reproductive tract. More research incorporating *in vivo* and *in vitro* sperm viability and motility are needed to understand the mechanism. One of the most potential fields of study in the reproductive technology may be molecular study of seminal plasma. Seminal plasma contains many proteins (Fujihara 1992). Seminal plasma are considered to be necessary for nourishing of sperm, maintaining buffering properties (Pilch and Mann, 2006), stimulating sperm motility (Ashizawa and Sano, 1990) and providing a safe environment to transfer sperm from male reproductive tract to the female reproductive tract (Robertson 2005; Rodríguez-Martínez et al. 2011; Juyena and Stelletta 2012). Still, the role of seminal plasma in avian species has not been fully elucidated and molecular study of seminal plasma may help to understand the mechanism of sperm maintenance in the male reproductive tract.

1.7 Scope and objectives

In internally fertilizing animals, sperm need to be surviving for a considerable period of time in male reproductive tract before ejaculation or in female reproductive tract before fertilization. The mechanism of sperm storage in the female reproductive tract seems to be somehow elucidated and studied widely. However, sperm storage and survival mechanisms in the male reproductive tract have given little attention, especially in avian species. The mechanism how sperm uphold its maturation and keep maintenance in the male reproductive tract remains unclear. The application of knowledge regarding physiological conditions existing in seminal fluid and seminal plasma components to *in vitro* study may help to understand the mechanism. The goal of modern poultry industry is to provide high quality meat and eggs. Nowadays, artificial insemination is being used widely in breeding farms to

propagate next generations. Artificial insemination is often limited due to poor survival of cryopreserved avian sperm. Thus, the viability and motility of sperm during *in vitro* liquid storage are important for successful fertilization. The objectives of the present study are:

a) To investigate sperm viability with various physiochemical conditions existing in seminal fluid of avian species.
b) To investigate the presence of seminal plasma protein in male reproductive tract.
c) To investigate role of seminal plasma protein on sperm viability.
d) To investigate role of seminal plasma dipeptides on sperm motility.

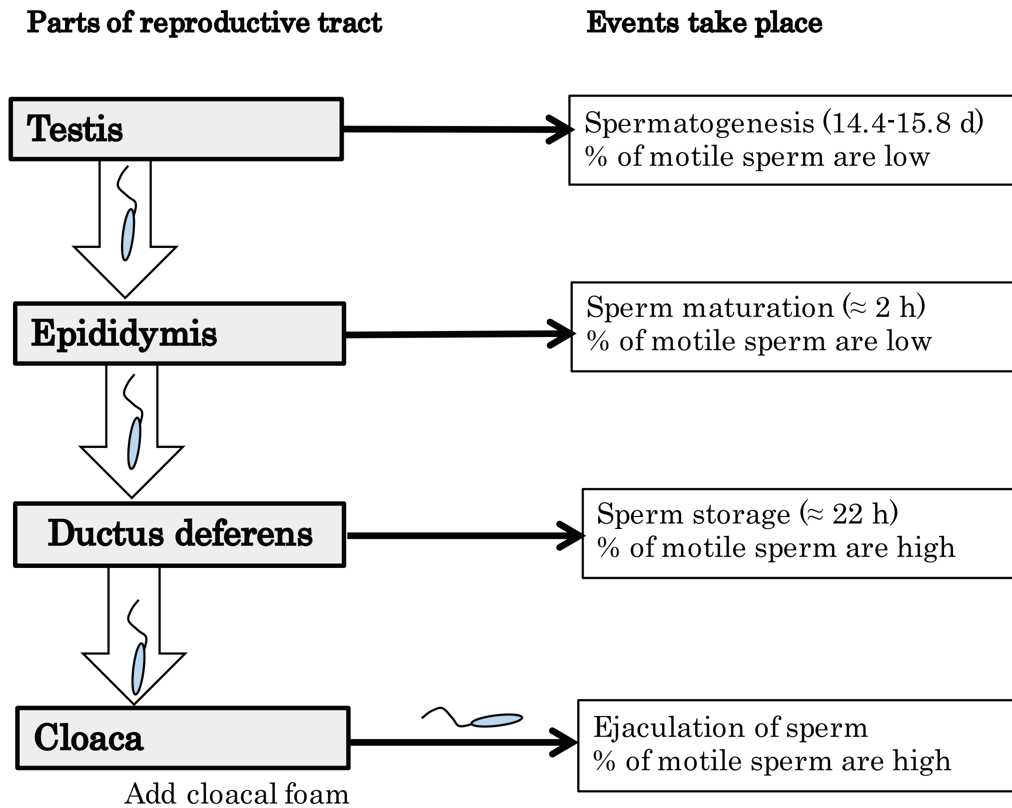

Fig. 1-1: **Sperm production and transportation process in male Japanese quail.** The figure is prepared from the findings of Clulow and Jones (1982). Sperm produces in testis, matures in epididymis and briefly store in ductus deferens. Sperm production, maturation and transportation is relatively quicker in Japanese quail. During ejaculation, cloacal foam is added with the semen. Testicular and epididymal sperm show low motility compare to sperm of ductus deferens and ejaculates.

Table 1-1: **Effects of seminal plasma on motility, viability and fertilizing ability of avian sperm.**

Types of SP	Species	Conditions applied	Roles	References
Fraction of SP (M_r <1000)	Chicken	washed spermatozoa at 41 °C for 15 min	stimulate sperm motility and oxygen consumption	Ashizawa and Okauchi, 1984
Fraction of SP (M_r <1 kDa and >50 kDa)	Chicken	sperm stored at 4 °C for 24 h	<1 kDa decreases fertility >50 kDa increases fertility	Blesbois and de Reviers, 1992
SP + PBS (1:2)	Chicken	sperm stored at 0 °C for 24 h	inhibit endogenous lipid peroxidation in sperm and improve fertility	Fujihara and Koga, 1984
SP albumin (1 or 4 mg/ml)	Chicken	sperm stored at 4 °C for 24 h	sperm mobility stimulating factor	Blesbois and Caffin, 1992
Whole SP	Turkey	sperm stored at 4 °C for 24 h	reduces fertility	Douard et al., 2005
SP dialyzed at 12-14 kDa	Turkey	sperm stored at 5 °C for 24-48 h	improve sperm viability, membrane integrity and sperm motility	Iaffaldano and Meluzzi, 2003

Chapter II: Sperm viability with various physiological conditions existing in seminal fluid of avian species

2.1 Introduction

In animals with internal fertilization, sperm can be stored and maintained in the specialized structure of female reproductive tract and may retain fertilizing ability even after single insemination (Orr and Brennan, 2015). Though sperm storage in female reproductive tract is higher than male reproductive tract, mature viable sperm are stored and maintained in male reproductive tract before ejaculation(Orr and Brennan, 2018). In avian species, sperm are stored and maintained in vas deferens of the male reproductive tract at body temperature 41°C before ejaculation (Beaupré et al., 1997).

During sperm production and transportation process, a complex fluid known as seminal fluid is added with sperm. In mammals, the seminal fluids are mainly derived from the accessory sex glands (seminal vesicle, prostate gland and bulbo-urethral gland) (Rodger, 1976). Unlike mammals, the accessory sex glands namely seminal vesicle, prostate and bulbo-urethral glands are absent in avian species. Thus, the fluids are derived from the testis, epididymis and lymph-like fluid and/or foam during ejaculation (Fujihara, 1992). Sperm are suspended in the seminal fluid derived from the male reproductive tract and matured sperm may retain viability and motility in the fluid *in vivo* condition. The seminal fluid is known to nourish and protect the sperm *in vivo* and *in vitro* condition. The physiochemical conditions existing in the seminal fluid is important as sperm are maintained in this condition in the reproductive tract. In avian species, sperm are highly concentrated in vas deferens and ejaculated semen (Donoghue and Wishart, 2000), pH of seminal plasma seems to be neutral to slight alkaline pH (Siudzińska and Łukaszewicz, 2008; Ondho, 2014; Getachew, 2016)

and osmolarity at isotonic condition (Siudzińska and Łukaszewicz, 2008; Dietrich et al., 2010).

The sperm remain in a quiescence state of motility in the male reproductive tract (Matsuzaki et al., 2017). The sperm motility imitation starts when sperm are diluted with a diluent. After motility imitation, sperm viability and motility decrease upon incubation time. The physiology of sperm maintenance in the vas deferens is not fully understood. As sperm are suspended in seminal fluid, the physiochemical conditions existing in seminal fluid may improve sperm viability. The aim of the study is to investigate sperm viability with various physiochemical conditions existing in seminal fluid. The study will give idea and a forward direction about the sperm maintenance in the male reproductive tract.

2.2 Materials and methods

Animal preparation and sperm collection

Male Japanese quails (*Coturnix japonica*), 20-40 weeks of age (Quail cosmos, Toyohashi, Japan) were maintained individually under a photoperiod of 14L: 10D (lights went on at 05:00). Birds were allowed to *ad libitum* water and a commercial diet (Toyohashi Feed Mills, Toyohashi, Japan). Hanks' balanced salt solution (HBSS) containing 136 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.26 mM $CaCl_2$, 4.2 mM $NaHCO_3$, 5.6 mM glucose, 60 mg/l benzylpenicillin potassium and 100 mg/l streptomycin sulfate salt was used as sperm diluents. HBSS at pH 7.4 was incubated at 37 °C for 10 minutes before semen collection. Semen was obtained from male quail during mating prior to ejaculation in accordance with the procedure of Kuroki and Mori (1997). All experimental procedures for the care and use of animals were carried out in accordance with approved guidelines of the Animal Care Committees of Shizuoka University (Approval number: 2018A-5).

Sperm processing and media preparation

Semen obtained from male quail was suspended in 1 ml HBSS and sperm concentrations were measured with hemocytometer under the microscope (BX 51, Olympus Optics, Tokyo, Japan) and were adjusted to various concentrations (2×10^7, 2×10^6 and 2×10^5 sperm/ml). The pH of HBSS was adjusted with 0.1 M HCl or 0.1 M NaOH and pH was measured by pH meter (Horiba, Model: B-712, LAQUAtwin, New Jersey, USA).

Preparation of reproductive fluids

Seminal plasma was prepared from ejaculated semen and fluid in vas deferens. To collect fluid from lumen of vas deferens, male Japanese quail was sacrificed, opened the abdomen, separated the vas deferens and fluid was collected by squeezing the vas deferens. In case of ejaculates, semen collected from male during mating prior to ejaculation. Semen were centrifuged at 15,000 × *g* for 10 min at 15 °C and collected the supernatant and considered as seminal plasma. Then, the seminal plasma was kept at -80 °C until use. For cloacal gland secretion, cloacal foam from male quails was obtained by pressing the cloacal gland. The foam collected and centrifuged at 15,000 × *g* for 10 min at 25 °C. After centrifugation, the supernatant was collected and considered as cloacal gland secretion. Then, the cloacal gland secretion was kept at -80 °C until use.

Seminal plasma (SP) like medium

The ions concentration of SP was measured by SPS 7800 plasma spectrometer, Seiko Instruments Inc. The SP like medium was adjusted with the ionic composition and concentration of SP, HBSSS was considered as control.

Sperm counting and determination of viability

Sperm viability was assessed by using dual fluorescence stain (Live/Dead® Sperm Viability Kit, Molecular Probes, Thermo Fisher Scientific K. K., Yokohana, Japan), SYBR 14 and propidium iodide (PI). 100 nM SYBR 14 and 2.4 µM PI were added to sperm suspension. Sperm suspension was mixed properly and then incubated at 37 °C for 5 minutes. After 5 minutes incubation, 5 µl sperm suspension was placed on a micro glass slide and covered with micro cover glass. The micro glass slide was then placed under a fluorescence microscope with 10 × objective and 2× magnifications (BX 51, Olympus Optics). Images were captured with the help of Microscope imaging software with camera (Leica Microsystems CMS GmbH, Switzerland Ltd.) with the filters NIBA and WIG (NIBA: excitation 470 nm ~ 490 nm, emission 510 nm ~ 550 nm and WIG: excitation 520 nm ~ 550 nm, emission 580 nm ~). When sperm were incubated with these two stains, live sperm emits bright green fluorescence and dead sperm emits red fluorescence. Sperm images of green and red fluorescence were analyzed with image analysis system (ImageJ v. 1.440, http://imagej.nih.gov/ij). For each treatment, three replications were maintained throughout the experiment and images from five microscopic fields were taken for each replication. For each microscopic field, both green fluorescence and red fluorescence images were analyzed, counted and calculated the sperm viability percentage.

Data analysis

Percentage data were arcsine transformed before analysis, and significance was based on transformed means. Data were analyzed for significant differences with one-way analysis of variance (ANOVA). Differences between treatments were analyzed with Tukey's honestly significant difference test. Declaration of significance were based on $P<0.05$.

2.3Results

Sperm viability at various physiological states existing in seminal fluid of avian species

Sperm were incubated with the knowledge existing at physiological states of seminal fluid to investigate sperm viability *in vitro* condition (Fig. 2-1). To investigate the effects of temperature on sperm viability, sperm were incubated with various temperatures (4 °C, 15 °C, 21 °C and 41 °C) and viability was evaluated after 24 h incubation (Fig. 2-1a). Sperm viability was found significantly ($P<0.05$) high at 4 °C compare to 21 °C and 41 °C. There were no significant differences between 4 °C and 15 °C. Sperm at 41 °C showed significantly low viability compare to 4 °C, 15 °C and 21 °C. The pH of seminal plasma in Japanese quail was 8.14 (Table 2-1). To know the effects of pH on sperm viability, various pH (between 4 to 9) of the extender was prepared for sperm incubation. After 24 h of incubation, statistically significant relationship ($r=0.53$, $p=0.03$) was found between pH and sperm viability. The scattered graph (Fig. 2-1b) showed that sperm viability declined at physiological pH of the extender. It was observed higher sperm viability at a pH approximately 5.0. Sperm were incubated at various concentrations (2×10^7, 2×10^6 and 2×10^5 sperm/ml) *in vitro* and found that higher the sperm concentrations, higher the sperm viability (Fig. 2-1c). Sperm viability was significantly higher at sperm concentration of 2×10^7sperm/ml than 2×10^6 and 2×10^5 sperm/ml. Sperm viability at concentration of 2×10^5 sperm/ml was significantly low than higher sperm concentrations. Osmolarity of seminal plasma was 363.60 (Table 2-1). *In vitro* sperm viability at various osmolarity (250, 305, 350, 400 and 450 mOsm/kg) were investigated, sperm viability was similar in a wide range of osmotic conditions (Fig. 2-1d). From the above results, sperm viability was high at 4 °C, pH approximately 5.0, and sperm concentration of 2×10^7sperm/ml. Sperm viability remains similar with a wide range of osmotic conditions.

Effects of reproductive tract fluids on *in vitro* sperm vivability

Ejaculated semen, fluids from vas deferens and, cloacal gland secretion were allowed to prepare supernatant. Sperm were incubated with the seminal plasma (0, 1, 3 and 10 %) obtained from ejaculated semen or vas deferens. After 24 h incubation, sperm viability was assessed. Seminal plasma from ejaculated semen (Figure 2-2a) and vas deferens fluid (Fig. 2-2b) did not improve the sperm viability *in vitro* condition. Sperm were also incubated with cloacal gland secretion (0, 1, 3 and 10 %) for 24 h.   After 24 h sperm storage, cloacal gland secretion (Fig. 2-3c) did not improve sperm viability significantly.

Sperm viability with seminal plasma (SP) like medium

The ions concentration of seminal plasma was measured with plasma spectrometer (Table 2-2). Sperm were incubated with SP like medium or HBSS. After 24 h of sperm incubation, viability was assessed. The viability of sperm was not improved compare to HBSS at pH approximately 5 (Fig. 2-3a) or 7.4 (Fig. 2-3b).

2.4 Discussion

In vitro sperm viability studies have been carried out in many species as well as poultry, but we have limited knowledge on sperm viability in Japanese quail. Due to diversified nature of semen and the physiology of poultry spermatozoa, many factors can independently be correlated with sperm viability. In this study, the knowledge available on physiochemical conditions existing in seminal fluid were investigated with *in vitro* sperm viability study. In physiological condition, avian sperm survive at body temperature 41 °C (Beaupré et al., 1997), with pH of SP seems to be neutral to slight alkaline condition (Siudzińska and

Łukaszewicz, 2008; Ondho, 2014; Getachew, 2016). Although sperm survives at body temperature 41 °C in both male and female reproductive tract, but the viability was lost when sperm were incubated at 41 °C *in vitro* condition. The viability of sperm at physiological pH was also decreased *in vitro* condition. During *in vitro* sperm incubation, probably the sperm motility and metabolic activity increases at high temperature (Bonato et al., 2012) and high pH (Holm and Wishart, 1998). At low pH, sperm flagellar quiescence happens with low sperm motility, and this condition might be associated with high sperm viability (Matsuzaki et al., 2015). But how sperm survive at high body temperature and alkaline pH *in vivo* is still a mystery. The ejaculate volume and sperm concentration varies among avian species (García-Herreros, 2016). Generally, avian sperm are highly concentrated, low in volume and containing 6 (roosters) to 12 (toms) billion sperm/ml (Donoghue and Wishart, 2000). During *in vitro* storage condition, the percentage of viable sperm is lower in undiluted semen as compare to semen diluted with extender (Dumpala et al., 2006). So, the undiluted sperm needs to be diluted for *in vitro* storage. Though the sperm viability was high at high sperm concentration of 2×10^7 sperm/ml, it was much lower than sperm concentration present in ejaculated semen. Sperm viability at low sperm concentration of 2×10^5 sperm/ml was significantly low. Probably, most detrimental effect of sperm dilution is known as dilution effect. It is possible that excessive dilution may destabilize sperm membrane which is detrimental for sperm viability (Maxwell and Johnson, 1999). Osmotic condition is associated with the intactness of sperm plasma membrane. In avian species, sperm viability was investigated with wide range of osmolarity, it reveals that sperm survive in a wide range of osmolarity (Sexton & Fewlass, 1978).

As sperm are suspended and maintained in seminal fluid, supernatant was prepared from the fluid and applied to investigate sperm viability *in vitro* condition. Both seminal plasma and cloacal gland secretion did not improve sperm viability. Then, sperm were incubated

with SP like medium. The SP like medium also did not improve the sperm viability compare to control. Semen is a complex fluid composed of spermatozoa and seminal plasma (Solomon & Duncan, 2007). The roles of seminal plasma (SP) on *in vitro* sperm viability are well studied in mammals and insects. The studies related to *in vitro* sperm viability and motility in avian species are limited. Probably, the first proteomic analysis of SP was investigated in rooster semen (Marzoni et al., 2013). The role of seminal plasma in avian sperm viability, motility and fertilizing ability seems to be contradictory. Blesbois and de Reviers, (1992) reported that fowl SP contains the fraction of higher molecular weight (>50 kDa) favours sperm fertilizing ability, whereas, fractions of lower molecular weight (<1 kDa) are toxic to sperm *in vitro* condition. In turkey, whole SP reduces fertility (Douard et al., 2005), whereas dialyzed SP at 12-14 kDa is beneficial for *in vitro* sperm viability (Iaffaldano and Meluzzi, 2003). Sexton, (1977) reported that removal of seminal plasma by centrifugation had no significant effect on fertilizing capacity of chicken sperm at 5 °C for 24 h. Seminal plasma contains several components and it is possible that all components are not beneficial for sperm viability, motility and fertilizing ability *in vitro* condition. In this experiment, whole seminal plasma was used to investigate sperm viability and found no significant improvement of sperm viability. Identification of seminal plasma proteins and application of seminal plasma proteins on sperm viability and motility study may give better understanding regarding the role of seminal plasma proteins in avian species.

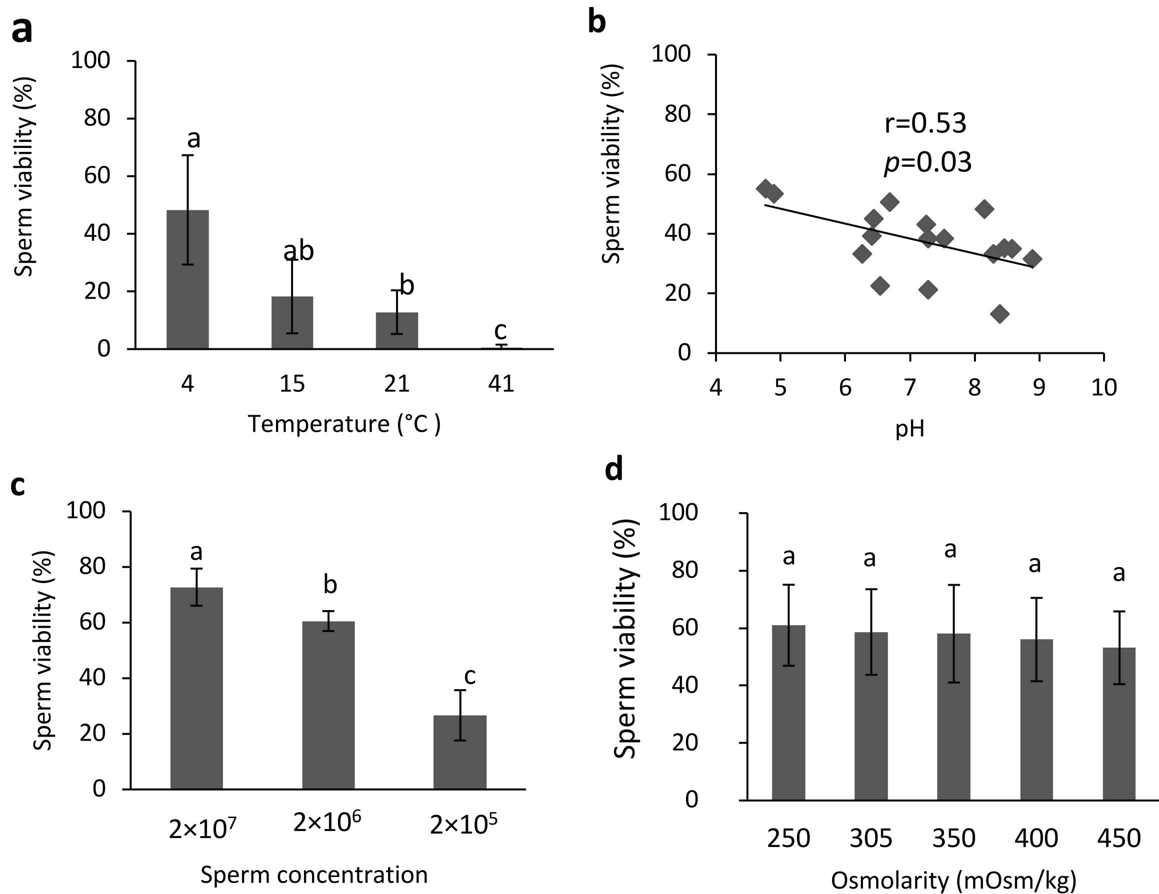

Figure 2-1. ***In vitro*** **sperm viability at various physiological states of Japanese quail. (a)** Effects of temperature on *in vitro* sperm viability. Sperm were suspended in HBSS at pH 7.4, 2×10^7 sperm/ml, and incubated at various temperatures. Sperm viability was assessed after 24 h of incubation. Data (n=3 independent experiments) are expressed as mean ± SD. Different superscript letters indicate significant differences at $p < 0.05$ among treatments. **(b)** Effects of pH on *in vitro* sperm viability. HBSS at various level of pH was applied for this experiment. Sperm were incubated at 4 °C with sperm concentration of 2×10^7 sperm/ml. Sperm viability was assessed after 24 h of incubation. Data (n=5 independent experiments) are expressed in this experiment. **(c)** Effects of sperm concentration on *in vitro* sperm viability. Various sperm concentrations were tested at pH approximately 5. Sperm were incubated at 4 °C and viability was assessed after 24 h of incubation. Data (n=5 independent experiments) are expressed as mean ± SD. Different superscript letters indicate significant

differences at $p < 0.05$ among treatments, and **(d)** Effects of osmotic conditions on *in vitro* sperm viability. Sperm were suspended in HBSS containing various osmolarity with pH approximately 5 and 2×10^7 sperm/ml. Sperm were incubated at 4 °C and viability was assessed after 24 h of incubation. Data (n=3 independent experiments) are expressed as mean ± SD.

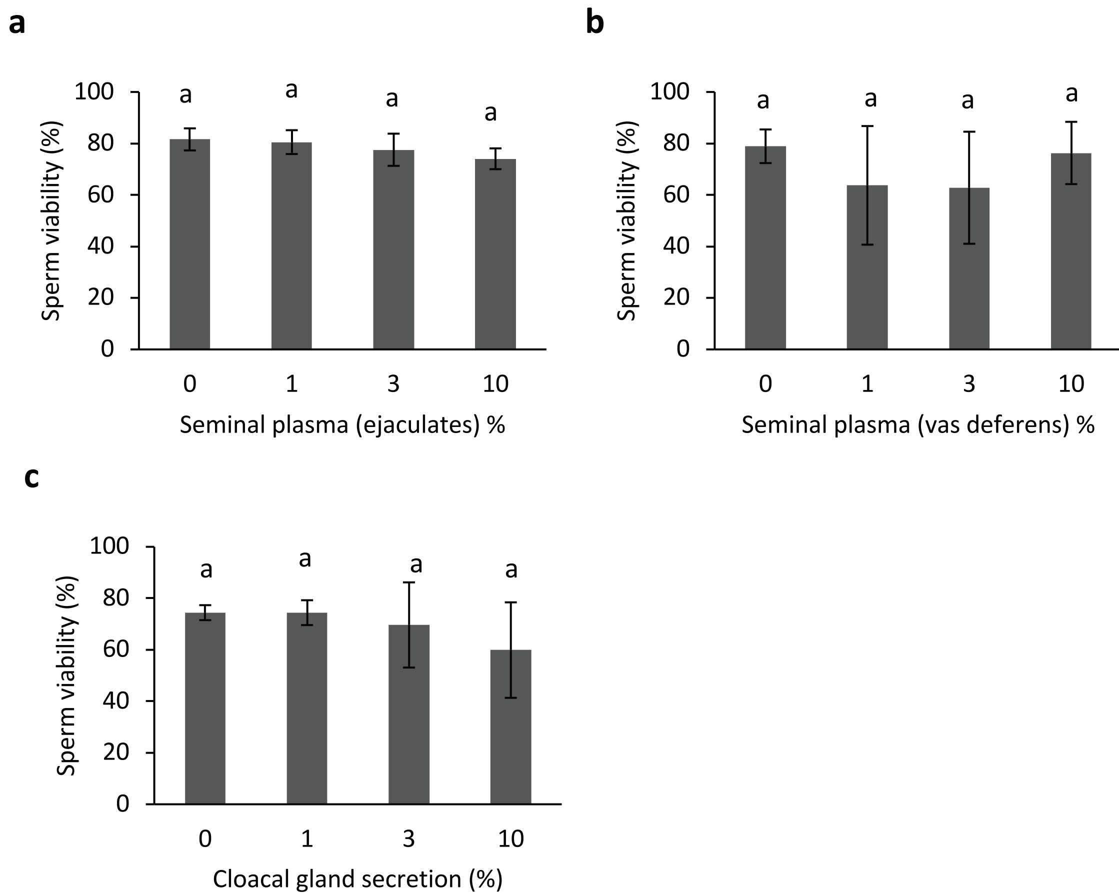

Figure 2-2. **Effects of fluids colleceted from reproductive tract on sperm viability.** Supernatant from ejaculated semen, supernatant from vas deferens fluid, and cloacal gland secretion were added with HBSS at pH approximately 5 and 2×10^7 sperm /ml. Sperm were incubated at 4°C and viability was assessed after 24 h of incubation. Data (n=3 independent experiments) are expressed as mean ± SD. Different superscript letters indicate significant differences at $p < 0.05$ among treatments. Seminal plasma prepared from ejaculated semen was applied to sperm suspension **(a)**, seminal plasma prepared from fluid of vas deferens was applied to sperm suspension **(b)**, and cloacal gland secretion prepared from cloacal foam was applied to sperm suspension **(c)**.

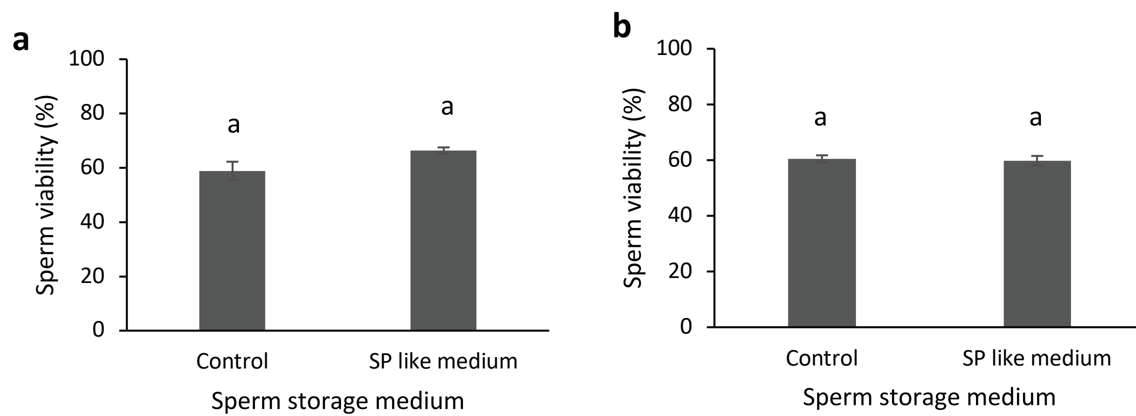

Figure 2-3. **Sperm viability with seminal plasma (SP) like medium.** SP like medium indicates the combination of ionic concentration present in seminal plasma of Japanese quail, whereas control indicates HBSS alone. Sperm were incubated at 4°C with 2×10^7 sperm /ml. Sperm viability were assessed after 24 h of incubation. Data (n=3 independent experiments) are expressed as mean ± SD. pH of sperm extender at approximately 5 **(a)**, and pH of sperm extender at 7.4 **(b)**. Different superscript letters indicate significant differences at p <0.05 among treatments.

Table 2-1: Osmolarity and pH of seminal plasma in Japanese quail

Sample #	Osmolarity (mOsm/kg)	pH
1	406	8.25
2	337	8.09
3	351	8.11
4	335	8.07
5	389	8.20
Average	363.60	8.14

Table 2-2: Ions concentration of seminal plasma in Japanese quail

Contents	Atomic weight	Measured (ppm)	Dilution factor	Conc. (ppm)	Conc. (%)	Conc. (g/l)	Conc. (mM)
Na	22.99	0.448	5000	2240	0.224	2.24	97.43
K	39.10	0.434	5000	2170	0.217	2.17	55.50
Ca	40.08	0.413	500	206.5	0.02065	0.2065	5.15
Mg	24.31	0.264	500	132	0.0132	0.132	5.43

Conc. means concentration. Ionic concentrations were measured with SPS 7800 plasma spectrometer, Seiko Instruments Inc.

Chapter III: Effects of albumin and transferrin on sperm viability

3.1 Introduction

Sperm are produced in testis, maintains in male reproductive tract and passes outside of the body during ejaculation. During sperm production and transportation process, a complex fluid known as seminal plasma (SP) is added with sperm from the male reproductive tract and accessory sex glands (Duncan and Thompson, 2007). Unlike mammals, avian species lack prostate, seminal and bulbourethral glands, and some avian species add lymph-like fluid and/or foam at the time of ejaculation (Fujihara, 1992). In avian species, thus, the fluid adds with the sperm mainly comes from the testis, epididymis and cloacal regions. In male Japanese quail, the processes of sperm production, maturation and transportation are comparatively quicker (Clulow and Jones, 1982), and male produces large quantities of cloacal foam during ejaculation (Seiwert and Adkins-Regan, 1998).

Seminal plasma (SP) is a complex fluid, serves as a nutrient media for sperm and some components of SP play important roles in sperm maintenance, viability and transportation into male and female reproductive tract (Poiani, 2006). In mammals, SP helps in sperm capacitation (Manjunath and Thérien, 2002), maternal immunity and pregnancy (THALER, 1989), *in vitro* fertilization success (Crawford et al., 2015), and acts as fertility marker (Killian, Chapman, & Rogowski, 1993; Moura, Chapman, Koc, & Killian, 2006; Viana et al., 2018, and Koppers, Reddy, & O'Bryan, 2011). In insects, the role of SP proteins in fertilization process have also been studied well (Bloch Qazi & Wolfner, 2003; Lung, Kuo, & Wolfner, 2001; Holman, 2009, and Bertram, Neubaum, & Wolfner, 1996).

The roles of SP proteins have been widely investigated in mammals and insects; little attention was paid in the avian species. Probably, the first proteomic analysis of SP was investigated through two-dimensional gel electrophoresis (2-DE) and mass spectrometry

MS/MS in rooster (Marzoni et al., 2013). The authors identified 17 spots in seminal plasma of ejaculated semen, and among the spot's serum albumin and ovotransferrin were stained more intensely. The presence and roles of these seminal proteins in male reproductive tract were not studied deeply. Previous research conducted from our laboratory revealed the presence of albumin and transferrin in the female reproductive tract of Japanese quail and their probable role in sperm longevity in female reproductive tract (Matsuzaki et al., 2019). To my knowledge, this is the first study in male Japanese quail to investigate the presence and localization of albumin (ALB) and transferrin (TF) in male reproductive tract. In this study, I tried to investigate the presence and localization of ALB and TF in ejaculates, cloacal foam and male reproductive tract. The effects of ALB and TF on sperm viability was also studied in this study.

3.2 Materials and methods

Animal care and management

Male Japanese quails (*Coturnix japonica*), 40-50 weeks of age (Quail cosmos, Toyohashi, Japan) were reared in individual cage and maintained a photoperiod of 14 h light and 10 h darkness (lights went on at 05:00). Birds were allowed to *ad libitum* commercial feed (Motoki Corporation) and safe drinking water. Animals were decapitated and opened the body for the collection of reproductive organs. Animal management and all experimental procedures were carried out in accordance with approved guidelines of the Animal Care Committees of Shizuoka University.

Preparation of seminal plasma and cloacal gland secretion

For the preparation of supernatant from ejaculates, mature male quails were used to collect ejaculated semen during mating prior to ejaculation (Kuroki and Mori, 1997). The ejaculates

were allowed to centrifuge at 50×100 *g* for 3 min at 4 °C and collected the supernatant. The supernatant was again centrifuged at 100×100 *g* for 3 min at 4 °C and collected the supernatant. For the preparation of supernatant from cloacal foam, cloacal foam was obtained by pressing the cloacal gland. The foam collected and centrifuged at 204×100 *g* for 5 min at 21 °C and collected the supernatant. The supernatant was again centrifuged at 100×100 *g* for 3 min at 4 °C and collected the supernatant. For collection of fluid from the lumen of male reproductive tract, abdomen was opened and collected fluid from inner tract of testis and vas deferens. Briefly, testis was cut down and collected the fluid from inside the testis by micropipette. For collection of fluid from lumen of vas deferens, it was divided into three equal parts (upper - adjacent to testis, lower - adjacent to cloaca, and middle - between upper and lower) and collected the fluid by hand squeezing to the vas deferens. The fluid obtained from testis and vas deferens was allowed to centrifuge at 150×100 *g* for 10 min at 4 °C and collected the supernatant. The supernatant was again centrifuged at 100×100 *g* for 3 min at 4 °C and collected the supernatant. The protein concentration for all sorts of supernatant was determined by Bradford assay (Quick start™ Bradford 1× Dye Reagent, Bio-Rad Laboratories, Inc., USA) by making a calibration curve with known concentration of a standard protein (bovine serum albumin).

Western blot analysis

Seminal plasma and cloacal gland secretion were diluted with PBS and added at 1 μg/lane for SDS-PAGE. Polyacrylamide gel of 10 and 5% (w/v) were used for resolving and stacking, respectively. The gel proteins were transferred onto a polyvinylidene fluid (PVDF) membranes through electrophoretic transfer. The PVDF membrane incubated for 1 hour with a blocking solution containing 5% (w/v) skim milk powder in Tris-buffered saline with 0.1% Tween 20. After blocking, the membrane was allowed to react with anti-ALB

(1:10,000) or anti-TF (1:10,000) for 1 hour as primary antibody. The primary anti-ALB and anti-TF used in this study was raised and described previously (Matsuzaki et al., 2019). After washing the primary antibody with TBS-T, the membrane was allowed to react with a second antibody HRP-conjugated anti-rabbit IgG (1:10,000) for 30 minutes. After washing with TBS-T, the membrane blot was visualized with a chemiluminescence technique using ImageQuant LAS 500 (GE Healthcare).

Immunohistochemistry

The immunohistochemical technique was performed in accordance with the procedure explained previously (Sasanami et al., 2002). The right testis, epididymis and vas deferens (upper, middle and lower) were fixed in Bouin solution (picric acid, formaldehyde solution and acetic acid, 15:5:1) for 24 h. To penetrate fixative solutions into testis, microwaving at 600 W (30 times, 10 sec each) was applied on ice condition. Bouin solution was replaced gradually by 70, 80, 90, 95, and finally 100% ethanol. The sections were embedded in paraffin, cut into 6 µm each and dried on hot plate at 40 ºC. After dewaxing and rehydration, the sections were incubated with 1% H_2O_2 for 20 min to inactivate endogenous peroxidase. After washing with PBS, the sections were blocked with PBS containing 1% BSA and 1% normal goat serum for 1 h. After blocking, the sections were allowed to react with anti-ALB or anti-TF or rabbit serum (RS) at 1:250 for 2h and 30 min. After washing with PBS, the sections were allowed to react with a second antibody HRP-conjugated anti-rabbit IgG (1:1000) for 1 h. After washing with PBS, 3,3´-Diaminobenzidine (DAB) peroxidase substrate solution (0.02% DAB, 0.016% H_2O_2 and 0.5 M Tris-HCl at pH 6.8) was used for the staining of protein. After staining, the sections were immediately washed in PBS. A drop of glycerol was placed on the slide and the slide was covered with a cover glass. Finally, the

sections were examined, and images were captured under a light microscope (BX51; Olympus Optics, Tokyo, Japan).

Determination of sperm viability

Sperm viability was assessed by using dual fluorescence stain (Live/Dead® Sperm Viability Kit, Molecular Probes, Thermo Fisher Scientific K. K., Yokohana, Japan), SYBR 14 and propidium iodide (PI). 100 nM SYBR 14 and 2.4 µM PI were added to sperm suspension. Sperm suspension was mixed properly and then incubated at 37 °C for 5 minutes. After 5 minutes incubation, 5 µl sperm suspension was placed on a micro glass slide and covered with micro cover glass. The micro glass slide was then placed under a fluorescence microscope with 10 × objective and 2× magnifications (BX 51, Olympus Optics). Images were captured with the help of Microscope imaging software with camera (Leica Microsystems CMS GmbH, Switzerland Ltd.). When sperm were incubated with these two stains, live sperm emits bright green fluorescence and dead sperm emits red fluorescence. Sperm images of green and red fluorescence were analyzed with image analysis system (ImageJ v. 1.440, http://imagej.nih.gov/ij). For each treatment, three replications were maintained throughout the experiment and images from five microscopic fields were taken for each replication. For each microscopic field, both green fluorescence and red fluorescence images were analyzed, counted and calculated the sperm viability percentage.

Data analysis

Percentage data were arcsine transformed before analysis, and significance was based on transformed mean. Data were analyzed for significant differences with one-way analysis of variance (ANOVA). Differences between treatments were analyzed with Tukey's Honestly Significant Difference test. Declaration of significance were based on $P<0.05$.

3.3 Results

Presence of albumin and transferrin in ejaculates, cloacal gland secretion and male reproductive tract

At first, seminal plasma prepared from ejaculated semen was investigated to know the presence of albumin (ALB) and transferrin (TF) in ejaculates. Seminal plasma was allowed to react with anti-ALB or anti-TF through western blot analysis. Anti-ALB and anti-TF revealed immunoreactive band at around 66, and 75 kDa, respectively. The western blot analysis results revealed the presence of ALB and TF in the seminal plasma of ejaculates (Fig. 3-1a). Then, I investigated cloacal gland secretion to know the presence of ALB and TF in cloacal foam, as cloacal foam added with semen during ejaculation. The western blot analysis also revealed the presence of ALB and TF in cloacal gland secretion (Fig. 3-1b). Then, I felt interest to know the presence of ALB and TF in the male reproductive tract. To investigate the presence of ALB and TF in male reproductive tract, supernatant from the reproductive tract (testis, and vas deferens) was allowed to react with anti-ALB or anti-TF through western blot analysis. ALB and TF were present in the supernatant of testis as well as upper, middle and lower vas deferens (Fig. 3-1c). Conclusively, ALB and TF were present in ejaculates, cloacal foam, and male reproductive tract of Japanese quail.

Localization of albumin and transferrin in the male reproductive tract

To know the localization and distribution of ALB and TF in male reproductive tract, immunohistochemical analysis on the section of testis, epididymis and vas deferens was performed with anti-ALB (Fig. 3-2b column) or anti-TF (Fig. 3-2c column). The expression of immunoreactive ALB and TF was localized in testis, epididymis and vas deferens (upper, middle and lower region). No intense signal was found in the sections of testis, epididymis

and vas deferens (upper, middle and lower) when the sections were incubated with rabbit serum (Fig. 3-2a column). Conclusively, both ALB and TF were localized in the male reproductive tract of Japanese quail.

Effects of ALB and TF on sperm viability

Sperm were incubated with 10% ALB or 10% TF for 24 h. After 24 h of incubation, significantly high sperm viability was observed between control and ALB or TF. There was no significant difference between sperm viability with ALB and TF. ALB and TF improved the sperm viability significantly *in vitro* condition.

3.4 Discussion

In avian species, most of the studies conducted so far were to investigate the effects of whole SP (Douard et al., 2005) and fraction of SP (Ashizawa and Okauchi, 1984, and Blesbois and de Reviers, 1992). Identification of specific SP proteins and their roles in sperm maintenance have been investigated limitedly. In this study, the presence of ALB and TF were identified in ejaculates, cloacal foam, and male reproductive tract of Japanese quail. I was unable to know the status of ALB and TF in the epididymis, as I could not separate supernatant from epididymis due to low volume of fluid collected from epididymis. To my knowledge, this is the first study where I have investigated the presence of ALB and TF in testis and vas deferens of male Japanese quail. Usually, ALB and TF are known as blood plasma protein, that are produced in liver. Like other species, ALB is the major component of serum in quail (HALEY, 1965). The roles of serum ALB and TF have been investigated in many species, but the roles of seminal plasma ALB and TF have been studied limitedly. The serum ALB and TF are known to maintain blood volume through oncotic pressure (Farrugia, 2010) and free iron (Yang et al., 1984), respectively. Immunohistochemical analysis reveals a scattered

expression of ALB and TF in testis, epididymis and vas deferens (upper, middle and lower). ALB and TF were localized in seminiferous epithelium of testis, and they were produced by primordial germ cells and Sertoli cells in rats (TOEBOSCH et al., 1987, and Gelly et al., 1994). In female quail, ALB and TF were localized in the sperm storage tubules (Matsuzaki et al., 2019). I was unable to separate ALB and TF from the seminal plasma due to low volume of semen. The ALB and TF separated from the UVJ mucosa and their effects were investigated during *in vitro* sperm incubation. ALB and TF improved sperm viability significantly compare to the control.

In avian species, studies on specific SP proteins related to sperm viability are limited. In turkey, bovine serum albumin increases the sperm motility as compare to diluent alone (Bakst and Cecil, 1992). The serum ALB and TF have been classified as a defense or immunity protein on the basis of their functions (Marzoni et al., 2013). In female Japanese quail, ALB and TF were present in the SSTs, and they play important role for maintaining sperm longevity (Matsuzaki et al., 2019). It is possible to improve sperm viability as ALB has antioxidant properties (Roche et al., 2008) and TF has anti-microbial properties (Valenti et al., 1983). Due to antioxidant properties of ALB and TF, the production of reactive oxygen species may be low, which may reduce the sperm damage and improve sperm viability. Recently, the over-expression of serum ALB and TF was observed in modern egg-laying chicken but their association with sperm viability was not evaluated (Atikuzzaman et al., 2017). As, ALB and TF are present in ejaculates, cloacal foam and male reproductive tract of Japanese quail, it is possible that they play important roles for sperm maintenance in the male reproductive tract as well as sperm viability *in vitro* condition.

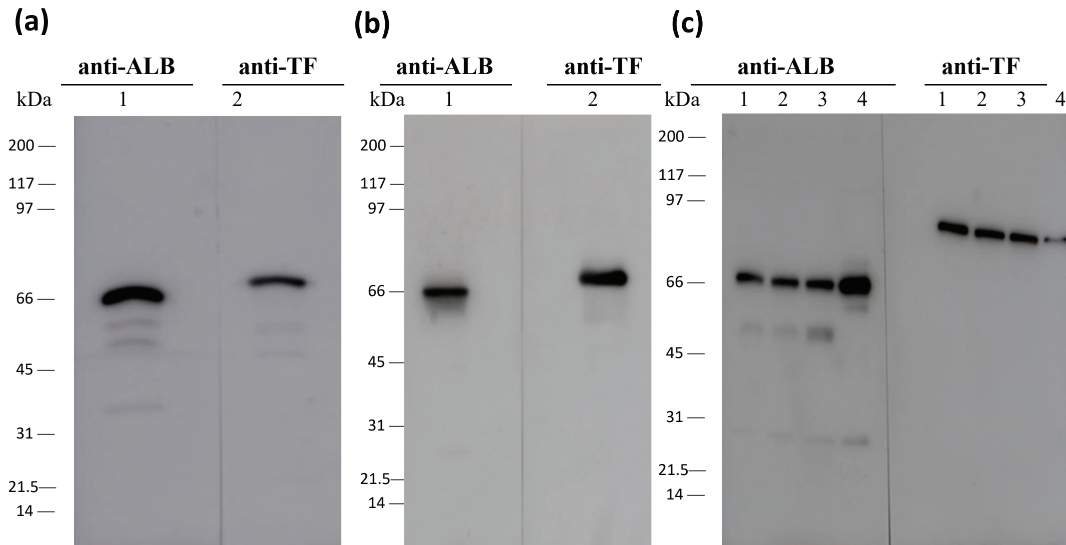

Figure 3-1. **Presence of albumin and transferrin in ejaculates, cloacal foam and male reproductive tract. (a)** The supernatant prepared from ejaculated semen (1 μg/lane) was separated on SDS-PAGE, transblotted onto PVDF membrane, and detected with anti-ALB (left lane 1) and anti-TF (right lane 2) through western blot. **(b)** The supernatant prepared from the cloacal gland foam (1 μg/lane) was separated on SDS-PAGE, transblotted onto PVDF membrane, and detected with anti-ALB (left lane 1) and anti-TF (right lane 2) through western blot. **(c)** The supernatant prepared from fluid present in vas deferens and testis (1μg/lane) was separated on SDS-PAGE, transblotted onto PVDF membrane, and detected with anti-ALB (left lane 1) and anti-TF (right lane 2) through western blot. The blot of 1, 2, 3 and 4 indicates upper vas deferens, middle vas deferens, lower vas deferens and testis, respectively.

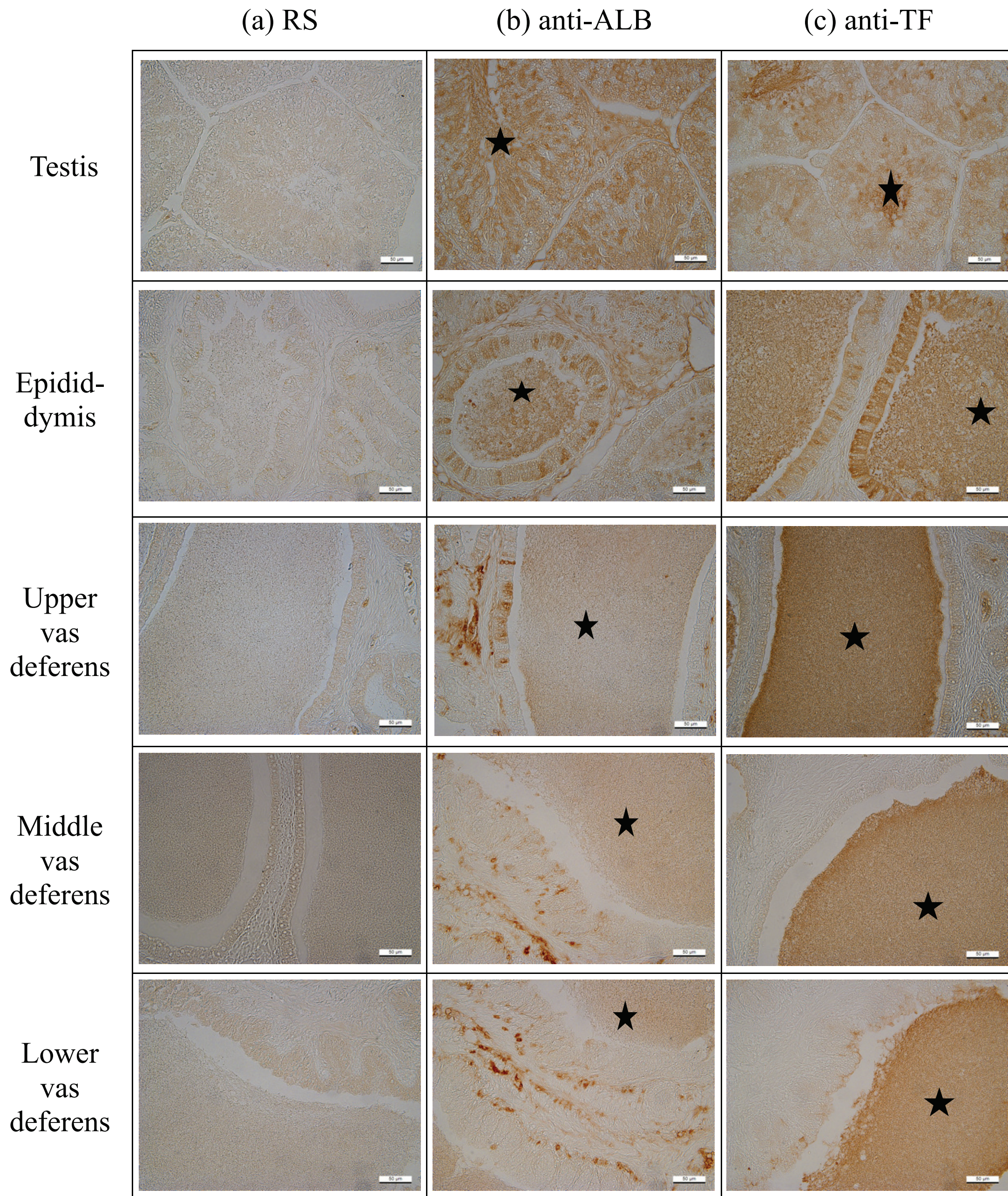

Figure 3-2. **Localization of albumin and transferrin in the male reproductive tract of Japanese quail.** Sections of testis, epididymis and vas deferens (upper, middle and lower) were processed for IHC staining. Scale bar = 50 μm. Column a, b and c represents the sections that were reacted with rabbit serum (RS), anti-ALB and anti-TF, respectively. The symbol 5-point star (★) indicates the positive staining of the sections. The results shown here represent a representative result of repeated experiments.

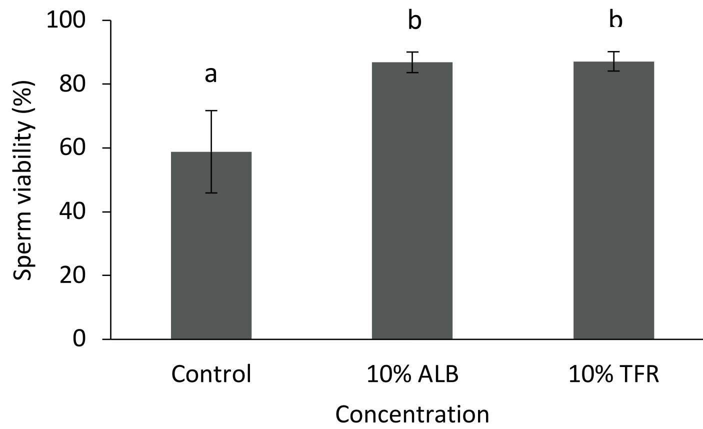

Figure 3-3. **Effects of albumin and transferrin on sperm viability.** Sperm were suspended with HBSS maintaining pH 7.4 and sperm concentration at 2×10^7 sperm/ml. Sperm were incubated with or without albumin or transferrin at 4°C for 24 h. After 24 h of incubation, sperm viability was assessed using a LIVE/DEAD viability kit. Data (n=3 independent experiments) are expressed as mean ± SD. Different superscript letters indicate significant differences at $p < 0.05$ among treatments.

Chapter IV: Effects of anserine and carnosine on sperm motility

4.1 Introduction

In animals, sperm motility is an important trait for successful fertilization (Florman and Ducibella, 2006). In birds, ejaculated sperm are stored in sperm storage tubules (SSTs) of female reproductive tract before fertilization (Sasanami *et al.*, 2013), and motility is considered to be an important factor responsible for sperm uptake into the SSTs (Froman, 2003). Actually, avian sperm encounter an intense selection process in the female reproductive tract, and immotile sperm are not capable of fertilizing the eggs (Allen and Grigg, 1957).

The modern poultry industry is promised to provide superior quality meat and eggs. To propagate next generation, artificial insemination (AI) is now being widely used in breeder farms (Łukaszewicz, 2010). Although cryopreservation has long been recognized for the preservation of germplasm in mammals including livestock and laboratory animals, this technique is not reliable due to low fertilizing ability of frozen/thawed sperm in birds (Long, 2006; Long *et al.*, 2014). It is reported that cryopreservation damages sperm plasma membrane (Bakst and Sexton, 1979), and decreases sperm morphological integrity (Blesbois *et al.*, 2005). To minimize fertility loss, liquid storage of sperm may be practiced in birds, but the motility of ejaculated sperm decreases upon time at liquid storage *in vitro* (Kotłowska et al., 2007).

Seminal plasma (SP), a complex fluid is added to sperm in the male reproductive tract (Duncan and Thompson, 2007). The SP serves as a nutrient rich medium to sperm (Juyena and Stelletta, 2012) and plays important roles in sperm maintenance, viability and transportation of sperm into female reproductive tract (Poiani, 2006). The role of SP

proteins, peptides and amino acids has been widely investigated in mammals (Manjunath *et al.*, 2007; Koppers *et al.*, 2011; Crawford *et al.*, 2015; Viana *et al.*, 2018). Of these, several studies emphasized the importance of imidazole dipeptides such as anserine and carnosine on influencing sperm motility and fertilizing ability in various species (Siems *et al.*, 2003; Tareq *et al.*, 2008; Bosler *et al.*, 2014). Carnosine is naturally occurring dipeptide consisting with β-alanine and histidine, and anserine is a methylated form of carnosine (Boldyrev *et al.*, 2013). Because both anserine and carnosine are histidine-containing dipeptides, it is thought that they play similar physiological roles (Boldyrev *et al.*, 2013) such as buffering capacity at neutral pH (Tanokura, 1983; Sale *et al.*, 2010). Actually, both imidazole dipeptides have been reported to maintain muscle buffering capacity and muscle functions (Begum *et al.*, 2005). In domestic birds such as chicken, it is also reported the presence of imidazole dipeptides in skeletal muscle, and that their role for anti-oxidant capacities (Sato *et al.*, 2008), however, as far as we know, there are no report describing the presence of these dipeptides in male reproductive tract in birds. Therefore, we felt interest to investigate their presence in the male reproductive tract. Here, we report the presence of imidazole dipeptides anserine and carnosine in the male reproductive tract of Japanese quail and also show the evidence that these dipeptides improved the sperm motility after *in vitro* storage in the liquid state.

4.2Materials and methods

Animal care and management

Male Japanese quails (*Coturnix japonica*), 40-60 weeks of age (Quail cosmos, Toyohashi, Japan) were maintained in individual cage under the photoperiod of 14 h light and 10 h darkness (lights went on at 05:00). Birds were allowed to eat commercial feed (Toyohashi Feed Mills, Toyohashi, Japan) and drinking water *ad libitum*. Animal management and all

experimental procedures were carried out in accordance with approved guidelines of the Animal Care Committees of Shizuoka University (Approval number: 2018A-5).

Preparation of testicular fluid, seminal plasma, cloacal gland secretion and blood serum

To collect testicular fluid, male quail was decapitated and opened the body for collection of testis. Briefly, testis was cut down and collected the fluid from inside the testis by micropipette. The fluid was centrifuged at 15,000 × *g* for 10 min at 4 °C and collected the supernatant as testicular fluid. For the preparation of SP, ejaculates from mature male quails were centrifuged at 5,000×*g* for 3 min at 4 °C and collected the supernatant. For the preparation of cloacal gland secretion, cloacal foam was obtained by pressing the cloacal gland. The foam was centrifuged at 20,400×*g* for 5 min at 21 °C. Blood was collected from wing vein and was centrifuged at 2,000×*g* for 20 min at 4 °C for the collection of blood serum.

Semen collection and processing

Hanks' balanced salt solution (HBSS) containing 136 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.26 mM $CaCl_2$, 4.2 mM $NaHCO_3$ and 5.6 mM glucose was used as sperm diluent. HBSS was incubated at 37 °C for 10 minutes before semen collection. Semen was obtained from mature male quail during mating prior to ejaculation in accordance with the procedure of Kuroki and Mori (1997). The semen was suspended in 500 µl HBSS. Sperm concentrations were measured with a hemocytometer under the microscope (BX 51, Olympus Optics, Tokyo, Japan), and prepared sperm concentrations of 1×10^8 sperm/ml. 100 µl of HBSS containing various concentration of anserine (L-Anserine Nitrate, Wako Pure Chemical Corporation, Japan) or carnosine (L-Carnosine, Wako Pure Chemical

Corporation) were prepared and added sperm suspension to get final concentration of 2×10^7 sperm/ml. Sperm were incubated at 15 °C up to 12 h.

Sperm motility test by computer assisted semen analysis (CASA)

Sperm suspension were taken from the droplets at 1 h, 3 h, 6 h, 9 h and 12 h of incubation. Sperm motility was analyzed by CASA system (SMAS, Ver. 3, DITECT Digital Image Technology, Japan). Glass slides were coated by 2% BSA and warmed on a hot plate at 39 °C. Sperm suspension was placed on warmed glass slide and covered with cover glass. Then, the glass slide was placed under microscope (Nikon ECLIPSE E200 , Japan). The following sperm motility parameters such as straight line velocity (VSL µm/s), curvilinear velocity (VCL µm/s), average path velocity (VAP µm/s), linearity (LIN) (LIN=VSL/VCL), straightness (STR) (STR=VSL/VAP), wobble (WOB) (WOB=VAP/VCL), amplitude of lateral head displacement (ALH µm), beat-cross frequency (BCF Hz) and motile rate (%) were recorded.

Detection of anserine and carnosine in the male reproductive tract

The testicular fluid, SP, cloacal gland secretion and blood serum were diluted 2 times with water and mixed with 3% sulfosalicylic acid at ratio of 1:1. Then, the mixture was centrifuged at 20,400×*g* for 10 min at 4 °C. The supernatant was analyzed with amino acid analyzer (JCL-500/V2; JEOL, Tokyo, Japan) according to the manufacturer instructions.

Data analysis

Percentage data were arcsine transformed before analysis, and significance was based on transformed means. Data were analyzed for significant differences with one-way analysis of

variance (ANOVA). Differences between treatments were analyzed with Tukey's Honestly Significant Difference test. Declaration of significance were based on *P*<0.05.

4.3 Results

Presence of anserine and carnosine in male reproductive tract

The presence of anserine, carnosine and their constituent β-alanine, 1-methylhistidine and histidine were investigated in male reproductive tract of Japanese quail (Table 4-1). In testicular fluid, we detected anserine, but carnosine was under detection limit. On the other hand, seminal plasma solely contained carnosine. The constituent histidine, β-alanine and 1-methylhistidine was present in both samples. In case of cloacal gland secretion, neither anserine nor carnosine was detected. Blood serum contains both imidazole dipeptides as well as the their constituents except for β-alanine.

Effects of anserine and carnosine on sperm motility

In the next experiments, we incubated the sperm with various concentration of anserine (0, 0.1, 0.3, 1 or 3 μM) or carnosine (0, 0.1, 0.3, 1 or 3 μM) (Table 4-2). Sperm motility did not show significant differences at concentration of 0.1 μM anserine or carnosine compare to control, but significant increase of sperm motility parameters was found at concentrations of 0.3, 1 or 3 μM of anserine or carnosine. The most effective dose of anserine and carnosine for sperm motility improvement was 1 μM and 0.3 μM, respectively. Then, sperm were treated with anserine (1 μM) or carnosine (0.3 μM) for various incubation time. A decreasing trend in sperm motility was observed upon sperm storage time in all treatments and there were no significant change in sperm motility up to 3 h of incubation. However, a significant improvement in sperm motility parameters was observed from 6 h of sperm incubation when

compared with control (Fig. 4-1A). The beneficial effects of anserine or carnosine on sperm motility were continued after 9 h of incubation. After 12 h of incubation, carnosine still improved the sperm motility parameters.

4.4 Discussion

In this study, I found that imidazole dipeptides, anserine and carnosine exist in the male reproductive tract in Japanese quail. Although the level of anserine and carnosine in male reproductive tract is far lower than that reported in the skeletal muscle in human (Mannion *et al*., 1992) and in chicken (Crush, 1970), this is the first report demonstrating the presence of imidazole dipeptides in the reproductive system of birds.

In my results on the analysis of the biological fluids derived from male reproductive tract, we found that testicular fluid contains anserine, but it does not contain carnosine. It is reported in chicken that carnosine is produced from β-alanine and histidine by carnosine synthase, and is further converted to anserine by the action of histamine N-methyltransferase-like protein (Boldyrev *et al.*, 2013). In addition, it is also known that carnosine is sensitive to carnosinase such as CNDP1 and 2, which is responsible for the degradation of carnosine to β-alanine and histidine. From these views, it is assumed that testicular anserine thought to be the product due to the methylation of carnosine by specific methylase (Boldyrev *et al.*, 2013), and remaining carnosine may be degraded by the action of CNDPs or by similar enzymes. To ascertain this, further experiments to confirm the expression and localization of these enzymes in the testis should be performed. If the testicular fluid is transported to epididymis and vas deferens as sperm pass through the male reproductive tract, anserine should be also present in the SP; however, in our results, SP solely contain carnosine (Table 4-1). The presence of carnosine in SP indicates the possibility that it is not derived from testis but is newly synthesized and added in the later

part of testis in male reproductive tract and that the enzymes responsible for either methylation and degradation of carnosine are absent. Similar situation that dominant localization of carnosine was reported in the muscle of horse and man (Harris et al., 1990), though exact mechanisms of how carnosine stabilized in the muscle are not known. Another possibility is that the demethylation of methylhistidine in later part of testis in reproductive tract occurs, and testicular anserine is converted to carnosine again during the passage through the male reproductive tract. Although the enzyme responsible for de-methylation of anserine is not demonstrated in any animals so far, it is interesting to explore novel demethylase in the male reproductive tract in birds.

In this study, carnosine and its derivatives anserine improved sperm motility after *in vitro* sperm storage at concentration of 0.3 μM and 1 μM, respectively. In physiological condition, avian species may uplift the production of antioxidant enzymes to protect the sperm (Mavi *et al.*, 2020), but the antioxidant defense mechanism may be disturbed *in vitro* due to oxidative stress (Sikka, 1996). Avian sperm contains high amount of polyunsaturated fatty acids (Surai *et al.*, 1998). The sperm integrity become susceptible with increased reactive oxygen species and lipid peroxidation (Khan, 2011). Several studies revealed the antioxidant properties of anserine and carnosine. The antioxidant activity of histidine and its derivatives is due to their imidazole moiety which decreases the rate of oxidation (Kohen *et al.*, 1988). The histidine containing compound is related with antioxidant ability, and the peptide linkage between β-alanine, histidine and 1-methylhistidine is associated with antioxidant properties of carnosine and anserine (Wu *et al.*, 2003). The antioxidant activity of carnosine is facilitated by chelation of metal ions, scavenging of reactive oxygen species and peroxyl radicals (Kohen *et al.*, 1988; Boldyrev *et al.*, 2013). The effects of carnosine on sperm motility have been investigated in mammals. For instance, carnosine improves the VSL of sperm in ram (Tan and Han, 1995), prevents oxidative stress in testis of rats (Aydın *et al.*,

2018), and improves sperm mitochondrial activity in human (Adami *et al.*, 2020). Carnosine acts as malondialdehyde scavenger, lower malondialdehyde concentration was observed with higher carnosine content in stallion semen (Rocha *et al.*, 2018). From above mentioned studies, we think therefore that the addition of anserine or carnosine may improve sperm motility due to the reduction of oxidative stress during sperm storage.

To conclude, this study indicate that the imidazole dipeptides exist in male reproductive tract and may improve sperm quality during *in vitro* sperm storage in the liquid states. Although I did not examine sperm membrane integrity of stored sperm, imidazole dipeptides could be one of the good candidates as the additive used for *in vitro* sperm storage to minimize the damage of sperm membrane.

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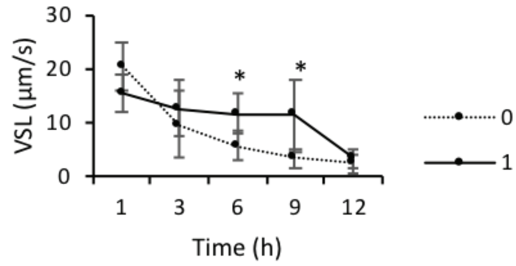

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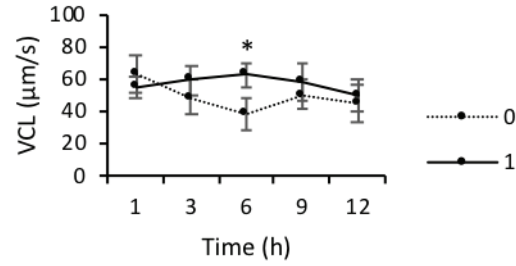

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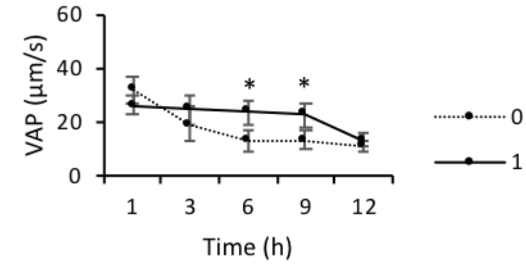

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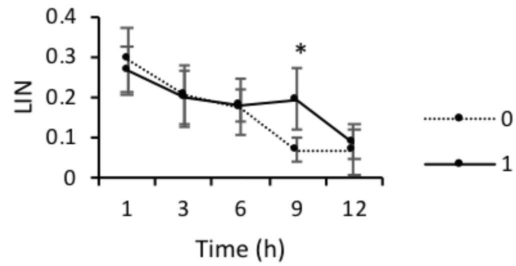

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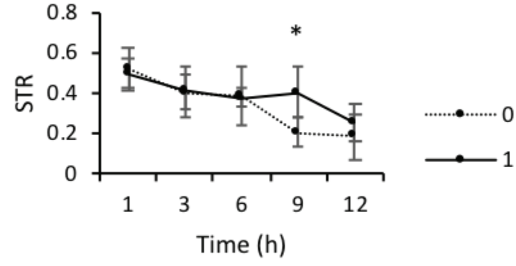

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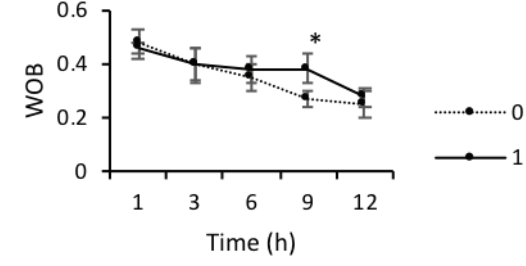

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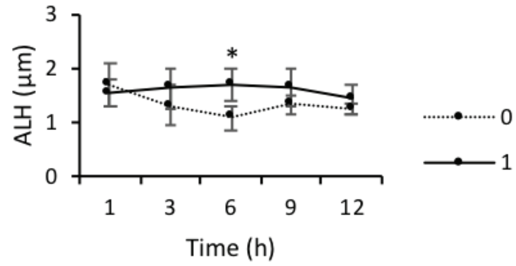

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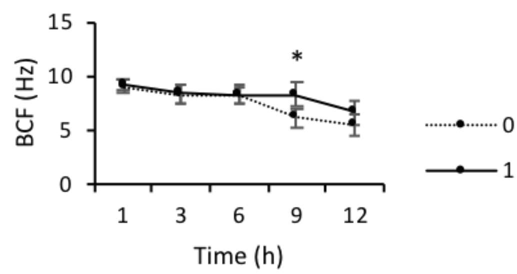

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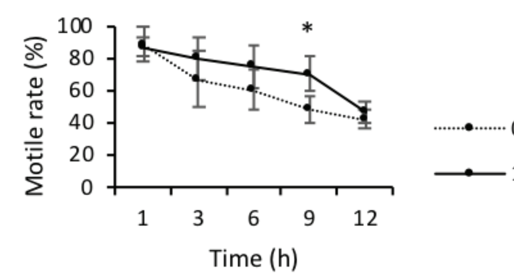

4-2

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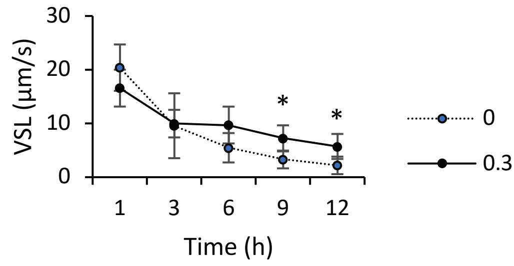

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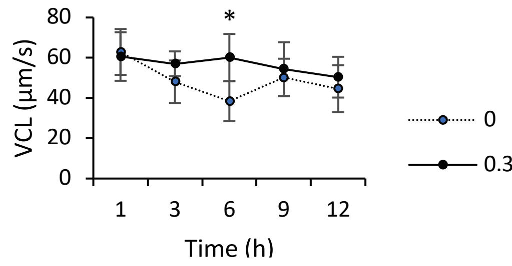

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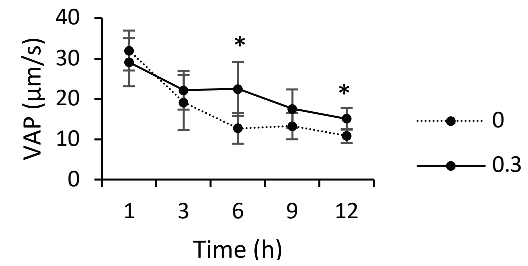

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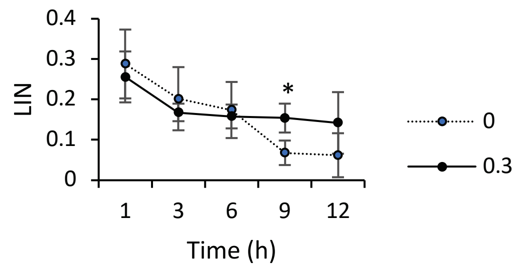

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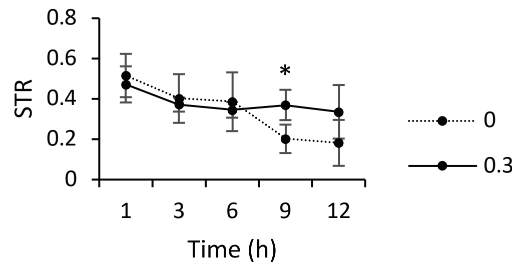

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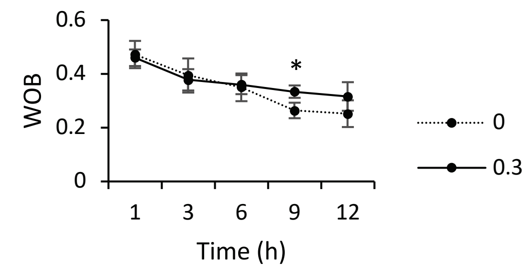

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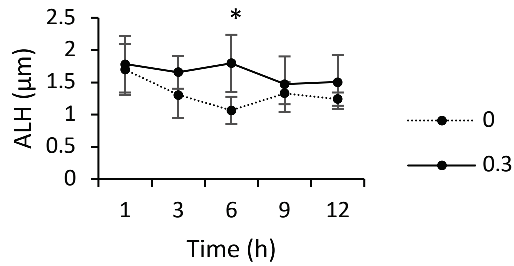

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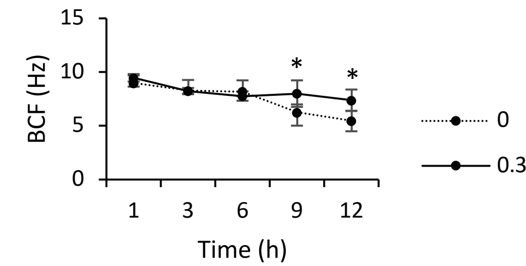

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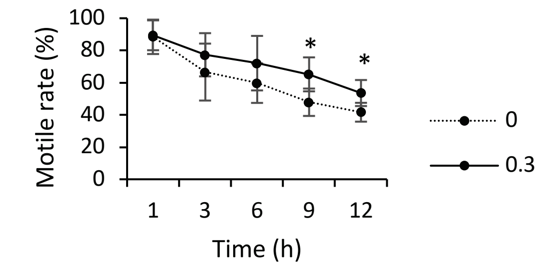

Fig. 4. **Effects of anserine and carnosine on sperm motility parameters**. **(1)** sperm were incubated with (1 μM) or without (0) anserine, and **(2)** sperm were incubated with (0.3 μM) or without (0) carnosine. Sperm motility was recorded at 1h, 3 h, 6 h, 9 h and 12 h of incubation with CASA. The graph shows (a) - straight line velocity (VSL); (b) - curvilinear velocity (VCL); (c) - average path velocity (VAP); (d) - linearity (LIN); (e) - straightness (STR); (f) - wobble (WOB); (g) - amplitude of lateral head displacement (ALH); (h) - beat-cross frequency (BCF); and (i) - motile rate. Data (n=5 independent experiments) are expressed as mean ± SD. *indicates significant differences at P<0.0 5.

Table 4-1: **Presence of anserine, carnosine, histidine, beta-alanine and 1-methylhistidine in the male reproductive tract of Japanese quail.**

Sources	**Anserine (µM)**	**Carnosine (µM)**	**Beta-alanine (µM)**	**Histidine (µM)**	**1-methylhistidine(µM)**
Testicular fluid	44.46	-	54.06	210.00	60.65
Seminal plasma	-	41.75	109.67	50.63	52.98
Cloacal gland secretion	-	-	51.30	18.70	7.48
Blood plasma	41.91	2.56	-	75.16	43.88

- indicates value under detection limit

Table 4-2: **Effects of various concentrations of anserine, carnosine on sperm motility after 6 h of sperm storage.**

Sperm motility parameters	Control (0)	Anserine (μM)				Carnosine (μM)			
		0.1	0.3	1	3	0.1	0.3	1	3
VSL (μm/s)	5.48±2.73[a]	5.81±2.66[ab]	10.47±2.40[ab]	11.58±3.62[b]	9.66±4.65[ab]	6.97±2.12 [a]	9.71±3.43 [a]	8.97±1.81[a]	9.67±4.57 [a]
VCL (μm/s)	38.41±9.93[a]	44.99±5.99[ab]	56.10±10.34[ab]	61.97±7.61[b]	56.49±12.54[ab]	54.98±3.78[ab]	60.10±11.69[b]	56.80±10.68[b]	60.34±12.87[b]
VAP (μm/s)	12.71±3.82[a]	14.04±2.24[ab]	22.33±4.50[b]	23.63±4.40[b]	21.75±8.56[b]	17.64±3.14[ab]	22.47±6.73[b]	20.07±3.92[ab]	22.81±8.53[b]
LIN	0.17±0.07[a]	0.16±0.06[a]	0.21±0.05[a]	0.18±0.04[a]	0.16±0.06[a]	0.14±0.02[a]	0.16±0.03[a]	0.17±0.05[a]	0.14±0.03[a]
STR	0.39±0.15[a]	0.34±0.09[a]	0.42±0.07[a]	0.37±0.05[a]	0.35±0.10[a]	0.34±0.04[a]	0.35±0.04[a]	0.38±0.10[a]	0.34±0.03[a]
WOB	0.35±0.05[a]	0.34±0.06[a]	0.41±0.04[a]	0.37±0.05[a]	0.37±0.07[a]	0.34±0.03[a]	0.36±0.04[a]	0.36±0.02[a]	0.36±0.05[a]
ALH (μm)	1.07±0.21[a]	1.10±0.19[ab]	1.64±0.28[b]	1.69±0.28[b]	1.57±0.53[ab]	1.57±0.17[ab]	1.79±0.44[b]	1.69±0.33[b]	1.74±0.42[b]
BCF (Hz)	8.19±1.04[a]	7.93±1.34[a]	8.98±1.08[a]	8.16±0.67[a]	8.15±0.99[a]	7.67±0.47[a]	7.77±0.44[a]	8.10±0.98[a]	7.67±0.62[a]
Motile rate (%)	59.69±12.27[a]	58.59±11.33[a]	68.05±19.37[a]	74.17±12.9[a]	71.05±20.67[a]	64.40±12.78[a]	72.11±16.93[a]	74.40±9.67[a]	77.65±11.70[a]

Data (n=5 independent experiments) are expressed as mean ± SD. Different superscript letters meaning significant differences at $P<0.05$ among treatments within the same row.

Chapter V: General Discussion

In physiological condition, avian sperm can be stored within the sperm storage tubules of female reproductive tract and may able to fertilize eggs up to 15 weeks. The long-term viability, motility and fertilizing ability of sperm is reduced when avian sperm are stored *in vitro* conditions. The viability, motility and fertilizing ability of avian sperm depends on *in vitro* storage conditions. Though sperm storage in the female reproductive tract is considerably high than male reproductive tract but sperm stores for a considerable period of time before ejaculation. Many factors can affect *in vitro* sperm motility, viability and fertilizing ability such as storage temperature, pH of extenders, osmolarity, sperm dilution rate, cloacal gland secretion and seminal plasma. Researchers are trying to extend longevity of avian sperm during *in vitro* condition by applying the knowledge of *in vivo* sperm storage mechanism(s) and sperm biology.

Sperm viability was studied *in vitro* with the knowledge of sperm maintenance at physiological states. Although sperm survives at body temperature 41 °C in both male and female reproductive tract, but the viability was lost when sperm were incubated at 41 °C *in vitro* condition. The viability of sperm at physiological pH was also low *in vitro* condition. During *in vitro* sperm incubation, probably the sperm motility and metabolic activity increases at high temperature (Bonato et al., 2012) and high pH (Holm and Wishart, 1998). Though the sperm viability was high at high sperm concentration of 2×10^7 sperm/ml, it was much lower than the sperm concentration of the ejaculated semen. Probably, most detrimental effect of sperm dilution is known as dilution effect. It is possible that dilution may destabilize sperm membrane which is detrimental for sperm viability (Maxwell and Johnson, 1999). Osmotic condition is associated with the intactness of sperm plasma membrane. In avian species, sperm viability was investigated with wide range of osmolarity. It reveals that sperm survive with a wide range of osmolarity (Sexton & Fewlass, 1978) *in*

vitro condition. Both seminal plasma and cloacal gland secretion did not improve sperm viability *in vitro* condition. Then, sperm were incubated with SP like medium but the SP like medium also did not improve the sperm viability. The role of seminal plasma in avian sperm viability, motility and fertilizing ability study seems to be contradictory. Blesbois and de Reviers, (1992) reported that fowl SP contains the fraction of higher molecular weight (>50 kDa) favours sperm fertilizing ability, whereas fractions of lower molecular weight (<1 kDa) are toxic to sperm *in vitro* condition. In turkey, whole SP reduces fertility (Douard et al., 2005), whereas dialyzed SP at 12-14 kDa is beneficial for *in vitro* sperm viability (Iaffaldano and Meluzzi, 2003). Sexton, (1977) reported that removal of seminal plasma by centrifugation had no significant effect on fertilizing capacity of chicken sperm at 5 °C for 24 h. Seminal plasma contains several components and it is possible that all components are not beneficial for sperm viability, motility and fertilizing ability *in vitro* condition. In this experiment, whole seminal plasma was used to sperm and found no significant improvement of sperm viability. Identification of seminal plasma proteins and investigation of seminal plasma proteins in sperm viability and motility study may give clearer scenario about the role of seminal plasma proteins and sperm maintenance in the reproductive tract of avian species.

In avian species, most of the studies conducted so far were to investigate the effects of whole SP (Douard et al., 2005) and fraction of SP (Ashizawa and Okauchi, 1984, and Blesbois and de Reviers, 1992). Identification of specific SP proteins and their roles in sperm maintenance have been investigated limitedly. In chapter III, the presence of ALB and TF were identified in ejaculates, cloacal foam, and male reproductive tract of Japanese quail. Usually, ALB and TF are known as blood plasma protein, that are produced in liver. Like other species, ALB is the major component of serum in quail (HALEY, 1965). The role of serum ALB and TF have been investigated in many species, but the roles of seminal plasma

ALB and TF have been studied limitedly. The serum ALB and TF maintains blood volume through oncotic pressure (Farrugia, 2010) and free iron (Yang et al., 1984), respectively. In female quail, ALB and TF were localized in the sperm storage tubules (Matsuzaki et al., 2019). I was unable to separate ALB and TF from the seminal plasma due to limited volume of semen. The ALB and TF separated from the UVJ mucosa were applied during *in vitro* sperm incubation. ALB and TF improved sperm viability compare to the control. ALB has antioxidant properties (Roche et al., 2008) and TF has anti-microbial properties (Valenti et al., 1983). Recently, (Atikuzzaman et al., 2017) observed the over-expression of serum ALB and TF in modern egg-laying chicken but they were unable to correlate these proteins with sperm viability. ALB and TF were present in ejaculates, cloacal foam and male reproductive tract of Japanese quail, and improved the sperm viability *in vitro* condition.

The presence and role of anserine and carnosine were investigated in the male reproductive tract of Japanese quail. The presence of carnosine and its derivates anserine were found in the male reproductive tract of Japanese quail. Anserine and carnosine at concentration of 1 μM and 0.3 μM, respectively improved sperm motility after 6 h of sperm incubation. The reason why anserine and carnosine improved the motility has not been investigated in this study. Naturally, avian semen contain antioxidants and enzymatic defenses that prolongs the sperm longevity *in vivo* and *in vitro* condition (Bréque et al., 2003; Partyka et al., 2012). However, avian sperm contains high amount of polyunsaturated fatty acids which is susceptible to reactive oxygen species and promotes lipid peroxidation (Khan, 2011). During *in vitro* storage condition, it is possible to occur lysis of lipid and peroxidation; and can modify the structure of spermatozoa (Blesbois et al., 1999). To overcome the problem of lipid peroxidation and achieving better fertility, experts tried to add different antioxidants during sperm storage period. The effects of carnosine on sperm motility have been investigated mainly in mammals. Carnosine improves the straight line velocity of sperm

motility parameters (Tan and Han, 1995), prevents oxidative stress in testis of rats (Aydın et al., 2018), and improves sperm mitochondrial activity in human (Adami et al., 2020). Carnosine works as a malondialdehyde scavenger in stallion seminal plasma (Rocha et al., 2018). It is thought that anserine and carnosine minimizes the damage of sperm during the sperm storage period. In this experiment, anserine and carnosine improved the sperm motility parameters in a dose-dependent and time-course manner.

In physiological condition, avian species may uplift the production of antioxidative enzymes that may protect the sperm *in vivo*. The antioxidative defense mechanism may be disturbed when sperm are incubated *in vitro*. Due to cellular metabolism, sperm abnormality, physiological stress, and infection, production of reactive oxygen species may be increased. The excess reactive oxygen species may imbalances antioxidant defense mechanism and oxidative stress occurs. The oxidative stress reduces sperm viability, motility and fertilizing ability. As, ALB, TF, anserine and carnosine have antioxidant properties, they may reduce the oxidative stress and improve sperm viability and motility. The presence of ALB, TF, anserine and carnosine in the male reproductive tract and their role on sperm viability and motility indicates they might play important roles for sperm maintenance *in vivo*.

I did not perform fertilizing ability of the stored sperm. Albumin and transferrin improved the sperm viability; anserine and carnosine improved the sperm motility. The combination of these proteins was not studied. In future, combination of these proteins may be studied for viability, motility and fertilizing ability of sperm.

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