STUDY ON PRODUCTIVE AND REPRODUCTIVE PERFORMANCES AND SEMEN QUALITY OF ASEEL CHICKEN

Dissertation submitted

By

Md. AHSAN HABIB PRAMANIK

Registration No.: 1805486

Doctor of Philosophy (PhD)
in
Genetics and Animal Breeding

Department of Genetics and Animal Breeding
Hajee Mohammad Danesh Science and Technology University
Dinajpur-5200, Bangladesh

January 2023

STUDY ON PRODUCTIVE AND REPRODUCTIVE PERFORMANCES AND SEMEN QUALITY OF ASEEL CHICKEN

Dissertation submitted

By

Md. Ahsan Habib Pramanik

Registration No.: 1805486

In partial fulfillment of the requirements for the degree of Doctor of Philosophy (PhD) in the Department of Genetics and Animal Breeding

Approved as to style and contents by

Supervisor **Professor Dr. Abdul Gaffer Miah** Department of Genetics and Animal Breeding HSTU, Dinajpur-5200, Bangladesh	**Co-supervisor** **Professor Dr. Ummay Salma** Department of Animal Science and Nutrition HSTU, Dinajpur-5200, Bangladesh

----

Chairman, Examination Committee
Professor Dr. Tahera Yeasmin
Department of Dairy and Poultry Science
HSTU, Dinajpur-5200, Bangladesh

Department of Genetics and Animal Breeding
Hajee Mohammad Danesh Science and Technology University
Dinajpur-5200, Bangladesh

January 2023

Preface

The Dissertation entitled **"Study on Productive and Reproductive Performances and Semen quality of Assel chicken"** was conducted during the period from July 2018 to June 2021 at the Department of Genetics and Animal Breeding, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, Bangladesh for partial fulfillment of requirements for the degree of Doctor of Philosophy (PhD). The research project for my PhD was funded by GARE, BANBEIS, the Ministry of Education, Government of the People's Republic of Bangladesh.

The main intervention areas of the research work were the productive and reproductive performance of chicken genetic resources of Bangladesh and germplasm cryobanking using a natural cryoprotective agent. The main objectives of the study were to know the productive and reproductive performance of chicken genetic resource of Bangladesh, sperm motility and biokinetic parameter of the Aseel rooster semen, improvement of sperm viability using honey as a natural antioxidant, and cryopreservation of Aseel semen using natural cryoprotectant i.e., honey. It is expected that the outcome of this PhD research would impact positively to conserve the chicken genetic resources available in Bangladesh and save them from extinction.

The Author

Declaration

This Dissertation has been written and composed by the author. It is a record of original research work carried out by the author under the guidance of supervisor Professor Dr. Abdul Gaffar Miah and Co-supervisor Professor Dr. Ummay Salma. This Ph.D. research work was funded by the project entitled "Conservation and Germplasm cryobanking of native chicken genetic resources of Bangladesh" project; aided by GARE, BANBEIS, Ministry of Education, and conducted at the Advanced Avian Research Farm, Laboratory of the Department of Genetics and Animal Breeding. This dissertation is an outcome of an original research work except where due reference is made to the contribution of others. All sources of such information are listed in the reference section. All kinds of support and assistance are sincerely appreciated and acknowledged.

None of the work has been presented elsewhere for the repeated degree.

The Author

Certification

This is to certify that the Dissertation entitled **"Study on Productive and Reproductive Performances and Semen quality of Assel chicken"** prepared by the examinee bearing registration No.: 1805486, session 2018-2019, Department of Genetics and Animal Breeding, Hajee Mohammad Danesh Science and Technology University, Dinajpur, to award the degree Doctor of Philosophy (PhD) in Genetics and Animal Breeding, is a record of original research work carried out by him under my close supervision.

The work is original and unique, to the best of my knowledge, no part of the Dissertation has been produced elsewhere for any degree.

Professor Dr. Abdul Gaffer Miah
Supervisor
Department of Genetics and Animal Breeding
Hajee Mohammad Danesh Science and Technology University
Dinajpur-5200, Bangladesh

Dedication

To all my family members

Acknowledgments

First, I would like to remember the blessings of Almighty Allah who enabled me to carry out the study successfully. Then I would like to thank my supervisor, Professor Dr. Abdul Gaffer Miah for his invaluable, competent, and kind academic support during challenging times until the last hour of dissertation submission. I desire to thank my co-supervisor Professor Dr. Ummay Salma for her motivating suggestion and invaluable discussions on the nutritive aspects of the study. Moreover, I am grateful to Professor Dr. Md. Rashedul Islam, Chairman, Department of Genetics and Animal Breeding, Nipa Rani Sarker, Assistant Professor, and Md. Hosne Mobarak, Assistant Professor, Department of Genetics and Animal Breeding for their continuous supports throughout the research journey. I am also indebted to Professor Dr. Fatema Zohora and Professor Dr. Md. Faruk Islam, Department of Medicine Surgery and Obstetrics, Professor Dr. Md. Sazzad Hossain Sarker, Department of Food Engineering and Technology, Professor Dr. Faruq Hasan, Department of Agriculture Extension, Dr. Md. Kamruzzaman, Department of Dairy and Poultry Science for their continuous suggestions and inspiration during my research. I want to acknowledge GARE, BANBEIS, the Ministry of Education, Government of the People's Republic of Bangladesh for their financial support endorsed for the research project entitled "Conservation and Germplasm cryobanking of native chicken genetic resources of Bangladesh". I am also grateful to Md. Azizul Hakim, Faruk (Staff of Advanced Avian Research Farm), Md. Bachchu and Ranajit Kormoker (Staff of the Department of Genetics and Animal Breeding) for providing generous support to complete this study.

I would like to thank Md. Asaduzzaman Sazal (former PhD fellow, Department of Genetics and Animal Breeding) and Khondoker Tawhidul Islam (PhD fellow, Department of Animal Science and Nutrition) for their skillful assistance in the lab and at the experimental site. My sincere thanks to all other teachers of the Faculty of Veterinary and Animal Science for their scholarly support and encouragement.

As this was a new research topic in Bangladesh, so many people whom I do not know, visited the research site and smiled at me or nice words which encouraged me a lot; I would like to say them, thank you.

Finally, my deepest gratitude and sincere thanks to my parents, wife, son, daughter, sisters, and sisters-in-law for their irreplaceable help, support, and encouragement throughout the PhD course.

The Author

ABSTRACT

Aseel, Naked Neck, and Red Junglefowl are the most important chicken genetic resources in Bangladesh. However, due to the unconsciousness of the farmers, uncontrolled crossing with other indigenous chickens, lack of proper conservation programs these are close to extinction. To conserve the extinct species the various ways were adopted in developed countries. As the first initiative of *in vitro* conservation of the poultry genetic resources, the present study was undertaken with four different distinct experiments to know the productive and reproductive performance of Aseel chicken and to study semen quality, sperm motility, biokinetic characteristics, sperm cryo-survivability by pre-freezing addition of honey as the possible cryo-protectant. Semen quality and the effect of honey on semen cryopreservation were evaluated by measuring sperm motility and biokinetic parameters with Computer Semen Analysis (CASA). The Aseel, Naked Neck and Red Junglefowl chickens were reared in open sided house in semi-scavenging condition at the Advanced Avian Research Farm, HSTU, Dinajpur. The Aseel sperm were cryopreserved using different level of honey where Glycerol and DMSO were also used as the positive controls. Considering productive and reproductive characteristics of the Aseel chicken showed almost similar performances of other chicken genetic resources but in some cases Aseel performed better. The semen color obtained from Aseel cocks was creamy white and showed a normal appearance and proved the semen quality was normal. The volume of semen per ejaculation and the pH were almost similar to others' semen. Sperm motility was also observed normal in relation to motility and progressive motility along with other biokinetic characteristics. Considering the motility and percentage of the progressively motile sperm, the semen samples might be stored for up to 2 hours in good condition for insemination. Pre-freezing addition of 3% honey showed the satisfactory results in maintaining cryo-survivability. The productive and reproductive characteristics did not vary in case of natural and artificial insemination with fresh and diluted semen. Moreover, the result showed that the addition of 3% honey in Ringer's solution performed as a cryoprotective agent which may help to cryopreserve Aseel roosters' semen as the native chicken genetic resource and revealed that honey might has the cryoprotective properties. Therefore, the study concluded that the pre-freezing addition of honey improved the cryo-survivability of Aseel's sperm and suggested for *in vitro* conservation of chicken genetic resources available in Bangladesh.

Key words: Aseel, productive and reproductive performance, semen quality, honey, cryopreservation

Page No.

Table of Contents

Contents Page

List of the Tables

List of the Figure

List of the Photo

Chapter I
GENERAL INTRODUCTION

1.1 Background

We are passing through the age of modern civilization. In the modern world, various developments that are beyond our imagination have taken place in every sector, such as in the industrial sector, the engineering sector, the medical sector, the agricultural sector, as well as the animal and poultry sectors. Modern high-yielding layers and broilers are part of the recent development in the poultry world. These recent modern developments are for the welfare of living beings, no doubt, but besides this development, we have lost some valuable resources like the purity of breeds and some breeds of animals and poultry are going close to extinction. That is why some brilliant scientists of the modern world are paying attention to conserving the breeds that are close to extinction and are going to save the world from the next mishaps before the cessation of development.

Some important chicken genetic resources available in Bangladesh, such as Aseel, Naked Neck, Red Junglefowl, Hilly chickens, etc., are still now found in different parts of the country. But day by day, these important chicken breeds are going close to extinction, i.e., they are considered endangered breeds of chicken. Now is the high time to save them, otherwise, these breeds will go to extinction.

The Aseel breed of chicken is one of the principal ancestors of the "Indian Sub-Continent Game Bird", which originated in Brahmanbaria, a district of Bangladesh, and is one of the oldest game fowls in Asia. It is a handsome, sprightly, and shapely bird with an upright and majestic gait and is bred for its highly valued meat, superior in taste and texture. This breed is close to extinction and is presently being used mainly for cockfighting (Bhatti *et al*., 1991; Khan, 2004; Rao and Preetem, 2009; Anonymous, 2009). Aseel is the recognized indigenous poultry breed found in Bangladesh as well as in India and Pakistan and is reared in backyard poultry rearing systems in rural areas (Khan, 2004). Aseel is characterized by its aggressive behavior, upright posture, small wattles, pea comb, prominent shoulders, narrow sternum, and hard muscular thighs with strong legs (Dohner, 2001), and is mainly known for its fighting tendency. In the last few decades, indigenous chicken has been paid great attention to by researchers all

over the world due to its better immunity and adaptability to local climatic conditions (Khan, 2015).

Efforts for the conservation and further improvement of Aseel chicken are still in progress for the revival of the rural poultry farming system (Haunshi *et al*., 2013). But in the Bangladesh context, minimum attention is paid to conserving these assets like Aseel as well as other local chicken resources, i.e., Naked Neck, Hilly, and Red Junglefowl, etc. Some breeds that are at risk of becoming extinct or may suffer from inbreeding depression and genetic drift due to increasing global use of high-yielding strains/varieties/breeds of farm animals have been coupled with a loss of genetic diversity in most species. So, it is a crying need to take a potential initiative for the conservation of these native chicken genetic resources.

1.2 Importance of the study

For large animals, scientists are considering different steps to conserve endangered species like cloning, embryo transfer, cryopreservation or vitrification, germ cell preservation, etc. Among the available steps, cloning and embryo transfer are not possible for poultry or any other birds as they produce eggs for reproducing next generation. Poultry birds are artificially reproduced through insemination and that is why semen cryopreservation and the use of cryopreserved semen may be the suitable method to conserve the Aseel chickens. Germplasm cryopreservation also may be used for conserving the chickens, but semen cryopreservation is preferred for this research. Rather this is for breeding purposes cryopreservation of poultry semen is not used widely in Bangladesh as well as in other countries of the world. Moreover, following this method, 50% of the genetic materials for the desired breed may be conserved and may have the possibility of getting 50% of genetic materials after extinction or a long period.

Semen evaluations by macroscopic and microscopic methods are the tools used to evaluate male fertility in roosters (Peters *et al.*, 2004). Macroscopic parameters like color, consistency, appearance score, and volume and microscopic traits like concentration, initial motility, abnormal sperms, and percentage of dead sperms are used in semen evaluation (Moce and Graham, 2008). Among all the characteristics of semen, initial motility is considered the single most reliable characteristic of semen for identifying the fertilizing ability of roosters (Charms, 1969). The semen characteristics

of avian species are different to a greater degree from those of mammals owing to the diverse physiology and anatomy, like the intra-abdominal placement of testes and the absence of accessory sex glands (Etches, 1996). Furthermore, chicken sperm differs morphologically from that of mammals (Etches, 1996) and is extremely sensitive to high environmental temperatures during collection and temperature fluctuations during cryopreservation (Graham, 1978). The semen of avian species is highly concentrated with very minimal seminal plasma (Etches, 1996). The semen quality in roosters was found to be varied among different genotypes like breed (Peters *et al.*, 2008), strain (Murugesan *et al.*, 2013), and line (Tarif *et al.*, 2013). The age of the roosters is also found to interact with semen quality parameters (Kelso *et al*., 1996). The environmental factors that influence semen quality are climate (Saeed and Al-Soudi, 1975), time of collection (Egbunike and Oluyemi, 1979), frequency of collection (Riaz *et al*., 2004), and nutrition (Kabir *et al*., 2007b). The existing literature provides little information on the semen quality of Aseel roosters, and in particular, there is a total paucity of information on the semen attributes of older roosters. The individual sperm motility of indigenous roosters was found to have a significant positive correlation with live sperm, sperm concentration, and sperm output in local chickens (Bah *et al*., 2001). The interrelationship of semen quality parameters has been reported for broiler breeders (Modupe *et al*., 2013), indigenous (Hu *et al*., 2013), hybrids (Chauhan *et al*., 2012), and a pool of several breeds (Peters *et al*., 2008), but little has been reported for the Aseel breed.

The reproductive potential of a breed or strain of chicken is assessed by the quality of the semen. Semen quality and quantity are affected by the breed and strain of chicken (Peters *et al*., 2008; Prieto *et al*., 2011; Shanmugam *et al*., 2012). Genetic selection for higher egg production affects semen quality (Shanmugam *et al*., 2013). Initial motility is considered the single reliable characteristic of semen for identifying the fertility of roosters, but other attributes like viability, membrane, and acrosome integrity also contribute to the fertility of semen. The semen quality parameters reported for White Leghorn (Tarif *et al*.,2013), Plymouth Rock (Elagib *et al*., 2012), RIR (Kabir *et al*., 2007), and indigenous Roosters (Hu *et al*., 2013), Synthetic, White Rock and Assel×RIR lines (Tarif *et al*.,2013), RIR rooster (Churchil *et al*., 2014), Lingnan, Bangkok, Kedu and Arabic (Almahdi *et al*., 2014), Nigerian indigenous (NI) and Isa White (IW) chickens (Mkpughe *et al*., 2015), Vanaraja and Indigenous Chicken of

Assam (Das *et al*., 2015), local Iraq breeds and ISA brown crosses (Hermiz *et al*., 2016) demonstrated a high degree of variation. It was pointed out that investigations are still required to establish baseline values for productive and reproductive parameters of this breed and characterize their performance. There is a scarcity of information on semen attributes and inter-relationship among the sperm parameters of Aseel Roosters.

Semen cryopreservation is a modern tool for the poultry industry to conserve and it was introduced in the 1960s as a means of preservation. Due to the difficult progress that has been made in this field, the biological and biochemical mechanisms involved in cryopreservation have not been thoroughly elucidated. Various factors during the freezing process, including sudden temperature changes, ice formation, and osmotic stress, have been proposed as reasons for poor sperm quality post-thaw. Though the cryopreservation of semen also protects germ plasm from the danger of loss and supports artificial insemination technology programs in livestock.

Generally, chicken sperm is more sensitive to the freezing process, and its quality of post-thawing ability is also relatively lower compared with that of other domestic livestock like cows, sheep, and goats (Mosca *et al*., 2016). Chicken sperm have a unique structure and differ in biochemical composition. The extremely high proportion of long-chain polyunsaturated fatty acids in the phospholipids fraction of spermatozoa is the most important feature of the lipid composition of rooster sperm. On one hand, the high polyunsaturated fatty acid proportion is a necessity to maintain specific membrane properties (fluidity, flexibility, etc.), and on the other hand, sperm become very susceptible to lipid peroxidation, and therefore the antioxidant defense is a key element in maintaining semen quality.

Therefore, it is necessary to improve protocols for freezing rooster semen for artificial insemination which will be suitable for Bangladesh as well as other parts of the developing world. The main cause of avian spermatozoa mortality during the process of freezing is the resistance to osmotic pressure and the membrane fluidity of the sperm (Blesbois *et al*., 2005; Morris *et al*., 2011). Overall, avian spermatozoa are recognized to be weaker for cryopreservation than mammalian sperm (Long, 2006).

The difficulty of preserving rooster spermatozoa was due to specific reproductive physiology and high variability from species and breed/strains (Rakha *et al*., 2016). Nevertheless, freezing-thawing processes result in dramatic damage to avian

spermatozoa membranes, resulting in the loss of more than 50% of spermatozoa (Lemoine *et al*., 2011; Long, 2006). The cryopreservation of semen typically involves the use of cryoprotectants to defend sperm from cold shock and cell damage due to the development of ice crystals. Cryopreservation of semen and procedures involving diverse cryoprotective agents has been studied. Procedures using cryoprotectants such as glycerol, N, N-dimethyl acetate-amide (DMA), and dimethyl-sulfoxide (DMSO) have been employed to cryopreserve chicken sperm (Blesbois *et al*., 2005). In general, cryoprotectants can be classified into two categories: penetrating and non-penetrating mediators of the membrane plasma of spermatozoa (Lemma, 2011). While Fuller (2004) revealed that sugars are a type of non-penetrating cryoprotectants that act extracellularly and change the osmotic gradient of the extended semen and allow water from the sperm to diffuse out. Honey is a natural material rich in nutrients such as simple sugars, antioxidants, proteins, vitamins, and minerals, all of which are beneficial to improving sperm quality (Saxena *et al*., 2010). Furthermore, honey comprises a large variety of simple sugars that serve as a source of nutrition and non-penetrating cryoprotectants (Fuller, 2004). Based on the description, honey can be added to the semen extender for the freezing of sperm in chickens. Because no prior research has tested the effects of supplementing honey *in vitro* into a skim milk-egg yolk extender as a chicken spermatozoa-freezing medium. Therefore, this research was designed to evaluate the effects of adding various concentrations of honey to a skim milk-egg yolk extender on the cryopreservation of chicken spermatozoa.

1.3 Research issues

The productive and reproductive characteristics of chicken genetic resources available in Bangladesh are not well defined, nor the semen quality reflects on fertility well studied, nor are the preservation techniques to conserve these chicken genetic resources well defined to save the chicken. So, the main questions were

i) What is the productive and reproductive performance of chicken genetic resources available in Bangladesh?
ii) What is the quality of semen produced by the rooster of chicken genetic resources?
iii) Possibility to improve the viability of sperm by adding natural antioxidants?
iv) Is it possible to preserve the semen of chicken genetic resources to conserve them?
v) Does honey work as a cryoprotective agent during cryopreservation?

1.4 Objectives of the study

The productive and reproductive behavior of Aseel and other genetic resources of chicken is not well defined, sperm quality of Aseel chickens is not widely studied, cryopreservation is not widely practiced in the case of rooster sperm, and honey has not been used as a cryoprotective agent in case of poultry until now. That is why the research was designed for the following objectives.

i) To be familiar with the productive and reproductive performance of Aseel and other genetic resources of chicken available in Bangladesh.

ii) To know the motility and bio-kinetic parameters of semen collected from Aseel rooster.

iii) To improve the viability of rooster sperm using a natural cryoprotective agent i.e., honey.

iv) To determine the level of honey suitable for cryopreservation of rooster semen.

v) To evaluate the breeding performance of the Aseel chicken using fresh and diluted semen.

1.5 Research conceptual framework

To address the objectives and research issues considering the problem statement and significance of the study, a conceptual research framework has been developed and presented in the Figure 1.1.

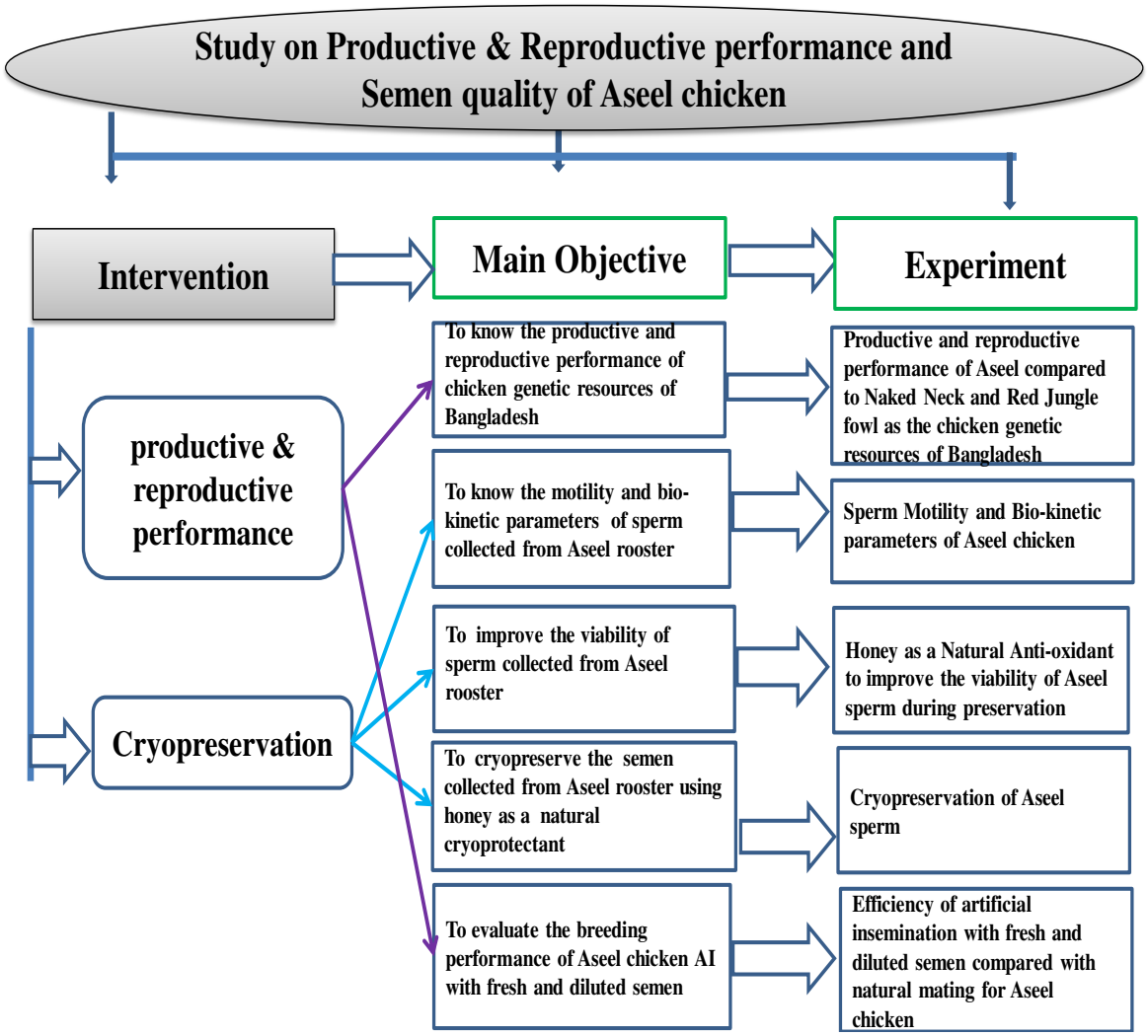

Figure 1. 1: Research conceptual framework

1.6 Dissertation organization

Four independent studies were conducted under the research framework. After chapter one, which presents the general introduction, this dissertation is presented with the following chapters:

- Chapter 2: Literature Review
- Chapter 3: Productive and reproductive performances of Aseel compare to Naked Neck and Red Junglefowl as chicken genetic resource of Bangladesh.
- Chapter 4: Sperm motility and bio-kinetic parameters of Aseel rooster
- Chapter 5: Honey as a natural antioxidant to improve the viability of Aseel sperm during preservation
- Chapter 6: Cryopreservation of Aseel Sperm
- Chapter 7: Efficiency of Artificial Insemination with fresh and diluted semen compare to natural mating for Aseel chicken
- Chapter 8: Summary and Conclusions
- References

Chapter II
LITERATURE REVIEW

The chapter provides a solid background for the research study through a review of the literature regarding the productive and reproductive performance of Aseel, Naked Neck, and Red Junglefowl chicken and regarding the sperm quality, storage of sperm, and cryopreservation of rooster sperm. The reviewed literature has been collected from books, publications, journals, reports, and other information from different websites.

2.1 History of the Aseel chicken

Aseel chicken (also spelled Asill or Asli) is an ancient breed of chicken from the Indian sub-continent (Bangladesh). These chickens are originally reared for cock fighting and ornamental purpose. Aseel chickens were brought from Europe around 1750 and were considered the strongest game birds in the world. The birds are very smart, and strongly muscled and contributed to the modern Cornish (broiler) breed. Sometimes, Aseel chickens become friendly when they are kept apart from other roosters. Aseel hens are not very good layers, but they are excellent as broody hens and mothers. Naked-neck chickens look like a cross between a turkey and a chicken with their completely featherless necks and faces. They are often referred to as turkens, Transylvania Naked Necks, bare necks, Hackle-less, and Rubber necks and are characterized by the Naked Neck trait, caused by a single autosomal dominant gene (Davenport, 1914).

2.2 Productive performance

It has been reported that the poor productivity of the Aseel breed of chickens might be attributed, at least partly, to the poor management and feeding system instead of inherent low productivity (Sarkar *et al.,* 2006).

Previous work on Aseel revealed that the Mushki variety performed better in terms of egg production, egg weight, egg mass, and FCR/dozen eggs, whereas the Peshawari variety showed a pointedly higher feed intake compared with Lakha, Mianwali, and Mushki Aseel (Usman *et al.,* 2013).

In another study, Usman *et al.* (2014) reported higher productive efficiency of Mushki Aseel followed by Lakha, Mianwali, and Peshawari varieties. Furthermore, the overall production performance remained better in the post-molt production phase compared with that of the pre-molt (Usman *et al.,* 2013).

2.2.1 Production of eggs

For the period of 22−44 weeks, the hen-day and hen-housed egg production of Aseel is reported to be about 54% and 54%, respectively (Haunshi *et al.,* 2013). The Naked Neck chicken produced more eggs than the Mushki, Lakha, Mianwali, and Peshawari varieties of Aseel, which could be attributed to genetic differences between the Aseel and the naked-neck chicken. The egg production of the Naked Neck is recorded at up to 138 eggs in 52 weeks of the production cycle, whereas for the Aseel chicken it is recorded at up to 92 eggs per annum by the Central Avian Research Institute, and similarly, ICar (2009) showed a great variation in the egg production of these two breeds. This comparative difference in the egg production of these breeds might be related to the difference in live weight of these breeds, as the egg production and live weight have a negative correlation with each other and the live weight of Aseel (2.0−2.5 kg of pullets) is remarkably higher than that of Naked Neck (1.5 kg for pullets) as reported by ICar (2009) and Grobbelaar *et al.* (2010) respectively. Moreover, it has also been stated by Korver *et al.* (2004) that different modern strains have higher egg production compared to older breeds, concluding that different breeds have different egg production performances. Similarly, the difference in egg production among varieties of Aseel has been explained by Usman *et al.,* 2013 who observed that the egg production of different varieties of Aseel is higher than the other varieties of Aseel. In another study, Hurwitz *et al.* (1998) reported that different breeds have different performances in terms of their egg production when the molting process was applied.

However, Mohan *et al.* (2008) recorded a total egg production of 160 eggs during a production period of 23 to 78 weeks for Aseel. Different egg production performances for different breeds were also explained by Akram (1998), who stated that the best egg production was observed in an exotic breed (Rhode Island Red) compared with the native genetic resources. However, after genetic improvement, India has developed two breeds, namely CARI-Shayama and CARI-Nirbheek, from Aseel ICar (2004), and reported an egg production of 92 eggs per annum with an average egg weight of 52 g. Highest hen day egg production (peak production) was reported at 67.57% in Aseel in the 31st week and 75.56% in Kadaknath at the 35th week of age (Haunshi *et al.*, 2013).

The total egg production was obtained at 160 eggs (50−55 eggs/annum) during the production period of 23−78 weeks from Aseel available in Bangladesh (Mohan *et al.,* 2008; Yoshimura *et al.* 1997). Haunshi *et al.* (2013) reported that egg persistency was

better during the initial production phase (24 to 36 weeks) for Aseel, whereas egg persistency during the later phase (40 to 44 weeks) and the egg production was higher in the Kadaknath breed.

Sohail *et al.* (2013) found that the egg production number and egg mass remained higher in the second production cycle as compared to the first and third production cycles of Peshawari (an Aseel strain). He reported the average annual egg production of Peshawari Aseel and recorded 53eggs/annum.

The Age of sexual maturity of Aseel (174 days) has been reported to be faster than that of Kadaknath (181 days) by Haunshi (2013), which is also comparable to the findings of Mohan *et al.,* 2008.

According to Islam and Nishibori (2009), the Naked Neck chicken has a good heat dissipation ability or mechanism, is well adapted to the harsh tropical environment and poor nutrition, and is highly resistant to disease and superior to indigenous full-feathered and exotic egg-type or exotic Naked Neck counterparts in terms of growth rate, egg production, egg quality, meat yield traits and can produce about double the number of eggs under improved nutrition and management conditions. The light brown phenotype might have a better genetic potential for egg production, as it is reported that egg production mainly depends on genetic makeup (Akhtar *et al.,* 2007).

Similarly, a study conducted on Naked Neck and normal plumage hens resulted in a variation in egg production with a higher number of eggs produced in Naked Neck hens compared to those of normal plumage. Adomako (2009) also claimed similarly and stated that variation in egg production was observed due to genotypic differences. Similarly, breed (Usman *et al.,* 2014) variety (Usman *et al.,* 2013) strain (Hanan, 2010) or genotype effect (Ahmad *et al.,* 2014) on egg production has already been reported in literature concluding that genotype is the major cause of variation in egg production (Akhtar *et al.,* 2007).

There was no significant difference between hatching egg production in natural mating and artificial insemination. Sayyazadeh *et al.* (2005) showed that artificial insemination significantly produced thinner-shelled eggs and a decreased average weight of the eggs. Besides, artificial insemination increased the production of bi-yolk egg percentage and in general, underweight and overweight egg production in the lines. The percentage of

broken eggs during production, in this case, was greater than with natural mating, compared to the control tester.

Ghanem *et al.* (2017) showed that the natural mating group had the lowest average egg production in number and body weight compared to the artificial insemination group. However, the AI group had a higher mortality percentage compared to the natural mating group. The outcomes of the study indicated that the AI group achieved higher values for average egg production number and average body weight compared to natural mating. This may be attributed to the large size of the Muscovy male compared to that of the Pekin female in the natural mating group. The large size may create reproductive problems like effects on the number of eggs produced, the size of the eggs, and consequently, the average live weight of the newly hatched chicks. Moreover, natural mating recorded lower fertility and hatchability percentages. Concerning mortality, this study showed that the AI group had the highest (about 41%) mortality compared to the natural mating group (about 14%). The latter may be attributed to the practical applied permanent stress on the flock, no wastage of the fertilized zygotes, and the high percentage of the inseminated fertilized eggs. Also, during the technique of AI or even syringe penetration, there may be uterine prolapse. These stressful factors before and after the technique of insemination can be minimized during the processes and the personnel who collect the semen should have adequate knowledge regarding proper collection methods that may help in excluding various defects or mortality. However, natural mating exhibits slow stages without any stress. However, the breeder should be conscious of overcoming the mortality problems. Brun and Larzul (2003), Gee *et al.* (2004) and Dhama *et al.* (2007) also recorded that AI exhibits superior fertility than natural mating. The authors suppose that these results enable rapid genetic improvement via increasing selection differentials, where thousands of females are mated with one highly selected superior sire. On the contrary, Koohpar (2010) indicated that the fertility and hatchability traits were not significantly different; the fertility percent in the AI flock was higher than in natural mating but without a reasonable level. It was suggested that both more accurate insemination and expected fertility were the principal cause.

Mohan *et al.* (2018) discussed using semen diluents, the services of a superior male can be used for AI, which is not possible under natural mating. From an economic perspective, diluents reduce the number of males required for AI, hence saving feed

costs due to having fewer males and saving space, maintenance, and operating costs. Natural mating fixes an upper limit of males to females at a ratio of about 1:10-12, whereas AI allows breeders to serve 100 hens from a single male. This increased use of males, combined with the ability to recognize hens not fit for laying eggs (10-30%) in the flock during the AI process, provides an excellent means to reduce (at least by 20%) the cost (Hammerstedt, 1996). Recently, Mohan and Sharma (2017) showed that fresh semen (5.34 x 109 sperm/ml) obtained from a male bird (0.5 ml) can be inseminated into 70 hens/day after dilution with CARI diluents (1:6) having 38 million sperm per dose for AI (0.05 ml diluted semen). Following this manner, nearly 90% or above fertility can be achieved. Using a single male on alternate days (four times a week), 280 hens could be covered, whereas, under natural mating, the cock and hen ratio of 1:8 was kept. AI in avian species results in better fertility than natural mating (Saeki and Nagomi, 1964; Brillard, 2003; Mohan *et al.*, 2016). Even though natural mating can lead to good fertility, rates can be enhanced further simply by adding AI to the reproductive program (Gee *et al.*, 2004). Advantages from the overall fertilization rate and hatchability result in a reduction of the cost per unit of day old chicks hatched (Brillard, 2003). Similar advantages in terms of reproductive success can be expected if avian AI techniques are extended to endangered birds.

2.2.2 Weight of the egg

Some previous studies also discussed the difference in egg weight between Naked Neck and Aseel breeds and reported that the average egg weight of Naked Neck was 55 g (Grobbelaar *et al.,* 2010) and 57 g (Lima *et al.,* 2012) and the egg weight of Aseel was 50 g ICar (2009). This showed the differences between these two breeds in terms of egg weight, which might be due to the different genetic potential abilities of these two breeds. The difference in egg weight of different varieties of Aseel has also been observed by Usman *et al.* (2013), who reported a higher egg weight for the Mushki variety compared to other varieties of Aseel.

Singh *et al*. (2000) studied various reproductive and productive performances of Aseel birds under field conditions and reported an average egg weight of Aseel of 41 g, which was almost similar to the Kadaknath birds. Evaluating the performance of Aseel at different ages Sohail *et al.* (2013) reported the egg weight of Peshawari, a strain of Aseel, in the 1st, 2nd, and 3rd production cycles were measured at 45.53, 44.13, and 42.67 g, respectively.

However, Sohail *et al.* (2013) reported that younger birds took higher feed intake, and produced a higher number of egg production with higher egg mass and FCR/dozen eggs were better as compared to older birds. Ahmad *et al.* (2013) reported an average egg weight of 45 g from Mushki Aseel, whereas, Singh *et al.* (2000) reported an egg weight of 47 g from Aseel. In another study, Usman *et al.* (2014) reported that the egg weight of Peshawari Aseel was 55.65g, which was almost similar compared with Lakha (54.03 g), Mushki (53.70 g) and Mianwali (51.62 g) Aseel.

According to Iqbal *et al.* (2012), the average egg weight for the Lakha, Peshawari, Mushki, and Mianwali varieties of Aseel is reported to be 41.8, 41.2, 43.5, and 45.9 g, respectively, which is in contradiction to the findings of the Central Avian Research Institute (CARI), where the egg weight for Aseel has been reported at 52 g. Moreover, an egg weight of 42.57, 44.65, 45.84, and 47.57 g for Aseel chickens at 28, 32, 36, and 40 weeks of age was observed by (Haunshi *et al.,* 2012).

2.2.3 Live weight and day old chick weight

Similar day old chick weight was reported for RIR chicks (Ashraf *et al.,* 2003; Das *et al.,* 2014a; Das *et al.,* 2014b), PL1 and PL2 strains of White Leghorn chicks (Choudhary *et al.,* 2009), CARI-Sonali and CARI-Debendra chicks (Das *et al.,* 2014b), and slightly lower day old chick weight was reported for RIR chicks (Malago and Baitilwake, 2009). Some strains demonstrated overall lower chick weight than the RIR control (Das *et al.,* 2015a) and selected lines (Das *et al.,* 2015b), though the second hatch performed as well as the RIR control line (Das *et al.*2015a). The live weights were comparable to the reports in RIR strains (Das *et al.,* 2014a; Das *et al.,* 2014b), and RIR male and female lines (Nwagu *et al.,* 2007a). The present strain demonstrated better body weights than the RIR control line (Das *et al.,* 2015a), but lower than the RIR selected line (Das *et al.,* 2015b). The chicken strain had these estimates better than the reports in other chicken breeds, lines, strains, and their crosses with RIR chicken as evident when compared to the available reports in White Leghorn (Ahmad and Singh, 2007; Jayalaxmi *et al.,* 2010; Qadri *et al.,* 2013), Gramapriya female line (Chatterjee *et al.,* 2010), Kadaknath and Aseel (Chatterjee *et al.,* 2007a), Giriraja (Adebambo *et al.,* 2006), CARI-Sonali (CARI Annual Report, 2011; Das *et al.,* 2014b), Fayoumi male × RIR female cross and its reciprocal (El-Maghraby *et al.,* 1975) and crosses of RIR × indigenous lines Bare-neck or Betwil or Large Beladi (Mohammed *et al.,* 2005); though few chicken lines (CARI Annual Report, 2011; Das *et al.,* 2015b) or crosses (Das *et*

al., 2014b) were found better than this present chicken strain. The difference might be due to strain, line, or breed differences and different management as well as rearing systems.

The least squares analysis of variance revealed that the different hatch significantly influenced the estimates of the chick weight and body weights up to the housing weight (BW20), though the body weight of 40-week-aged pullets had no inter-hatch difference at a significant level. Significant hatch differences have previously been reported in different chicken genotypes (Das *et al.,* 2014b), as well as in different tester×line crosses between exotic tester (viz. RIR, Bovans, and Fayoumi cockerels) and indigenous lines (viz. large Beladi, Bare-neck, and Betwil hens) at various ages in the Sudanese environment (Mohan *et al*., 2005) however, Ashraf *et al.* (2003) did not find any significant hatch difference in the chick weight of Lyallpur Silver Black and Rhode Island Red chickens. When Nwagu *et al.* (2007b) studied RIR male and female lines, they reported that the hatching effect may have contributed to the variable response in different economic traits achieved from generation to generation, as well as the varying season of hatching across generations.

The least squares analysis also revealed that the sex of the chicks had a significant effect on the body weights from eight weeks onwards; the males were heavier than the females throughout the ages.

Significant sex differentiation in live weights and male birds being heavier than females were also observed in RIR selected lines at the sixth week onwards (Das *et al.,* 2015b), Libyan chicken (El-Safty, 2012) and Giriraja, WLH and Nigerian improved indigenous chicken genotype at 12 weeks onwards (F_1, F_2 and B-α chickens) (Adebambo *et al.,* 2006).

The live weight of Aseel at 0, 1, 2, 3, and 4 weeks of age was 29, 40, 55, 76, and 107 g, respectively (Sarkar *et al.,* 2006); the live weight of day old Aseel chicks have also been reported as 33 g (Singh *et al.,* 1999).

The live weights of Nicobari fowl at 4 weeks were 74 g and 53 g under the intensive system and backyard system, respectively (Chatterjee *et al.,* 2002). Moreover, the live weight of Aseel at the age of 6, 8, 10, and 12 weeks is reported as 161, 234, and 317and 408 g, respectively (Gurung and Sing, 1999). At 10 weeks Singh *et al.* (1999) observed

a 552 g live weight of the the Aseel breed. At 40 weeks of age, the live weight of Aseel was reported 1853 g (Haunshi *et al.,* 2011).

The live weight of Aseel at 6, 7, 8, and 9 months of age was1033, 1244, 1551, and 1743 g, respectively (Sarkar *et al.,* 2006), which was found higher than Kadaknath (Gurung and Sing, 1999). At 10, 11, and 12 months of age, the live weight of Aseel was found to be 1964, 2249, and 2590 g, respectively, whereas the weekly live weight gain from 0−52 weeks was found to be 53g (Gurung and Sing, 1999).

Kalita *et al.* (2013) worked on the productive performance of the Peshawari variety of Aseel and found differences in live weight at 0, 1, 2, and 3 weeks of age and also reported the highest live weight (1819.43 g) in the third production cycle compared with that of the second and first production cycles, which were 1607.71 and 1534.43 g, respectively. Babar *et al.* (2012) reported that the live weight of Pakistani varieties of Aseel chickens like Lakha, Mushki, Mianwali, Peshwari, and Sindhi varieties of Aseel ranges between 3.0−3.8, 3.0−3.5, 2.5−3.0, 2.8−3.2, 4.0−7.0kg, respectively for males; whereas, the female chicken of these varieties, in the same order, were found 2.5−3.2, 2.5−3.0, 2.0−2.2, 2.2−2.5, 3−4.5 kg heavier. Likewise, the live weight of Indian Aseel at the age of 4, 8, 12, and 16 weeks of age has been reported to be 65.1, 154, 393, 796, and 1218 g, respectively (Chatterjee *et al.,* 2007). However, Chatterjee *et al*. (2007) have reported that the standard live weight of Aseel ranges from 3−5 kg for cocks and 2−4 kg for hens.

Growth appears to be slow in Aseel chickens during the early period of life and birds continued to grow up to 40 weeks of age. The growth was faster in the later period, up to 40 weeks of age. The live weight up to 16 weeks of age was comparable to the findings of Chatterjee *et al*. (2007) in Aseel chickens. Sex had an effect on live weight and shank length in the Aseel. Cocks had firm and compact bodies with broad chests and upright postures. Similar observations on sex effects were reported by Haunshi *et al.* (2011) in the Aseel. Aseel cocks have been selected for their fighting abilities with longer and stronger shanks and legs either naturally or by farmers, leading to longer and stronger shanks in the birds. The annual live weight of cocks 3.79 kg and hens 2.33 kg were similar to the observations of 3 to 4 kg cocks and 2 to 3 kg hens, reported by Singh (2001).

Deeb and Cahaner (1999) observed the highest potential benefit in Naked Neck birds compared to fast-growing broilers in a hot climate. Male birds were heavier with lower feed conversion ratios (FCR) than their female counterparts. IFF and INN male and female mature live weights were 876.2, 803.4, and 1092.1, 890.0 g, respectively (Islam and Weil 2000). Other authors have reported that the mature live weights of IFF and INN chickens were 833.0 and 1214 g, respectively. Darmstadt *et al.* (2005) reported live weights of 1.02 and 1.00 kg for adult male and female INN chickens respectively, which coincided with the findings of a report demonstrating the better adaptability of Naked Neck chickens in stressful environments by its higher survival rate (Horst, 1988).

2.2.4 The quality of eggs

The egg quality parameters are under the influence of several facts or the major one of which is the breed or variety of the observed chicken. The internal quality of the egg is very important from the consumer's point of view, but it cannot be assessed without breaking the egg. The difference in haugh unit scores might be attributed to the differences in albumen height of observed birds, which is in agreement with the results of different albumen heights for different breeds. These results are also in agreement with those of Ali *et al.* (2012) who also observed different Haugh unit scores of different breeds.

However, a higher shell percentage of the Mushki variety of Aseel has been observed by Shafiq *et al.* (2013). In this study, the Mianwali variety of Aseel showed higher yolk% than other varieties of Aseel and Naked Neck whereas the yolk index showed differences in all varieties of Aseel and Naked Neck. Similar findings have also been reported by Haunshi *et al.* (2011) who observed differences in haugh unit scores among the Vanaraja and White Leghorn breeds of chickens.

However, Shafiq *et al.* (2013) also reported a higher yolk index of the Mianwali and Peshawari varieties than the other varieties of Aseel. This contradiction might be due to the performance of Aseel birds in different habitats, as Aseel is famous for its better adaptability to local environmental conditions and the performance efficiency of birds might be influenced by different living conditions.

Haunshi *et al.* (2011) also observed differences in the yolk index of different close-bred stocks and different breeds of chickens. Mushi chickens had the highest albumen%,

followed by Lakha, Mianwali, Peshwari, and Naked Neck chicken. Similar findings have also been reported by Shafiq *et al.* (2013), who reported the lowest albumen% of the Peshawari variety than other varieties of Aseel.

As far as the egg quality is concerned, Usman *et al.* (2014) reported that a higher shell percentage was observed in Peshawari Aseel (13.57), Albumen% in Mushki Aseel (61.83), yolk% in Mianwali Aseel (31.28), Haugh Unit score in Peshawari Aseel (81.95) and no differences in yolk index among different varieties of Aseel.

Shafiq *et al.* (2013) worked on the egg quality parameters of Aseel and reported that the yolk Index was better in Mushki and Mianwali varieties of Aseel, the Haugh unit score in Mushki, the shell thickness in Lakha, the higher yolk and albumen pH in the Mianwali variety, whereas the albumen height was better in both Lakha and Mianwali varieties. Evaluating the egg quality parameters influenced by molting, Shafiq *et al.* (2013) reported that albumen and yolk p^H, albumen%, yolk index, shell thickness, and haugh unit score improved in response to molting whereas albumen height was not influenced by molting.

Sohail *et al.* (2013) observed differences in the shell%, yolk index, egg shape index, and albumen height of three production cycles of Peshawari Aseel. It was observed that the highest egg surface area and egg volume were in the second production cycle, followed by the first and third production cycles of Peshawari Aseel. However, a higher yolk index at different ages in the Aseel breed has also been reported in another study (Haunshi *et al.,* 2011).

Sohail *et al.* (2013) worked on the egg geometry parameters of Aseel and reported that egg length, egg surface area, and egg volume were higher in the Lakha and Mushki varieties of Aseel, whereas the egg shape index remained better in both the Peshawari and Mianwali varieties of Aseel.

Sohail *et al.* (2013) found differences in age in the egg surface area, egg shape index, and egg volume of Aseel whereas egg breadth remained lowest in younger birds as compared to older Aseel. Naked Neck hen's eggs were stronger in terms of shell-breaking strength than normal feathered. The effect of the Na gene on egg quality measurements under moderate or high ambient temperatures was studied by Bordas *et al.* (1980), who reported that the homozygous NaNa genotype had more albumen than

those of the Nana and nana genotypes at both temperatures. Albumen height was higher for the nana genotype (6.81 mm) than for NaNa (6.06 mm) and Nana (6.28 mm) birds. The heterozygous genotype was intermediate for most traits but was closer to the nana for the yolk-to-albumen ratio. Under hot environmental conditions (31°C), Bordas *et al.* (1980) showed that egg weight, yolk weight, albumen weight, and albumen height for nana and Nana genotypes were 48.70 vs. 53.20 g, 14.60 vs. 16.10 g, 29.14 vs. 31.05 g and 7.05 vs. 6.22 mm, respectively.

The heterozygous genotype was generally intermediate but closer to NaNa birds for albumen height. Under Egyptian environmental conditions, Zein El-Dein (1981) concluded that the eggshell weight for the Nana genotype was superior to that of NaNa (6.25 vs. 5.84 g), while the nana genotype was intermediate (6.16 g). Eggshell percentage and eggshell weight exhibited the same trend.

Fathi (1987) found that albumen and shell percentages were higher for Nana hens than for normal feathered chickens. Conversely, under natural and improved environmental conditions, Abdel-Rahman (1990) showed that the Na gene reduced eggshell percentage by about 0.8% and 4.4%, for Nana and NaNa genotypes, respectively. Fathi (1992) stated that there were differences among NaNa, Nana, and nana genotypes for most of the egg quality measurements. Shell thickness was higher for the nana genotype than for NaNa and Nana. The shell percentage was lower for both the NaNa and Nana genotypes. There was no advantage associated with the Na allele for yolk height.

Zulkifli *et al.* (1992) reported that the combination of Na and F genes within a dwarfed genotype (dw-Ff-Nana) did affect albumen height at 60 and 76 weeks of age, yolk weight at 76 weeks and shell-breaking strength at 60 weeks. They added that the combination of Na and F in non-dwarfed genotype background (Dw-Nana-Ff) appeared to have a positive interactive effect on several egg quality traits.

Galal (1995) found that the highest values of albumen weight occurred in the Nana genotype in Mandara, Golden Montazah and Gimmizah strains when compared with their normal plumage counterparts. Under Bangladesh conditions, Islam *et al.* (2001) reported that the Desi Naked Neck genotype produced better quality eggs than its Desi full-feathered sibs.

Alvarez *et al.* (2002) showed that the specific gravity, Haugh units, albumen height, and shell thickness were better in the Nana genotype compared with NaNa and nana.

Under the subtropical climatic conditions (30.1°C) of Maputo, Garces and Casey (2003) found that the Na gene increased yolk weight and reduced albumen height compared to its recessive allele. Egg quality is based on the characteristics of the egg that affect its acceptability. Each of the main components of the egg (shell, albumen, and yolk) has a natural variability that is not in line with modern requirements (De Ketelaere *et al.,* 2004).

Nowadays, concern about egg quality is growing steadily (Kemps *et al.,* 2006). The overall quality of the chicken egg is determined by the egg's external and internal quality. Both of them are of paramount importance to the egg industry (Roberts, 2004).

The appearance of the egg is important for consumer appeal. Egg shell quality is based on egg size, egg specific gravity, and shell color, shell breaking strength, shell deformation, shell weight, percentage shell, shell thickness, and shell ultra-structure (Roberts, 2004).

For table eggs, shells must be strong enough to prevent failure during packing and/or transportation (Narushin *et al.,* 2004). For hatching eggs, shells must be initially thick and strong to preserve the embryo, and then they must become thin and weak later during incubation to allow gas exchange as well as easier cracking when hatching (Narushin and Romanov, 2002). Interior egg quality is based on albumen quality, yolk quality, and the presence of blood or meat spots (Jacob *et al.,* 2000).

2.3 Reproductive efficiency

2.3.1 Age at sexual maturity

Li *et al.* (2018) examined the laying performance and mortality of fast-feathered Huainan partridge chickens. The results showed that NM birds had a significantly earlier age at first egg than AI ones. This result is probably because the existence of mated roosters stimulates the sexual maturation of hens in the same cage. The laying rate during the flock NM was less than AI**.** Considering the laying rate, which is affected by various factors, the reason for this discrepancy is not completely understood. However, the result insinuates an obvious deficiency in natural mating, and it is necessary to optimize the parameters of the natural mating system to achieve the best

possible output levels. This phenomenon is perhaps best explained by the permanent insemination stress of AI flocks. NM hens are inferior to AI hens in body weight. These results are largely consistent with those of Koohpar *et al.* (2010). This is explainable as NM hens are kept in larger cages and have more physical activity, but in the AI, small cages restrict the behavioral activities of laying hens, which might cause an increase in body weight.

Age at sexual maturity was observed to be between 165 days in Rajasri birds. This was similar to the findings of Czech Hughes and Dunkin (1984) in various breeds of birds in farmers' backyards. However, some of the beneficiaries (15.2%) reported birds reaching sexual maturity at more than 6 months, which might be due to poor vegetation in that area. The lower age at sexual maturity is desirable, which may lead to an increase in the laying period, thus improving egg production. The early age at sexual maturity and the greater live weight were observed in the majority of (84.80%) of Rajasri birds. This might be attributed to the supplementary feeding (10−15%) and availability of scratching grains from paddy fields. The age at sexual maturity in Rajasri birds was lower than in Aseel (187.43) and Kadaknath (196.12).

2.3.2 Fertility and hatchability

Koohpar *et al.* (2010) explained that no differences were observed in the egg weight, fertility, hatchability, and mortality of roosters and were unaffected by reproduction techniques of Natural mating and Artificial insemination. Habibullah *et al.* (2015) observed higher hatchability, production, total mortality, uniformity percent, and puberty weight (gm) of the birds after AI than before. They showed that hatchability percent, production percent, and puberty weight were significantly higher after AI than before AI. However, the mortality and uniformity rates were significantly lower after AI than before AI. In the study, hatchability percent, production percent, body weight, and puberty weight were significantly higher in post-insemination than in pre-insemination. On the other hand, total mortality percent, uniformity was significantly lower in post-insemination than in pre-insemination. Ghanem *et al.* (2017) attributed this to AI and natural mating of the males and females having no mating increases in hatchability percentage. Sayyazadeh *et al.* (2005) found that the average hatchability percentage in natural mating for Arian and Ross 308 lines was 82.7 and 83.1, respectively. But when artificial insemination was done, these values increased and

were 87.2% and 89.4%, respectively. The test showed that the hatchability percentage increased by artificial insemination. A similar result was also observed by Hocking and Bernared (1997), Christensen (2001), and Surai *et al.* (1996). Robinson (1996) reported that when artificial insemination is practiced, hatchability and production percentages are increased compared to natural mating. Similar results were shown by Schramm (2005). The development of suitable methods to collect sperm as well as insemination, dilution, and preservation of sperm has resulted in a useful and significant method in poultry breeding. This method of mating is done in the breeding of laying hens, guinea fowls, and carina moschata, but it has the most important role in the reproduction of modern heavy lines of turkeys.

Because of AI, it is possible to increase the proportion of mating, store semen from 6 to 24 h and longer without any loss of fertility and perform semen transfer. It is also possible to arrange semen depots for genetic resources by deep freezing conservation. The manual work and the need for weekly repetition of AI to achieve satisfactory results in fertility (90 to 95%) have resulted in the rampant use of AI in poultry. Artificial inseminations also increase production and puberty weight but decrease mortality and uniformity percentage of the birds.

Das *et al.* (2018) stated that the highest estimates were in the first hatch, followed by the third and second hatch. The first hatch showed a significant percent difference with the second hatch in terms of percent fertility and TES basis hatchability, while the other inter-hatch differences were non-significant by the normal deviate test. The present fertility estimates were well comparable to the available reports in RIR chicken (Malago and Baitil-wake, 2009; Das *et al.,* 2014b) and better than the reports in CARI-Sonali and CARI-Debendra chicken (Das *et al.,* 2014b). The present TES basis hatchability estimates were by the report in RIR control and white strains (Das *et al.,* 2014b) and the reviewed range of 60 to 88% in African indigenous chickens (Mengesha, 2012), but also better as evident when compared to the reports in RIR selected strains, CARI-Sonali and CARI-Debendra chickens (Das *et al.,* 2014b). The present FES basis hatchability estimates were comparable to the earlier reports in RIR strains (CARI Annual Report, 2011; Das *et al.*2014b), CARI-Sonali, and CARI-Debendra chickens (Das *et al.,* 2014b). Malago and Baitilwake (2009) reported a hatchability estimate of 64.0 ± 2.16% in an RIR chicken. The difference in the estimates

might be due to a different strain, line, or breed being investigated and a different incubation system being adopted.

The fertility and hatchability are influenced by the breed, nutrition, age, and management of birds. The mean fertility rate was 67.18% in Aseel chickens. The hatchability percentage was low, 44.71% on the total egg set (TES) and 80.87% on the fertile egg set (FES), respectively. High fertility (86.96%) and hatchability (FES: 81.21% and TES: 70.74%) were reported in Aseel birds, which were under selection for improved growth (Mohan *et al.,* 2008). The variations in fertility and hatchability might be due to the differences in the age of the birds and environmental conditions. The low reproductive performance might be because the birds were brought from the field and reared under captive conditions under a new environment, leading to reduced fertility and hatchability among the birds. Fertility and hatchability are major parameters of reproductive performance which are most sensitive to environmental and genetic influences (Stromberg, 1975).

Fertility refers to the percentage of incubated eggs that are fertile, while hatchability is the percentage of fertile eggs that hatch (Sapp *et al.,* 2004). The main effect of varying levels of egg storage temperature (10, 16, and 23°C); fumigation (fumigated and not fumigated); broiler strain, and the interaction effect of these factors on fertility indicated that there was no difference among the treatment groups in fertility. The result indicated that fertility is not dependent on egg storage treatments. The mean value for the fertility of indigenous chickens in the eastern Hararge Zone of Ethiopia showed a higher percentage (91.46) of fertile eggs (Abdurehman and Urge, 2016). The result reported indicates that fertility is not dependent on egg storage treatments employed. The result is expected because fertility mainly depends on the sex ratio of the parent stock, season, flock age, and other factors in the laying room other than storage temperature and fumigation. The result is expected because fertility mainly depends on the sex ratio of the parent stock, season, flock age, and other factors in the laying room other than storage temperature and fumigation. Candling was conducted on days 8 and 15 to detune fertility.

Ahmad *et al.* (2013) studied the hatching performance of Aseel varieties and reported that Lakha, Mianwali, Mushki, and Peshawari varieties of Aseel do not differ based on the percentage of fertility, hatchability, infertile, dead germ, and cracked eggs, whereas

dead in the shell was higher in the Lakha variety of Aseel. It has been reported that Peshawari Aseel has higher hatchability and hatch of fertility in the 1st production cycle followed by the 3rd and 2nd production cycles. Hatchability% and hatch of fertile percent were found to be 53.02, 15.96, 43.18 and 84.20, 39.56, and 58.20%, respectively in the first, second, and third production cycles (Sohail *et al.,* 2013).

Sohail *et al.* (2013) reported that hatchability% increased with increasing age while hatching efficiency remained better in younger birds of Aseel. The fertility rate was reported to be highest in the 3rd production cycle (74.54), followed by the 1st (64.61) and 2nd (41.58) production cycles. Hatchability from set eggs (%) was also highest for Kadaknath, followed by Aseel.

Kamble *et al.* (1996) reported 85% fertility in Indian full-feathered Kadakanath chickens, 78% in Dahlem Red and 66% in Indian Naked Neck and Aseel hen eggs, and the lowest in Frizzle breeds. They also found a similar trend for hatchability, but they observed poor fertility and hatchability among the crosses of those chickens.

Darmstadt *et al.* (2005) reported the significant differences between IFF, Naked Neck, and 'Hilly' type chicken for hatchability. Postembryonic chick mortality did not differ between Naked Neck and IFF flocks, except when exposed to heat stress above 40°C, where survival rates of 51.4% for INN and 38.8% for IFF chickens were reported. The authors reported hatchability values for Naked Neck and normal-feathered chickens to be 87.40% and 86.98%, respectively compared to 48% for Naked Neck chickens and 74% for normal-feathered chickens in this study.

2.4 Sperm storage

The assessment of semen quality characteristics of poultry birds is an excellent indicator of their reproductive potential and has been reported to be a major determinant of fertility and subsequent hatchability of eggs (Peters *et al*., 2004). Fertility and hatchability, on the other hand, are the major determinant of profitability in the hatchery enterprise. Therefore, it is necessary to find a suitable, harmless protocol for rooster spermatozoa handling. For the poultry industry to take advantage of modern AI techniques, proper storage of poultry semen is necessary. As poultry semen is viscous, highly concentrated, and of low volume, containing 6 (chicken) to 12 (turkey) billion spermatozoa/ml, the extension of neat semen with proper diluents is required before AI and storage. Diluents are buffered salt solutions used to extend semen, maintain the

viability of spermatozoa *in vitro*, and maximize the number of hens that can be inseminated. Semen diluents are based on the biochemical composition of chicken and turkey seminal plasma (Lake, 1995). Several factors play a role in maintaining the quality of semen over storage. For example, the diluents used for semen extension and storage conditions such as time, aeration, and holding temperature play a major role (Dumpala *et al*., 2006). There are many diluents available for poultry semen, both published recipes, and commercially available products. Several investigators have compared the composition of various diluents and summarized fertility data across studies (Howarth, 1983; Bakst, 1990; Bootswala and Miles, 1992). What is evident from these reviews is that there are no standard diluents for poultry semen and that studies are so variable in experimental design including time of insemination, vaginal depth, frequency of AI, several spermatozoa inseminated, and duration of fertility analyzed that discerning the benefits of different diluents is difficult (Donoghue and Wishart, 2000). Similarly, it is difficult to compare results reported in the literature involving the long-term liquid storage of poultry semen because of the variation in experimental design (Donoghue and Wishart, 2000). Such variation includes differences in AI schedules, semen dose, storage interval which can range from 6 to 48 hours, and analysis of fertility results reported. One phenomenon that appears to be consistent in results with liquid semen storage is the decline in fertility with semen stored longer than 6 hours after the first 5 to 8 weeks of egg production. Furthermore, when semen is stored for 24 hours or longer *in vitro*, fertility problems are magnified (Donoghue and Wishart, 2000). Sperm motility and the fertilizing ability of undiluted neat fowl semen stored *in vitro* usually decreases within 1 hour of collection (Carter *et al*., 1957). Therefore, to store fowl semen, the type of diluents and storage temperature is very important to avoid a reduction in sperm quality (Dumpala *et al*., 2006). Sperm metabolism is not completely arrested during liquid storage at a reduced temperature; the main changes which occur include an irreversible reduction in motility, morphological integrity, and fertility of spermatozoa (Maxwell and Stojanov, 1996). Semen storage is always accompanied by deterioration in spermatozoa quality and fertility (Thurston, 1995). Some of the detectable features of decreased spermatozoa quality could also be apoptotic-like characteristics. The changes in the plasma membrane permeability and mitochondrial membrane potential constitute an essential stage of apoptosis (Baccetti *et al*., 1996; Blanc-Layrac *et al*., 2000). The most significant changes related to apoptosis are the externalization of the phosphatidylserine

(PS), DNA fragmentation, caspase activation, loss of mitochondrial membrane potential, and increase in sperm membrane permeability (Bratton *et al*., 1997; Glander and Schaller, 1999; Martin *et al*., 2004). Apoptosis detection using YoPro-1 staining was previously tested on somatic cells (Idziorek *et al*., 1995) and later boar (Pexa *et al*., 2005; Trzcinska *et al*., 2008) and bull (Martin *et al*., 2004) spermatozoa and showed to be inexpensive, quick and easy to perform.

Peters *et al*. (2008) observed that the motility of spermatozoa in fresh semen of seven different chicken strains varied from 60 to 90%. We assumed that the surprisingly low sperm motility values in samples with avian diluents could be due to the specific composition of the commercial extender. The diluents might decrease sperm motility to preserve enough energy for the moment when spermatozoa will reach the female reproductive system. Exposing sperm to sub-physiological temperatures, especially in the range of 0–20°C, may cause damage, or "chilling injury" (Watson and Morris, 1987). This may occur immediately upon cooling (direct chilling injury), or as an indirect chilling injury inflicted over an extended storage interval (Arav *et al*., 1996). On the contrary, our experiments showed no negative effects of the low temperature (4°C) and the length of storage (0.5, 1, or 2 hours) on the motility values of rooster spermatozoa independently of the used diluents. However, the total and progressive motility of spermatozoa diluted in saline solution was significantly higher at each storage interval in comparison to avian diluents. For stored semen, Clarke *et al*. (1982) reported that sperm motility from both undiluted and diluted chicken semen is lowest when stored at a higher temperature (41°C), which is near the body temperature of the hen. Also, Slanina *et al*. (2011) reported that the use of a low temperature (4–8°C) for long-term storage of turkey spermatozoa could maintain better motility than higher storage temperatures. This could be due to several factors. One of them may be a low metabolic rate since it is known that every 10 °C reduction in storage temperature halves the metabolic rate of any cell type (Hammerstedt and Andrews, 1997). At low metabolic rates, consumption, and exhaustion of the limited sperm bioenergetics resources (ATP) are reduced. As demonstrated in chicken and turkey sperm, ATP consumption is reduced by 75 to 80% when semen is stored at 5°C compared to 40°C (Wishart, 1984). This would be associated with lower oxygen consumption at low temperatures, as reported in chicken and turkey sperm stored at 5°C compared to 15, 25, or 41°C for 3 or 6 hours (Clarke *et al*., 1982). Better sperm quality after storage at

low temperatures could also be due to lower lipid peroxidative damage at 5 and 10°C compared to 20°C, as reported for turkey semen (Cecil, 1993). One of the reasons for suboptimal spermatozoa quality may also be apoptosis-like changes. In male reproduction, abnormal spermatozoa may be eliminated via apoptosis (Sun *et al*., 1997). Those abnormal or immature spermatozoa and leukocytes are the two main sources of reactive oxygen species (ROS) in semen which are one of the common forms of free radicals. Pathologically, free radicals induce lipid peroxidation, DNA damage, and apoptosis, (Kothari *et al*., 2010). Avian spermatozoa are characterized by high proportions of polyunsaturated fatty acids, which are associated with increased susceptibility to ROS and lipid peroxidation. Recent advances in avian reproduction have focused on the potential of ROS as one of the prime mediators of infertility. Although ROS are involved in many physiological functions of spermatozoa, their excessive production may result in oxidative stress (Khan, 2011). The production of ROS is enhanced during unfavorable environmental and stressful conditions, thus proper handling conditions (type of diluents, storage time and temperature, etc.) are required to maintain the optimal quality of semen. Under fluorescent microscopy, YOPRO-l-fluorescent cells demonstrate the morphological features of cells undergoing apoptosis such as nuclear cell shrinkage and fragmentation. An important characteristic of the dye that differs from all other nuclear dyes previously used for the detection of apoptosis is that it does not label living cells (Idziorek, 1995). In other study observed the effect of diluent type and storage time on the proportion of apoptotic (Yo-PRO-1 positive) and dead (PI positive) cells in the spermatozoa samples. A negative effect of avian diluents and storage time was noticed since the proportion of YoPRO-1+ cells significantly increased after 1 hour of storage at 4°C, and the proportion of PI+ cells also significantly increased after 2 hours in comparison to saline solution. Dump all *et al*. (2006) also observed a slight linear increase in the percentage of dead sperm over storage time for undiluted neat semen, semen diluted with Beltsville Poultry Semen Extender (BPSE) or minimum essential medium (MEM) and stored at 4°C. In contrast, other experiments showed no significant differences in the proportion of Yo-PRO-1+ and PI+ spermatozoa over the storage time for samples diluted in saline solution. Thus, the saline solution seems to better maintain the plasma membrane integrity of the rooster spermatozoa stored for a longer time at a low temperature. The fertilizing ability of diluted and stored spermatozoa in the present study was estimated from the results of intravaginal artificial insemination of hens and was expressed as fertility (i.e.,

percentage of fertile per incubated eggs). Similar fertility values were maintained over the storage time using the saline solution for the spermatozoa dilution. On the other hand, there was great variability in fertility results over the storage time using avian diluents for spermatozoa dilution. At first, the fertility significantly decreased after 1 hour of storage in comparison to the saline solution and then insignificantly increased after 2 hours of storage. Therefore, due to these variable fertility values, could not recommend commercial avian diluents for long-term storage. However, further experiments are required to prove this assumption. Although according to Parker *et al*. (2000), the fertilization potential of the broiler breeder rooster is dependent upon several semen quality characteristics, including sperm concentration, viability, and motility, our results from *in vitro* tests (CASA and apoptosis assay *in situ*) were not correlated with the *in vivo* fertility results (data not shown). In conclusion, the comparison of two different diluents showed that the saline solution maintained the better quality of rooster spermatozoa in terms of their motility values and the proportion of apoptotic and dead cells (*in vitro*) than the commercial avian diluents when stored at 4°C for 2 hours. Although the fertility potential of spermatozoa (*in vivo*) was not correlated with *in vitro* results, the saline solution seems to be suitable for semen dilution and long-term storage at a low temperature, since similar fertility results were obtained over the whole storage time. Thus, the commercial avian diluents might be replaced by the saline solution under certain conditions. Furthermore, the saline solution is cheaper and more accessible for practical use than commercial avian diluents.

2.5 Cryopreservation of semen

Rooster sperm cryopreservation has been successfully demonstrated by various researchers and also shows extremely variable fertility (Lake, 1986; Hammerstedt and Graham, 1992; Buss, 1993; Long *et al.*, 2010). In the United State and Canada, approximately 40% or 238 lines of unique poultry lines have been lost since 1984 (Pisenti, 1999). Relatively more fertility for cryopreserved rooster sperm is desired, and if improved cryopreservation techniques are developed, they can be used to efficiently preserve these valuable poultry genetics and enable sharing of these genetics around the world, through shipping cryopreserved sperm.

2.5.1 Cryopreservation principles

Research over the past half-century has developed various techniques and principles of low-temperature biology to effectively preserve gametes (Lake, 1986; Foote, 1998; Foote, 1999). As stated previously, rooster spermatozoa were frozen at low temperatures (-76°C), using a carbon dioxide-alcohol mixture (Shaffner *et al.*, 1941). Many other research studies have focused on increasing the cryosurvivability of sperm by understanding the cryodamage caused by osmotic stress, intracellular and extracellular ice formation, and plasma membrane responses to cooling throughout the freezing process (Tselutin *et al.*, 1999; Purdy and Graham, 2004a; Squires *et al.*, 2004).

The steps to cryopreservation of rooster spermatozoa are: 1) cooling the sperm to 5°C; 2) adding the cryoprotectants to the sperm, 3) freezing the sperm; 4) storing the sperm in liquid nitrogen; and 5) thawing and diluting the sperm (Buss, 1993). In the first step, fresh sperm is diluted with the diluents immediately after collection (Burrows and Quinn, 1937; Lake *et al.*, 1978; Sasaki *et al.*, 2010). This dilution keeps sperm alive for a long period, and the sperm are then cooled from body temperature (41.5°C) to 5°Cover ice (Mazur, 1984). Temperature-induced changes to the plasma membrane and in this phase transition changes occur to the sperm. During this step, these changes can result in membrane damage (Hammerstedt *et al.*, 1990). This is the point where most membrane damage occurs to the sperm cells, although in rooster sperm, this is not the case since they do not undergo cold shock (Steponkus *et al.*, 1983; Parks and Lynch, 1992). Cold shock is defined as the insult a sperm undergoes when cooled too quickly before freezing (Pickett and Komarek, 1967). During the second step, the cryoprotectants are added to the sperm cells, and the sperm will face osmotic stress as the cryoprotectants create an extracellular hyperosmotic environment and water will move out of the sperm, causing the cell to shrink (Hammerstedt *et al.*, 1990). It is important during this time to make sure that adequate time is given for cryoprotectants equilibration. Cryoprotectants do not cross the membrane as fast as water, and therefore time must be allowed for water and cryoprotectants' equilibration (Amann and Pickett, 1987). Equilibration is completed within 2 (two) minutes, and the sperm is packaged into either 0.25-mL or 0.5-mL polyvinylchloride straws, depending on the method being utilized (Hammerstedt *et al.*, 1976, Lake *et al.*, 1978 and Sasaki *et al.*, 2010). During the third step, the rooster sperm are frozen in liquid nitrogen vapor (Lake *et al.*, 1978). Cooling between -6°C and -15°C is the most critical part of freezing when the

water in the diluents is transitioning to the crystalline state (Mazur and Cole, 1989; Hammerstedt, 1995). Unfrozen channels remain between the ice crystals which contain high salt concentrations and sperm that can tolerate the high salt environment survive in these unfrozen channels. Since the spermatozoa that will survive freezing are all found in these unfrozen channels, the volume is also critical to the survival of the sperm (Amann and Pickett, 1987; Mazur and Cole, 1989). The volume of the sperm cell or other type of cell must be large enough to support cell function intracellularly, but small enough to keep the membrane phospholipids in a bi-layer configuration (Amann and Graham, 1993). Again, high salt concentrations in the unfrozen channels damage the sperm (Quinn, 1989; Hammerstedt, 1990). Cryoprotectants either lower the freezing point of the diluents or increase the volume of the unfrozen fraction and decrease high salt concentrations in this fraction and ultimately alter the formation of ice crystals (Amann and Pickett, 1987). This ultimately increases the cryo survival of sperm residing in this high-salt environment. Once the spermatozoa reach -50°C, the unfrozen fraction and the sperm vitrify and become inert. Therefore, sperm can be plunged into liquid nitrogen after reaching -50°C and can be held in storage at -196°C indefinitely (At fourth step; Graham, 1996). Most methods require rooster sperm to be thawed in 5^0C water before use (Lake *et al.*, 1978; Sasaki *et al.*, 2010). The fifth step is the most critical in the cryopreservation process, due to the drastic changes the sperm will face during dilution (Griffiths *et al.*, 1979). In this step, sperm will face osmotic stress as well as membrane damage. As the sperm is thawed, its membranes will leave their vitrification state, as well as undergo the membrane phase transition to a more fluid state. Here, membrane damage from previous steps will be manifest when the plasma membrane reverts to its normal state (Hammerstedt *et al.*, 1990). After thawing, the sperm would be diluted. This extracellular hypoosmotic environment causes water influx, and the sperm swell (Griffiths *et al.*, 1979). Depending on the cryoprotectant used, water enters the cell beyond the point of membrane volume expansion tolerance (membrane tolerance), and the sperm cell will lyse. This is due to unequal movement of the cryoprotectant and water across the plasma membrane (Amann and Pickett, 1987). Sperm that are improperly diluted, with more water being added to the sperm's extracellular environment, can also cause membrane volume expansion past the tolerance level to the point of lysing.

2.5.2 Osmotic stress

Adding or removing cryoprotectants from the sperm cells induces osmotic gradients across sperm membranes, which cause the sperm to shrink or swell, respectively, which can ultimately irreversibly damage the cell and possibly cause cell death (Blanco *et al.*, 2008). When cryoprotectants are added to the sperm cells, a hyperosmotic environment is created, and at that time water crosses the membrane faster than does the cryoprotectant, water quickly exits from the sperm due to osmosis (Amann and Graham, 1993). As the cryoprotectant reaches equilibrium, water equilibrates with it, and cell volume is restored. When a cryoprotectant-loaded sperm is diluted without glycerol in the diluents or exposed to the female reproductive tract, it is exposed to a hypoosmotic environment. As described above, because cryoprotectants cross the membrane slower than water, water will enter the sperm, causing the sperm to swell. Spermatozoa will return to their normal size once the cryoprotectant leaves the sperm cell (Amann and Graham, 1993). However, if membrane tolerance is exceeded, the sperm plasma membrane will rupture, causing cell lysis. This not only can affect the plasma membrane by irreversible damage of cellular swelling and shrinking but also internal cell organelles may be affected similarly (Willoughby *et al.*, 1996). Rooster sperm has a lower osmotic tolerance limit of 17 mm, but its upper osmotic tolerance limit has not been determined or characterized (Watson *et al.*, 1992). This critical osmolality of 17 mOsm for rooster sperm is defined as the osmolality at which 50% of the cells have swollen to the point of rupture of the plasma membrane. The critical osmolality of sperm varies from species to species, and the ability of sperm to survive these osmotic stresses and volume changes affects their ability to survive freezing (Hammerstedt *et al.*, 1990). Theoretically, the rooster sperm's cylindrical-tapered shape should accommodate larger amounts of cell volume change to a sphere shape without rupturing (Watson *et al.*, 1992). In contrast, it has been observed that a rooster plasma membrane swells over both the head and tail (Bakst, 1980), and when one part of the membrane does not accommodate larger amounts of cell volume, this leads to the plasma membrane bursting sooner than theorized (Watson *et al.*, 1992). Since the upper limit of osmotic tolerance for rooster sperm is unknown, both theories could prove true until this limit is uncovered.

2.5.3 Cryoprotectants

When the sperm are cooled, ice is formed both extracellular and intracellular. Although intracellular ice is a major cause of cell death during freezing for most cells, it does not have the same effect on sperm (Mazur, 1984; Morris *et al.*, 2007). There are two types of cryoprotectants that are used to protect the cells from freezing damage. These are penetrating and non-penetrating (Doebbler, 1966; Meryman, 1971). Non-penetrating cryoprotectants are sugars such as lactose and sucrose, proteins, and polymers such as methylcellulose. These cryoprotectants do not cross the sperm plasma membrane, and therefore increase the extracellular hyperosmotic gradient and dehydrate the cells, limiting the chance of intracellular ice formation (Morris *et al.*, 2007). Penetrating cryoprotectants include glycerol, dimethylacetamide, and dimethyl sulfoxide, which cross the plasma membrane and enter the cell. These penetrating cryoprotectants protect the sperm by displacing intracellular water, thus minimizing the formation of intracellular ice. Furthermore, penetrating cryoprotectants also increase the unfrozen fraction of the solution and reduce the salt concentration in this fraction (Amann and Pickett, 1987). Non-penetrating cryoprotectants are usually used in high concentrations when freezing rooster sperm to lower the freezing point of the diluents and increase the resistance of the plasma membrane to temperature-induced (Phillips *et al.*, 1996). These cryoprotectants release water from the cell and prevent the formation of intracellular ice. However, the mechanisms that occur are not fully understood (Amann and Pickett, 1987). Glycerol is a commonly used cryoprotectant to cryopreserve rooster spermatozoa (Hammerstedt and Graham, 1992; Buss, 1993; Gill *et al.*, 1996; Sasaki *et al.*, 2010). Though glycerol is commonly used for rooster sperm when the sperm are diluted in a glycerol-containing solution, they have motility and membrane integrity that is comparable to unfrozen or fresh rooster sperm, and glycerol also severely reduces sperm fertilizing potential (Tajima *et al.*, 1989; Hammerstedt and Graham, 1992; Phillips *et al.*, 1996). Glycerol has a molecular weight of 92 and it is much larger than water. That is why, crosses the plasma membrane more slowly than water (Gao *et al.*, 1995). During glycerol addition and removal, the sperm cells face osmotic stress that leads to membrane damage by cell shrinkage and swelling (Hammerstedt and Graham, 1992). In contrast, cryoprotectants that are smaller than glycerol crosses the plasma membrane more rapidly than glycerol, and subsequently fewer osmotic excursions occur (Squires *et al.*, 2004; Purdy *et al.*, 2010; Sasaki *et al.*, 2010). These are cryoprotectants having a low molecular weight and cryoprotectants having

molecular weights less than 90 can increase mammalian sperm cryo survival rates and fertility compared to glycerol (Nagase *et al.*, 1972; Hanada and Nagase, 1980; Schmehl *et al.*, 1986; Dalimata and Graham, 1997; Squires *et al.*, 2004). Alternative cryoprotectants have been used to cryopreserve rooster spermatozoa, with limited success (Lake and Ravie, 1984; Mitchell and Buckland, 1975; Westfall and Harris, 1975; Graham *et al.*, 1982; Hammerstedt and Graham, 1992, Phillips *et al.*, 1996). Most alternative cryoprotectants do not protect the rooster sperm at a level equal to that of glycerol during freezing, with a few exceptions. Recently, Sasaki *et al.* (2010) utilized methyl acetamide to cryopreserved rooster sperm and achieved relatively high fertility rates of greater than 80%. Further benefits of the use of alternative cryoprotectants will be discussed further in succeeding sections.

2.5.3.1 Use of glycerol as cryoprotectants

Chicken sperm cryopreservation was practiced in 1941 and chicks were produced using artificial insemination of frozen sperm (Shaffner *et al.*, 1941), but Polge *et al.* (1949) successfully cryopreserved rooster semen in 1949 using glycerol as the cryoprotectants. These cryoprotectants would eventually become the most commonly used cryoprotectants to preserve sperm from all livestock species (Davis *et al.*, 1963; Foote, 1998; Squires *et al.*, 2004; Purdy *et al.*, 2010; Malo, 2011). Glycerol exhibits contraceptive effects on rooster spermatozoa when inseminated into the female reproductive tract, and that is why it must be reduced to a concentration of less than 1% (v:v) before the sperm are artificially inseminated (Lake, 1986; Hammerstedt and Graham, 1992). There are currently three methods used to remove glycerol from the sperm before insemination: 1) gradient centrifugation; 2) dialysis; and 3) stepwise dilutions with centrifugation (Long *et al.*, 2004; Lake, 1986; Tajima *et al.*, 1989; Phillips *et al.*, 1996). All of these methods have a detrimental effect on the rooster spermatozoa and are laborious and cost-intensive, making the implication impractical (Lake *et al.*, 1984; Phillips *et al.*, 1996; Purdy *et al.*, 2009).

2.5.3.2 Other cryoprotectants

Nowadays, the dairy industry has been incorporating artificial insemination using cryopreserved semen for reproduction management for decades since artificial insemination was first introduced in the 1930s (Foote *et al.*, 2002). In contrast, the poultry industry has not been integrated on a large scale using cryopreserved rooster spermatozoa due to the sperm's poor fertility after cryopreservation (Fulton, 2006).

That is why new techniques need to be developed to improve rooster sperm cryopreservation. Moreover, rooster sperm possess a unique shape and membrane composition (membrane-related lipid/protein) that makes these sperm particularly susceptible to cryopreservation damage (Bakst, 1980; Watson, 1990; Parks and Lynch, 1992; Etches, 1996). The cylindrical shaped head and their unique lipid composition, restrict the membranes from undergoing the large volume changes needed to survive cryopreservation efficiently (Parks and Lynch, 1992; Blanco *et al.*, 2008), as the cell experiences different osmotic environments (Amann and Pickett, 1987; Blanco *et al.*, 2008). Before cryopreservation, cryoprotectants are added to sperm cells and when the sperm cells are thawed and diluted in a solution containing no cryoprotectants, isosmotic conditions occur, causing the cells to swell which can damage the membrane (Hammerstedt *et al.*, 1990; Gao *et al.*, 1995). Lower molecular weight cryoprotectants (MW = 92) cross membranes faster than glycerol, equilibrate faster, and thus do not cause as much cell volume change (Hammerstedt and Graham, 1992; Buss, 1993; Gill *et al.*, 1996; Sasaki *et al.*, 2010). Alternative cryoprotectants have been used to cryopreserve stallion sperm, which also have minimum osmotic tolerances, and these alternative cryoprotectants induce minimum osmotic excursions, inducing less cell damage due to volume changes (Squires *et al.*, 2004). As a solution freezes, ice is formed from pure water, with unfrozen channels between the ice crystals, and all the salts in the solution become concentrated in the unfrozen channels. In addition, sperm reside in the unfrozen channels to survive cryopreservation (Mazur and Cole, 1989; Hammerstedt, 1995). Effective cryoprotectants must be non-toxic to sperm cells, protecting against freezing damage. Penetrating cryoprotectants are used widely to cryopreserve rooster sperm. Penetrating cryoprotectants work by increasing the extracellular unfrozen channel volume and decreasing the salt concentration of the unfrozen channels (Amann and Graham, 1993). By using alternative cryoprotectants, limited success was found for cryopreserving rooster sperm (Westfall and Harris, 1975; Mitchell and Buckland, 1976; Graham *et al.*, 1980; Lake and Ravie, 1984; Hammerstedt and Graham, 1992; Phillips *et al.*, 1996). Dimethyl-acetamide (MW=73) has been used and does not need to be removed from the sperm before insemination (Purdy *et al.*, 2009). Rooster sperm cryopreserved with dimethylacetamide does not have high fertility and hens must be inseminated multiple times per week due to the extensive process, which limits its use outside the laboratory (Lake and Ravie, 1984; Lake, 1986; Purdy *et al.*, 2009). Ethylene glycol and dimethylformamide are other

cryoprotectants that have been used to cryopreserve stallion sperm as well as bovine embryos and oocytes (Squires *et al.*, 2004). Both cryoprotectants have lower molecular weights than glycerol (ethylene glycol=MW 62, methyl formamide = MW 59), and they equilibrate across the cell membrane more quickly than glycerol (Hammerstedt and Graham, 1992). That is why, these cryoprotectants have not been studied extensively for cryopreserving rooster sperm (Hammerstedt and Graham, 1992). Frozen sperm with ethylene glycol and dimethylformamide without dilution before insemination have resulted in higher fertility rates than sperm frozen with glycerol as cryoprotectants (Squires *et al.*, 2004). In two recent studies, methyl-acetamide has been used as a cryoprotectant to cryopreserve rooster sperm, and the sperm were inseminated after thawing without removing the cryoprotectant, resulting in 82% fertility and 90% hatchability (Hanzawa *et al.*, 2010; Sasaki *et al.*, 2010). These fertility rates are sufficiently high to be adopted by the commercial poultry industry to allow for the genetic preservation of important genetic lines (Sasaki *et al.*, 2010). Several different cryoprotectants, dimethylacetamide, dimethylformamide, methyl formamide, and ethylene glycol, may protect rooster sperm from cryopreservation damage without having to be removed from the cells before insemination.

Alternative cryoprotectants have been used in cryopreserving spermatozoa from other species to reduce the osmotic stresses the sperm encounter during cryoprotectant addition and dilution (Squires *et al.*, 2004). Cryoprotectants with lower molecular weights than glycerol cross the sperm membranes faster than glycerol does and, therefore, do not induce the amount of osmotic damage that glycerol does, as the length of time sperm is exposed to osmotic imbalances is reduced, as well as the magnitude of the imbalance (Squires *et al.*, 2004). The objective of the last part of this thesis was to determine if utilizing an alternative cryoprotectant (honey) could increase the cryo survival of rooster sperm. Cooling spermatozoa induces a plasma membrane phase transition, causing irreversible membrane damage (Amann and Pickett, 1987). Sperm that contain high levels of cholesterol and/or unsaturated fatty acids do not readily undergo this phase transition and do not exhibit membrane damage (Parks and Lynch, 1992). New technologies that incorporate cholesterol and/or unsaturated fatty acids into the sperm membrane ultimately increase the cryosurvivability of the spermatozoa (Purdy and Graham, 2004b; Moore *et al.*, 2005; Mocé *et al.*, 2010; Spizziri *et al.*, 2010). The environment in which sperm are maintained after ejaculation plays an important

role in their survival (Wishart, 1989). Many different rooster sperm cryopreservation diluents have been developed, and each requires its specialized protocol, utilizing different freezing rates, straw size, and sperm concentration (Lake and Stewart, 1978; Chaudhuri and Lake, 1988; Tajima *et al.*, 1989; Sasaki *et al.*, 2010). The cooling rate has been correlated with sperm membrane damage, as it is partially responsible for the osmotic stresses placed on the spermatozoa (Oldenhof *et al.*, 2012). For a freezing protocol to be effective, the sperm must cool sufficiently slowly enough to allow cryoprotectants and water equilibration to occur (Hammerstedt *et al.*, 1990). This ultimately leads to less membrane damage due to smaller osmotic gradients created as the cryoprotectants equilibrate into the sperm cell (Amann and Pickett, 1987).

2.5.3.3 Structure of rooster sperm

Spermatozoa are haploid cells having highly condensed chromatin and limited capacity for biosynthesis and cell repair. Rooster spermatozoa are homogametic. At the width point, the cells are about 0.5 μm, in length 100 μm, and the volume about 10 μm (Etches, 1996). A sperm has four different regions: head, midpiece, principal piece, and endpiece. Each region of the sperm plays an important role in fertilizing the oocyte. The rooster sperm head contains an acrosomal cap, acrosomal spine, and nucleus. Both the acrosomal cap and acrosomal spine originate from the Golgi apparatus during spermatogenesis, and the acrosome contains the proteolytic enzymes needed during fertilization (Amann and Graham, 1993). The acrosomal cap sits at the top of the acrosomal spine and surrounds it. At the posterior acrosomal region, the nucleus is present, which contains highly condensed chromatin (Barth and Oko, 1989). The shape of the nucleus gives the shape of the sperm. The rooster sperm's nucleus is thin, long, and cylindrical, giving the head of the cell a bullet-like shape (Etches, 1996). The midpiece, principal piece, and endpiece contain the mitochondria and cytoskeleton which are responsible for the motility of the functional spermatozoa (Etches, 1996). The head and midpiece are attached by the proximal centriole, which is tightly fused to the distal centriole. The distal centriole makes up the core of the midpiece and anchors the two central fibers (axoneme) that are part of the endpiece. The axoneme is surrounded by nine tubules that form the axial filament complex. This complex is surrounded by mitochondria, which provide sperm energy metabolism capabilities through ATP production (Etches, 1996). The annulus at the end of the mitochondrial ring discriminates the transition from the caudal midpiece to the principal piece. The

axial filament complex extends into the principal piece, and the rigidity of these fibers is needed for normal spermatozoon tail motion (Amann and Graham, 1993). The axial filament complex contains only the axoneme and plasma membrane, which distinguishes the caudal principal piece from the end piece.

2.5.3.4 Plasma membrane

The outer membrane of the sperm cell is the plasma membrane, and it surrounds the sperm (Barth and Oko, 1989). It consists of a lipid layer made up of polar phospholipids, cholesterol, and proteins, a phospholipid-H_2O interface, the exterior part of the cell, and the glycocalyx, the outer-most region of the plasma membrane (Hammerstedt and Graham, 1992). Individual phospholipids layers are composed of a hydrophilic headpiece and hydrophobic fatty acid chains, with the arrangement of hydrophilic heads on external surfaces and hydrophobic chains on the internal surface of the membrane (Amann and Graham, 1993). The phospholipid-H_2O interface is the primary site for all membrane exchange, while the glycocalyx contains adsorbed proteins, and is the primary site for cell contact recognition (Hammerstedt and Graham, 1992). The rooster sperm membrane is composed of about 50% protein (W/W), considered either intrinsic or extrinsic proteins (Amann and Graham, 1993). Integral proteins are strongly bound with the membrane, with some stretching across the lipid layer. Some integral proteins function as pores, channels, or receptors, depending on their placement with the membrane (Amann and Pickett, 1987). Peripheral proteins have little permanent binding with the membrane (Robertson, 1983), which can be easily removed from the membrane via mild salt or chemical treatments (Amann and Graham, 1993). The outer glycocalyx changes with the different environments, as some proteins are loosely bound by net negative charge attraction to the membrane. These adhered proteins are integral to the glycocalyx's response to its environment (Amann and Graham, 1993). Sperm plasma membranes are composed of heterogeneous membrane domains, and each part of the sperm membrane has a unique lipid and protein composition that is responsible for fertilization (Israelachvili, 1978; Hammerstedt and Graham, 1992). Within every membrane domain, phospholipids are arranged randomly, forming a bi-layer that allows the non-polar region inside of the bi-layer to act as a barrier, hindering the passage of water-soluble molecules. Membrane phospholipids can move laterally in the cell membrane within their bi-layer domain (Amann and Graham, 1993). This lateral movement allows for the random arrangement

of phospholipids at any time. Rooster sperm plasma membranes consist of different phospho-lipid, which are distinct from their mammalian counterparts. They have a lower protein-to-phospho-lipid ratio of 0.46 (w/w), compared to boar (1.26), bull (0.80), and stallion sperm (0.86), and therefore are more resistant to cold shock when reduced to temperatures around 5°C, than are mammalian spermatozoa (Wales *et al.*, 1959; Pickett *et al.*, 1967; Parks and Lynch, 1992). The rooster sperm plasma membrane is stable and inelastic (Roberston, 1984) and membranes can easily rupture when the sperm are exposed to hypotonic conditions. Temperature plays a crucial role in the behavior of the plasma membrane (Mazur, 1984), at body temperature the membrane allows the lipids and proteins to move freely throughout their domains. When membranes are cooled to 5°C, the lipids undergo the phase of transition and become rigid. At the same time, Phospho-lipid and protein rearrangement occurred (Hammerstedt and Graham, 1992). This is very important because phospholipids are associated with specific membrane proteins which dissociate from their associated proteins and make proteins nonfunctional (Amann and Graham, 1993). When the transition occurs in this phase, the membrane becomes rigid, fragile, and more susceptible to damage. In addition, the sperm cells become cooled and lipid/protein rearrangements occurred. Again, when the sperm cells become warmed return to the fluid state but the phospholipids and proteins may not return to their original arrangements and may lead to cell dysfunction and death (Hammerstedt *et al.*, 1990). Membrane fluidity is also partially determined by the cholesterol: phospholipid ratio of the plasma membrane. Sperm membranes with high cholesterol: phospholipid ratios (rabbit 0.88; human 0.99) are responsible for more rigid membranes at room temperature compared to low cholesterol: phospholipid ratios at low temperature. As the sperm cells are cooled during cryopreservation, the high cholesterol content makes the sperm membranes more fluid, and incurred less membrane damage compared to species having low cholesterol: phospholipid ratios e.g., boar: 0.26; bull: 0.45; rooster: 0.30; stallion: 0.23 (Darin-Bennett and White, 1977; Parks and Lynch, 1992). Recently, Morris *et al.* (2011) have found that the combination of intracellular protein content and osmotic shrink causes intracellular vitrification of sperm during cooling and also leads to osmotic shock when sperm is thawed and subsequent membrane damage. When the sperms are exposed to a new outside environment, the membrane faces first and becomes altered negatively and ultimately altering the sperm's intracellular environment and cell dysfunction occurred. But when the sperm plasma membrane is

altered positively, by increasing plasma membrane fluidity at lower temperatures, increases the cell cryosurvivability. So, plasma membrane manipulation and alternative cryoprotectants are the key factors for improving rooster sperm cryosurvivability.

2.6 Cryopreservation of rooster sperm

As mentioned previously, the rooster sperm that were cryopreserved in 1941 was not able to produce live chicks from frozen/thawed rooster sperm before 1949 and succeeded by Polge *et al.*, 1949. This work led to further research on rooster sperm cryopreservation. It was also during that research that glycerol was discovered to be an effective cryoprotective agent, and that cooled rooster spermatozoa exposed to glycerol showed the same high motility as fresh rooster sperm controls (Polge *et al.*, 1949). Smith and Polge, 1950 discovered that cooled rooster sperm exposed to 10 to 15% glycerol did not survive when placed into the hen reproductive tract by artificial insemination though high percentages of motile cells were observed. This contraceptive effect caused by glycerol on rooster sperm has since been well documented (Neville *et al.*, 1971; Westfall and Howarth, 1977; Hammerstedt and Graham, 1992). Polge (1951) developed a dialysis method to reduce the amount of glycerol from the sperm and improved the fertility of the frozen/thawed rooster sperm. Shaffner, (1964) presented a new method for removing glycerol from the sperm by slowly diluting the cells and after that re-concentrating, the sperm by centrifugation resulting in higher fertility rates compared to dialysis, but it was time-consuming and labor intensive. Again, another research stated that the metabolic activity of rooster sperm decreased when glycerol-treated sperm were diluted, centrifuged, and re-suspended in fresh diluents (Clark and Shaffner, 1960). This dilution procedure exposes the rooster sperm to a hypotonic environment and that is why care must be taken to protect the sperm membrane from rupture during osmotic swelling. Sexton, (1981) stated that highly concentrated sperm should be frozen when cryopreserved and during insemination concentrated (500 to 700 million) semen samples are required compared to fresh sperm (50 to 100 million) to achieve similar fertility (Lake, 1986). The high concentration of sperm has a positive effect on sperm cryosurvivability, which must be diluted before insemination in a suitable environment for sperm to survive (Shannon, 1965).

Since 1970, rooster sperm were cryopreserved using glycerol or dimethyl-acetamide as cryoprotectants, and glycerol was removed from sperm samples before artificial insemination (Bacon *et al.*, 1986; Chalah *et al.*, 1999; Woelders *et al.*, 2006; Blesbois

et al., 2007). When glycerol was replaced with dimethyl-acetamide removal of cryoprotectants was not required as the dimethyl-acetamide readily crosses the rooster sperm membrane and high fertility rates were observed when insemination was performed multiple times per week (Lake and Ravie, 1984; Lake, 1986; Purdy *et al.*, 2009). On the other hand, though the use of dimethyl-acetamide showed high fertility rates, dimethyl-acetamide had a toxic effect on sperm and requires time-sensitive procedures. This problem makes this technique impractical for the commercial poultry industry.

Table 2.1: Fertility of poultry semen reported after freezing in different extenders containing different cryoprotectants in different concentrations.

Breed	Extender	Cryoprotectants	Fertility%	References
Rooster	Modified Beltsville Poultry semen extender	Dimethyle sulfoxide (4%, v/v)	38.2±2.5	Ansari *et al*., 2017
Rooster	Modified Extender	DMSO (4%, v/v)	41.6±0.0	Zandani *et al.*, 2017
Rooster	Lake and Ravie mediun	Dimethyleacetamide (DMA, 6% v/v)	10.8±0.0	Abouelezz *et al*., 2015
Rooster	Lake and Ravie mediun	Dimethyleacetamide (DMA, 6% v/v)	33.6±13.4	Santiago-Moreno *et al*., 2011
Rooster	Beltsville extender	Glycerol (2%, v/v)	29.2±2.8	Shahverdi *et al*., 2015
Rooster	Lake and Ravie mediun	Glycerol (11%, v/v)	2.1±0.0	Abouelezz *et al*., 2015
Rooster	Lake and Ravie mediun	Glycerol (8%, v/v)	31.3±0.0	Abouelezz *et al*., 2017
Gander	EK diluent	Dimethyle formamide (DMF, 6% v/v)	83.78±9.5	Lukaszewicz, 2000
Turkey	Lake diluent	DMA (6%, v/v)	8.6±2.11	Long *et al*., 2014
	Lake diluent	Glycerol (11%, v/v)	10.7±2.3	

2.7 Spermatozoa concentration, diluents and straw size on rooster spermatozoa cryopreservation

Cryopreservation is a modern procedure in that sperm permits the transport of unique genetics to the next generation, decreases genetic drift in populations, and allows the establishment of the cryobank, be used to restore lost populations, and produces facilities for insemination at any time (Blesbois, 2007). No step has been taken concerning the poultry industry to take these benefits as cryopreserved rooster sperm possess low fertility and require extensive and expensive methods to achieve that fertility (Lake, 1986; Hammerstedt and Graham, 1992; Buss, 1993; Long, 2010). To improve the cryo survival rate and fertility many methods have been developed for rooster sperm time, but these methods showed limited success (Lake, 1986; Hammerstedt and Graham, 1992; Buss, 1993; Long *et al.*, 2010). To optimize the rooster sperm cryo survival the researchers investigated diluents composition, cooling rate, straw volume and different cryoprotectants (Lake, 1986; Hammerstedt and Graham, 1992; Buss, 1993) and glutamate-based diluents (LLT; Lake *et al.*, 1978) has been one of the most commonly used diluents to freeze rooster sperm and trehalose-based diluents were recently developed to cryopreserve rooster sperm using methyl acetamide, alternative cryoprotectants (Hanawa *et al.*, 2010; Sasaki *et al.*, 2010).

Sperm concentration has physiological and practical effects on sperm cryopreservation and insemination. If sperm are not cryopreserved in a straw, at a sufficiently high sperm concentration using glycerol there will not be sufficient sperm for insemination after thawing, dilution, and centrifugation when using (Sexton, 1981; Lake, 1986) though cryo survival rates are affected due to cryopreservation at high concentrations of sperm cells (Shannon, 1965; Alvarez *et al.*, 2012). When the rooster sperm are frozen using cryoprotectants that do not need to be slowly dilution before insemination and sperm concentrations are recognized as an important factor (Sasaki *et al.*, 2010).

2.8 Sperm quality evaluation methods

Over the past millennia, various methods have been developed to evaluate sperm function *in vitro* and correlate these data with the fertilizing capability of sperm (Williams and Savage, 1927; Moench and Holt, 1931; Graham *et al.*, 1990; Graham and Mocé, 2005). Although the only way to truly assess sperm fertilizing potential is to perform a fertility trial, this assay is often inaccessible due to time or cost constraints.

Therefore, *in vitro* sperm quality evaluation methods have been developed to measure the quality parameters by measuring the spermatozoa for motility, morphology, and cell characteristics by flow cytometry, perivitelline and oocyte membrane binding and penetration assays, and by *in vitro* fertilization (Graham *et al.*, 1990; Bramwell and Howarth, 1992; Carrell, 2000; Graham and Mocé, 2005).

2.8.1 Motility

A sperm should have the normal motility to fertilize an oocyte *in vivo* (Graham, 2001). Not only the motility level is highly correlated with fertility but also many other factors contribute to a sperm's ability for fertilizing an oocyte (Graham *et al.*, 1980). There are two types of motility measurements commonly accessed: 1) total motility; and 2) progressive motility. The percentage of total motility of sperm in a sample is most used to evaluate the quality of rooster spermatozoa. Motility should be assessed at 37°C or may be assessed visually through a high-power microscope or with a Computer Assisted Sperm Analysis (CASA) system (SCA, Hamilton Thorne Motility Analyzer; IVOS, Beverly and MA, etc.). CASA can assess many different parameters including motility, such as straightness and velocity of sperm. Although many of these evaluated parameters are not correlated with fertility. For example, evaluated motility can easily hide the sperms that are dead versus alive. The CASA system assessed relatively huge numbers of spermatozoa in the shortest possible time and it is performed non-biased (Graham and Mocé, 2005). To evaluate spermatozoa using CASA, sperm must be diluted to between 20 and 30 million sperm/ml (Vantman, 1988). At this time of dilution, rooster sperm quickly become immotile, so care must be taken to dilute rooster sperm in diluents containing 1 mg/ml bovine serum albumin (BSA), which mitigates this problem (Harrison *et al.*, 1982; White *et al.*, 1984).

2.8.2 Flow cytometric evaluation

Flow cytometry is a procedure that can evaluate different parameters of individual sperm cells in a sperm population that are not related to each other, e.g., membrane viability, mitochondrial function, and intracellular calcium levels. By simultaneously measuring multiple sperm attributes in a population, the researcher can easily evaluate the spermatozoa which can fertilize, and which are not (Graham, 2001; Gillan *et al.*, 2005; Graham and Mocé, 2005). The flow cytometry procedure includes staining sperm with fluorescent dyes, incubation, and injecting the samples into a chamber through which fluid flows. The fluid flows through a nozzle that breaks the fluid stream into

individual droplets containing one cell. These droplets pass through a laser beam, which excites the dyes in the sperm. Photomultiplier tubes, with filters allowing only specific wavelengths of light to pass them, detect whether each sperm cell has a specific dye(s) (Garner *et al.*, 1986; Graham, 2001; Gillan *et al.*, 2005). The efficiency of the procedure is that the multiple sperm attributes can be measured simultaneously and several thousand cells can be evaluated accurately and precisely in less than a minute (Graham *et al.*, 1990; Gillan *et al.*, 2005) but current rooster spermatozoa cryodiluents contain no egg yolk, milk proteins and other particulates that are approximately similar in size to a sperm, which can create doubt to evaluate sperm through flow cytometry (Lake and Stewart, 1978; Chaudhuri and Lake, 1988; Tajima *et al.*, 1989; Sasaki *et al.*, 2010). Flow cytometry is used to assess sperm's plasma membrane integrity which damages the sperm's plasma membrane and decreases its integrity, and these sperm cells are more likely to be nonfunctional or dead. On the other hand, membrane integrity evaluated using fluorescent dyes, enters the cytoplasm and nucleus of cells that compromised plasma membranes. Propidium iodide of the dyes intercalates into the cell's DNA, binding the nucleic acids and impervious to the intact plasma membranes (Garner *et al.*, 1986, Graham *et al.*, 1990). That is, propidium iodide only entered cells that have plasma membrane damage properties, that bind the DNA and when excited by laser light and emitted a red fluorescence that is greater than 610nm (Grogan and Collins, 1990; Harrison and Vickers, 1990). Although a high correlation (r=0.68) has been reported between the percentages of live stallion sperm and the fertility of inseminated mares when the same samples were used by flow cytometry (Wilhelm *et al.* 1998). A large difference was seen between membrane integrity measurements and observed fertility in the hen, and an accurate measurement for fertilizing capabilities would be to perform a fertility trial (Hammerstedt and Graham, 1992).

2.9 Insemination protocols

Rooster spermatozoa are artificially inseminated through the everted cloaca of the hen, and sperm is placed inside the vagina near the sperm storage tubules. Approximately 50-µL of sperm can be inseminated without having large portions of the inseminate efflux from the hen's reproductive tract (Amann *et al.*, 1997). An insemination dose should contain 100 million fresh sperm or 200 million frozen/thawed sperm (Phillips *et al.*, 1996). Sperm can be inseminated every other day or even multiple times a day, but maximum fertility can be achieved using fresh semen, by inseminating hens once per

week (Amann *et al.*, 1997). Technologies that require inseminating hens more than once a week will preclude them from being used in the industry (Sasaki *et al.*, 2010).

2.10 Effect of glycerol on sperm inside hen's reproductive tract

A small percentage of fertility for rooster sperm was observed by Polge (1951) when sperm were exposed to glycerol. The glycerol was diluted with the sperm and removed before insemination and fertility was measured. Shaffner (1964) reported that glycerol should be slowly removed from the sperm to avoid osmotically induced damage and reduced its contraceptive effect. Shaffner (1964) developed a stepwise dilution method to remove glycerol, followed by centrifugation, to reconcentrate the sperm. A stepwise method to dilute sperm after thawing is required to freeze the rooster sperm at very high concentrations to maintain adequate sperm numbers for artificial insemination (Lake, 1986). This is a time-consuming and laborious process (Hammerstedt and Graham, 1992). When rooster sperm diluted with glycerol are placed into the hen reproductive tract, they are transferred from an environment of 1300 mOsm to one of 300 mOsm which causes immediate sperm cell swelling and possible damage, making the sperm infertile (Hammerstedt *et al.*, 1990). In contrast, when rooster sperm in presence of glycerol are deposited into the hen's shell gland using surgical or intravaginal insemination, less contraceptive effect occurred and fertility is achieved (Allen and Bobr, 1955). This technique is very challenging, time-consuming, and expensive, making it impractical outside of a laboratory setting. For commercial use, rooster spermatozoa must remain viable and fertilize oocytes for at least 7 days after insemination (Amann *et al.*, 1997). Rooster sperm need approximately 72 hours to fertilize a single oocyte as compared to mammalian sperm which makes a negative impact on successful cryopreservation techniques for chickens (Lake, 1986; Hammerstedt and Graham, 1992). On the other hand, the hen's reproductive tract is smaller and narrower and only one ovary has a functional state compared to mammalian species. Side by side the hen's reproductive tract is a single long tube containing an infundibulum, magnum, isthmus, shell gland, and vagina (Jacob *et al.*, 2011). A chicken can store sperm in host glands near the junction of the vagina and the shell gland and sperm are released over a period of 10 to 21 days and allowing for sequential fertilization of ova (Gilbert *et al.*, 1968).

2.11 Fertility trials

To accurately measure rooster spermatozoa fertility, a fertility trial is a must. Although *in vitro* assessment may give the idea about sperm physiology, which is unable to measure the sperm fertilizing ability clearly (Graham, 2005). Fertility measurements for poultry are the ratio of some fertile eggs compared to the number of eggs incubated and the hatchability is calculated as the number of chicks produced compared to the number of fertile eggs (Wilson, 1997). Blackburn *et al.*, 2009 assessed the longevity of the cryopreserved sperm by inseminating once every 18 days in the female reproductive tract, and eggs were collected daily. Chicken eggs were incubated between 37°C to 38°C for 7 days and then candled to determine fertility and embryonic growth (Wilson, 1991). Eggs that do not show embryo development should be cracked to check for early dead embryos (Phillips *et al.*, 1996).

Chapter III

Productive and Reproductive performance of Aseel compared to Naked Neck and Red Junglefowl as the chicken genetic resources of Bangladesh

3.1 Introduction

Now, we are passing an era of modern developments. In various sectors i.e., in the industrial sector, the medical sector, the agricultural sector, and the livestock sector the developments are directed to the peak and some of these developments are beyond our knowledge. The modern high-yielding layers and broilers are parts of the recent development in the poultry world. These recent modern developments are for the welfare of living beings, no doubt here but besides this development, we lost some valuable resources like the purity of breeds and some breeds of animal and poultry are going close to extinction. That is why some brilliant scientists of the recent modern world are paying back their attention to conserving the breeds which are close to extinction and going to save the world from the next mishaps after the cessation of development.

The Aseel breed of chicken is one of the principal ancestors of the "Indian Sub-continent Game bird" originated in Brahmanbaria a district of Bangladesh and is one of the oldest game fowls of Asia, a handsome, sprightly, and shapely birds with upright and majestic gait and bred for its highly valued meat superior in taste and texture. This breed is close to extinction and is presently being used mainly for cockfighting (Bhatti *et al*., 1991; Khan, 2004; Rao and Preetem, 2009; Anonymous, 2009). Aseel is the recognized indigenous poultry breed found in Bangladesh as well as in India and Pakistan and reared in backyard poultry rearing systems in rural areas (Khan, 2004). Aseel is characterized by its aggressive behavior, upright posture, small wattles, pea comb, prominent shoulders, narrow sternum, and hard muscular thighs with strong legs (Dohner, 2001) and is mainly familiar for its fighting tendency. In the last few decades, indigenous chicken has been paid great attention to by researchers all over the world due to its better immunity and adaptability to local climatic conditions (Khan, 2015).
Efforts for the conservation and further improvement of Aseel chicken are still in progress for the revival of the rural poultry farming system (Haunshi *et al*., 2013). But In the Bangladesh context, minimum attention is paid to conserving the Aseel as well as other local chicken resources i.e. Naked Neck, Hilly, Red Junglefowl, etc. Some

breeds that are at risk of becoming extinct or may suffer from inbreeding depression and genetic drift due to increasing global use of high-yielding strains/varieties/breeds of farm animals have been coupled to loss of genetic diversity in most species. So, it is a crying need to take a potential initiative for the conservation of these native chicken genetic resources.

In the Bangladesh context, Sonali chicks are mostly reared for meat purposes up to 65-70 days of age and these birds are used in various social festivals for preparing roasts as an item of a dish. Native chicken meat is favorable for its intense flavor, firm texture, low fat, and rich nutrients (Zhao *et al*., 2007; Chen *et al*., 2008). The Aseel and Naked Neck chicks may be a good replacement for Sonali chicks for the same purpose and the local resources may be saved. Assessing and documenting the meat quality and its composition, especially proximate composition in Aseel is very important in terms of its nutrient composition and consumer interest.

Therefore, these points keeping in mind, the research has been designed with the following objectives.

i) To know the growth performance of Aseel compared to Naked Neck and Red Junglefowl

ii) To know the reproductive performance of Aseel compared to Naked Neck and Red Junglefowl

3.2 Methods and Materials

3.2.1 Experimental site

The study was carried out in Advance Animal Breeding and Nutrition Farm of the Department of Genetics and Animal Breeding at Hajee Mohammad Danesh Science and Technology University, Dinajpur under the project entitled "Conservation and germplasm cryobanking of native chicken genetic resources of Bangladesh" aided by the Grants for Advance Research in Education (GARE), Ministry of Education, Government of the Peoples Republic of Bangladesh.

Photo 3.1: Advance Animal Breeding and Nutrition Farm, HSTU, Dinajpur

3.2.2 Experimental birds

The chicks of different breeds i.e. Aseel, Naked Neck and Red Junglefowl were collected just after hatching (at day old) on the same day and obtained from the incubator situated in the laboratory of the Genetics and Animal Breeding department. Fifty (50) chicks of each breed were collected. The birds have grown after upto the experimental period.

3.2.3 Brooding of chicks

After hatching, the healthy chicks were selected and kept in the brooder at a suitable temperature (35^0C). The feeder, waterer, and other required equipments were also placed before placing the chicks. The brooding activities were done up to the 6^{th} week of age of the chicks.

Table 3.1 Brooding temperature provided to the chicks

Age of the birds (Week)	Temperature confirmed (^{0}C)
1st	35
2nd	32
3rd	29
4th	27
5th	24
6th	21

The next period of rearing 21^{0}C was continued.

3.2.4 Housing of the birds

In the brooding stage, the same brooder was used to rear all the chicks, and the same management also was provided for the chicks. After the brooding period, the birds were kept in different pens but provided the same management. The size of the pen was 28 sq feet (7ft×4ft).

3.2.5 Feeding management

The birds were fed routinely twice a day i.e., feeds were supplied to birds in the morning between 8.00−8.30 AM and in the afternoon between 4.30−5.00 PM. A similar diet was supplied all over the experimental period for the chicks. The broiler starter-ready feed was supplied to the birds. The feed consists of ME-3000- 3100 Kcal/kg, Crude Protein-21-22%, Calcium-0.95%, Phosphorus-0.45%, Crude Fat- 5% and Humidity-12%. The feed ingredients which were used to prepare the feed were Maize, Rice Police, Soybean Meal, Full Fat Soybean, Animal protein, Vitamin-mineral premix, Salt, Toxin binder, Amino acids, Toxin binder, Coccidiostat and antioxidant. Feed was supplied according to Table 3.2.

3.2.6 Water management

The clean and safe water was supplied twice daily i.e., in the morning and afternoon. Water-soluble vitamins were supplied to water from time to time. Some medicines were also used when needed. The amount of water supplied was about double the feed supplied to the birds also shown in Table 3.2.

Table 3.2 The amount of feed and water used with growing age to the bird

Age of the birds (Week)	Amount of Feed consumed (g/ bird/day)	Amount of Water (mi/bird/day)
1	8	15
2	13	25
3	17	35
4	21	40
5	25	50
6	30	60
7	40	80
8	45	90
9	50	100
10	50	100

3.2.7 Immunization

To increase the immunity of the birds' Fowl Pox and RDV vaccines were used with the growing age of the birds. The vaccination schedule is given below with the age of the birds in Table 3.3.

Table 3.3 Vaccination program which was followed with the age of birds

Age of the birds	Name of vaccine	Route of administration	Dose
1-3 day	*BCRDV	Eye drop	One drop in one eye
10 days	Gumboro	Eye drop	One drop in one eye
16th days	*BCRDV	Eye drop	One drop in one eye
21st days	Gumboro	Eye drop	One drop in one eye
35th days	Fowl Pox	Wing web punching	Just touch through punching
9th week	**RDV	***I/M	1 ml/bird

**BCRDV means Baby Chicks Ranikhet Disease Vaccine, **RDV means Ranikhet Disease Vaccine, ***I/M means Intramuscular*

3.2.8 Bio-security measure

A strict bio-security measure was taken to rear the birds. While working personal protection was taken and disinfectants like GPC 8, and TH_4 were used with water in the footbath at the entry path of the room and on the farm. The entry was restricted for unexpected people. The case, the room, and all over the farm were sprayed with the Virkon-S solution from time to time.

3.2.9 Lighting management

In the starting period, 24 hours of light was provided and lessened gradually to normal lighting hours as daylight and in the growing period, no extra light was provided with the natural light to the experimental chicks and during the laying period, 16 hours of light and 8 hours of darkness was confirmed.

3.2.10 The medication of the birds

To overcome the roundworm infestation, deworming was carried out. To prevent mycoplasma infection the antibiotic (Tylorest, a product of Incepta Pharmaceuticals Ltd.) was provided to the experimental birds. The water-soluble vitamins and broad-spectrum antibiotics were administered to the experimental birds according to the veterinarian's suggestion. The medicines used are shown in Table 3.4.

Table 3.4 Medicines used in the experimental bird

Trade name of medicines	Dose	Duration of administration	Route of application	Time of application\
Lysovit	1g/1litre	3 days	With drinking water	Two times in every month
EL plex (B_1, B_2 and B_6)	1ml/1litre	3 days	With drinking water	Two times in every month
ESel (E and Selenium)	1ml/1litre	3 days	With drinking water	One time in every month
Renalytes	1g/1litre	During hot season	With drinking water	During hot season
Renaflox	1g/1litre	3 days	With drinking water	According to physicians' suggestion
Renamox	1g/1litre	3 days	With drinking water	According to physicians' suggestion

3.2.11 Environmental management

3.2.11.1 Temperature management

The birds were kept in the open-sided house. The environmental temperature was almost satisfactory but during the summer season when the temperature became high (more than 25^0C) the ceiling fan was used to make the birds comfortable and

electrolytes (Renalyte) along with lemon juice were used with the drinking water to reduce the heat stress.

3.2.11.2 Humidity control

Over the experimental period, the humidity level was almost satisfactory. So, humidity control was not required. The satisfactory level of humidity for the birds is 60−65% but the observed level of humidity was 55−75% which was almost in the desired level.

3.2.11.3 Ventilation

As the open-sided house was used for the experimental birds, air control was not required. Natural air was allowed to move inside freely so that adequate ventilation was made possible when required.

3.2.12 Postmortem examination

Dead birds were brought to the laboratory and a postmortem was carried out to investigate the actual cause(s) of death. The carcass was subsequently disposed of properly.

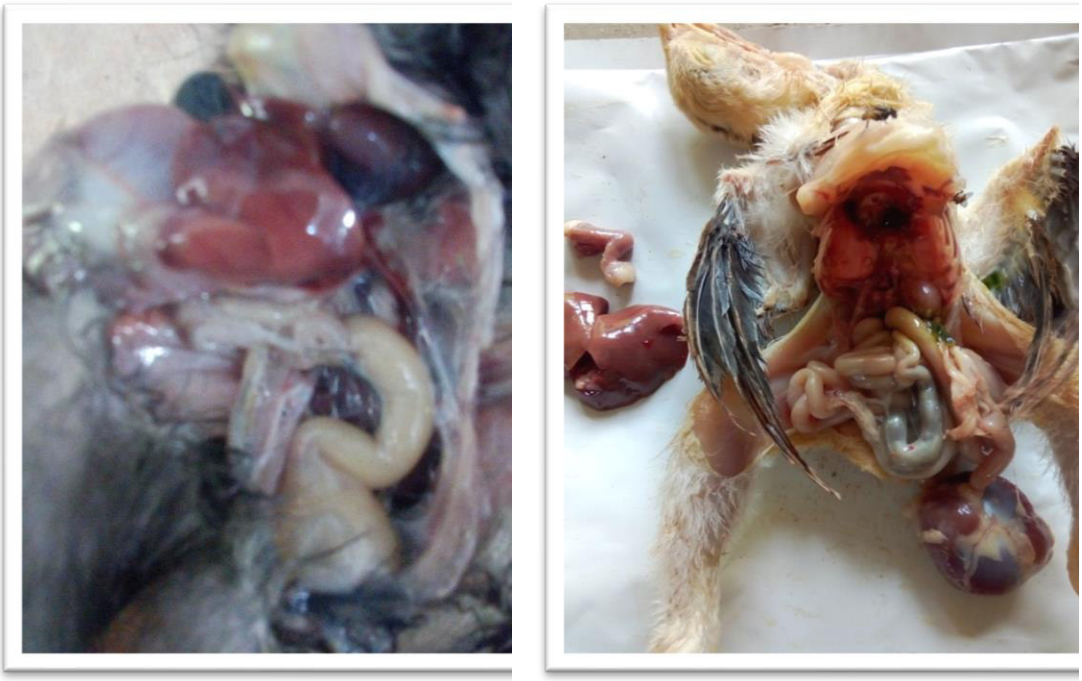

Photo 3.2: Postmortem examination of dead birds

3.2.13 Record keeping

3.2.13.1 Live weight

Live weights were recorded weekly. From the records of live weight, live weight gain was calculated.

$$\text{Live weight Gain} = \textit{F}\text{inal Live weight} - \text{Initial live weight}$$

3.2.13.2 Feed intake

Feed intake is also recorded weekly and then converted to grams per bird per day.

3.2.13.3 Feed conversion ratio

The feed conversion ratio was calculated from the records of feed intake and live weight gain.

$$\text{FCR} = \frac{\text{Feed intake}}{\text{Live weight gain}}$$

3.2.13.4 Egg production

Eggs were collected just after being laid by the birds. An egg laid by the birds was collected from each pen and recorded and finally the number of eggs laid by per bird was calculated.

3.2.13.5 Egg weight

After collection, the eggs were weighed and marked on the shell of the eggs, and records were kept every day. Then eggs were kept in the egg tray and stored in an air-conditioned room.

3.2.13.6 Survivability

Mortality was recorded accordingly, and survivability was calculated from the record book.

3.2.13.7 Fertility of eggs

After first candling infertile and clean eggs were identified and carried out. From the setting eggs, the infertile eggs were deducted, and fertility was calculated.

$$\text{Fertility (\%)} = \frac{\text{No. of fertile eggs}}{\text{No. of eggs set}} \times 100$$

3.2.13.8 Hatchability of eggs

After hatching, the chicks were carried out from the incubator. The healthy and abnormal chicks were sorted first and then counted. The abnormal chicks were then

counted according to abnormal type, and unhatched eggs were checked for dead in shell, late embryonic mortality, or sticky chicks. Then hatchability was calculated.

$$\text{Hatchability \%} = \frac{\text{No. of chicks hatched}}{\text{No. of fertile eggs}} \times 100$$

3.2.14 Calculation of data

The following variables were determined after calculations

i) Egg production per bird
ii) Egg weight
iii) Broken egg percent
iv) Fertility percent
v) Hatchability percent
vi) Dead in shell, early embryonic mortality, late embryonic mortality, etc. in percent
vii) Survivability percent
viii) Day old chick weight

3.2.15 Statistical analysis

The effect of treatment on fertility, hatchability, egg production, egg weight, day old chick weight, embryonic mortality, and normal and abnormal chicks was analyzed using the One-way ANOVA procedure following a Completely Randomized Design (CRD) following the GLM procedure of SPSS computer software 22.00 (SPSS, 2013). The significance of differences among the means of treatments was compared by using Duncan's Multiple Range Test (DMRT) of the same package. All data were expressed as Mean±standard error of Mean (SEM). Significant differences were considered at the level of $P<0.01$ and $P<0.05$. The following linear model summarizes the statistics employed to analyze the data:

$Y_i = \mu + TR_i + E_i$,

Where, Y_i is the dependent variable, μ is the overall mean, TR_i is the treatment effect, and E_i is the error.

3.3 Results

3.3.1 Productive performance

3.3.1.1 Egg production and egg weight

The annual egg production and egg weight of Aseel, Naked Neck, and Red Junglefowl are presented in figure 3.1. The annual egg production number of Aseel, Naked Neck, and Red Junglefowl were 49.00±2.30, 65.00±2.43, and 57.00±3.04 respectively. The annual egg production varied significantly (P<0.01) among the three chicken genetic resources available in Bangladesh. The lowest egg production was observed for Aseel, and the highest number of egg production was observed for Naked Neck among the three genetic resources.

The average egg weight varied significantly (P<0.01) among the three important chicken breeds. The highest egg weight was observed for the Aseel breeds and the lowest egg weight was observed for the Red Junglefowl. The observed egg weights were 43.39±1.23, 38.29±2.41, and 33.21±1.23 g respectively for the Aseel, Naked Neck, and Red Junglefowl.

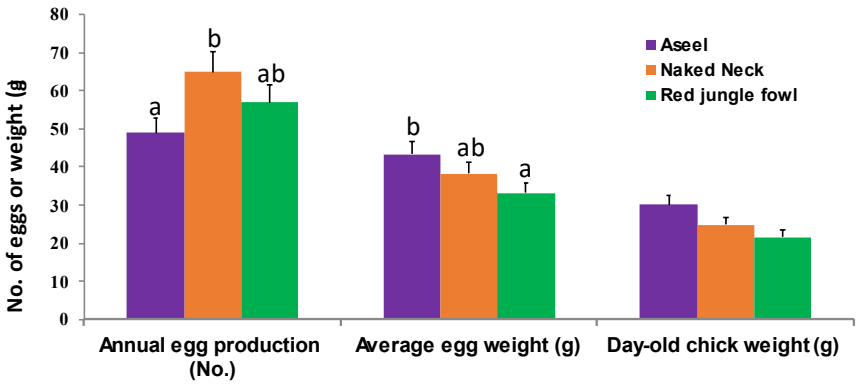

Figure 3.1 Annual egg production, average egg weight, and day-old chicks' weight of Aseel, Naked Neck, and Red Junglefowl genotype. Each bar with the error bar represents the Mean±SEM; different letters indicate statistically significant differences (P<0.05).

3.3.1.2 Live weight

The live weight of three genetic resources of Bangladesh is presented in figure 3.2. The figure showed that the live weight of Aseel chickens was significantly higher (P<0.05) than Naked Neck and Red Junglefowl from the very beginning of the brooding stage. The day old chicks weight of Aseel (30.07±2.02g) was higher than the Naked Neck (21.45±1.30g) and Red Junglefowl (21.3±1.26g). At the end of the brooding stage (6th

week of age) the difference became higher and varied significantly (P<0.01) and the live weights were 460.33±26.07, 369.89±22.59 and 293.00±16.44 g respectively for Aseel, Naked Neck, and Red Junglefowl breeds. At the 10th week of age, these differences stand at 996.44±69.71, 713.00±47.04 and 550.25± 34.02 g and also differed significantly (P<0.01) and at 15th weeks of age, the live weights were 1663.83±113.10, 1056.00±79.48 and 880.00±60.40 g and varied significantly (P<0.01) and at the onset of maturity the differences were increased and varied significantly (P<0.01) the weights at maturity (20th weeks) were 2107.40±138.59, 1577.00±106.16 and 983.00±73.64 g respectively for Aseel, Naked Neck, and Red Junglefowl.

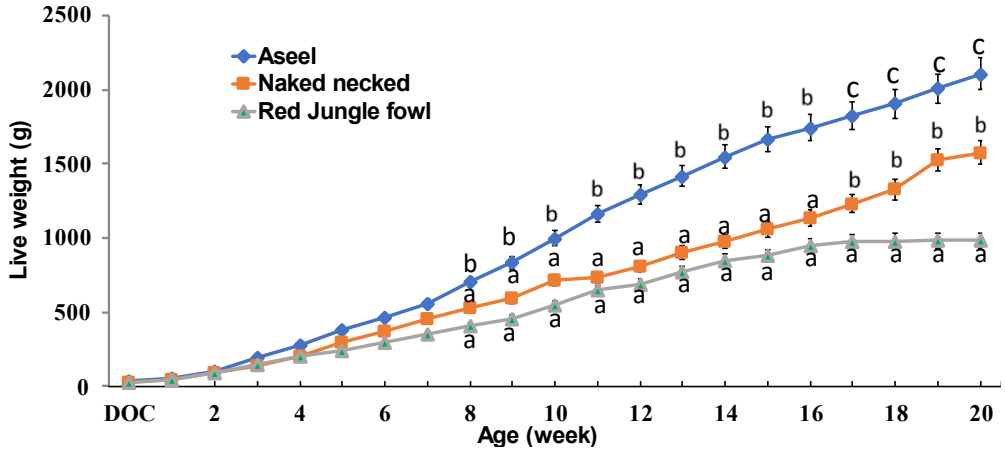

Figure 3.2 Average live weights (up to 20 weeks of age) of Aseel, Naked Neck, and Red Junglefowl genotypes available in Bangladesh. Each line with error bars represents the Mean±SEM; different letters indicate statistically significant differences (P<0.05 or P<0.01).

3.3.1.3 Live weight gain

The live weight gain of three important chicken breeds of Bangladesh are shown in figure 3.3. The figure stated that from the very beginning of chicks live weight gain varied significantly. The trend of live weight gain was significantly (P<0.01 and P<0.05) higher for the Aseel breed except at 6 and 16 weeks of age, compared to the other two chicken breeds available in Bangladesh. Naked Neck chicken showed medium growth trends compared to Aseel and Red Junglefowl and the Red Junglefowl breed showed the slowest trends of live weight gain. From 5-16 weeks of age, the Aseel breed showed a continuous higher live weight gain and ultimately live weight reached higher than the other two breeds.

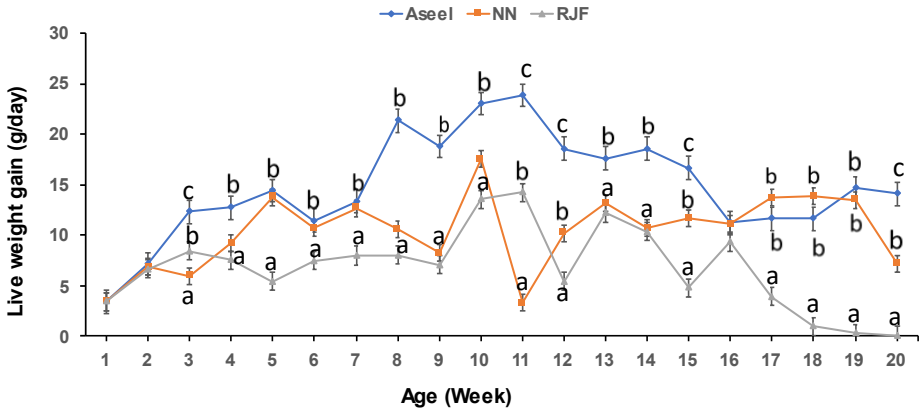

Figure 3.3 Live weight gain (g/day) of Aseel, Naked Neck, and Red Junglefowl genotype of Bangladesh. Each line with error bars represents the Mean±SEM; different letters indicate statistically significant differences (P<0.05 or P<0.01).

3.3.1.4 Feed conversion efficiency (FCE)

The feed conversion efficiency of Aseel, Naked Neck, and Red Junglefowl chicken breeds of Bangladesh are shown in the following figure 3.4. The figure expressed that the feed conversion efficiency was not significant except in the last stage (17-20 weeks (P<0.01) of age.) In the last stage, the live weight gain of Red Junglefowl was static but Aseel and Naked Neck were not. Up to 16 weeks of age, though the feed efficiency was not varied significantly, the feed efficiency of Aseel chickens was better than the other two chicken breeds.

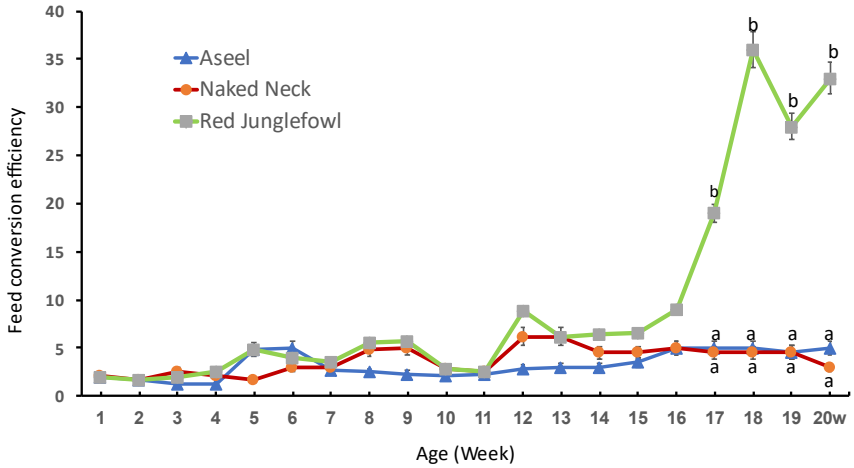

Figure 3.4 Feed conversion Efficiency of Aseel, Naked Neck, and Red Junglefowl genotype of Bangladesh. Each line with error bars represents the Mean±SEM; different letters indicate statistically significant differences (P<0.05 or P<0.01).

3.3.2 Reproductive performance

3.3.2.1 Age at maturity

Age at first egg was presented in the following Table 3.5 for Aseel compared with Naked Neck and Red Junglefowl. The Aseel chicken reached maturity significantly ($P<0.01$) later than the Naken neck and Red Junglefowl. The age at maturity recorded was 215 days for Aseel chickens whereas the age at maturity for Naked Neck and Red Junglefowl was recorded at 135 and 122 days respectively were faster than Aseel chickens (Table 3.5).

3.3.2.2 Weight at maturity

The weight at maturity varied significantly ($P<0.01$) within the chicken genetic resources. The weight at maturity was significantly higher in Aseel chicken compared with the Naked Neck and Red Junglefowl. The weight at maturity was observed at 3274.80 and 2174.70 g for Aseel male and female chicken, 1848.00 and 1205.00 g for Naked Neck male and female chicken, and 1115.50 and 830.00 g for Red Junglefowl male and female chickens respectively. On the other hand, the weight at maturity for male and female chickens also differed significantly among all chicken genetic resources (Table 3.5).

3.3.2.3 Day old chick weight

The day old chicks weight also differed significantly ($P<0.05$) among the chicken genetic resources (Figure 3.1). The day old chicks weight of Aseel (30.03±1.88) chickens was significantly ($P<0.05$) higher compared to the day old chicks weight of the Naked Neck (24.88±1.08) and Red Junglefowl (21.57±0.88). The day old chicks weight was positively correlated with the weight of the eggs. As the weight of eggs laid by Aseel chickens was higher, the day old chicks' weight was also higher and the weight of eggs laid by Naked Neck chickens was medium and the day old chicks weight were medium. On the other hand, the weight of eggs laid by Red Junglefowl was lowest and the chicks weight of Red Junglefowl was lowest (Table 3.5).

Table 3.5 The reproductive traits of Aseel chicken compared with Naked Neck and Red Junglefowl genotype

Parameter	Observed Value			
	Aseel	Naked Neck	Red Junglefowl	Levels of significant
Age at maturity (days)	215.00±13.00[c]	135.00±8.90[b]	122.00±6.10[a]	**
Weight at maturity (g):				
Male	3274.80±190.21[c]	1848.00±95.06[b]	1115. 50±48.05[a]	**
Female	2174.70±121.01[c]	1205.00±82.76[b]	830.00±46.86[a]	**
Fertility (%)	65.68±2.48[a]	80.75±4.67[b]	76.00±3.56[b]	**
Hatchability (%)	75.68±.5.01[b]	56.81±0.48[a]	57.89±0.40[a]	**
Early embryonic mortality (%)	9.00±0.71	6.12±0.24	4.00±0.23	
Late embryonic mortality (%)	4.50±0.23	5.61±0.40	8.00±0.46	
Dead in shell (%)	10.81±0.75	7.08±0.46	8.00±0.24	
Dead chick (%)	1.75±0.08	1.83±0.05	4.00±0.23	

3.3.2.4 Fertility

The fertility percent of eggs laid by Aseel chickens was significantly ($P<0.01$) lower than eggs laid by Naked Neck and Red Junglefowl. The fertility percent of eggs were 65.68±2.48, 80.75±4.67, and 76.00±3.56 respectively for eggs laid by Aseel, Naked Neck, and Red Junglefowl. The result showed that the fertility percentage was higher in the eggs laid by Naked Neck and lowest in the eggs laid by Aseel chickens (Table 3.5).

3.3.2.5 Hatchability

The hatchability percentage was calculated based on fertile eggs. The hatchability percentage of eggs laid by chicken genetic resources varied significantly (P<.01). The hatchability percentage was better in eggs laid by Aseel (75.68±.5.01) chickens compared with the eggs laid by the Naked Neck (56.81±0.48) and Red Junglefowl (57.89±0.40) though the fertility percentage was higher in eggs laid by Naked Neck chickens. The hatchability of eggs laid by Naked Neck chickens and the Red Junglefowl was almost similar (Table 3.5).

3.3.2.6 Other hatchability traits

Early embryonic mortality of eggs laid by Aseel chickens was comparatively higher than other chicken genetic resources but did not differ significantly. The early embryonic mortality was 9.00±0.71%, 6.12±0.24%, and 4.00±0.23% in the case of eggs laid by Aseel, Naked Neck, and Red Junglefowl respectively. The result showed the late embryonic mortality for the eggs laid by Aseel compared with the eggs laid by Naked Neck and Red Junglefowl. The percent of late embryonic mortality was 4.50±0.23, 5.61±0.40, and 8.00±0.46. Dead in the shell of the embryo was higher in Aseel breeds than the other two breeds. Embryos were observed as dead in shell percentages as 10.81±0.75, 7.08±0.46, and 8.00±0.24 respectively in Aseel, Naked Neck, and Red Junglefowl. Dead chicks just after hatching were observed 1.75±0.08, 1.83±0.05, and 4.00±0.23% respectively in Aseel, Naked Neck, and Red Junglefowl and which did not differ significantly (Table 3.5).

3.4 Discussion

3.4.1 Productive performance

3.4.1.1 Egg production and egg weight

The annual egg production number of Aseel, Naked Neck and Red junglefowl chicken breeds varied significantly and recorded egg production stated that the annual egg production was 49.00±2.30, 65.00±2.43, and 57.00±3.04 respectively. Bangladesh Livestock Research Institute (BLRI, 1999) revealed that the egg production number of Aseel and Naked Neck was 33 and 50-55/year respectively (Yoshimura, *et al*., 1997; Sazzad *et al*., 1990; Hoque *et al*., 1975; Ahmed and Islam, 1985; Huque *et al*., 1990) which was lower for the said breed when compared with the present study. The annual egg production didn't vary significantly among the three chicken genetic resources

available in Bangladesh. The lowest egg production was observed for Aseel, and the highest number of egg production was observed for Naked Neck among the three genetic resources.

The average egg weight stated that egg weight varied significantly among the three important chicken breeds. The highest egg weight was observed for the Aseel breeds and the lowest egg weight was observed for the Red Junglefowl. The observed egg weights were 43.39±1.23, 38.29±2.41, and 33.21±1.23 g respectively for the Aseel, Naked Neck, and Red Junglefowl. Hamid (2019) found that the egg weight of Aseel and Naked Neck was 40.69 and 42.00 respectively among them egg weight of Aseel was similar but the egg weight of Naked Neck was higher in the present study. Ahmed and Islam (1985) showed that the egg weight of the Naked Neck was 42 g which was close to the observed value of the current research (Sazzad, 1986).

3.4.1.2 Live weight before maturity

The live weight of Aseel chickens was significantly higher ($P<0.05$) than Naked Neck and Red Junglefowl. The day old chicks weight of Aseel (30.07±2.02g) was higher than the other two chicken breeds (NN 21.45±1.30g and RJF 21.3±1.26g). Miazi *et al*. (2020) reported that the weight of day old chicks was 31.69 and 23.78 g respectively for Aseel and Red jungle Hilly which was like this study. At the end of the brooding stage (6^{th} week of age) the difference became highly significant ($P<0.01$) and the live weight were 460.33±26.07, 369.89±22.59 and 293.00±16.44 g respectively for Aseel, NN and RJF breeds. At the 10^{th} week of age these differences stand at 996.44±69.71, 713.00±47.04 and 550.25± 34.02 g respectively for Aseel, NN, and RJF and differed significantly. Miazi *et al*. (2020) reported weight of Aseel and Red jungle Hilly at 3 months of age were 926.5 and 505.5 g respectively which also support this study. At 15^{th} weeks of age, the live weights were 1663.83±113.10, 1056.00±79.48 and 880.00±60.40 g for Aseel, Naken Neck, and Red Junglefowl genotipes respectively, and varied significantly ($P<0.01$) and at the onset of maturity the differences were increased and varied significantly ($P<0.01$) the weights at maturity (20^{th} weeks) were 2107.40±138.59, 1577.00±106.16 and 983.00±73.64 g respectively. Miazi *et al*. (2020) reported that at the onset of maturity live weight was 1995 and 850 g respectively which was almost similar to this study. Hamid (2019) expressed that the live weight at maturity of Aseel, Naked Neck, and Jungle fowl of 3166±457, 969±132, and 961±150 g respectively and these findings were almost similar for Aseel and Red Junglefowl with an exception of Naked Neck where they showed lower value.

3.4.1.3 Live weight gain

From the very beginning, the chicks live weight gain varied significantly. The trend of live weight gain was significantly higher for the Aseel breed except at 6 and 16 weeks of age compared to the other two chicken breeds available in Bangladesh. NN chicken showed medium growth trends compared to Aseel and RJF and the RJF breed showed the slowest trends of live weight gain. Faruque *et al*. (2017) revealed that the live weight gain of Naked Neck chickens of 3.39, 8.39, and 7.48 g/day at 0-4, 4-20, and 0-20 weeks of age, and these values were lower than the present study. From 5-16 weeks of age, the Aseel breed showed a continuous higher live weight gain and ultimately live weight reached higher than the other two breeds.

3.4.1.4 Feed conversion efficiency (FCE)

The feed conversion efficiency was not significant except in the last stage (17-20 weeks of age.) In the last stage, the live weight gain of Red Junglefowl was static but Aseel and Naked Neck were not. Up to 16 weeks of age, though the feed efficiency was not varied significantly, the feed efficiency of Aseel chickens was better than the other two chicken breeds. Faruque *et al*. (2017) stated the feed conversion ratio of Naked Neck chickens as 3.56, 5.40, and 5.15 at 0-4, 4-20, and 0-20 weeks of age which were almost like this study.

3.4.2 Reproductive performance

3.4.2.1 Age at maturity

The age at maturity recorded 215 days for Aseel chickens whereas the age at maturity for Naked Neck and Red Junglefowl was recorded as 135 and 122 days respectively. Miazi *et al.* (2020) reported that the age at sexual maturity for Aseel was 210, and which was almost similar to this present study, but in the case of Red jungle Hilly, they reported 180 days which differed from the present study (122 days).

3.4.2.2 Weight at maturity

The weight at maturity varied significantly within the chicken's genetic resources. The weight at maturity was significantly higher in Aseel chicken compared with the Naked Neck and Red Junglefowl. At maturity, Aseel males and females weighed 3274.80±190.21 and 2174.70±121.01 g, Naked Neck males and females weighed 1848.00±95.0 and 1205.00±82.7 g Red Junglefowl males and females weighed 1115. 50±48.0 and 830.00±46.86 g, respectively. Miazi *et al*. (2020) reported that the weight

of mature Aseel males and females were 4000 and 2200 g respectively, where they reported increasing trend of male weights than the present study. It might be due to the later stage of maturity. The Aseel female weights were almost similar to the previous study. On the other hand, Red Jungle Hilly weighed 900 and 850 g respectively and male weight was reported lower than the present study and female weight was almost similar. Hamid (2019) reported that the adult weight of Aseel, Naked Neck, and red jungle were 3749±83,83 (male) and 2062±105.26 (female); 1500-2000 (male) and 1300-1500 (female); 2500-3000 (male) and 1800-2000 (female) respectively and the findings were close to the output of the present study. Jahan *et al*. (2017) stated that the weight at sexual maturity of Naked Neck was 782.85±31.56 g which was lower than the current findings. BLRI (1999) showed the live weight of Naked Neck and Aseel as 1171 g and 1700-4500 g respectively and almost similar results were obtained from other study (Okada *et al*., 1988; Huque and Asaduzzaman 1990).

3.4.2.3 Weight of day old chicks

The day old chicks weight (30.03±1.88) of Aseel chickens was significantly higher compared with the day old chicks weight of Naked Neck (24.88±1.08) and Red Junglefowl (21.57±0.88). Miazi *et al*. (2020) revealed that the weight of day old chicks was 31.69 and 23.78 g respectively for Aseel and Red jungle Hilly which were like this study. Singh *et al*. (1999) stated the weights at day old chicks for Aseel (33±0.30gm) and Naked Neck (34±0.36gm) chicks under farm conditions, which were also similar for Aseel chicks and higher in the case of Naked Neck chicks. Haunshi *et al*. (2011) also reported a similar day old chicks weight (33.19±0.20 g) for Aseel chicks. Faruque *et al*. (2010) also reported the day old chicks weight for a Naked Neck of 29.49 g which was slightly higher than this study, and Jahan *et al*. (2017) reported the day old chicks weight of 24.78±0.29 g which was similar to the present study. The day old chicks weight was positively correlated with the weight of the eggs. The weight of the eggs of Aseel chickens was higher and produced higher weighed day old chick. On the other hand, the weight of eggs of Red Junglefowl was lower and they produced lower-weighed day old chicks. Similarly, medium weight day old chicks were obtained from Naked Necks due to the eggs with medium weight.

3.4.2.4 Fertility

The fertility percent of eggs laid by Aseel chickens was significantly lower than eggs laid by Naked Neck and Red Junglefowl. The fertility percentage of eggs laid by Aseel, Naked Neck, and Red Junglefowl was found to be 65.68±2.48, 80.75±4.67, and

76.00±3.56, respectively. The result showed that the fertility percentage was higher in the eggs laid by Naked Neck and lowest in the eggs laid by Aseel chickens. Hamid (2019) got the fertility percent for the Naked Neck 81.93-88.09%, which was almost like this study. Faruque *et al*. (2017) stated the hatchability percent for eggs laid by a Naked Neck was 54.52% and this value was lower than the present study. Pramanik *et al*. (2020) also showed the fertility for the overweight and underweight broiler parent stock of 77.50 and 80.76 and that was similar to the present study.

3.4.2.5 Hatchability

The hatchability percent was calculated based on fertile eggs. The hatchability percentage of eggs laid by the chicken's genetic resources varied significantly. The hatchability percent was better in eggs laid by Aseel (75.68±5.01) chickens compared with the eggs laid by the Naked Neck (56.81±0.48) and Red Junglefowl (57.89±0.40) though the fertility percent was higher in eggs laid by Naked Neck chickens. The hatchability of eggs laid by Naked Neck chickens and the Red Junglefowl was almost similar. The hatchability of the Naked Neck observed by Hamid (2019) was 71.68±9.59 and 68.99±1.49% respectively which were like this study, and the hatchability percent of the Naked Neck obtained by Faruque *et al*. (2017) was 75.62% and which was higher than this study. Pramanik *et al*. (2020) showed the hatchability percent for broiler parents of 79.30–81.60 which was almost like the hatchability percent of Aseel chickens of the present study.

3.4.2.6 Other hatchability traits

Early embryonic mortality showed by eggs laid by Aseel chickens was comparatively higher but did not differ significantly. The early embryonic mortality was observed at 9.00±0.71%, 6.12±0.24%, and 4.00±0.23% in the case of eggs laid by Aseel, Naked Neck, and Red Junglefowl respectively. The late embryonic mortality for the eggs laid by Aseel was better compared with the eggs laid by Naked Neck and Red Junglefowl respectively. The rate of late embryonic mortality was 4.50±0.23, 5.61±0.40, and 8.00±0.46, respectively, and did not differ significantly. Faruque *et al*. (2017) stated that 27.59% of the embryo was dead in germ conditions which was much higher than this study. Dead in the shell of the embryo was worst in nature for Aseel breeds than the other two breeds and did not differ significantly and were almost similar. Dead in shell percentages observed were 10.81±0.75, 7.08±0.46, and 8.00±0.24, respectively.

Dead chicks just after hatching were observed as 1.75±0.08, 1.83±0.05, and 4.00±0.23% and did not differ significantly. Pramanik *et al*. (2020) observed the dead in shell, sticky chicks, and other abnormal chicks of 5.49, 7.14, and 8.93 which were somehow like this study.

3.5 Conclusion

The production and reproduction performance of Aseel was significantly better compared with Naked Neck and Red Junglefowl chickens. The yearly egg production performance of Aseel (49.00±2.30) was lower than Naked Neck (65.00±2.43) and Red Junglefowl (57.00±3.04); egg weights were higher in Aseel (43.39±1.23 g) compared to Naked Neck (38.29±2.41 g) and Red Junglefowl (33.21±1.23 g); day old chicks weights was also higher in Aseel (30.03±1.88 g) compared to Naked Neck (24.88±1.08 g) and Red Jungle fowl (21.57±0.88 g); Aseel chicken got maturity at a later stage (215 days old) than Naked Neck (135 days old) and Red Jungle fowl (122 days old) respectively. Weight at maturity was better in Aseel male (3274.80±190.21 g) and female (2174.70±121.01 g) whereas Naked Neck male and female weighed 1848.00±95.0 and 1205.00±82.7 g and Red Jungle male and female weighed 1115.50±48.0 and 830.00±46.86 g at maturity. Fertility, hatchability, and related traits were satisfactory for all the breeds and did not differ significantly in maximum cases.

Chapter IV

Sperm Motility and Bio-Kinetic parameters of Aseel chicken

4.1 Introduction

The word Aseel is from Arabic, and it means "pure" or "thoroughbred". The Aseel chicken breed has some advantages as compared to other indigenous chicken breeds. The Aseel is an important native chicken breed in Bangladesh along with the available six native chickens (Aseel, Hilly, Naked Neck, Non-descript deshi, and native dwarf type). This breed of chicken is one of the principal ancestors of the "Indian Sub-continent Game bird" which originated in Brahmanbaria, a District of Bangladesh. It is one of the oldest game fowls of Asia, a handsome, sprightly, and shapely bird with an upright and majestic gait and bred for its highly valued meat superior in taste and texture. The Aseel breed is known for its stamina, pugnacity, majestic gait, and dogged fighting qualities (Jabbar *et al*., 2015; Panda and Mahapatra, 1989). The Aseel chickens are also found in other countries of the Indian subcontinent e.g., India, Pakistan, and Srilanka. This breed is characterized by its hardiness and ability to thrive under adverse climatic conditions, and its meat is considered to have a desirable taste and flavor. Its immune system is far superior to that of other native breeds (Jatoi *et al*., 2014) and its eggs and meats are high in protein, iron, and amino acids (Mohan *et al*., 2008). Aseel breed is facing the problems of poor growth rate, late maturity, less persistence and number of egg production, broodiness, and low fertility and hatchability rates (Amjad *et al*., 2012) and these breeds are under threat of extinction (Singh, 2009).

Semen evaluations by macroscopic and microscopic methods are the tool to evaluate male fertility in roosters (Peters *et al*., 2004). Macroscopic parameters like color, consistency, appearance score, and volume and microscopic traits like concentration, initial motility, abnormal sperms, and percentage of dead sperms are used in semen evaluation (Moce and Graham, 2008). Among all, initial motility is considered the single reliable characteristic of semen for identifying the fertilizing ability of roosters (Charms, 1969). The semen characteristics of avian species are different to a greater degree from that of mammals owing to the diverse physiology and anatomy like the intra-abdominal placement of testes and absence of accessory sex glands (Etches, 1996). Moreover, the sperms of chickens are morphologically different from those of mammals (Etches, 1996) and very fragile to high environmental temperature and

temperature fluctuations at cryopreservation (Graham, 1978). The semen of avian species is highly concentrated with very minimal seminal plasma (Etches, 1996). The semen quality in roosters was found to be varied among different genotypes like breed (Peters *et al*., 2008), strain (Murugesan *et al*., 2013), and line (Tarif *et al*., 2013). The age of roosters is also found to interact with semen quality parameters (Kelso *et al*., 1996). The environmental factors that influence semen quality are climate (Saeed and Al-Soudi, 1975), time of collection (Egbunike and Oluyemi, 1979), frequency of collection (Riaz *et al*., 2004), and nutrition (Kabir *et al*., 2007b). The literature provides little information on the semen quality of Rhode Island Red (RIR) roosters and especially there is a total paucity of semen attributes of older RIR roosters. The individual sperm motility of indigenous roosters was found to have a significant positive correlation with live sperm, sperm concentration, and sperm output in local chickens (Bah *et al*., 2001). The interrelationship of semen quality parameters has been reported for broiler breeders (Modupe *et al*., 2013), indigenous (Hu *et al*., 2013), hybrid (Chauhan *et al.,* 2012), and a pool of several breeds (Peters *et al*., 2008), but little has been reported for RIR breed.

The reproductive potential of a breed or strain of chicken is assessed by the quality of semen. The semen quality and quantity are affected by the breed and strain of chicken (Peters *et al*., 2008; Prieto *et al*., 2011; Shanmugam *et al*., 2012). Genetic selection for higher egg production affects semen quality (Shanmugam *et al*., 2013). Initial motility is considered the single reliable characteristic of semen for identifying the fertility of roosters, but other attributes like viability, membrane, and acrosome integrity also contribute to the fertility of semen. The semen quality parameters reported for White Leghorn (Tarif *et al*., 2013), Plymouth Rock (Elagib *et al*., 2012), RIR (Kabir *et al*., 2007), and indigenous Roosters (Hu *et al*., 2013), Synthetic, White Rock and Assel RIR lines (Tarif *et al*., 2013), Rhode Island Red roosters (Churchil *et al*., 2014), Lingnan, Bangkok, Kedu and Arabic (Almahdi *et al*., 2014), Nigerian indigenous (NI) and Isa White (IW) chickens (Mkpughe *et al*., 2015), Vanaraja and Indigenous Chicken of Assam (Das *et al*., 2015), local Iraq breeds and ISA brown crosses (Hermiz *et al*., 2016) demonstrated a high degree of variation. Aseel and Kadaknath are two important indigenous chicken breeds of the Indian subcontinent. Aseel is a meat-type game bird with brown feathers and long shanks. There is a significant demand for the products of native chickens like Aseel. Literature reveals

that a considerable variation exists in the production traits of these native chicken breeds. It was pointed out that investigations are still required to establish baseline values for the production parameters of these breeds and characterize their performance. These breeds differ in various growth, production, egg, and semen quality traits (Huanshi *et al*., 2011). Aseel breed with higher body weights had higher semen volume and sperm motility but had lower seminal plasma cholesterol as compared to other local chickens. There is a scarcity of information on semen attributes, inter-relationship among the sperm parameters, and the fertility of Aseel Roosters. Therefore, these points keeping in mind, the research proposal has been designed with the following objectives

i) To determine the macroscopic characteristics and pH of semen and

ii) To determine the motility and bio-kinetic parameters of semen obtained from Aseel cock

4.2 Methods and Materials

4. 2.1 Study location and duration

The research work was conducted at Advanced Avian Research Farm, Hajee Mohammad Danesh Science and Technology University, Dinajpur. The duration of the study was six months.

4.2.2 Experimental birds

The mature Aseel chickens were selected from the birds available in Advanced Animal Research Farm and reared from the early growing stage to matured stage. A total of 20 male chickens were selected for this purpose.

Photo 4.1: Experimental Aseel cocks for semen collection

4.2.3 General management

The Aseel chickens (cocks) were reared in deep litter pens and demarcated all birds. They were maintained in a semi-intensive system under standard management practices following the existing rules and regulations of the Bangladesh Veterinary Council regarding animal care and management. All birds were treated equally in all respects.

A similar diet was supplied to all birds. Feed and water were supplied in plastic feeders and drinkers. Throughout the experimental period, *ad libitum* clean drinking water was made available all day by using hanging drinkers. Rice husk was used as litter materials. Each cock was marked with permanent markers for proper identification. Both natural and mechanical procedures were followed to maintain proper ventilation and illumination in the chickens' houses. The average environmental temperature was between 21−35°C and the light and dark pattern was 16 h light and 8 h dark. During the summer season, when the environmental temperature was too hot, lemon juice, and electrolytes (Renalytes, a Renata ltd. Product) were used with the drinking water, and water was changed frequently with the cool water. A breeder diet was supplied for the experimental cocks. The diet was made with the feed ingredients maize, rice polish, soybean meal, animal protein, vitamin-mineral premix, amino acid, salt, toxin binder, and antioxidant where the ingredient composition was not expressed due to the company's business secrecy. The nutrient composition of the diet is presented in Table 4.1.

Table 4.1 Nutrient composition of experimental diet

Nutrients	**Amount (%)**
DM (%)	88.00
Crude protein (%)	21.00
Calcium (%)	2.00
Phosphorus (%)	0.45
Crude Fat (%)	5.00
ME (Kcal/kg)	3000−3100

Source: Aftab Bahumukhi Farms Ltd. Bangladesh

4.2.4 Training of cocks for semen collection

At the age of twenty-eight weeks, all the cocks were trained for semen collection by using the abdominal massage technique as described by Burrows and Quinn (1937). The training was performed twice a week until the experiment started. By thirty-two weeks of age, all the cocks had become almost equally ready for semen collection.

Photo 4.2: Training of cocks for semen collection

4.2.5 Semen collection

From the matured, Aseel cock semen was collected through massaging method. A special type of wooden chair was made with keeping facilities of locking two legs of cocks: and comfortable sitting and massaging options for the semen collector. The main goal of the semen collection procedure was to obtain the maximum amount of clean high-quality semen with minimum handling and stress. The testes located at the dorsum were stroked and massaged gently until the protrusion of the cloaca. The researcher himself collected semen every week at the same time, and under the same conditions to minimize stress and maximize the quality of semen. In the cooler period of daytime i.e. in the morning (8.00–9.00 A.M.) the collection was performed and the collection was carried out twice a week. However, the following steps were performed for semen collection:

i. At first, cocks were taken gently on the lap of the collector and two legs of cocks were locked on the arm of the chair
ii. To start massaging the left hand is placed on the back of the tail and the right hand on the ventral part of the tail or rear part of the abdomen
iii. The cock was stimulated by stroking the abdomen and pushing the tail upward and toward the bird's head with the right hand
iv. The cocks responded and the copulatory organ enlarged and partially protruded from the vent. If the copulatory organ had not protruded, it seemed that the cock was probably not sexually responsive and needs more time and massage

v. When cock responded and the copulatory organ was exposed, the left hand was placed at the cloaca with the forefingers. At the same time, the right hand was used to provide inward and upward pressure beneath the cloaca
vi. When semen was ejaculated, it was then squeezed out by a short, sliding, downward movement of the left hand and the upward pressure of the right hand. Care was taken so that the copulatory organ was not touched and harmed during collection. Individual ejaculates were collected into 1 ml microtubes
vii. After each collection, the ejaculates were examined visually as well as microscopically. Special care was taken to avoid contamination of semen with feces, urates, and transparent fluid, which lower semen quality
viii. Just after the collection of semen the macroscopic parameters were observed and recorded and transported to the laboratory for the next analysis using a CASA analyzer

4.2.6 Semen dilution

The collected semen was diluted using previously made diluents which are also known as modified Ringer's solution. Before selecting the dilution ratio for CASA analysis using Ringer's solution, the practice was done previously and the dilution ratio was selected at a ratio of 1:20, which ratio was given better results for analysis. This ratio (1:20) was used during the final analysis using CASA. For this study, semen diluents were prepared by Akcay *et al.* (2006). The composition of the diluents is given in Table 4.2.

Table 4.2 Composition of the semen diluents

Ingredients	**Amount**
Sodium chloride (g)	9.50
Potassium chloride (g)	0.20
Calcium chloride (g)	0.26
Sodium bicarbonate (g)	0.20
Distilled water (ltr.)	1.00
Glucose (g)	1.00

Source: Akcay *et al*., 2006

4.2.7 Macroscopic analysis

Just after the collection of the semen sample the macroscopic parameters such as semen volume, and color was observed and transported to the laboratory spending minimum time and pH was measured using a pH meter. The observed values were recorded as well. Then another analysis was performed.

4.2.8 Motility analysis

A new, fresh, clean Liza slide was taken, and then 0.5µl diluted semen was taken in a clean and disinfected micropipette and carefully placed on the slide within the boundaries of the loading area. The process was done so carefully that the coverslip gets no touch by the hand so that no premature filled occurred. The piston of the micropipette was pushed with such care that the chamber was filled well by the capillary force and the loading area was filled well. The chamber was filled within 3−20 seconds as the viscosity of the samples was all right. The excess semen samples were taken away with the help of cotton bud tips to prevent the floating of cells. After resting for 5 seconds the analysis was done promptly preventing evaporation.

The motility analysis was done using the green filter, the condenser was selected for chicken (animal species) i.e. Ph-1, and 10X negative phase contrast was used for contrasting the object of the microscope. Then select the program SCA Motility, the motility was measured using Computer Assisted Semen Analyzer (CASA).

Photo 4.3: Motility Analysis using CASA

4.2.9 Statistical analysis

Effect of treatment on sperm concentration, motility, progressiveness, velocity, linearity index, lateral head displacement and beat cross frequency was analyzed using the One-way ANOVA procedure by a Completely Randomized Design (CRD) following the GLM procedure of SPSS computer software 22.00 (SPSS, 2013). The significance of differences among the means of treatments was compared by using Duncan's Multiple Range Test (DMRT) of the same package. All data were expressed as Mean ± standard error of Mean (SEM). Significant differences were considered at the level of $P<0.01$ and $P<0.05$. The following linear model summarizes the statistics employed to analyze the data:

$$Y_i = \mu + TR_i + E_i,$$

Where; Y_i is the dependent variable, μ is the overall mean, TR_i is the treatment effect, and E_i is the error.

4.3 Results

4.3.1 Semen color, volume, and pH

The semen color was creamy or milky white and showed a normal appearance. First of all, the color indicated a normal appearance because there was no abnormal color or no blood observed with the ejaculated semen. The foreign particles were not present with the collected semen (Table 4.3).

Semen was normally collected in the cooler part of the daytime i.e. in the morning (8.00–9.00 A.M.). Ejaculated semen volume was 0.50 ml., and this is the normal volume for a matured and healthy cocks. As the semen was collected in a measuring plastic micro-tube, it was measured as soon as collected and observed normal volume (Table 4.3).

The semen is transported to the laboratory shortly after collection, where the pH was measured and found to be 7.15. The pH value also showed normal. The Aseel cock's semen showed normal color, volume, and pH (Table 4.3).

Table 4.3 Semen quality of Aseel cock

Parameter recorded	**Observed Value**
Semen volume/ejaculation (ml)	0.50
Semen color	Creamy White
pH of semen	7.15

4.3.2 Concentration of semen

Semen concentration is a very important characteristic of a cock. Just after collection, semen was transported to the laboratory and semen concentration along with another bio-kinetic parameter of semen quality was measured using a Computer-assisted semen analyzer (CASA). The semen concentration was 5.18×10^9/ml which also indicated the normal for a cock (Table 4.4).

Table 4.4 Concentration and motility of semen obtained from Aseel cocks

Characters	**Observed value**
Concentration (M/mL)	5.18±1.44
Motility (%)	67.08±3.50

Values are expressed as Mean±SEM

4.3.3 Motility

Figure 4.1 (a) and (b) represent the motility of sperm obtained by the Aseel rooster. Semen motility was observed at 67.08±3.50% (Figure 4.1a) among which 2.83±0.12% were rapidly motile, 11.90±0.59% were medium motile and 52.35±1.49% were slow motile (Table 4.5) that is 32.92±1.63% of sperm showed the immotile.

Progressiveness is an important character of sperm, indicating the good quality of semen. Among the 67.08±3.50% of motile sperm obtained from Aseel cock, 5.8% were progressively motile and 61.23±3.89% were also motile but not progressive (Figure 4.1a). Again, among the progressively motile sperm 0.66±0.25% was rapidly progressive and 5.19±0.25% was medium progressive (Table 4.5).

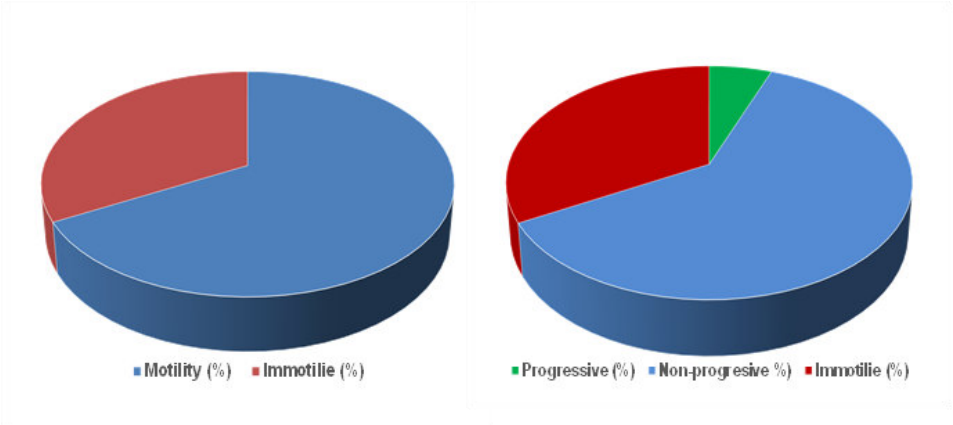

Figure 4.1 Motility and progressive motility of sperm obtained from Aseel rooster.

Table 4.5 Sperm motility and progressive motility obtained from Aseel cocks.

Characters	Motility (%)	Progressivity (%)
Rapid	2.83±0.12	0.66±0.25
Medium	11.90±0.59	5.19±0.25
Slow	52.35±1.49	61.23±3.89

Values are expressed as Mean±SEM

4.3.4 Curvy linear, Straight-line, and Average path velocity

Figure 4.2 represents the curvilinear, straight line, and average path velocity of sperm obtained from the Aseel rooster. The average curvilinear velocity of Aseel sperm was 45.06±2.60µm/s. Among them there were some sperm have slowed in nature having a velocity of 33.88±1.27 µm/s, some sperm were medium in nature having a velocity of 102.75±7.22 µm/s and some sperm were rapid in nature having a velocity of 131.92±9.55 µm/s.

The average straight-line velocity was 16.86±1.03 µm/s. Like curvilinear velocity some sperm were slow in nature, some were medium in nature and some of them were rapid in nature and the speed were 13.42±1.03, 33.76±2.07 and 51.68±2.41 µm/s respectively.

Like the above traits it was also slow, medium, and rapid in nature. The average value of this trait was 27.35±1.28 µm/s, and slow, medium, and rapid speeds of average path velocity were 21.68±1.37, 56.97±3.55 and 67.54±2.54 µm/s.

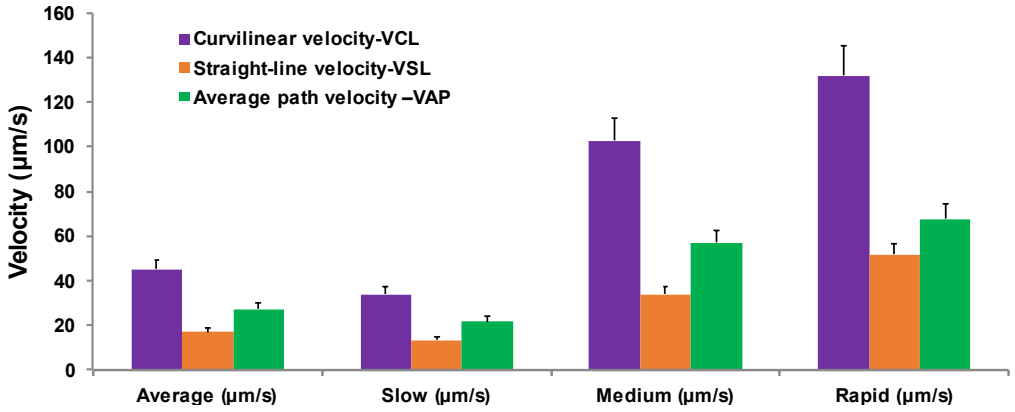

Figure 4.2 Sperm velocity (VCL, VSL, and VAP) obtained from Aseel rooster. Each bar with an error bar represents the Mean ± SEM values.

43.5 Linear index, Straightness index, and Oscillation index

Figure 4.3 represents the linear index, straightness index, and oscillation index of sperm obtained from the Aseel rooster. The average linear index for semen obtained from Aseel cock was 47.82±2.38% among them 49.18±2.93% were slow, 40.39±2.33% were medium and 41.42±1.33% were rapid in nature.

On the other hand, average straightness was 63.72±4.09% among them 63.65±4.09, 62.86±4.02 and 76.20±5.09% were slow, medium, and rapid in nature respectively. And average oscillation index was 67.31±3.58%, among them 67.31±3.58, 60.20±3.81 and 53.75±3.40% were similarly slow, medium, and rapid in nature.

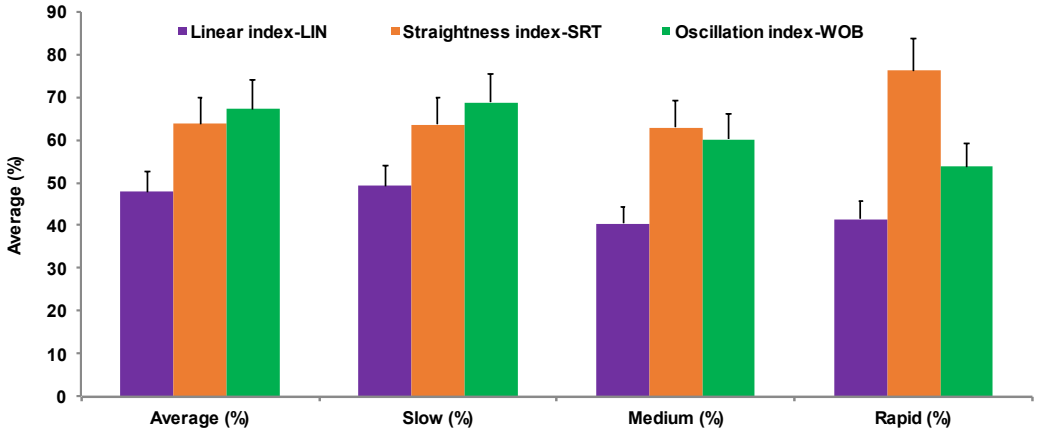

Figure 4.3 Linear index (LIN) straightness index (SRT) and oscillation index (WOB) for semen obtained from Aseel rooster. Each bar with an error bar represents the Mean ± SEM values.

4.3.6 Amplitude lateral head displacement and Beat cross frequency

Figure 4.4 represents the amplitude lateral head displacement and beat cross frequency of sperm obtained from the Aseel rooster. The average amplitude lateral head displacement was 2.56±0.20 µm among them which were medium and rapid in nature were 5.51±0.34 and 7.16±0.25 µm. The beat cross frequency was an average of 2.93±0.13 Hz and among them which were medium and rapid in nature were 3.23±0.12 and 4.99±0.23 Hz.

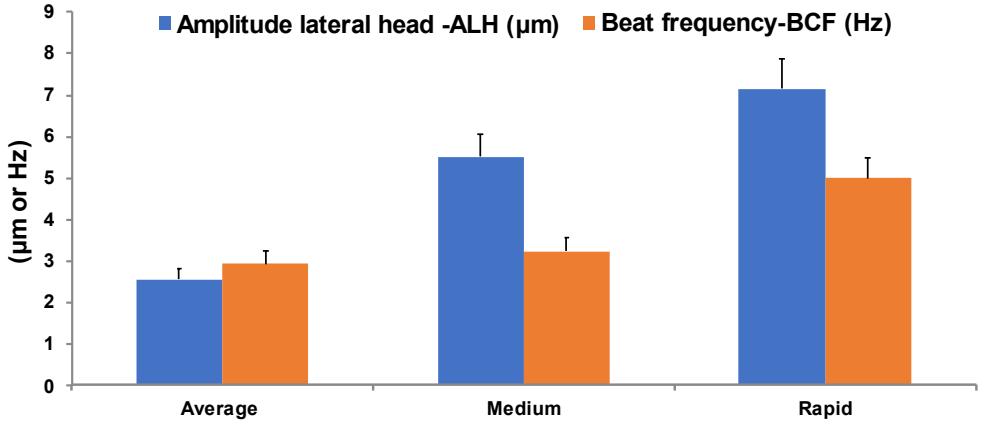

Figure 4.4 Amplitude lateral head displacement-ALH and Beat frequency-BCF for semen obtained from Aseel rooster. Each bar with an error bar represents the Mean ± SEM values.

4.4 Discussion

4.4.1 Semen color, Volume, and pH

The semen color was creamy white and showed a normal appearance. First, the color indicated a normal appearance because no abnormal color or blood was observed with the ejaculated semen. The foreign particles were not present with the collected semen. Mavi *et al*. (2017) reported that the similar semen color of Aseel and Kadaknath were creamy white. Das *et al*. (2015) also reported the creamy white semen for both the Vanaraja and indigenous chickens. Mavi *et al*. (2019) reported the semen color of RIR, Punjab Red, and RIR cross with indigenous chickens and got creamy white color for every case of their study, which was similar to this study.

Semen was collected twice a week after being matured from Aseel cocks and ejaculated semen volume was 0.50±0.0 ml. This was the normal volume for matured and healthy Aseel cocks. Mavi *et al*. (2017) reported that the semen volume obtained from Aseel and Kadaknath were 0.36 and 0.30 ml respectively were slightly lower than in this study. Mosca *et al*. (2020) stated that the mean volume of sperm from fresh ejaculates was 0.18 ml in case chickens and Yousaf *et al*. (2016) observed a semen volume of 0.34 ml for molted and got 0.16 ml for non-molted birds. Das *et al.* (2015) also reported 0.13 ml for indigenous and 0.223 ml for Vanaraja chickens in India, these volumes were less than the result obtained by the author and this may be due to the high ambient temperature in India or for the indigenous type of chicken was there. Mavi *et al*. (2019) reported the volume for RIR, Punjab Red and RIR cross were 0.27, 0.30, and 0.51 ml, in this case, RIR cross-given semen volume were similar to this study, and Sayed *et al*. (2017) reported that the volume of ejaculated semen from Dandarawi, Fayoumi, and Sharkasi chickens were 0.54, 0.46 and 1.04 ml respectively which were almost similar to this study. Chrchil *et al*. (2014) also showed the volume of semen obtained from the RIR roaster was 0.38 (0.21-0.78) ml and the range of the volume supports the result obtained by this author.

Just after collection, the semen was transported to the laboratory and the pH was measured and showed a pH of 7.15 and the pH value also showed normal. Das *et al*. (2015) reported 6.95 for Indigenous and 7.13 for Vanaraja chickens, Mavi *et al*. (2019) the pH value of RIR, Punjab Red, and RIR cross were 7.11, 7.17, and 7.05 respectively and Mavi *et al*. (2017) reported that pH value of Aseel and Kadaknath were 7.11 and 7.17 respectively, all of these values were similar to the result obtained by this study and on the other hand, Okoro *et al*. (2016) reported the pH of Koekoek cocks was 8.00 and the reported value was more than that of the result obtained by this study.

4.4.2 Concentration of semen

The semen concentration observed in the Aseel cock in this study was 5.18×10^9/ml which also indicated the normal for a cock. Das *et al*. (2015) reported a similar result (5.30×10^9/ml) for the indigenous chicken but got a lower concentration for Vanaraja chickens and Chrchil *et al*. (2014) showed the semen concentration obtained from the RIR roaster was 4.03×10^9 /ml (1.52×10^9–5.63×10^9)/ml and this range of the semen concentration supports the result obtained by this study. Mosca *et al*. (2020) stated the semen concentration of chicken was 3.70×10^9/ml, and Yousaf *et al*. (2016) reported 3.86×10^9/ml for molted and 1.87×10^9 /ml for non-molted birds which were slightly lower than obtained value, it was after molting that is middle age or old age bird but we got from newly matured cocks and Sayed *et al*. (2017) reported that the semen concentration from Dandarawi, Fayoumi and Sharkasi chickens were 3.22×10^9, 2.85×10^9 and 2.89×10^9/ml respectively, these study results were slightly lower than this study, on the other hand, Mavi *et.al*. (2019) stated that the concentrations of semen of RIR, Punjab Red and RIR cross with local chickens were 1.83×10^9, 2.13×10^9 and 2.80×10^9/ml respectively and they reported the lower value related to this study. Okoro *et al*. (2016) reported 65.00×10^6, $73.33\times10^{6,}$ and 58.33×10^6/ml.

4.4.3 Motility

Sperm motility was observed at 67.08±3.50%, among the motile sperm 2.83±0.12% were rapidly motile, 11.90±0.59% were medium motile and 52.35±1.49% were slow motile that is 32.92±1.63% of sperm showed the immotile but Mavi *et al*. (2019) stated that the sperm motility 66.60, 55.38 and 51.95% respectively for RIR, Punjab Red and RIR cross with local chickens and Yousaf *et al*. (2016) got 75% from molted and 64% from non-molted birds and this result was almost similar to the result obtained by the authors. Mosca *et al*. (2020) reported motility of 87.7% for fresh chicken semen, and Sayed *et al*. (2017) reported that the sperm motility of Dandarawi, Fayoumi, and Sharkasi chickens were 85.50, 91.71 and 86.16% and immotile sperm were 14.50, 8.29 and 13.84% respectively, Mavi *et al*. (2017) reported that individual motility of Aseel and Kadaknath was 75.87 and 75.78% respectively. Chrchil *et al*. (2014) showed the initial sperm motility obtained from the RIR roaster was 80.34%. Das *et al*. (2015) reported a motility percentage of 84.38 percent for both the indigenous and Vanaraja chickens which was higher than this study, the other hand, Okoro *et al*. (2016) reported 93.00, 94.33 and 68.33% immotile sperm cells and this value were inferior to the result obtained by this study.

Progressiveness is an important characteristic of semen which also indicates the good quality of semen. Among the 67.08±3.50% of motile semen obtained from Aseel cock, 5.8±0.34% of sperm were progressively motile, and 61.23±3.89% were also motile but not progressive. Again, among the progressively motile sperm 0.66±0.25% was rapidly progressive and 5.19±0.25% was medium progressive. Okoro *et al*. (2016) reported progressive motile cells at 3.33, 4.67, and 15.00% in different inclusion levels upon which 4.67% were like this study. Mosca *et al*. (2020) reported a progressive motility of 23.1% for fresh chicken semen. Sayed *et al*. (2017) reported that the progressiveness of sperm motility of Dandarawi, Fayoumi, and Sharkasi chickens was 27.08, 23.36 and 24.81% and non-progressive sperm were 58.42, 68.38 and 61.22% respectively and these showed the inferior result than this study. Mavi *et al*. (2017) reported that the viable sperm of Aseel and Kadaknath was 82.04 and 78.35% respectively and Churchil *et al*. (2014) showed the live sperm obtained from RIR roaster was 83.18% and abnormal sperm 4.52% which also reported the higher value than this study.

4.4.4 Curvilinear, Straight-line, and Average path velocity

The average curvilinear velocity of Aseel sperm was 45.06±2.60μm/s. Among them there were some sperm have slow in nature whose speed was 33.88±1.27, some sperm were medium speed whose speed was 102.75±7.22 and some sperm were rapid in nature whose speed were 131.92±9.55 μm/s. Mosca *et al*. (2020) reported the Curvy liner velocity of chicken sperm was 55.5 μm/s for fresh semen which reported almost similar to this study, on the other hand, Sayed *et al*. (2017) reported that the average curvy liner velocity of sperm from Dandarawi, Fayoumi, and Sharkasi chickens were 79.13, 68.17 and 63.00 μm/s respectively which showed higher than this study.

The average straight-line velocity was 16.86±1.03 μm/s. Like curvilinear velocity some sperm were slow in nature, some were medium in nature and some of them were rapid in nature and the speed were 13.42±1.03, 33.76±2.07 and 51.68±2.41 μm/s respectively. Mosca *et al*. (2020) reported the Straight-line velocity of chicken sperm was 23.6 μm/s for fresh semen the result stated almost similar but Sayed *et al*. (2017) reported that the average straight-line velocity of semen from Dandarawi, Fayoumi, and Sharkasi chickens were 27.37, 22.44 and 21.35 μm/s respectively which were higher than this study.

The average path velocity was 27.35±1.28 μm/s, and slow, medium, and rapid speeds of average path velocity were 21.68±1.37, 56.97±3.55 and 67.54±2.54 μm/s. Mosca *et al*. (2020) reported a higher Average path velocity of chicken sperm which was 36.8 μm/s for fresh semen and Sayed *et al*. (2017) reported that the average path velocity of sperm from Dandarawi, Fayoumi, and Sharkasi chickens was 38.33, 32.31 and 29.40 μm/s respectively which were slightly higher but almost similar to this study.

4.4.5 Linear index, Straightness index, and Oscillation index

The average linear index of sperm obtained from Aseel cock was 47.82±2.38%, for slow sperm it was 49.18±2.93%, for medium sperm it was 40.39±2.33% and for rapid sperm it was 41.42±1.33%. Mosca *et al*. (2020) reported a similar linear index of chicken sperm was 42.6% for fresh semen.

The average straightness was 63.72±4.09% and 63.65±4.09, 62.86±4.02 and 76.20±5.09% were for slow, medium, and rapid sperm respectively. Mosca *et al*. (2020) reported a similar Straightness of chicken sperm was 64.2% for fresh semen.

And average oscillation index was 67.31±3.58%, and 67.31±3.58, 60.20±3.81 and 53.75±3.40% were for slow, medium, and rapid sperm respectively. Mosca *et al*. (2020) reported a similar Oscillation index of chicken sperm was 66.3% for fresh semen and the result obtained supported the result obtained by this study.

4.4.6 Amplitude lateral head displacement and Beat cross frequency

Average amplitude lateral head displacement was 2.56±0.20 μm among them which were medium and rapid in nature were 5.51±0.34 and 7.16±0.25 μm. Mosca *et al*. (2020) reported a similar Average amplitude lateral head displacement of chicken sperm was 2.8 μm for fresh semen.

The beat cross frequency was an average of 2.93±0.13 Hz among them which were medium and rapid in nature were 3.23±0.12 and 4.99±0.23 Hz. Mosca *et al*. (2020) reported the higher beat cross frequency of chicken sperm was 7.5 Hz for fresh semen

4.5 Conclusion

Considering the macroscopic characters i.e. color, appearance, semen volume (0.50±0.0 ml.), pH (7.15), motility (67.08%), progressive motility (5.8%), non-progressive motility (61.23%) and bio-kinetic parameters i.e. curvilinear velocity (45.06±2.60 μm/s), straight-line velocity (16.86±1.03 μm/s), average path velocity (27.35±1.28 μm/s), linear index (47.82±2.38%), straightness index (63.72±4.09%), oscillation index (67.31±3.58%), amplitude lateral head displacement (2.56±0.20 μm) and beat cross frequency (2.93±0.13 Hz) of semen obtained from Aseel chicken showed the normal state and values. So, the semen quality of Aseel was normal in nature.

Chapter V

Honey as a natural Anti-oxidant to improve the Viability of Aseel sperm during preservation

5.1 **Introduction**

Semen cryopreservation is a useful tool for poultry breeding and development introduced in the 1960s as a way of preservation. Due to the extensive progress that has been made in this field, the biological and biochemical mechanisms involved in cryopreservation have not been thoroughly elucidated. Various factors during the freezing process, including sudden temperature changes, ice formation, and osmotic stress, have been proposed as reasons for poor sperm quality post-thaw. Though the cryopreservation of semen also protects germ plasma from the danger of loss and supports artificial insemination technology programs in livestock.

Chicken sperms are unique in structure and different in chemical composition. The rooster semen's lipid composition is distinguished by an extremely high proportion of long-chain polyunsaturated fatty acids (PUFAs) in the phospholipids fraction of spermatozoa. On one side, the high PUFA proportion is a necessity to maintain specific membrane properties (fluidity, flexibility, etc) and on the other side, sperms became very susceptible to lipid peroxidation therefore the antioxidant defense is a key element in maintaining semen quality.

In general, chicken sperm is more sensitive to the freezing process, and their quality post-thawing ability is also relatively less compared with that of other domestic livestock like cows, sheep, and goats (Mosca *et al*., 2016). Therefore, it is necessary to improve protocols for freezing roaster semen for artificial insemination programs. The primary cause of avian spermatozoa death during the process of freezing is resistance to osmotic pressure and the membrane fluidity of the sperm (Blesbois *et al*., 2005; Morris *et al*., 2011). Overall, avian spermatozoa are recognized to be weaker in cryopreservation than mammalian sperm (Long, 2006).

The difficulty of preserving chicken spermatozoa was due to specific reproductive physiology and high variability from species and breed/strains (Rakha *et al*., 2016). Nevertheless, freezing-thawing processes result in dramatic damage to avian spermatozoa membranes, resulting in the loss of more than 50% of spermatozoa

(Lemoine *et al*., 2011; Long, 2006). The cryopreservation of semen typically involves the use of a cryoprotectant to defend sperm from cold shock and cell damage due to the development of ice crystals. Cryopreservation of semen was a procedure involving diverse cryoprotective agents that have been studied. Procedures using cryoprotectants such as glycerol, N, N-dimethyl acetate-amide (DMA), and dimethyl-sulfoxide (DMSO) have been employed to cryopreserve chicken sperm (Blesbois *et al*., 2005). In general, cryoprotectants can be classified into two categories: penetrating and non-penetrating mediators of the membrane plasma of spermatozoa (Lemma, 2011). While Fuller (2004) revealed that sugars are a type of non-penetrating cryoprotectant that acts extracellularly and changes the osmotic gradient of the extended semen and allow water from the sperm to diffuse out.

Vitamin E was discovered as a "vitamin of reproduction" in 1922. In recent years it has been shown that vitamin E is in spermatozoa and provides antioxidant protection, especially in stress conditions of *in vitro* semen manipulation, including dilution, storage, and deep freezing of spermatozoa. Furthermore, it was shown that vitamin E provides additional protection in the case of fatty acid manipulation of the semen (Surai *et al*., 2000; Zinini *et al*., 2003; Cerolini *et al*., 2005). However, in some studies, the dose-response of vitamin E in cockerels was found to be non-linear (Lin *et al*., 2005). Se supplementation is known to affect the antioxidant defenses of chicken semen (Surai *et al*., 1998). Furthermore, Edens (2002) showed that, when cockerels were fed on a basal diet containing 0.28 ppm Se without additional dietary supplementation of this trace element, the percentage of normal spermatozoa was only 57.9% and two major abnormalities seen were bent midpiece (18.7%) and corkscrew head (15.4%). When this diet was supplemented with an additional 0.2 ppm Se in the form of selenite, the percentage of normal spermatozoa increased to 89.4%, and abnormalities in the form of bent midpiece and corkscrew head were decreased down to 6.2 and 1.8% respectively. However, when organic Se was included in the cockerel's diet in the same amount, semen quality was further improved, and those abnormalities decreased to 0.7 and 0.2% and the percentage of normal spermatozoa increased up to 98.7%. These results clearly showed that the form of dietary Se supplementation is a crucial factor in its efficiency, with organic Se being much more effective in comparison to selenite. Therefore, Se deficiency is associated with midpiece damage to spermatozoa. The midpiece of spermatozoa of the Se-deficient male is broken. In such conditions, sperm

motility and fertilizing capacity would be compromised. Organic selenium can also improve fertility and, more importantly, increase the duration of fertility (Agate *et al*., 2000). Preliminary observations in female chickens have also revealed the effectiveness of dietary supplementation with vitamin E, organic Se, or both, to sustain fertility in aging flocks (Breque *et al*., 2003). Thus, avian spermatozoa might be expected to have systems that will maintain stability throughout this period. Indeed, recent results have confirmed the existence of a complex antioxidant system in the uterovaginal portion of the fowl oviduct (Breque and Brillard, 2002). GSH-Px activity in the uterovaginal junction was 12-fold higher than in the liver.

Honey is a natural material rich in nutrients such as simple sugars, antioxidants, proteins, vitamins, and minerals, all of which are beneficial to improving sperm quality (Saxena *et al*., 2010). Furthermore, honey comprises a large variety of simple sugars that serve both as a source of nutrition and a non-penetrating cryoprotectant (Fuller, 2004). Based on the description, honey can be used to be added to the semen extender for the freezing of the sperm of a chicken. No research has been performed to test the effects of supplementing honey to the extender to freeze the chicken spermatozoa. Therefore, this research was designed to evaluate the effects of adding various concentrations of honey to extender the cryopreservation of chicken spermatozoa.

Objectives:

i) To determine the ability of honey as a natural antioxidant to improve the viability of sperm
ii) To fix up the levels of honey to improve the viability of the sperm at room temperature

5.2 Methods and Materials

5.2.1 Study location and duration

The research work was conducted under the project entitled "Conservation and Germplasm cryobanking of native chicken genetic resources of Bangladesh" at Advanced Avian Research Farm, Hajee Mohammad Danesh Science and Technology University, Dinajpur. The duration of the study was seven months.

5.2.2 Experimental birds

The mature Aseel chickens were selected from the birds available in Advanced Animal Research Farm and reared from the early growing stage to matured stage. A total of 16 male chickens were selected for this purpose.

5.2.3 General management

The Aseel chickens (cocks) were reared in deep litter pens and demarcated all birds. They were maintained in a semi-intensive system under standard management practices following the existing rules and regulations of the Bangladesh Veterinary Council regarding animal care and management. All birds were treated equally in all respects.

A similar diet was supplied to all birds. Feed and water were supplied in plastic feeders and drinkers. Throughout the experimental period, *ad libitum* clean drinking water was made available all day by using hanging drinkers. Rice husk was used as litter materials. Each cock was marked with permanent markers for proper identification. Both natural and mechanical procedures were followed to maintain proper ventilation and illumination in the chickens' houses. The average environmental temperature was between 21−35°C and the light and dark pattern was 16 h light and 8 h dark. During the summer season, when the environmental temperature was too hot, lemon juice, and electrolytes (Renalytes, a Renata ltd. Product) were used with the drinking water, and water was changed frequently with the cool water. A breeder diet was supplied for the experimental cocks. The diet was made with the feed ingredients maize, rice polish, soybean meal, animal protein, vitamin-mineral premix, amino acid, salt, toxin binder, and antioxidant where the ingredient composition was not expressed due to the company's business secrecy. The nutrient composition of the diet is presented in Table 5.1.

Table 5.1 Nutrient composition of experimental diet

Nutrients	Amount (%)
DM (%)	88.00
Crude protein (%)	21.00
Calcium (%)	2.00
Phosphorus (%)	0.45
Crude Fat (%)	5.00
ME (Kcal/kg)	3000−3100

Source: *Aftab Bahumukhi Farms Ltd. Bangladesh*

5.2.4 Training of cocks for semen collection

At the age of twenty-eight weeks, all the cocks were trained for semen collection by using the abdominal massage technique as described by Burrows and Quinn (1937). The training was performed twice a week until the experiment started. By thirty-two weeks of age, all the cocks had become almost equally ready for semen collection.

5.2.5 Semen collection

From the matured, Aseel cock semen was collected through massaging method. A special type of wooden chair was made with keeping facilities of locking two legs of cocks: and comfortable sitting and massaging options for the semen collector. The main goal of the semen collection procedure was to obtain the maximum amount of clean high-quality semen with minimum handling and stress. The testes located at the dorsum were stroked and massaged gently until the protrusion of the cloaca. The researcher himself collected semen every week at the same time, and under the same conditions to minimize stress and maximize the quality of semen. In the cooler period of daytime i.e., in the morning (8.00-9.00 A.M.) the collection was performed, and the collection was carried out twice a week. However, the following steps were performed for semen collection:

i) At first, cocks were taken gently on the lap of the collector and two legs of cocks were locked on the arm of the chair

ii) To start massaging the left hand is placed on the back of the tail and the right hand on the ventral part of the tail or rear part of the abdomen

iii) The cock was stimulated by stroking the abdomen and pushing the tail upward and toward the bird's head with the right hand

iv) The cocks responded and the copulatory organ enlarged and partially protruded from the vent. If the copulatory organ had not protruded, it seemed that the cock was probably not sexually responsive and needs more time and massage

v) When cock responded and the copulatory organ was exposed, the left hand was placed at the cloaca with the forefingers. At the same time, the right hand was used to provide inward and upward pressure beneath the cloaca

vi) When semen was ejaculated, it was then squeezed out by a short, sliding, downward movement of the left hand and the upward pressure of the right hand. Care was taken so that the copulatory organ was not touched and harmed during collection. Individual ejaculates were collected into 1 ml microtubes

vii) After each collection, the ejaculates were examined visually as well as microscopically. Special care was taken to avoid contamination of semen with feces, urates, and transparent fluid, which lower semen quality

viii) Just after the collection of semen the macroscopic parameters were observed and recorded and transported to the laboratory for the next analysis using a CASA analyzer

5.2.6 Preparation of Ringer's solution

Ringer's solution was used as an extender and the extender was prepared using the following component. The components were measured first in the beaker and then distilled water was added to make it 1 liter. For this study, semen diluents were prepared by Akcay *et al.*, (2006). The Composition of the diluents is given in Table 5.2.

Table 5.2 Composition of the semen diluents

Ingredients	**Amount**
Sodium chloride(g)	9.50
Potassium chloride(g)	0.20
Calcium chloride(g)	0.26
Sodium bicarbonate(g)	0.20
Distilled water (ltr.)	1.00
Glucose(g)	1.00

Source: *Akcay et al., 2006*

Photo 5.1: Preparation of Ringer's Solution in Genetics and Animal Breeding Laboratory, HSTU, Dinajpur

5.2.7 Use of Honey as natural anti-oxidant in Ringer's solution

Normally Ringer's solution was used as a control extender and the other six (6) types of extenders were prepared using honey in different levels instead of glucose used in the Ringer's solution. In the other extenders instead of glucose 1%, 2%, 3%, 4%, and 5% honey was used to prepare the extenders. Here Honey was used as a natural antioxidant.

5.2.8 Semen dilution

The collected semen was diluted using previously made diluents also known as modified Ringer's solution and the extenders were 1%, 2%, 3%, 4%, and 5% honey was used. Before selecting the dilution ratio for CASA analysis using Ringer's solution, the practice was done previously and the dilution ratio was selected at a ratio of 1:20, which ratio was given a better result for analysis. This ratio (1:20) was used during the final analysis using CASA.

Photo 5.2: Ringer's solution and the extenders were 1%, 2%, 3%, 4%, and 5%, honey

5.2.9 Macroscopic analysis

Just after collection, the semen sample macroscopic parameters such as semen volume, and color were observed and transported to the laboratory spending minimum time and pH was measured using a pH meter. The observed values were recorded as well. Then another analysis was performed.

5.2.10 Motility analysis

Samples were preserved in a hot water bath at 37.5^0C and analysis was carried out at 0, 2, and 4 hours of preservation following the procedure mentioned below. A new, fresh, clean Liza slide was taken, and then 0.5µl diluted semen was taken in a clean and disinfected micropipette and carefully placed on the slide within the boundaries of the loading area. The process was done so carefully that the coverslip gets no touch by the hand so that no premature filled occurred. The piston of the micropipette was pushed with such care that the chamber was filled well by the capillary force and the loading area was filled well. The chamber was filled within 3−20 seconds as the viscosity of the samples was all right. The excess semen samples were taken away with the help of cotton bud tips to prevent the floating of cells. After resting for 5 seconds the analysis was done promptly preventing evaporation.

The motility analysis was done using a green filter, the condenser was selected for chicken (animal species) i.e., Ph-1, and 10X negative phase contrast was used for contrasting the object of the microscope. Then select the program SCA Motility, the motility was measured using Computer Assisted Semen Analyzer (CASA).

5.2.11 Statistical analysis

The effect of treatment on sperm concentration, motility, progressiveness, velocity, and immotility was analyzed using the One-way ANOVA procedure following with a Completely Randomized Design (CRD) following the GLM procedure of SPSS computer software 22.00 (SPSS, 2013). The significance of differences among the means of treatments was compared by using Duncan's Multiple Range Test (DMRT) of the same package. All data were expressed as Mean ± standard error of Mean (SEM). Significant differences were considered at the level of $P<0.01$ and $P<0.05$. The following linear model summarizes the statistics employed to analyze the data:

$$Y_i = \mu + TR_i + E_i,$$

Where Y_i is the dependent variable, μ is the overall mean, TR_i is the treatment effect, and E_i is the error.

5.3 Results

5.3.1 Sperm concentration

Figure 5.1 represents the sperm concentration at 0, 2, and 4 hours of storage. The normal sperm concentration in Aseel semen observed in the experiment were $5.18\pm0.31\times10^9$, $5.07\pm0.30\times10^9$, $5.16\pm0.31\times10^9$, $5.00\pm0.30\times10^9$, $4.99\pm0.30\times10^9$ and $4.94\pm0.30\times10^9$ per ml at 0 hours for T_0 (Control), 1% honey (T_1), 2% honey (T_2), 3% honey (T_3), 4% honey (T_4) and 5% honey (T_5) treatment groups and did not differ significantly when 1%, 2%, 3%, 4% and 5% of honey was added with Ringer's solution except control group. At 2 hours of storing in a hot water bath containing 37.8^0C the concentration of sperm decreased except T_3 group where 3% honey was used with Ringer's solution and the concentration was increased from $5.00\pm0.30\times10^9$ to $5.09\pm0.29\times10^9$ per ml and for the control group though the concentration was decreased it was minimum, which decreased $5.18\pm0.31\times10^9$ to $5.00\pm0.30\times10^9$ per ml. The sperm concentration was increased in that case. The difference was significant ($P < 0.05$) for T_0 and T_3 with other treatment groups. Again, after 4 hours of storing the concentration of semen decreased drastically for all the treatment groups and the semen concentration were $3.76\pm0.23\times10^9$, $3.55\pm0.25\times10^9$, $3.50\pm0.27\times10^9$, $4.75\pm0.30\times10^9$, $3.67\pm0.27\times10^9$ and $3.05\pm0.30\times10^9$ per ml for T_0, T_1, T_2, T_3, T_4, and T_5 treatment groups respectively and did not vary significantly (Figure 5.1). After 4 hours of storing, the semen samples were usable for all the cases considering sperm concentration.

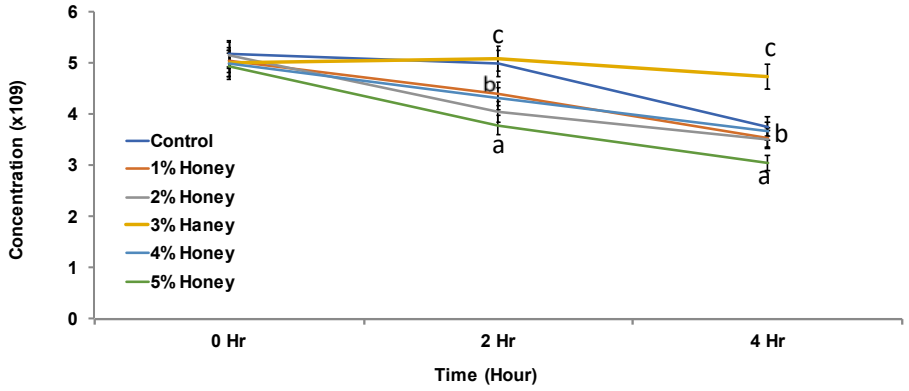

Figure 5.1 The sperm concentration ($\times 10^9$) after 0, 2, and 4 hours of storing when different levels of honey were used with Ringer's solution. Each line with error bars represents the Mean ± SEM values; different letters above the error bars of the same feeding period indicate statistically significant differences ($P < 0.05$).

5.3.2 Sperm motility and progressiveness

Figure 5.2 represents the motility of sperm when the different levels of honey were used and stored for up to 4 hours. The motility percent of sperm in the semen samples were 87.08±5.96, 87.81±6.52, 79.52±4.63, 87.80±6.20, 82.43±5.95 and 84.87±5.67% for T_0, T_1, T_2, T_3, T_4, and T_5 treatment groups respectively at 0 hours and this motility percent was decreased drastically after 2 hours of storing except control and T_3 treatment groups where 0 and 3% of honey were used. In the case of the control group, the motility percentage decreased but not drastically and for the T_3 group, it increased. Again 4 hours of storing, sperm motility was decreased for all the cases though the motility of the control and T_3 groups was satisfactory.

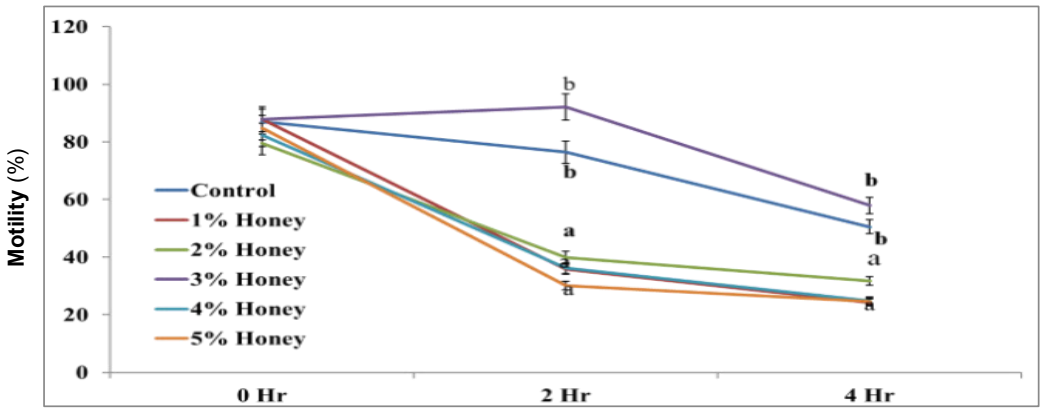

Figure 5.2 The sperm motility (%) after 0, 2, and 4 hours of storing when different levels of honey were used with Ringer's solution. Each line with error bars represents the Mean ± SEM values; different letters above the error bars of the same feeding period indicate statistically significant differences ($P < 0.01$).

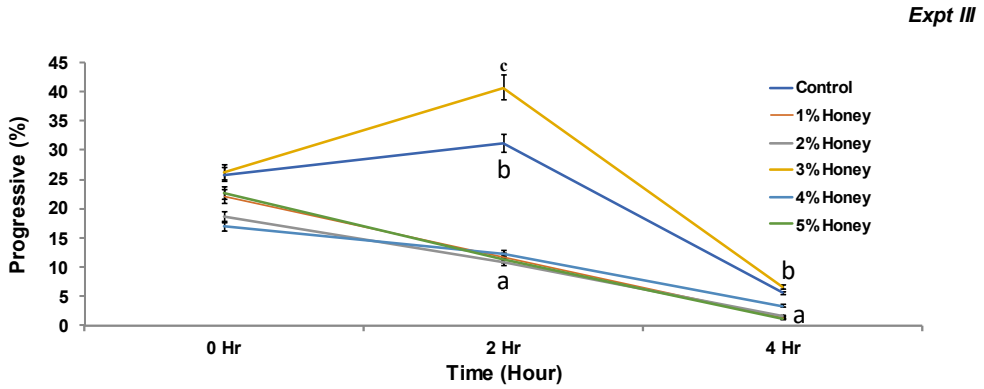

Figure 5.3 The progressive sperm (%) after 0, 2, and 4 hours of storing when different levels of honey were used with Ringer's solution. Each line with error bars represents the Mean ± SEM values; different letters above the error bars of the same feeding period indicate statistically significant differences ($P < 0.05$).

5.3.3 Non-progressive and immotile of sperm

Figure 5.4 represents the non-progressiveness of sperms. Non-progressiveness of sperm and immotile sperm are the negative characteristics of sperm. The non-progressive sperm is considered to be live sperm but has no progressiveness on the other hand immotile sperm are considered dead sperm. At 0 hours of storing the non-progressive sperm were 61.23±3.98, 65.70±4.52, 60.98±4.78, 61.64±4.39, 65.46±4.68 and 62.17±4.79% respectively for T_0, T_1, T_2, T_3, T_4, and T_5 and did not vary significantly on the other hand after 2 and 4 hours of storing the percent of non-progressive sperm were decreased with the time duration because the non-progressive sperm become immotile so fast and varied significantly within the treatment groups ($P < 0.05$).

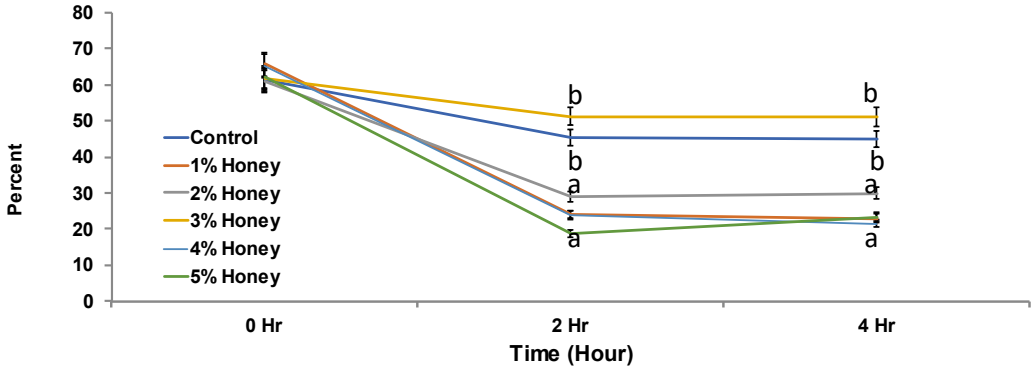

Figure 5.4 The non-progressiveness of sperm (%) after 0, 2, and 4 hours of storing when different levels of honey were used with Ringer's solution. Each line with error bars represents the Mean ± SEM values; different letters above the error bars of the same feeding period indicate statistically significant differences ($P < 0.05$).

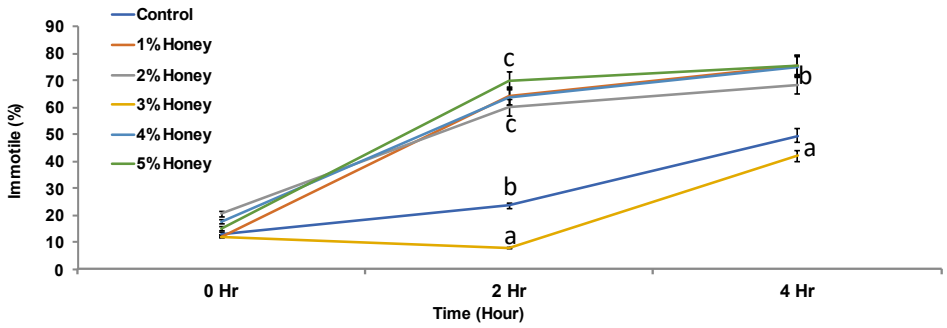

Figure 5.5 The immotile sperm (%) after 0, 2, and 4 hours of storing when different levels of honey were used with Ringer's solution. Each line with error bars represents the Mean ± SEM values; different letters above the error bars of the same feeding period indicate statistically significant differences ($P < 0.01$).

Figure 5.5 represents the immotility of sperm up to 4 hours of storing period. Immotile sperm percent were 12.92±0.93, 12.19±0.75, 20.48±1.36, 12.20±0.76, 17.57±1.04 and 15.13±1.12% at 0 hours of storing and did not differ significantly within the treatment groups (T_0, T_1, T_2, T_3, T_4, and T_5) and these percents were increased with the storing time and it was 23.60±0.88, 64.22±4.76, 60.07±4.08, 7.97±0.36, 63.88±4.11 and 69.95±4.95% after 2 hours of storing and 49.48±2.99, 75.75±5.60, 68.27±4.61, 42.14±2.73, 75.11±5.88 and 75.39±5.03% after 4 hours of storing and differed significantly among the treatment groups (T_0, T_1, T_2, T_3, T_4, and T_5) in both the cases ($P < 0.01$).

5.4 Discussion

5.4.1 Sperm concentration

The normal concentration of sperm in Aseel semen observed in the experiment were $5.18\pm0.31\times10^9$, $5.07\pm0.30\times10^9$, $5.16\pm0.31\times10^9$, $5.00\pm0.30\times10^9$, $4.99\pm0.30\times10^9$ and $4.94\pm0.30\times10^9$ per ml respectively at 0 hours of storing for T_0, T_1, T_2, T_3, T_4, and T_5 treatment groups. At 2 hours of storing in a hot water bath containing 37.8 ^{0}C the concentration of sperm decreased except for the extender where 3% honey (T_3) was used with Ringer's solution where the concentration was increased from $5.00\pm0.30\times10^9$ to $5.09\pm0.29\times10^9$ per ml and for the control group though the concentration was decreased, it was minimum, which decreased $5.18\pm0.31\times10^9$ to $5.00\pm0.30\times10^9$ per ml Vasicek *et al*. (2015) reported that the concentration of sperm after 2 hours of storing at 4^{0}C were 4.95±0.70 and 3.53±0.53 when they used the saline solution and avian diluents, respectively and this result closer and supports the result of this study with

time duration though the semen were stored in 4^0C and without any anti-oxidants. Again, after 4 hours of storing the concentration of semen decreased drastically for all the treatment groups and the semen concentration were $3.76\pm0.23\times10^9$, $3.55\pm0.25\times10^9$, $3.50\pm0.27\times10^9$, $4.75\pm0.30\times10^9$, $3.67\pm0.27\times10^9$ and $3.05\pm0.30\times10^9$ per ml for T_0, T_1, T_2, T_3, T_4, and T_5 treatment groups respectively. After 4 hours of storing, the semen samples were usable for all the cases considering sperm concentration. Breque *et al*., 2003; Partyka *et al*., 2012 stated that avian semen that contained antioxidants and enzymatic defense prolongs sperm longevity both *in vivo* and *in vitro* conditions and honey has an anti-oxidant property which prolonged sperm longevity and Shimizu *et al*., 2008, Das *et al*., 2011 also stated that Avian sperm also contain beta-defensins which is antimicrobial peptides protect the sperm from microbial infection. However, avian sperm contains a high amount of polyunsaturated fatty acids, is susceptible to reactive oxygen species, and promotes lipid peroxidation (Khan, 2011). During *in vitro* storage conditions, the total lipid contents of spermatozoa decrease significantly at 2-5 °C after 48 h in chicken. It is possible to occur lysis of lipids and peroxidation; and can modify the structure of spermatozoa (Blesbois *et al*., 1999).

5.4.2 Sperm motility and progressiveness

Sperm motility in semen is an important criterion that determines the quality and ability to fertilize the ovum after insemination in the uterus of a female animal. The motility percent of sperm in the semen samples were 87.08±5.96, 87.81±6.52, 79.52±4.63, 87.80±6.20, 82.43±5.95 and 84.87±5.67% for T_0, T_1, T_2, T_3, T_4, and T_5 treatment groups respectively at 0 hours and this motility percent was decreased drastically after 2 hours of storing except control and T_3 treatment groups where 0 and 3% of honey were used Vasicek *et al*., 2015 resulted that the motility of sperm after 2 hours of storing at 4^0C were 77.38±1.56 and 21.15±2.60% for saline solution and avian diluents and the motility of sperm percent was increased with the storing time (62.61±2.83, 74.60±2.64 and 77.38±1.56% at 0.5, 1 and 2 hours of storing, respectively) when the saline solution was used and though the result slightly lower supports the result obtained in this study. In the case of the control group, the motility percentage decreased but not drastically and for the T_3 group, it increased. Again 4 hours of storing, sperm motility was decreased for all the cases though the motility of the control and T_3 groups was satisfactory.

At 0 hours of storing the progressive sperm were 25.85±1.68, 22.11±1.67, 18.54±0.84, 26.16±1.92, 16.97±0.53 and 22.70±1.18% respectively for T_0, T_1, T_2, T_3, T_4 and T_5

treatment groups. After 2 hours of storing, the percent of progressive sperm was decreased for all the treatments except control and T_3 groups where the percent of progressive sperm was increased (31.20 and 40.72%) Vasicek *et al*., 2015 resulted that the progressive motility of sperm after 2 hours of storing at 4^0C were 64.95±2.26 and 15.70±2.21 for saline solution and avian diluents and the motility of sperm percent was increased with the storing time (45.48±4.00, 61.80±3.53 and 64.95±2.26 at 0.5, 1 and 2 hours of storing, respectively) when the saline solution was used and though the result higher but supports the result obtained in this study. Again, the percentage of progressive sperm was fall at a critical stage where the control and T_3 groups have a minimum percentage of progressive sperm.

5.4.3 Non-progressiveness and Immotile of sperm

Non-progressiveness of sperm and immotile sperm are the negative characteristics of sperm. The non-progressive sperm is considered to be live sperm but has no progressiveness on the other hand immotile sperm are considered dead sperm. At 0 hours of storing the non-progressive sperm were 61.23±3.98, 65.70±4.52, 60.98±4.78, 61.64±4.39, 65.46±4.68 and 62.17±4.79%, respectively for the treatment groups (T_0, T_1, T_2, T_3, T_4, and T_5) and after 2 and 4 hours of storing the percent of non-progressive sperm were decreased with the time duration because the non-progressive sperm become immotile so fast. Mohamed *et al*., 2012 showed that 26.83±1.62, 26.77±1.10 and 27.74 ± 1.99% of non-progressive bovine sperm when stored at 37^0C after 1, 2, and 3 hours, respectively, and the value showed lower than this study this is because the rooster sperm are more sensitive than bovine sperm.

Immotile sperm percent were 12.92±0.93, 12.19±0.75, 20.48±1.36, 12.20±0.76, 17.57±1.04 and 15.13±1.12% at 0 hours of storing, and these percents were increased with the storing time and it was 23.60±0.88, 64.22±4.76, 60.07±4.08, 7.97±0.36, 63.88±4.11 and 69.95±4.95% after 2 hours of storing and 49.48±2.99, 75.75±5.60, 68.27±4.61, 42.14±2.73, 75.11±5.88 and 75.39±5.03% after 4 hours of storing among treatment groups (T_0, T_1, T_2, T_3, T_4, and T_5). Mohamed *et al*., 2012 also showed 22.90±1.65, 50.60±2.46 and 69.79±2.26% of Immotile bovine sperm when stored at 37^0C after 1, 2, and 3 hours, respectively, and the result is like this study in case of 3% honey after 2 hours and after 3 hours of storage, showed immotile slightly higher than this study resulted after 3 hours.

5.5 Conclusion

Considering the motility percent and percent of progressive sperm the semen samples may be stored for up to 2 hours in the good condition for insemination Simple Ringer's solution and Ringer's solution prepared with 3% honey and indicate that the honey supplementation improves the sperm quality and longevity relation without honey showed in the previous experiment. Poultry breeders are using different kinds of antioxidants *in vitro* sperm storage conditions to improve sperm quality. It can be said that antioxidant improves the motility, viability, and fertilizing ability of avian sperm in a dose-dependent manner.

Chapter VI

Cryopreservation of Aseel chicken sperm

6.1 Introduction

The American Museum of Natural History suggested that there are about 18,000 avian species in the world (George *et al*., 2016). The International Union for Conservation of Nature's (IUCN) Red List of threatened species expressed that among the number of animal and avian species, 28,000 species are under threat of extinction, among which about 14% are avian species in May 2019. With the changes in climates and habits, thousands of species of wild animals are in an endangered situation and disappearing day by day. Many scientists and agencies are paying attention to finding a solution to meet emergencies that could lead to the rapid extinction of species. The situation is complicated, keeping in mind that, captive breeding, and the exchange of individuals between zoos are common practices around the world. However, the scope is limited, and it is quite impossible to maintain sufficient numbers of each extinct species. Germplasm cryobanking may be an effective solution that may facilitate the cryobanking of sperm, oocytes, or even embryos of many species (Pukazhenthi and Wildt 2004; Prieto *et al*., 2014). Again, the semen characteristics of avian species are somehow different from those of mammals considering the different physiology and anatomy, like the intra-abdominal placement of testes and the absence of accessory sex glands (Etches, 1996). Moreover, the sperm of chickens are different morphologically from those of mammals (Etches, 1996) and very fragile to high environmental temperatures as well as temperature fluctuations during cryopreservation (Graham, 1978). Since the storage of eggs or embryos from birds poses a difficult challenge because of their large size and complexity, sperm cells are a good alternative (Long, 2013). Cryopreservation of sperm is a fairly easy and inexpensive way and means compared with the oocyte or embryo, because of the small volume of water inside of the sperm (Rosen *et al*., 2019). Furthermore, existing technologies allow the semen of many animal species to be collected and used (FAO, 2012) and frozen semen can be a very important part of *ex-situ* conservation efforts since it allows for the storage of genetic material from multiple species for a long period. Stored semen can be used in reintroduction to enlarge the genetic pool of a fragmented population. It also allows for the selection of individuals of genetic interest and the exchange of samples between distant locations (Brock *et al*., 1983; Villaverde-Morcillo *et al*., 2017). In birds, frozen

semen could become an increasingly relevant tool, because captive breeding programs are often slowed down by incompatibility between individuals or by stress, leading to the absence of mating (Bailey and Lierz, 2017). Cryopreservation of semen is a technique largely used in large animals, but, in birds, there are still some limitations. Avian spermatozoa are filiform-shaped, which makes them more susceptible to injury from manipulation (Madeddu *et al*., 2009). Additionally, they are highly sensitive to the changes in osmolarity that occur during the freezing and thawing process (Long, 2006; Blesbois, 2012).

The semen of avian species is more concentrated, with minimal seminal plasma (Etches, 1996). The semen quality in roosters also varied among different genotypes like breed (Peters *et al*., 2008), strain (Murugesan *et al*., 2013), and line (Tarif *et al*., 2013). The semen quality parameters reported for different breeds like White Leghorn (Elagib *et al*., 2012), Plymouth Rock (Tarif *et al*., 2013), Rhode Island Red (RIR) (Kabir *et al*., 2007a) and indigenous roosters (Hu *et al*., 2013) stated a high degree of variation. The age of roosters is also a factor that may interact with semen quality parameters (Kelso *et al*., 1996). The environment also influences semen quality, i.e., climate (Saeed and Al-Soudi, 1975), time of collection (Egbunike and Oluyemi, 1979), frequency of collection (Riaz *et al*., 2004) and nutrition (Kabir *et al*., 2007b). The literature provides little information on the semen quality of Aseel roosters and, there is a total paucity of semen attributes of older Aseel roosters. The individual sperm motility of indigenous roosters was found to have a significant positive correlation with live sperm, sperm concentration, and sperm output in local chickens (Bah *et al*., 2001). The interrelationship of semen quality parameters has been reported for indigenous (Hu *et al*., 2013) and a pool of several breeds (Peters *et al*., 2008), but little has been reported for the Aseel breed. Therefore, the experiment was performed with the following objectives:

i) To observe the sperm quality after cryopreservation using different cryoprotective agents
ii) To observe the sperm quality after cryopreservation using a gradual reduction of sperm sample temperature

6.2 Methods and Materials

6.2.1 Study location and duration

The research work was conducted under the project entitled "Conservation and Germplasm cryobanking of native chicken genetic resources of Bangladesh" at Advanced Avian Research Farm, Hajee Mohammad Danesh Science and Technology University, Dinajpur. The duration of the study was eight months.

6.2.2 Experimental birds

The mature Aseel chickens were selected from the birds available in Advanced Animal Research Farm and reared from the early growing stage to the mature stage. A total of 18 male chickens were selected for this purpose.

6.2.3 General management

The Aseel chickens (cocks) were reared in deep litter pens and demarcated all birds. They were maintained in a semi-intensive system under standard management practices following the existing rules and regulations of the Bangladesh Veterinary Council regarding animal care and management. All birds were treated equally in all respects.

A similar diet was supplied to all birds. Feed and water were supplied in plastic feeders and drinkers. Throughout the experimental period, *ad libitum* clean drinking water was made available all day by using hanging drinkers. Rice husk was used as litter materials. Each cock was marked with permanent markers for proper identification. Both natural and mechanical procedures were followed to maintain proper ventilation and illumination in the chickens' houses. The average environmental temperature was between 21−35°C and the light and dark pattern was 16 h light and 8 h dark. During the summer season, when the environmental temperature was too hot, lemon juice, and electrolytes (Renalytes, a Renata ltd. Product) were used with the drinking water, and water was changed frequently with the cool water. A breeder diet was supplied for the experimental cocks. The diet was made with the feed ingredients maize, rice polish, soybean meal, animal protein, vitamin-mineral premix, amino acid, salt, toxin binder, and antioxidant where the ingredient composition was not expressed due to the company's business secrecy. The nutrient composition of the diet is presented in Table 6.1.

Table 6.1 Nutrient composition of experimental diet

Nutrients	Amount (%)
DM (%)	88.00
Crude protein (%)	21.00
Calcium (%)	2.00
Phosphorus (%)	0.45
Crude Fat (%)	5.00
ME (Kcal/kg)	3000−3100

Source: Aftab Bahumukhi Farms Ltd. Bangladesh

6.2.4 Training of cocks for semen collection

At the age of twenty-eight weeks, all the cocks were trained for semen collection by using the abdominal massage technique as described by Burrows and Quinn (1937). The training was performed twice a week until the experiment started. By thirty-two weeks of age, all the cocks had become almost equally ready for semen collection.

6.2.5 Semen collection

From the matured, Aseel cock semen was collected through massaging method. A special type of wooden chair was made with keeping facilities of locking two legs of cocks: and comfortable sitting and massaging options for the semen collector. The main goal of the semen collection procedure was to obtain the maximum amount of clean high-quality semen with minimum handling and stress. The testes located at the dorsum were stroked and massaged gently until the protrusion of the cloaca. The researcher himself collected semen every week at the same time, and under the same conditions to minimize stress and maximize the quality of semen. In the cooler period of daytime i.e., in the morning (8.00-9.00 A.M.) the collection was performed, and the collection was carried out twice a week. However, the following steps were performed for semen collection:

i) At first, cocks were taken gently on the lap of the collector and two legs of cocks were locked on the arm of the chair

ii) To start massaging the left hand is placed on the back of the tail and the right hand on the ventral part of the tail or rear part of the abdomen

iii) The cock was stimulated by stroking the abdomen and pushing the tail upward and toward the bird's head with the right hand

iv) The cocks responded and the copulatory organ enlarged and partially protruded from the vent. If the copulatory organ had not protruded, it seemed that the cock was probably not sexually responsive and needs more time and massage

v) When cock responded and the copulatory organ was exposed, the left hand was placed at the cloaca with the forefingers. At the same time, the right hand was used to provide inward and upward pressure beneath the cloaca

vi) When semen was ejaculated, it was then squeezed out by a short, sliding, downward movement of the left hand and the upward pressure of the right hand. Care was taken so that the copulatory organ was not touched and harmed during collection. Individual ejaculates were collected into 1 ml microtubes

vii) After each collection, the ejaculates were examined visually as well as microscopically. Special care was taken to avoid contamination of semen with feces, urates, and transparent fluid, which lower semen quality

viii) Just after the collection of semen the macroscopic parameters were observed and recorded and transported to the laboratory for the next analysis using a CASA analyzer

Photo 6.1: Collection of semen in plastic microtubes

6.2.6 Preparation of Ringer's solution

Ringer's solution was used as an extender and the extender was prepared using the following component. The components were measured first in the beaker and then distilled water was added to make it 1 liter. For this study, semen diluents were prepared by Akcay *et al*. (2006). Composition of the diluents is given in Table 6. 2.

Table 6.2 Composition of the semen diluents

Ingredients	**Amount**
Sodium chloride(g)	9.50
Potassium chloride(g)	0.20
Calcium chloride(g)	0.26
Sodium bicarbonate(g)	0.20
Distilled water (ltr.)	1.00
Glucose(g)	1.00

Source: Akcay *et al*., 2006

6.2.7 Use of Honey as cryoprotective agents

As cryoprotective agents 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0% honey, 4.0, 8.0, and 11.0% DMSO, and 4.0, 8.0 and 11.0% glycerol were used for the cryopreservation of Aseel sperm. The dilution was prepared in such a way that the dilution was content of the above-mentioned level before plunging the semen dilution into the liquid nitrogen container.

Photo 6.2: Use of honey as cryoprotective agents

6.2.8 Cryopreservation of Aseel semen

After preparing the semen dilution, the tubes containing sperm with the cryoprotective agent were equilibrated by keeping them at 4^0C in a Refrigerator for 30 minutes, then the tubes were placed in liquid nitrogen vapor for the next 30 minutes, and finally, the tubes were plunged into a liquid nitrogen container for 2 days.

Photo 6.3: Nitrogen cane where the cryopreservation was performed

6.2.9 Thawing of sperm samples

Before analysis of sperm quality after cryopreservation, the sperm samples were thawed for 45 seconds at 37.5^0C in a hot water bath. After thawing, the sperm samples were diluted at a 1:20 ratio, and then analysis was performed using CASA software which consisted of a microscope, and a computer with a monitor where SCA software was installed.

6.2.10 Cryopreserved semen analysis

A new, fresh, clean Liza slide was taken, and then 0.5µl diluted semen was taken in a clean and disinfected micropipette and carefully placed on the slide within the boundaries of the loading area. The process was done so carefully that the coverslip got no touch by the hand so that no premature filling occurred. The piston of the micropipette was pushed so carefully that the chamber was filled well by the capillary force and the loading area was filled well. The chamber was filled within 3−20 seconds as the viscosity of the samples was all right. The excess semen samples were taken

away with the help of cotton bud tips to prevent the floating of cells. After resting for 5 seconds, the analysis was done promptly preventing evaporation.

The motility analysis was done using a green filter, the condenser was selected for chicken (animal species) i.e., Ph-1, and 10X negative phase contrast was used for contrasting the object of the microscope. Then select the program SCA Motility, the motility was measured using Computer Assisted Semen Analyzer (CASA).

6.2.11 Statistical analysis

The effect of treatment on sperm concentration, motility, progressiveness, velocity, linearity index, lateral head displacement and beat cross frequency was analyzed using the One-way ANOVA procedure following a Completely Randomized Design (CRD) following the GLM procedure of SPSS computer software 22.00 (SPSS, 2013). The significance of differences among the means of treatments was compared by using Duncan's Multiple Range Test (DMRT) of the same package. All data were expressed as Mean ± standard error of Mean (SEM). Significant differences were considered at the level of $P<0.01$ and $P<0.05$. The following linear model summarizes the statistics employed to analyze the data:

$$Y_i = \mu + TR_i + E_i,$$

Where, Y_i is the dependent variable, μ is the overall mean, TR_i is the treatment effect, and E_i is the error.

6.3 Results

6.3.1 Sperm concentration

Figure 6.1 represents the sperm concentrations in different cryoprotectants at different levels. After the cryopreservation procedure sperm concentration showed significantly better when 11% glycerol and 8% DMSO were used as cryoprotectants and showed the concentration level of sperm was $1.99\pm0.13\times10^9$ and $1.88\pm0.10\times10^9$/ml respectively. Considering sperm concentration 1% and 3% honey and 8%of glycerol showed significantly good results compared to other levels of honey as cryoprotectants and other cryoprotectants agents and sperm concentration levels were 1.71±0.10, 1.74±0.10 and $1.75\pm0.10\times10^9$ /ml, respectively ($P < 0.05$).

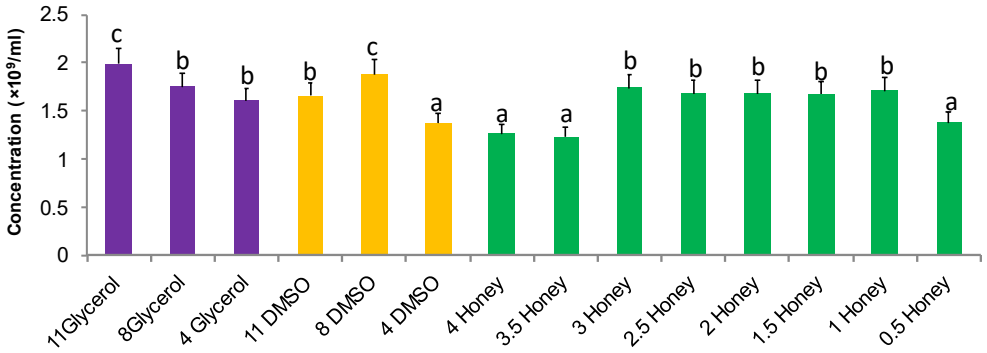

Figure 6.1 The sperm concentration (×10⁹/ml) after cryopreservation with different levels of cryoprotective agents. Each bar represents the Mean±SEM values; different letters above the error bars statistically significant differences ($P < 0.05$).

6.3.2 Total motility and Progressive motility of sperm

The figure represents the total and progressive motility of sperm in different cryoprotectants at different levels. Motility of sperm was showed significantly better for 11% glycerol and 8% DMSO as cryoprotectants and motility of sperm was 58.32±3.66 and 53.32±3.27%, respectively. The motility of rooster sperm for other cryoprotective agents except honey used in the experiment was less than 25.32±1.92%. For honey higher percent of motility, the level was observed when 3% honey was used as cryoprotectants and the sperm motility was 28.83±2.03% and other levels of honey were observed to be lower than 23.00±1.48% of sperm motility though the levels were significantly similar except for 0.5% levels honey which showed poor state.

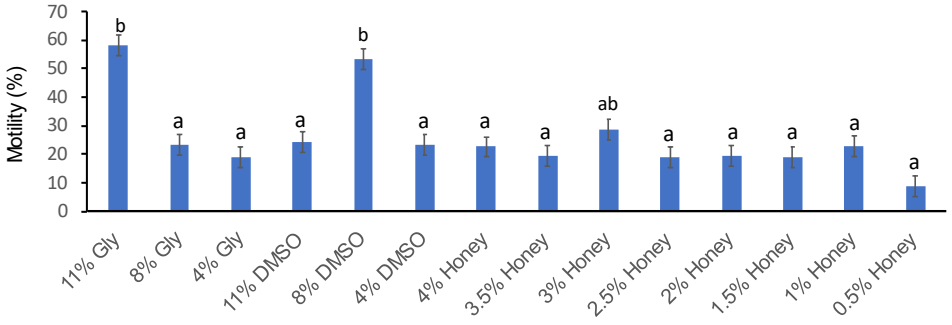

Figure 6.2 The motility (%) after cryopreservation with different levels of cryoprotective agents. Each bar represents the Mean±SEM values; different letters above the error bars indicate statistically significant differences ($P < 0.05$).

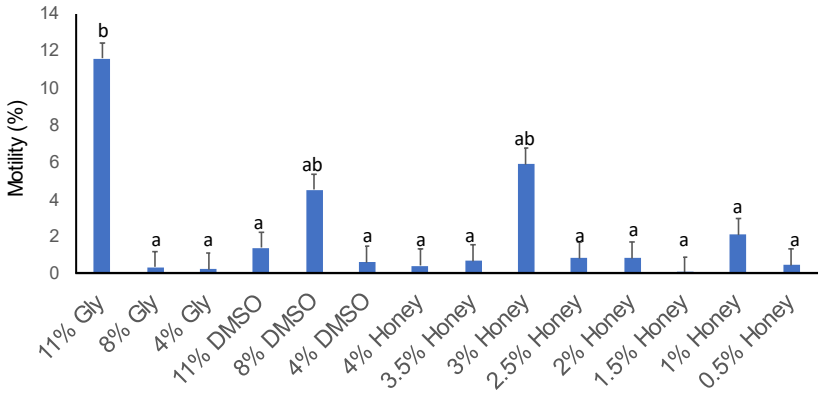

Figure 6.3 The motility (%) and progressive motility (%) after cryopreservation with different levels of cryoprotective agents. Each bar represents the Mean ± SEM values; different letters above the error bars indicate statistically significant differences ($P < 0.05$).

Progressive motility of sperm was observed significantly better when 11% glycerol, 8% DMSO, and 3% of honey were used as cryoprotectants compared to other levels of glycerol, DMSO, and honey. The progressive motility of sperm was 11.54±0.32, 4.48±0.25, and 5.66±0.28% where 11% glycerol, 8% DMSO, and 3% of honey were used as cryoprotectants, respectively.

6.3.3 Velocity

The velocity of sperm in different cryoprotectants at different levels. Considering velocity 5.07±0.40, 15.23±1.12 and 38.02±2.40% sperm were rapid, medium, and slow in nature for the level of 11% glycerol; 2.58±0.06, 7.31±0.48 and 40.09±3.02% sperm were rapid, medium and slow in nature for the level of 8% DMSO and 3.11±0.14, 5.13±0.14 and 23.12±1.48% sperm were rapid, medium and slow in nature for the level of 3% of honey when used as cryoprotective agents. For the other levels of cryoprotectants of glycerol, DMSO and honey the sperm velocity was too lower than the levels mentioned above.

6.3.4 Velocity and progressiveness

Figure 6.4 depicts the velocity and progressiveness of sperm in various cryoprotectants at various levels. Considering the velocity and progressiveness at a time cryoprotective agents of 11% glycerol level showed the best performance that is 1.59±0.10% of sperm showed rapidly progressive, 9.96±0.68% of sperm showed medium progressive and

46.78±3.47% of sperm showed non-progressive, in case of 8% DMSO levels resulted in 0.38±0.03% sperm rapidly progressive, 4.10±0.23% sperm medium progressive and 35.51±2.48% sperm showed non-progressive and in case of 3% honey when used as cryoprotective agent resulted in 0.31±0.02% sperm showed rapidly progressive, 4.44±0.35% sperm medium progressive and 18.18±1.37% sperm showed non-progressive. Except for these three, other levels of glycerol, DMSO and honey showed insignificant results regarding velocity and progressiveness at a time.

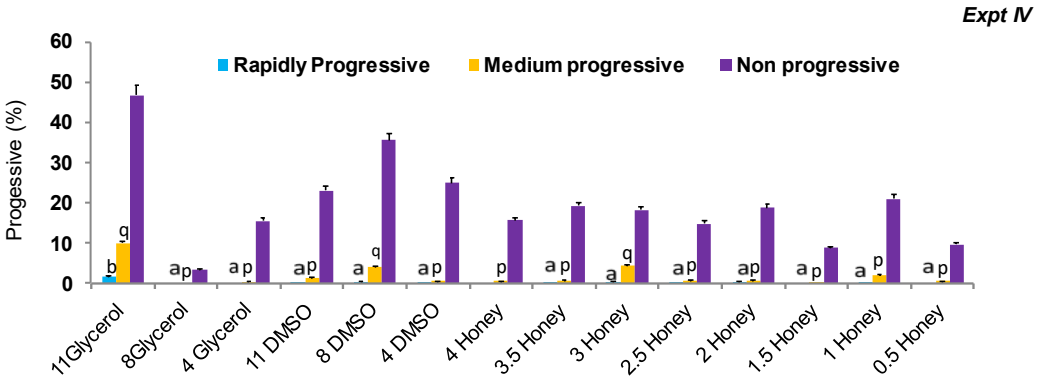

Figure 6.4 The progressiveness along with velocity (%) after cryopreservation with different levels of cryoprotective agents. Each bar epresents the Mean±SEM values; different letters above the error bars indicate statistically significant differences ($P < 0.05$).

6.3.5 Curvilinear, Straight-line, and Average path velocity

Figure 6.5 represents the curvilinear, straight-line, and average path velocity of sperm in different cryoprotectants at different levels. The average curvilinear velocity of Aseel sperm after cryopreservation showed 45.99±2.76, 39.45±2.51 and 44.37±3.45µm/s, respectively for 11% glycerol, 8% DMSO, and 3% honey when used as cryoprotective agent. In the case of other levels of cryoprotective agents, the speed showed significantly lower than these three levels. The straight linear velocity was 15.93±1.17, 10.17±0.71 and 12.69±1.00 µm/s, respectively for 11% glycerol, 8% DMSO, and 3% honey when used as a cryoprotective agent and as curvilinear velocity other levels of cryoprotective agents, the straight linear speed showed significantly lower than these three levels. The average path velocity was 26.90±1.52, 19.63±1.47 and 22.03±1.67µm/s, respectively for 11% glycerol, 8% DMSO, and 3% honey when used as a cryoprotective agent and like curvilinear and straight linear velocity other levels of cryoprotective agents, the average path velocity showed significantly lower than these three levels.

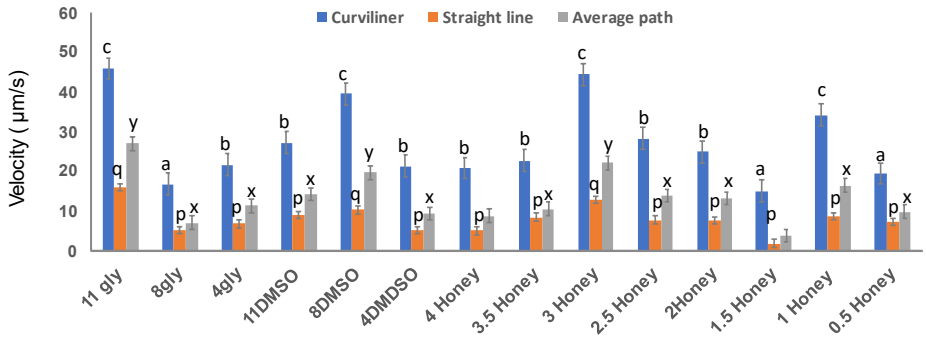

Figure 6.5 The Curvilinear (µm/s), Straight line (µm/s), and Average path velocity (µm/s) after cryopreservation with different levels of cryoprotective agents. Each bar represents the Mean±SEM values; different letters above the error bars indicate statistically significant differences ($P < 0.05$).

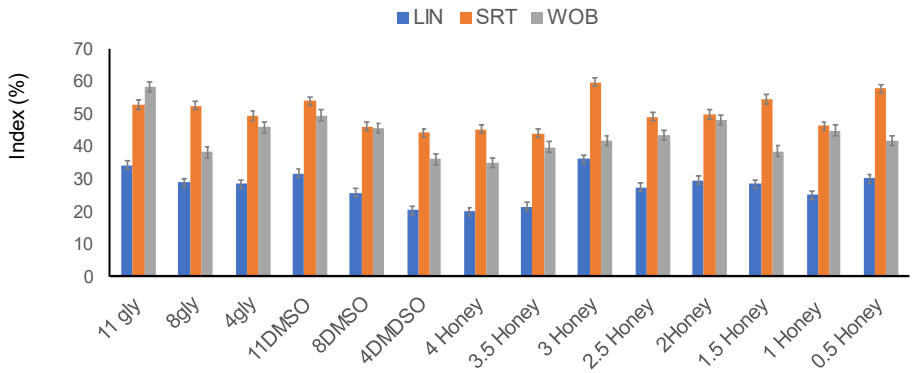

Figure 6.6 The Linear index (%), Straightness index (%), and Oscillation index (%) after cryopreservation with different levels of cryoprotective agents. Each bar with an error bar represents the Mean±SEM values.

6.3.6 Linear index, Straightness index and Oscillation index

Figure 6.6 represents the linear index, straightness index, and oscillation index of sperm in different cryoprotectants at different levels. The linear index, straightness index, and oscillation index did not vary significantly among the levels used as cryoprotective agents. The linearity index ranges from 36.08±2.64—20.09±1.06%, the straightness index ranges from 59.73±4.48—44.02±3.25% and the oscillation index ranges from 58.27±4.16—35.02±2.08%. For different glycerol levels linearity index ranges from 34.30±2.47—28.41±2.17%, for different levels of DMSO it ranges from 20.44±1.36—31.46±2.15% and for different levels of honey it ranges from 36.08±2.64—20.09±1.06%. For different glycerol levels straightness index ranges from

52.83±3.64—49.42±3.59%, for different levels of DMSO it ranges from 53.94±4.13—44.26±3.45% and for different levels of honey it ranges from 57.72±4.16—44.02±3.25%. For different glycerol levels oscillation index ranges from 58.27±4.16—38.23±2.84%, for different levels of DMSO it ranges from 49.54±3.69—36.06±2.48% and for different levels of honey it ranges from 48.08±3.48—35.02±2.08%.

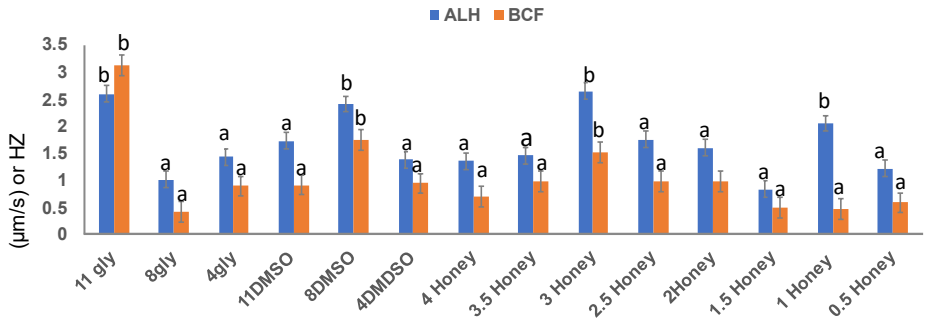

Figure 6.7 The amplitude of lateral head displacement (µm/s) and beat cross frequency (Hz) after cryopreservation with different levels of cryoprotective agents. Each line with the error bar represents the Mean ± SEM values; different letters above the error bars of the same feeding period indicate statistically significant differences ($P < 0.05$).

6.3.7Amplitude lateral head displacement and Beat cross frequency

Figure 6.7 represents the amplitude lateral head displacement and beat cross frequency of sperm in different cryoprotectants at different levels. The amplitude of lateral head displacement was 2.60±0.02, 2.41±0.12 and 2.65±0.12 µm/s, respectively for 11% glycerol level, 8% DMSO level, and 3% honey level, and other levels of cryoprotective agents the value showed significantly lower. The beat cross frequency was observed at 3.12±0.12, 1.74±0.12 and 1.51±0.10 Hz respectively for 11% glycerol level, 8% DMSO level, and 3% honey level, and other levels of cryoprotective agents the value showed significantly lower.

6.4 Discussion

6.4.1 Sperm concentration

After the cryopreservation procedure sperm concentration showed significantly better when 11% glycerol and 8% DMSO were used as cryoprotectants and showed the concentration level of sperm was $1.99\pm0.13\times10^9$ and $1.88\pm0.10\times10^9$/ml respectively. Considering sperm concentration 1% and 3% of honey and 8% of glycerol showed significantly good results compared to other levels of honey as cryoprotectants and other cryoprotectants agents and sperm concentration levels were 1.71±0.10, 1.74±0.10 and $1.75\pm0.10\times10^9$ /ml, respectively. Gill *et al*., 1996 stated that the sperm concentration in the thawed semen was lower than the fresh semen and the statement supports all the treatments and sperm concentration was lowered compared to fresh semen.

6.4.2 Total motility and Progressive motility of sperm

Total motility of sperm was showed significantly better preserved with 11% glycerol and 8% DMSO as cryoprotectants and motility of sperm was 58.32±3.66 and 53.32±3.27%, respectively but Mphaphathi *et al*. (2016) resulted in that total motility 46.0% for Venda chicken sperm cryopreserved with 8% DMSO which was lower than this experiment and, in this experiment, cryopreserved with 8% DMSO resulted in 53.32±3.27%.

The total motility of rooster sperm for other cryoprotective agents except honey used in the experiment was less than 25.32±1.92%. Honey was found to have a higher percent of motility when 3% of honey was used as a cryoprotectant and the sperm total motility was 28.83±2.03%, while other levels of honey were found to be lower than23.00±1.48% of sperm total motility, though the levels were significantly similar except for the 0.5% of honey, which showed poor state. Mphaphathi *et al*. (2016) showed that total motility and progressive motility were cryopreserved without CPA and showed 5.6 and 1.7% of total and progressive motility which supports the result obtained in the experiment. Malik *et al*. (2019) reported the total motility of cryopreserved native chicken sperm were 31.27± 0.62, 35.13±1.16, 34.47±0.07, 33.15±1.03 and 26.67±1.12 which were almost similar to this study.

Progressive motility of sperm was observed significantly better when 11% glycerol, 8% DMSO, and 3% of honey were used as cryoprotectants compared to other levels of glycerol, DMSO, and honey. The progressive motility of sperm was 11.54±0.32, 4.48±0.25 and 5.66±0.28% for where 11% glycerol, 8% DMSO, and 3% of honey were

used as cryoprotectants, respectively. Shanmugam and Mahapatra (2020) got the progressive motility after cryopreservation 12.14 and 9.28% when they used 2% DMSO with Sasaki diluents and Lake and Ravie diluents, respectively and this result was closer to this study. Mphaphathi *et al*. (2016) resulted that cryopreserved with 8% DMSO for Venda chicken sperm showed progressive motility of 25.4% again they reported the progressive motility for six different Venda cocks were 23.3, 27.5, 22.9, 14.3, 29.1 and 20.0 which supports the result obtained by cryopreserved with 11% glycerol but higher than another level of cryoprotectants and Shanmugam and Mahapatra (2019) showed that the effect of DMA and sucrose on post-thaw semen parameters and observed 25.45 and 18.18% progressive motility when they used 6% and 9% DMA as a cryoprotective agent which is slightly higher than this study but when they used sucrose with DMA and got 15.90 and 14.09% progressive motility for 6% and 9% DMA which were almost similar to this study though they had preserved by pellet method.

6.4.3 Curvilinear, Straight-line and Average path velocity

The average curvilinear velocity of Aseel sperm after cryopreservation showed 45.99±2.76, 39.45±2.51 and 44.37±3.45 μm/s, respectively for 11% glycerol, 8% DMSO, and 3% honey when used as cryoprotective agent. In the case of other levels of cryoprotective agents, the speed showed significantly lower than these three levels Mphaphathi *et al*. (2016) reported the curvilinear velocity cryopreserved with 8% DMSO was 83.9 μm/s and six different Venda chickens showed 62.5–110.5 μm/s which supported this study and value perhaps closer to this study obtained in case of cryoprotectants 11% glycerol and 38.9 μm/s for CPA free preservation which supports for the curvilinear velocity observed using 3% honey which was almost similar to this study. The straight linear velocity were 15.93±1.17, 10.17±0.71 and 12.69±1.00 μm/s, respectively for 11% glycerol, 8% DMSO, and 3% honey when used as a cryoprotective agent and as curvilinear velocity other levels of cryoprotective agents the straight linear speed showed significantly lower than these three levels but Mphaphathi *et al*. (2016) reported for Venda chickens the straight velocity was 50.1 μm/s for 8% DMSO and six different Venda chickens this value showed 33.7–69.5 μm/s and 24.6 for CPA free cryopreservation and these result showed a lower level of straight linear velocity. The average path velocity were 26.90±1.52, 19.63±1.47 and 22.03±1.67 μm/s, respectively for 11% glycerol, 8% DMSO, and 3% honey when used as an agent and similar to curvilinear and straight linear velocity other levels of cryoprotective agents the average path velocity showed significantly lower than these three levels but average path

velocity observed by Mphaphathi *et al*. (2016) showed 60.0μm/s for 8% DMSO and six different chickens it was 41.4–80.1μm/s this result was higher than this study and 28.8 μm/s for CPA free cryopreservation which supports for the 3% honey.

6.4.4 Linear index, Straightness index and Oscillation index

The linear index, straightness index, and oscillation index did not vary significantly among the levels used as cryoprotective agents. The linearity index ranges from 36.08±2.64—20.09±1.06%, the straightness index ranges from 59.73±4.48—44.02±3.25% and the oscillation index ranges from 58.27±4.16 to 35.02±2.08%. For different glycerol levels linearity index ranges from 34.30±2.47—28.41±2.17%, for different levels of DMSO it ranges from 20.44±1.36—31.46±2.15% and for different levels of honey it ranges from 36.08±2.64—20.09±1.06%. For different glycerol levels straightness index ranges from 52.83±3.64—49.42±3.59%, for different levels of DMSO it ranges from 53.94±4.13—44.26±3.45% and for different levels of honey it ranges from 57.72±4.16—44.02±3.25%. For different glycerol levels oscillation index ranges from 58.27±4.16—38.23±2.84%, for different levels of DMSO it ranges from 49.54±3.69—36.06±2.48% and for different levels of honey it ranges from 48.08±3.48—35.02±2.08%. Mphaphathi *et al*. (2016) reported 58.7, 82.7, and 70.6% linearity, straightness, and oscillation index, respectively for 8% DMSO and six different rooster linearity indexes varied from 51.3–65.6% and straightness index varied from 77.7–87.2% which were higher than this study.

6.4.5 Amplitude lateral head displacement and Beat cross frequency

The amplitude of lateral head displacement was 2.60±0.02, 2.41±0.12 and 2.65±0.12 μm/s, respectively for 11% glycerol level, 8% DMSO level, and 3% honey level, and other levels of cryoprotective agents the value showed significantly lower. The beat cross frequency was observed at 3.12±0.12, 1.74±0.12 and 1.51±0.10 Hz for 11% glycerol level, 8% DMSO level, and 3% honey levels respectively, and was significantly lower for other levels of cryoprotective agents. According to Mphaphathi *et al*. (2016) the amplitude of lateral head displacement and beat cross frequency of 2.3 and 13.5 for 8% DMSO were supported in terms of the amplitude of lateral head displacement and showed higher values for beat cross frequency.

6.5 Conclusion

The honey has also cryoprotective properties and 3% of honey may act as a cryoprotective agent. As a cryoprotective agent 11% glycerol is the best and 8% DMSO also has good cryoprotective ability during cryopreservation of Aseel rooster sperm.

Chapter VII

The efficiency of Artificial insemination with Fresh and Diluted semen compared with Natural mating for Aseel chicken

7.1 Introduction

Artificial insemination (AI) involves the deposition of semen into the female reproductive tract manually. It starts with the collection of the semen from the male and its evaluation in terms of motility, viability and concentration followed by its deposition into the female reproductive tract. Sexual maturity in both male and female birds occurs at 18 weeks of age. One ejaculate of a male can cover up 20 female birds by using AI. A dose of 50 microliter of volume semen should contain 100–200 million spermatozoa per insemination Kharayat *et al*. (2016). Artificial insemination was first practiced in America during the 1920s and then used widely in Australia with the introduction of laying cages during the late 1950s. For geneticists, AI is the method of choice for maintaining pedigreed mating. Artificial insemination (AI) is considered a valuable tool for the poultry industry (Benoff *et al*., 1981) due to the efficient utilization of males, which is not possible under natural mating. This decreases the cost of poultry production directly by reducing the number of cockerels needed for male gamete production (Benoff *et al*., 1981). AI was the first biotechnological tool applied to increase poultry production, as it allowed wider use of genetically superior cockerels with high productive performance (Benoff *et al*., 1981). That is, the benefits of artificial insemination in the poultry are as follows: i) artificial insemination increases mating ratio at about fourfold, ii) Older males with outstanding performance can be used for several generations, iii) Valuable male birds with the leg injuries can still be used for artificial insemination, iv) Preferential mating is eliminated, and v) AI aids in successful cross breeding.

The problems of native chickens are slow growing, producing a few eggs, the higher percentage of broodiness, light-weight body and their late maturing age compared with the industrial strains. On the other hand, native chickens have some advantages and these include high quality eggs, thicker eggshells better in taste and flavor, a high percentage of meat, resistance to heat and cold and some disease (Makarechian, 2002). To improve local chickens like Aseel and other important genetic resources, artificial insemination may play an important role. There is an opportunity to develop the local

chickens using breeding technologies using artificial insemination and the local genetic chickens may be used to develop a modern strain.

Fertility is an important parameter in chickens and reflects the total actual reproductive capacity of females and males expressed by their ability when mated together to produce offspring. An egg is said to be infertile when it fails to show any evidence of developing an embryo (Miazi *et al* 2012). Hatchability is a trait of economic importance in the chicken industry because it has a strong effect on chick output (Wolc *et al* 2010). It is influenced by several factors such as egg weight, turning of eggs, storage, humidity, shell strength, egg size and genetic factors within the chickens kept. The ability of the embryo to successfully escape from the shell is called hatchability (Tarek 1992). To some extent, good hatchability of eggs is heritable but is determined by a complicated genetic constitution and the environment. Artificial insemination has a positive effect on improving fertility and hatchability traits also.

It is well established that artificial insemination in avian species has an advantage over natural mating (Surai and Wishart, 1996). AI resulted in better fertility than natural mating in poultry. Gee *et al*. (2004) reported that even when under natural mating 80–85% of eggs are fertile, fertility can be increased by another 5-10% simply by applying AI. Egg production of native genetic resources is lower than that of improved strains normally used at farm leves. So, hatching procedures are very important to increase the number of chicks (Kaygısız, *et al*., 1994).

The application of AI along with natural mating is widely used in developed countries. But no initiative has yet been undertaken in Bangladesh to investigate the suitability and efficiency of this technique. Fresh semen should be inseminated within 15-30 minutes after collection to achieve better fertility (Brillard, 2003). But within such a short period, it is difficult to inseminate a large number of hens. For this reason, in addition to fresh semen, an attempt was made under this study, to investigate the performance of diluted semen. Therefore, keeping in mind the above points, the study was undertaken with the following objectives:

i) To compare the fertility and hatchability traits of eggs laid by Aseel through natural mating and AI using fresh and diluted semen; and
ii) To determine the effect of natural mating and AI using fresh and diluted semen on egg weight and day old chick weight for Aseel chickens.

7.2 Methods and Materials

7.2.1 Study location and duration

The research work was conducted under the project entitled "Conservation and Germplasm cryobanking of native chicken genetic resources of Bangladesh" at Advanced Avian Research Farm, Hajee Mohammad Danesh Science and Technology University, Dinajpur. The duration of the study was 10 months.

7.2.2 Experimental birds

The mature Aseel chickens were selected from the birds available in Advanced Animal Research Farm and reared from the early growing stage to a mature stage. A total of 54 hens and 18 males were selected for this study i.e. each treatment group contained 6 hens and 2 cocks and each treatment group had 3 (three) replications.

7.2.3 Experimental design

Treatment → **Replication↓**	**Natural mating**	**AI with Fresh semen**	**AI with diluted semen**
R_1	6♀ 2 ♂	6♀ 2 ♂	6♀ 2 ♂
R_2	6♀ 2 ♂	6♀ 2 ♂	6♀ 2 ♂
R_3	6♀ 2 ♂	6♀ 2 ♂	6♀ 2 ♂
Breeding system	Cocks and hens were kept together and were allowed to have natural mating	Cocks were kept in different places, semen was collected and fresh semen was inseminated	Cocks were kept in different places, Semen was collected, diluted and inseminated

7.2.4 General management

Each treatment group of chickens were reared in a deep litter system, demarcated well and kept in a separate pen. They were maintained in a semi-intensive system under standard management practices following the existing rules and regulations of the Bangladesh Veterinary Council regarding animal care and management. All birds were treated equally in all respects, except the mating system and in case of artificial insemination, fresh and diluted semen were used.

A similar diet was supplied to all birds. Feed and water were supplied in plastic feeders and drinkers. Throughout the experimental period ad libitum clean drinking water was made available all day by using hanging drinkers. As for litter, rice husk were used as a litter material. Each pair of chickens was marked with permanent markers for proper identification. Both natural and mechanical procedures were followed to maintain proper ventilation and illumination in the chickens' house. The average environmental temperature was between 21−35°C and the light and dark pattern was 16 h light and 8 h dark. During the summer season, when the environmental temperature was too hot, lemon juice, Renalytes (Renata Ltd. products) were used with the drinking water and water was changed frequently with the cool water. A breeder diet was formulated for the experimental groups. The diet was made out of the feed ingredients maize, rice polish, soybean meal, animal protein, vitamin-mineral premix, amino acid, salt, toxin binder, and anti-oxidant. The composition of the diet is presented in Table 7.1.

Table 7.1 Nutrient composition of experimental diet

Nutrients	**Amount (%)**
DM (%)	88.00
Crude protein (%)	21.00
Calcium (%)	2.00
Phosphorus (%)	0.45
Crude Fiber (%)	5.00
ME (Kcal/kg)	3000−3100

Source: *Aftab Bahumukhi Farms Ltd. Bangladesh*

7.2.5 Training of cocks for semen collection

At the age of twenty-eight weeks, all the cocks were trained for semen collection by using the abdominal massage technique as described by Burrows and Quinn (1937). The training was performed twice a week until the experiment started. By thirty-two weeks of age, all the cocks had become almost equally ready for semen collection. Then

the semen collection was carried out and just after collection the semen sample was transported to the laboratory.

7.2.6 Semen collection

From the matured Aseel cock semen was collected through the massaging method. A special type of wooden chair was made with keeping facilities for locking two legs of cocks; and comfortable sitting and massaging options for the semen collector. The main goal of the semen collection procedure was to obtain the maximum amount of clean and quality semen with minimum handling and stress. The testes located at the dorsum were stroked and massaged gently until protrusion of the cloaca. The researcher himself collected semen every week at the same time, and under the same conditions to minimize stress and maximize the quality of semen. The cooler period of the day, i.e. at morning (4.00-5.00 P.M.) the collection was performed and the collection was carried out twice a week. However, the following steps were performed for semen collection:

i) At first, cocks were taken gently onto lap of the collector and two legs of cocks were locked onto the arm of the chair.

ii) To start massaging, the left hand is placed on the back of the tail and the right hand on the ventral part of the tail or rear part of the abdomen.

iii) The cock was stimulated by stroking the abdomen and pushing the tail upward and toward the bird's head with the right hand.

iv) The cocks responded and the copulatory organ enlarged and partially protruded from the vent. If the copulatory organ had not protruded, it seemed that the cock was probably not sexually responsive and needed more time and massage.

v) When the cock responded and the copulatory organ was exposed, the left hand was placed at the cloaca with the forefingers. At the same time, the right hand was used to provide inward and upward pressure beneath the cloaca.

vi) When semen was ejaculated, it was then squeezed out by a short, sliding, downward movement of the left hand and an upward pressure of the right hand. Care was taken so that the copulatory organ was not touched and harmed during collection. Individual ejaculates were collected into 1 ml micro tubes.

vii) After each collection, the ejaculates were examined visually as well as microscopically. Special care was taken to avoid contamination of semen with feces, urates, and transparent fluid, which lower semen quality.

viii) Just after the collection of semen, the macroscopic parameters were observed and recorded and transported to the laboratory for next analysis using the CASA analyzer.

7.2.7 Semen examination

Before using the semen, the collected semen was examined first using CASA (Computer Assisted Semen Analyzer). The cocks that produced better semen, were used for artificial insemination.

7.2.8 Semen dilution

The collected semen was diluted using previously made diluents, which are also known as modified Ringer's solution. For artificial insemination, one group was inseminated with fresh semen, just after collection and another group was inseminated with diluted semen, which was diluted at a 1:2 ratio. For the control group, natural mating was practiced. For the study, semen diluents were used following the composition used by Akcay *et al*., (2006). The composition of the diluents is given in Table 7.2.

Table 7.2 Composition of the semen diluents

Ingredients	**Amount**
Sodium chloride(g)	9.50
Potassium chloride(g)	0.20
Calcium chloride(g)	0.26
Sodium bicarbonate(g)	0.20
Distilled water (ltr.)	1.00
Glucose(g)	1.00

Source: *Akcay et al., 2006*

7.2.9 Artificial insemination (AI)

The females were inseminated by "venting" as described by Hafez (1985). Venting was done by applying pressure to the left side of the abdomen around the vent in such a way that it causes the cloaca to come out and the oviduct to protrude. Then a 1 ml plastic syringe without a needle with an appropriate amount of semen was inserted into the oviduct and semen was delivered at a depth of 1.5 to 2 cm. AI was performed twice in

a week between 4-5 P.M. to avoid the presence of a hard-shelled egg in the uterus. Timing of hen insemination is very important to achieve a high rate of fertility. Because it is generally recognized that AI should be carried out when no hard-shelled egg is likely to be present in the uterus or at least not within 3 hours of an oviposition (Giesen *et al*., 1980). As the semen was expelled into the vagina, pressure around the vent was released and then the vent area was massaged, which assisted the hen in retaining sperm in the vagina or the oviduct. AI was performed within 30 minutes of semen collection.

- **AI with fresh semen:** Freshly collected undiluted pooled semen was drawn with a 1 ml syringe and deposited into the vagina @ 0.02 ml for each hen.
- **AI with diluted semen:** Pooled diluted semen was drawn with a 1 ml syringe and deposited into the vagina @ 0.2 ml for each hen.

Rough handling of the hens was avoided carefully before, during and after the insemination process. Hens were released gently after insemination to prevent semen regurgitating from the vagina, which might result in lower fertility. However, the following precautions were taken during the insemination:

i) To avoid contamination, exteriorized vent of the hen was not touched with hands without hand gloves
ii) Syringes used for insemination were cleaned, washed and disinfected
iii) The vents of cocks and hens were properly cleaned with cotton wool moistened with physiological saline before semen collection and insemination and
iv) Contaminated semen was not used for insemination.

7.2.10 Data collection

7.2.10.1 Collection, cleaning, weighing and storing of eggs

The eggs obtained from the different groups were collected and just after collection the eggs were cleaned with wet cloths and weighed according to the treatment groups and the cracked, broken and off-shaped eggs were culled. The weights of eggs were recorded treatment wise. Then the eggs were stored differently (treatment wise) in the Genetics and Animal Breeding Laboratory where the air conditioning facility was available. The healthy eggs were stored for no more than seven days.

7.2.10.2 Incubation, candling and hatching of eggs

From the storage, the well-shaped and healthy eggs were selected, kept at a normal temperature and then incubated in an automatic forced-air incubator where temperature, humidity and turning were performed automatically. The candling was done twice during the incubation period, on the seventh and eighteenth days of incubation. The eggs were transferred to the hatching tray after 2nd candling and after the hatching chicks were weighed and recorded treatment wise.

7.2.10.3 Fertility

After first candling, infertile and clean eggs were identified and carried out for culling and recorded treatment wise and the rest of the fertile eggs were kept for hatching. From the set eggs, the infertile eggs were deducted and fertility was calculated.

$$\text{Fertility (\%)} = \frac{\text{No. of eggs set} - \text{No. of infertile eggs}}{\text{No. of eggs set}} \times 100$$

$$= \frac{\text{No. of fertile eggs}}{\text{No. of eggs set}} \times 100$$

7.2.10.4 Hatchability

After hatching, the chicks were carried out from the incubator. The healthy and abnormal chicks were sorted first and then counted. The abnormal chicks were then counted according to abnormal type, and the eggs which were not hatched were checked for dead in shell, late embryonic mortality or sticky chicks. Then hatchability was calculated.

$$\text{Hatchability (\%)} = \frac{\text{No. of chicks hatched}}{\text{No. of fertile eggs}} \times 100$$

7.2.11 Statistical analysis

The effect of treatment on fertility, hatchability, egg production, egg weight, day old chick weight, embryonic mortality, normal and abnormal chicks were analyzed using the One-way ANOVA procedure in accordance with a Completely Randomized Design (CRD) following the GLM procedure of SPSS computer software 22.00 (SPSS, 2013). The significance of differences among the means of treatments was compared by using Duncan's Multiple Range Test (DMRT) from the same package. All data were presented as Mean±standard error of Mean (SEM). Significant differences were

considered at the level of P<0.01 and P<0.05. The following linear model summarizes the statistics employed to analyze the data:

$$Y_i = \mu + TR_i + E_i,$$

Where; Y_i is the dependent variable, μ is the overall mean, TR_i is the treatment effect, and E_i is the error.

7.3 Results

7.3.1 Age at maturity

Figure 7.1 represents the age at maturity in different breeding situations. The age at maturity obtained by this study did not vary significantly. The hens which were managed by natural mating laid their first egg at 215±15.05 days of age. The hens which were managed by AI with fresh and diluted semen laid eggs at 213±13.07 and 216±13.98 days of age, respectively.

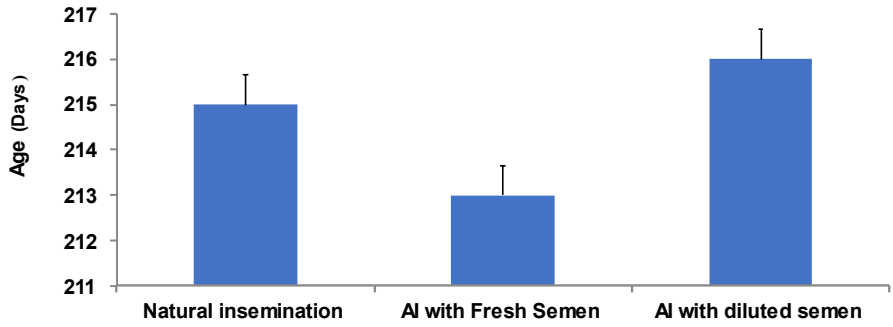

Figure 7.1 The age at maturity (days) in different breeding situations. Each bar with an error bar represents the Mean±SEM values.

7.3.2 Egg weight

Figure 7.2 represents the egg weight and day old chick weight in different breeding situations. The egg weight produced by the different treatment groups did not differ significantly. The egg weights were 43.39±2.97, 43.03±2.94 and 43.21±2.95 g respectively. The result indicates that the breeding system (Normal mating and Artificial Insemination by fresh and diluted semen) had no effect on egg weight.

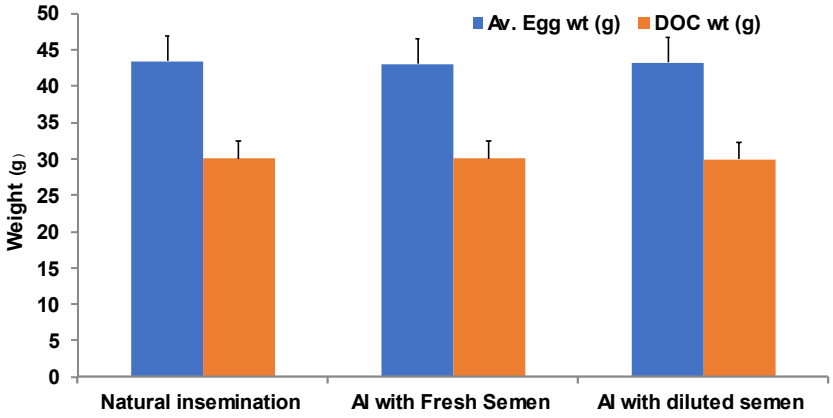

Figure 7.2 The egg weight (g) and day-old chick weight (g) in different breeding situation. Each bar with an error bar represents the Mean±SEM values.

.

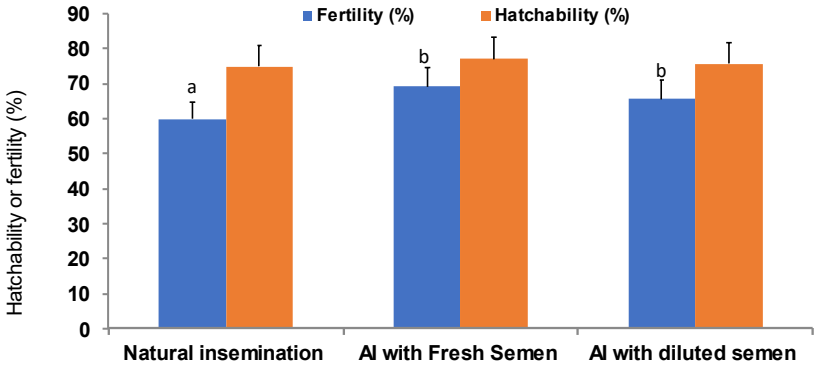

Figure 7.3 The fertility (%) and Hatchability (%) in different breeding situation. Each bar represents the Mean±SEM values. Different letters above the error bars indicates statistically significant differences (P<0.05)

7.3.3 Fertility and hatchability

Figure 7.3 represents the fertility and hatchability in different breeding situations. Fertility is determined on the basis of the number of fertile eggs obtained after first candling compared with set eggs. The breeding system (Normal mating and Artificial Insemination by fresh and diluted semen) had the minimum effect on fertility. The insemination by the fresh and diluted semen (69.25±5.04, 65.68±4.75) showed significantly better (P<0.05) fertility than the fertility observed by natural mating (60.00±4.3) and insemination by fresh semen showed significantly better fertility (P<0.05) than the diluted semen also.

The hatchability is determined on the basis of fertile eggs. The breeding system (Normal mating and Artificial Insemination by fresh and diluted semen) had no effect on the hatchability (75.00±5.50; 77.21±5.67; 75.68±5.55) of eggs among the different treatment groups, i.e. hatchability did not differ significantly.

7.3.4 Other hatchability traits

Figure 7.4 represents the other hatchability traits in different breeding situations. Early embryonic mortality (10.67±0.75, 8.02±0.54 and 9.00±0.6 percent) ; late embryonic mortality (5.00±0.2, 4.70±0.27 and 4.50±0.26 percent); dead in shell (8.33±0.45, 9.32±0.54 and 10.81±0.56 percent) and dead chicks (8.33±0.45, 9.32±0.54 and 10.81±0.56 percent) did not differ significantly between the different breeding system and insemination with fresh and diluted semen.

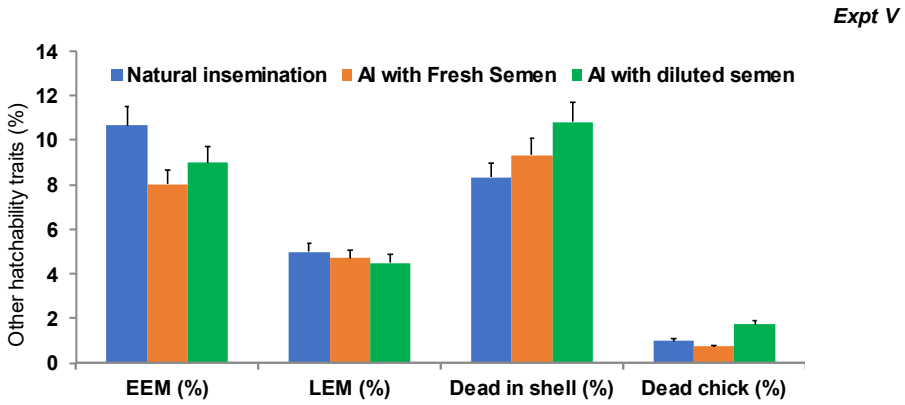

Figure 7.4 The other hatchability traits (%) in different breeding situation. Each bar with an error bar represents the Mean ± SEM values

Figure 7.5 represents the normal and abnormal chick production in different breeding situations. Normal (99.00±6.42, 99.99±6.99 and 98.50±6.88 percent) and abnormal (1.00±0.05, 0.01±0.00 and 1.50±0.09 percent) chicks production did not differ significantly among the treatment groups.

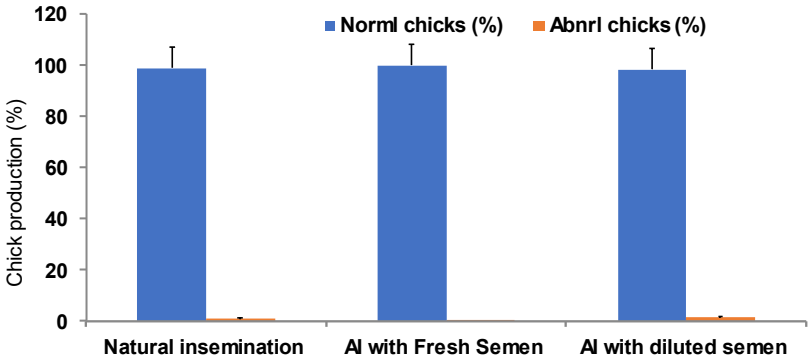

Figure 7.5 The normal and abnormal chicks production (%) in different breeding situation. Each bar with an error bar represents the Mean±SEM values.

7.3.5 Day old chicks weight

Figure 7.2 represents the day old chicks weight in different breeding situations. The day old chicks weight did not vary significantly among the treatment groups. The day old chicks weight were almost similar (30.03±1.91, 29.98±1.93 and 29.90±1.86) within the treatment groups.

7.4 Discussion

7.4.1Age at maturity

The hens which were managed by natural mating laid their first eggs at 215±15.05 days of age. On the other hand, those which were managed by AI with fresh and diluted semen laid eggs at 213±13.07 and 216±13.98 days of age, respectively (Table 3.1) and did not vary significantly. Li *et al*. (2018) commented that birds managed with natural mating showed a significantly earlier age at their first egg compared with AI, but in this study the result showed no significant variation among the treatment groups.

7.4.2 Egg weight

The egg weight (43.39±2.97, 43.03±2.94 and 43.21±2.95 g respectively) produced by the different treatment groups did not differ significantly and similar result was observed by Li *et al*. (2018) when they studied artificial insemination and natural mating.

7.4.3 Fertility and hatchability

The breeding system (Normal mating and Artificial Insemination by fresh and diluted semen) had the minimum effect on fertility. The insemination by the fresh and diluted semen (69.25±5.04, 65.68±4.75) showed significantly better (P<0.05) fertility than the fertility observed by natural mating (60.00±4.3) and insemination by fresh semen showed significantly better fertility (P<0.05) than the diluted semen also (Table 3.1). Li *et al*. (2018) observed no differences among the birds managed with AI and natural mating. Though this experiment was not the same as the previous one, the result somehow supported the result of this study. Vermal *et al*. (2018) stated that the fertility (%) were 87.18±2.92a, 80.00±3.00b for Kadaknath and Aseel chickens, respectively

The hatchability is determined on the basis of fertile eggs. The breeding system (Normal mating and Artificial Insemination by fresh and diluted semen) had no effect on the hatchability (75.00±5.50; 77.21±5.67; 75.68±5.55) of eggs among the different treatment groups, i.e. hatchability did not differ significantly (Table 3.1). Habibullah *et al*. (2015) observed significantly higher hatchability after AI than before AI and this result disagreed with the result of this study. Sayyazadeh *et al*. (2005) showed that the hatchability percentage increased by artificial insemination. The average hatchability percentage in natural mating for Arian and Ross 308 lines were 82.7 and 83.1 respectively. But when artificial insemination was done, these values were increased and that was 87.2% and 89.4% respectively. A similar result was also observed by Hocking and Bernared (1997), Christensen (2001) and Surai *et al*., (1996). Robinson (1996) reported that when artificial insemination is practiced, hatchability and production percentages are increased compared to natural mating.

7.4.4 Other hatchability traits

The comparison in relation to Early Embryo mortality (10.67±0.75, 8.02±0.54 and 9.00±0.6 percent); the Late Embryonic mortality (5.00±0.2, 4.70±0.27 and 4.50±0.26 percent); Dead in shell (8.33±0.45, 9.32±0.54 and 10.81±0.56 percent) and Dead chick (1.00±0.06, 0.75±0.05 and 1.75±0.11 percent) did not differed significantly among the different treatment groups of breeding system and insemination with fresh and diluted semen (Figure 1). Similar result were reported by Keith, (2008) and Bramwell *et al*. (1996). The results of this study were lower than Morz *et al*. (2010) who reported 13.0¬23.0% and also lower than Khan *et al*. (2013) who reported 7.5, 13.2 and 19.3% deaths as early, mid and late embryonic mortality, respectively.

Normal (99.00±6.42, 99.99±6.99 and 98.50±6.88 percent) and abnormal (1.00±0.05, 0.01±0.00 and 1.50±0.09 percent) chick production did not differ significantly among the treatment groups.

7.4.5 Day old chicks weight

The day old chicks weight did not vary significantly among the treatment groups. The day old chicks weight was almost similar (30.03±1.91, 29.98±1.93 and 29.90±1.86) within the treatment groups. Habibullah *et al*. (2015) observed thea similar result in the case of broiler parent stock.

7.5 Conclusion

The age at maturity for natural (215±15.05 days) and artificial insemination with fresh and diluted semen (213±13.07 and 216±13.98 days) did not vary significantly. The egg weight (43.39±2.97, 43.03±2.94 and 43.21±2.95 g respectively) and day old chicks' weight (30.03±1.91, 29.98±1.93 and 29.90±1.86) also did not vary significantly. The fertility percent (69.25±5.04, 65.68±4.75) demonstrated by artificial insemination using fresh and diluted semen was significantly better ($P<0.05$) than natural mating (60.00±4.3) but hatchability (75.00±5.50; 77.21±5.67; 75.68±5.55) did not differ significantly. The other hatchability traits did not vary significantly.

Chapter VIII

SUMMARY AND CONCLUSIONS

Aseel, Naked Neck, and Red Junglefowl are the important chicken genetic resources in Bangladesh and these breeds are close to extinction. The productive characteristics which were observed were egg production, egg weight, day old chicks weight, etc. The egg production varied significantly among the three genetic resources, but the Naked Neck chickens showed higher egg production per annum (65.00±2.43) compared to the Aseel (49.00±2.30) and Red Junglefowl (57.00±3.04), i.e., considering egg production among Aseel, Naked Neck, and Red Junglefowl, Naked Neck chicken produced a significantly higher number of eggs followed by Red Junglefowl and Aseel. Considering the egg weight, the Aseel and Naked Neck chickens produced significantly higher weighed eggs compared to Red Junglefowl chickens and the egg weight was 43.39±1.23, 38.29±2.41 and 33.21±1.23 g, respectively for Aseel, Naked Neck and Red Junglefowl. Similarly, to egg weight, the day old chick weights of Aseel chicken weighed higher followed by chicks obtained from Naked Neck and then Red Jungle fowl. The weights were 30.03±1.88, 24.88±1.08, and 21.57±0.88 g, respectively for Aseel, Naked Neck, and Red Junglefowl. The Red Junglefowl and Naked Neck chickens became mature compared to Aseel chickens. Normally, most of the chickens became mature at 126 to 140 days, and the Red Junglefowl and Naked Neck chickens showed the normal phenomena the Aseel chickens became mature later than the normal phenomena and their age at maturity were 215,135 and 122 days, respectively, for Aseel, Naked Neck and Red Junglefowl. The mature live weight of Aseel chickens and cock was about three times higher than Naked Neck and Red Junglefowl. Weight at maturity was 3274.80 and 2174.70 g for Aseel male and female, 1848.00 and 1205.00 g for Naked Neck male and female, and 1115.50 and 830.00 g for Red Jungle male and female, respectively. The trend of live weight gain for Aseel chickens was significantly higher up to maturity compared to Naked Neck and Red Junglefowl chickens and the Aseel breed showed continuously higher live weight than the other two genetic resources. The feed efficiency for the three chicken genetic resources did not vary significantly except in the later stage of maturity.

The fertility rate was significantly lower for Aseel chickens compared to the other two breeds and the percent fertility observed for the chicken breeds were 65.68±2.48,

80.75±4.67 and 76.00±3.56, respectively for Aseel Naked Neck and Red Junglefowl breeds. On the other hand, the hatchability rate was significantly higher for Aseel breeds compared to the other two chicken genetic resources. The fertility percentage showed 75.68±.5.01, 56.81±0.48, and 57.89±0.40, respectively for Aseel, Naked Neck, and Red Junglefowl breeds. The other related traits were satisfactory for all the breeds and did not vary significantly in most cases.

The semen color obtained from Aseel's cock was creamy white and showed a normal appearance. There was no abnormal color or no blood observed with the ejaculated semen. The volume of semen per ejaculation was 0.50±0.0 ml and the pH was 7.15.

Sperm motility was observed to be 67.08% and 32.92% of sperm were immotile. Among the motile semen obtained from Aseel's cock, 5.8% were progressively motile, and 61.23% were also motile but not progressive. Again, among the progressively motile sperm, 0.66% were rapidly progressive and 5.19% were medium progressive. The average curvilinear velocity of Aseel sperm was 45.06±2.60 μm/s and the average straight-line velocity was 16.86±1.03 μm/s. The average path velocity was 27.35±1.28 μm/s. The average linear index of sperm obtained from Aseel cock was 47.82±2.38 %. The average straightness was 63.72±4.09% and the average oscillation index was 67.31±3.58%, the average amplitude lateral head displacement was 2.56±0.20 μm and the beat cross frequency was an average of 2.93±0.13 Hz.

Considering the sperm characteristics, the Aseel semen showed normal macroscopic and bio-kinetic parameters. Considering the motility percent and percent of progressive sperm, the semen samples may be stored for up to 2 hours in good condition for insemination. The honey supplementation with the Ringer's solution improves the sperm quality and longevity relation without honey. Poultry breeders use various antioxidants during *in vitro* sperm storage situations to improve sperm quality. It can be said that antioxidants improve the motility, viability, and fertilizing ability of avian sperm in a dose-dependent manner.

Considering sperm concentration, total and progressive motility of sperm, velocity and progressiveness, Curvilinear, straight-line, and average path velocity, and Liner, straightness, and oscillation index the 3% honey showed the result satisfactory and may be possible to use as a cryoprotective agent for cryopreservation of rooster semen. As a cryoprotective agent, 11% glycerol is the best and most well-recognized by scientists,

and 8% DMSO also proved a good cryoprotective ability. So, the honey also has cryoprotective properties and 3% of the honey may act as a cryoprotective agent.

The chickens were bred through insemination with fresh and diluted semen compared with natural mating. The egg weight (43.39±2.97, 43.03±2.94 and 43.21±2.95 g, respectively) and day old chicks' weight (30.03±1.91, 29.98±1.93 and 29.90±1.86) also did not vary significantly among the breeding treatment groups. The age at maturity for natural (215±15.05 days) and artificial insemination with fresh and diluted semen (213±13.07 and 216±13.98 days) did not vary significantly. The fertility percent (69.25±5.04, 65.68±4.75) demonstrated by artificial insemination using fresh and diluted semen was significantly better ($P<0.05$) than natural mating (60.00±4.3) but hatchability (75.00±5.50; 77.21±5.67; 75.68±5.55) did not differ significantly. The other hatchability traits did not vary significantly.

Therefore, the result showed that the addition of 3% honey in Ringer's solution performed as a cryoprotective agent which may help to cryopreserve Aseel roosters' semen as the native chicken genetic resource and revealed that honey might has the cryoprotective properties. Therefore, the study concluded that the pre-freezing addition of honey improved the cryo-survivability of Aseel's sperm and suggested for *in vitro* conservation of chicken genetic resources available in Bangladesh.

REFERENCES

Abdel-Rahman, A. M. 1990. Significance of some major genes in relation to the productive adaptability of four German sublines compared with local Egyptian chickens under Assiut subtropical conditions. Ph. D. Thesis, Assiut University Egypt.

Abdurehman, A. and Urge, M. 2016. Evaluation of fertility, hatchability, and egg quality of rural chicken in gorogutu district, Eastern Hararghe, Ethiopia. Asian Journal of Poultry Science.10:111−116.

Abouelezz, F.; Castaño, C.; Toledano-Díaz, A.; Esteso, M.; López-Sebastián, A.; Campo, J. and Santiago-Moreno, J. 2015. Effect of the interaction between cryoprotectant concentration and cryopreservation method on frozen/thawed chicken sperm variables. Reproduction in Domestic Animals 50:135−141.

Abouelezz, F.; Sayed, M. and Santiago-Moreno, J. 2017. Fertility disturbances of dimethylacetamide and glycerol in rooster sperm diluents: Discrimination among effects produced pre and post freezing-thawing process. Animal Reproduction Science 184:228−234.

Adebambo, A. O.; Ozoje, M. O.; Adebambo, F. and Abiola, S. S. 2006. Genetic variations in growth performance of Giriraja, Indian White Leghorn, and improved indigenous chicken breeds in southwest Nigeria. *Nigerian* Journal of *Genetics*. 20:7−16.

Adomako, K. 2009. Egg production in naked-neck Creole (Nana) hens and those with normal plumage (nana) in the region of the Mexican plateau. Ph.D. A thesis submitted to the Department of Animal Science, Kwame Nkrumah.

Aguero, A.; Miragaya, M. H. and Mora, N. G. 1995. Effect of vitamin E addition on equine sperm preservation. Communication Biology, 13:343−356.

Ahmad, M. and Singh, P. K. 2007. Estimates of genetic parameters for some economic traits in White Leghorn. Indian Journal of Poultry Science, 42(3):311−312.

Ahmad, S.; Hussain, J.; Akram, M.; Aslam, F.; Mahmud, A.; Mahmood, S. and Mustafa, G. 2013. Comparative study on productive performance and hatching traits of three age groups of indigenous Mushki Aseel chickens. Agricultural Advances, 2:146−149.

Ahmad, Z.; Sahota, A. W.; Akram, M.; Khalique, A.; Jatoi, A. S.; Shafique, M.; Usman and Khan, U. 2014. Pre- and post-moult productive efficiency in four varieties of indigenous Aseel chicken during different production cycles. Journal of Animal and Plant Science, 24:1276−1282.

Akhtar, N. S.; Mehmood, M.; Hassan and Yasmeen, F. 2007. Comparative Study of Production Potential and Egg Characteristics of Lyallpur Silver Black, Fayoumi and Rhode Island Red Breeds of Poultry. Pakistan Veterinary Journal, 27:184−188.

Akiyama M. *In vivo* scavenging effect of ethylcysteine on reactive oxygen species in human semen. Nippon Hinyokika Gakkai Zasshi, 90:421–428.

Akram, M. 1998. Effect of induced moult on the subsequent second production cycle performance of commercial layers reared under various lighting and feeding regimes, Department of Poultry Husbandry. vol. Ph.D.

Ali, A.; Akram, M. and Hussain, J. 2012. Evaluation of egg Quality traits among different breeds and varieties of chicken locally available in Pakistan. In Book of abstract 'National science conference of agriculture and food security issues in global environment perspective. University of Ponch Rawalakot AJ and K (291).

Allen, T. E. and, Bobr, L. W. 1955. The fertility of fowl spermatozoa in glycerol diluents after intrauterine insemination. Poultry Science, 34:1167−1169.

Alvarez, M.; Tamayo-Canul, J.; Anel, E.; Bioxo, J. C.; Mata-Campuzano, M.; Martinez-Pastor, F.; Anel, L. and de Paz, P. 2012. Sperm concentration at freezing affects post-thaw quality and fertility of ram semen. Theriogenology, 77:1111−1118.

Alvarez, T.; Carrasco, E.; Tato, P. and Téllez, G. 2002.Comparison of production parameters and egg quality between laying hen's indigenous Naked Neck (Na) and commercial Babcock B-380. Proceedings of 91st Poultry Science annual meeting, Newark, University of Delaware, USA, 11−14 Aug.

Amann, R. P.; Gill, S. P. S. and Hammerstedt, R. H. 1997. Maximizing Genetic Impact of Roosters: Effective Handling, Extension and Utilization of Rooster Semen and Artificial Insemination. BioPore, Inc. State College, pp:141−156.

Amann, R. P.; Graham, J. K. Spermatozoal Function. In: McKinnon VO, Voss JL. 1993. Equine Reproduction. Lea and Febiger. Philadelphia, London:715−745.

Amann, R. P. and Pickett, B. W. 1987. Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. Journal of Equine Veterinary Science. 7:145−173.

Ansari, M.; Zhandi, M.; Kohram, H.; Zaghari, M.; Sadeghi, M. and Sharafi, M. 2017. Improvement of post-thawed sperm quality and fertility of arian rooster by oral administration of d-asparticacid.Theriogenology, 92 :69−74.

Arav, A.; Zeron, Y.; Leslie, S. B.; Behboodi, E.; Anderson, G. B. and Crowe, J. H. 1996. Phase transition temperature and chilling sensitivity of bovine oocytes. Cryobiology, 33: 589− 599.

Ashraf, M.; Mahmood, S. and Ahmad, F. 2003. Comparative re-productive efficiency and egg quality characteristics of Lyallpur Silver Black and Rhode Island Red breeds of poultry. International Journal of Agriculture and Biology, 5(4):449−451.

Askari, H.A.; Check, J. H. and Peymer, N. 1994. Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freez-thaw process. Archives of Italian Urology and Andrology, 33:11−15.

Aurich, J. E.; Schonherr, U. and Hoppe, H. 1997. Effects of antioxidants on motility and membrane integrity of chilled stored stallion semen. Theriogenology, 48:185−192.

Babar, M. E.; Nadeem, A.; Hussain, T.; Wajid, A.; Shah, S. A.; Iqbal, A. and Akram, M. 2012. Microsatellite marker based genetic diversity among four varieties of Pakistani Aseel Chicken. Pakistan Veterinary Journal, 32:237−241.

Baccetti, B.; Collodel, G. and Piomboni P. 1996. Apoptosis in human ejaculated sperm cells (notulaeseminologicae). Journal Submicroscopic Cytology and Pathology, 28: 587−596.

Bacon, L. D.; Salter, D. W.; Motta, J. V. and Crittenden, L. B. 1986. Cryopreservation of chicken semen of inbred or specialized strains. Poultry Science, 65:1965−1971.

Bakst, MR. 1980. Fertilizing capacity and morphology of fowl and turkey spermatozoa in hypotonic extender. Journal of Reproduction and Fertility, 60:121−127.

Bakst, M. R. 1990. Preservation of avian cells. In: Crawford, R.D. (ed.), Elsevier, Amsterdam. Poultry Breeding, pp. 91−108.

Ball, B. A.; Medina, V. and Gravance, C. G. 2001. Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5°C. Theriogenology, 56:577−589.

Barth, A. D. and Oko, R. J. 1989. Abnormal Morphology of Bovine Spermatozoa. Iowa State University Press. Ames, IA:31−43.

Behne, D.; Weiler, H. and Kyriakopoulos, A. 1996. Effects of selenium deficiency on testicular morphology and function in rats. Journal of Reproduction and Fertility, 106:291− 297.

Biswas, A.; Mohan, J. and Sastry, K. V. H. 2007. Effect of dietary vitamin E on the cloacal gland, foam and semen characteristics of male Japanese quail. Theriogenology, 67:259–263.

Bjorveboe, A.; Bjorveboe, B. E. A. and Drevon, C. A.1990. Absorption, transportation and distribution of Vitamin E. Journal of Nutrition, 120:233–42.

Blackburn, H. D.; Silversides, F. and Purdy, P. H. 2009. Inseminating fresh or cryopreserved semen for maximum efficiency: implications for gene banks and industry. Poultry Science, 88:2192−2198.

Blanc-Layrac, G.; Bringuier, A. F.; Guillot, R. and Feldmann, G. 2000. Morphological and biochemical analysis of cell death in human ejaculated spermatozoa. Cell MolecularBiolology (Noisy-legrand), 46: 187−197.

Blanco, J. M.; Long, J. A.; Gee, G.; Donoghue, A. M. and Wildt, D. E. 2008. Osmotic tolerance of avian spermatozoa: Influence of time, temperature, cryoprotectant and membrane ion pump function on sperm viability. Cryobiology, 56:8−14.

Blesbois, E.; Seigneurin, F.; Grasseau, I.; Limouzin, C.; Besnard, J.; Gourichon, D.; Coquerelle, G.; Rault, P. and Tixier-Biochard, M. 2007. Semen

cryopreservation for *ex situ* management of genetic diversity in chicken: Creation of the French avian cryobank. Poultry Science, 86:555−564.

Blesbois, E. 2007. Current status in avian semen cryopreservation. World's Poulty Science Journal, 63:213−222.

Boonsorn, T.; Kongbuntad, W. and Narkkong, N. A. 2010. Effects of catechin addition to extender on sperm quality and lipid peroxidation in boar semen. American-Eurasian Journal of Agricultural and Environmental Sciences, 7:283−288.

Bootswala, S. M. and Miles, R. D. 1992. Development of diluents for domestic fowl semen. Worlds Poultry Science Journal, 48: 121−128.

Bordas, A.; Monnet, L. E. and Mérat, P. 1980. Gene county, performances efficacies alimentaireselon la temperature chez la poule.Annual Genetics Selection of Animal, 12:343−361

Bramwell, R. K. and Howarth, Jr. B. 1992. Preferential attachment of cock spermatozoa to the perivitelline layer directly over the germinal disc of the hen's ovum. Biology of Reproduction, 47:1113−1117.

Bratton, DL.; Fadok, V. A.; Richter, D. A.; Kailey, J. M.; Guthrie, L. A. and Henson, P. M. 1997. Appearance of phosphatidylserine on apoptotic cells requires calciummediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. Journal of Biological Chemistry, 272: 26159−26165.

Brezezinska-Slebodzinska, E.; Slebodzinski, A. B. and Pietras, B.1995. Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. Biological Trace Element Research, 47:69–74.

Brillard, J. P. 2003. Practical aspects of fertility in poultry. World's Poultry Science Journal 59: 441−446.

Brun, J. M. and Larzul, C. 2003. Inheritance of the reproductive traits of female common ducks in pure breeding and inter-generic cross breeding with Muscovy ducks. British Poultry Science, 44: 40−45.

Bucak, M. N.; Atessahin, A. and Vas, O. 2008. The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen, microscopic and oxidative stress parameters after freeze-thawing process. Theriogenology, 67:1060−1067.

Burrows, W. H. and Quinn, J. P. 1937. The collection of spermatozoa from the domestic fowl and turkey. Poultry Science, 14:19−24.

Buss, E. G. 1993. Cryopreservation of rooster sperm. Poultry Science, 72:944−954.

CARI Annual Report. 2011. Central Avian Research Institute. Izatnagar, India.

Carrell, D. T. 2000. Semen analysis at the turn of the century: an evaluation of potential uses of new sperm function assays. Archives of Andrology, 44:65−75.

Carter, R. D.; McCartney, M. G.; Chamberlin, V. D. and Wyne, J. W. 1957. The effect of storage time and temperature on fertilising capacity of turkey semen. Poultry Science, 72: 1370−1378.

Cecil, H. C. 1993. *In vitro* lipid peroxidation of turkey spermatozoa. Poultry Science, 72: 1370−1378.

Cerolini, S.; Maldjian, A. and Surai, P. 2000. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. Animal Reproduction Science, 58: 99−111.

Cerolini, S.; Zaniboni, L. and Maldjian, A. 2006. Effect of docosahexaenoic acid and α-tocopherol enrichment in chicken sperm on semen quality, sperm lipid composition and susceptibility to peroxidation. Theriogenology, 66:877–886.

Chalah, T.; Seigneurin, F.; Blesbois, E. and Brillard, J. P. 1999. *In vitro* comparison of fowl sperm viability in ejaculates frozen by three different techniques and relationship with subsequent fertility *in vivo*. Cryobiology, 39:185-191.

Chatterjee, R. N.; Sharma, R. P.; Reddy, M. R.; Niranjan, M. and Reddy, B. L. N. 2007a. Growth, body conformation and immune responsiveness in two Indian native chicken breeds. Livestock Research for Rural Development (http://www.cipav. org.co/lrrd/lrrdhome.html.)

Chatterjee, R. N.; Ahlawat, S. P. S.; Yadav, S. P.; Senani, S.; Kundu, A.; Jeyakumar, S. and Bharati, D. 2002. Comparative growth performance of Nicobari fowl and their cost effectiveness under backyard and intensive system. Indian Journal of Poultry Science, 37:63−66.

Chatterjee, R. N.; Niranjan, M.; Sharma, R. P.; Dange, M. and Bhatta-charya, T. K. 2010. Estimation of genetic heterogeneity of chicken germplasm being used for development of rural varie-ties utilizing DNA markers. Journal of Genetics, 89(2),33−37.

Chatterjee, R. N.; Sharma, R.P.; Reddy, M.R.; Niranjan, M. and Reddy, B. L. N. 2007. Growth, body conformation and immune-responsiveness in two Indian native chicken breeds. Livestock Research for Rural Development, Poultry Genetics and Breeding, Rajendranagar, Hyderabad (Andhra Pradesh), India. 19:151

Chaudhuri, D. and Lake, P. E. 1988. A new diluent and methods of holding fowl semen for up to 17 hours at high temperatures. Proceedings in XVIII World's Poultry Congress, Nagoya, Japan, 591−593.

Choudhary, M. L.; Brah, G. S. and Khurana, S. 2009. Inheritance of body weight and body weight ratio and their relationship with economic traits in White Leghorn chicken. Indian Journal of Poultry Science, 44(2), 167−171.

Christensen, V. L. 2001. Factors associated with early embryonic mortality. World Poultry Science Journal, 57: 359−372.

Clark, C. E. and Shaffner, C. S. 1960. The fertilizing capacity of frozen chicken sperm and the influence of related *in vitro* processes. Poultry Science, 39:1213−1220.

Clarke, R. N.; Sexton, T. J. and Ottinger, M. A. 1982. Effects of holding temperature and storage time on respiratory rate, motility, and fertility of chicken and turkey semen. Poultry Science, 61: 1912−1917.

Dalimata, A. M. and Graham, J. K. 1997. Cryopreservation of rabbit spermatozoa using acetamide in combination with trehalose and methyl cellulose. Theriogenology, 48:831−841.

Darin-Bennett, A. and White, I. G. 1977. Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. Cryobiology, 14:466−470.

Darmstadt, G.L.; Ahmed, A. N. U.; Saha, S. K.; Chowdhury, M. A.; Alam, M. A.; Khatun, M.; Black, R. E. and Santosham, M. 2005. Infection control practices reduce nosocomial infections and mortality in preterm infants in Bangladesh. Journal of Perinatology, 25:331.

Das, A. K.; Kumar, S.; Rahim, A.; Kokatate, L. S. and Mishra, A. K. 2015b. Genetic analysis of body conformation and feed efficiency characteristics in a selected line of Rhode Island Red chicken. Asian Journal of Animal Sciences 9(6): 434−440.

Das, A. K.; Kumar, S. and Mishra, A. K. 2018. Characterization of Production and Reproduction Performances in Rhode Island Red-White Strain Chicken. Iranian Journal of Applied Animal Science, 6(3): 707−713.

Das, A. K.; Kumar, S. and Rahim, A. 2015a. Genetics of body conformation and feed efficiency characteristics in a control line of Rhode Island Red chicken. Iranian Journal of Applied Animal Sciences 5(4),965−973.

Das, A. K.; Kumar, S.; Rahim, A. and Mishra A. K. 2014b. Genetic variability in immunocompetence and performance status of Rhode Island Red chicken strains and its crosses, International Journal of Bio-resource and Stress Management, 5(2):246−254.

Das, A. K.; Kumar, S.; Rahim, A.; Kokatate, L. S. and Mishra, A. K. 2014a. Assessment of body conformation, feed efficiency and morphological characteristics in Rhode Island Red-White strain chicken. Indian Journal of Animal Sciences, 84(9): 984−991.

Davenport, C. B. 1914. The bare necks. Journal of Heredity, 5:374.

Davis, I. S.; Bratton, R. W. and Foote, R. H. 1963. Livability of bovine spermatozoa at 5, -25, and -85°C in tris-buffered and citrate-buffered yolk-glycerol extenders. Journal of Dairy Science, 46:333−336.

De Ketelaere, B.; Bamelis, F.; Kemps, B.; Decuypere, E. and De Baerdemaeker, J. 2004. Non-destructive measurements of the egg quality. World's Poultry Science Journal, 60:289−302.

Deeb, NADER. and Cahaner, AVIGDOR. 1999. The effects of Naked Neck genotypes, ambient temperature, and feeding status and their interactions on body temperature and performance of broilers. Poultry Science, 78:1341−1346.

Dhama, K. M.; Mahendran, M. and Tomar, S. 2007. Artificial insemination in poultry: Health care and managemental approaches. Poultry World, 2: 33−36.

Doebbler, G. F. 1966. Cryoprotective compounds: Review and discussion of structure and function. Cryobiology, 3:2−11.

Donoghue, A. M. and Wishart, G. J. 2000. Storage of poultry semen. Animal Reproduction Science, 62: 213−232.

Dumpala, P. R.; Parker, H. M. and McDaniel, C. D. 2006. The effect of semen storage temperature and diluent type on the sperm quality index of broiler breeder semen. International Journal of Poultry Science, 5: 838−845.

El-Safty, S. A. 2012. Determination of some quantitative and qualitative traits in Libyan native fowls. Egyptian Poultry Science 32(2): 247−258.

Etches, R. J. 1996. Reproduction in poultry. Centre for Agriculture and Biosciences International Wallingford, UK.

Fathi, M. M. 1987. Effect of Naked Neck gene on some quantitative characters in chickens, M. Sc. Thesis, Ain Shams University, Egypt.

Fathi, M. M. 1992. Estimating some genetic parameters of Naked Neck gene under different environmental conditions, Ph. D. Thesis, Ain Shams University, Egypt.

Foote, R. H.; Brockett, C. C. and Kaproth, M. T. 2002. Motility and fertility of bull sperm in whole milk extender containing antioxidants. Animal Reproduction Science,71:13–23.

Foote, R. H. 1988. Artificial insemination to cloning: Tracing 50 years of research. Published by RH Foote; Ithaca, NY.

Foote, R. H. 1999. Development of reproductive technologies in domestic animals from artificial insemination to cloning: A perspective. Cloning, 1:133−142.

Franchini, A.; Bergonzoni, M. L. and Melotti, C. 2001. The effect of dietary supplementation with high doses of vitamin E and C on the quality traits of chicken semen. Archiv fur Geflugelkunde, 65:76−81.

Fulton, J. E. 2006. Avian genetic stock preservation: An industry perspective. Poultry Science, 85:227−231.

Galal, A. 1995.Effect of Naked Neck gene on growth and other productive traits of chicken under hot climatic conditions. M. Sc. Thesis, Ain shams University, Egypt.

Gao, D. Y.; Liu, J.; Liu, C.; McGann, L. E.; Watson, P. F.; Kleinhans, F.W.; Mazur, P.; Critser, E.S. and Crister, J. K. 1995. Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol. Human Reproduction, 10:1109−1122.

Garces, A. and Casey, N. H. 2003. Oviposition and egg quality traits of dwarf and Naked Neck layers. South African Journal of Animal Science, 33:105−111.

Garner, D. L.; Pinkel, D.; Johnson, L. A. and Pace, M. M. 1986. Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analysis. Biology of Reproduction, 34:127-138.

Gee, G. F.; Bertschinger, H.; Donoghue, A. M.; Blanco, J. M. and Soley, J. 2004. Reproduction in nondomestic birds: Physiology, semen collection, artificial insemination and cryopreservation. Avian Poultry Biological Reviews, 15: 47−101.

George, F.; Barrowclough, Joel C.; John, K. and Robert, M. Z. 2016. How Many Kinds of Birds Are There and Why Does It Matter? PLOS ONE, 11 (11):1-15

Ghanem, H. M.; Ateya, A. I.; Saleh, R. M. and Hussein, M. S. 2017. Artificial Insemination vs Natural Mating and Genetic PRL/PstILocus Polymorphism and Their Effect on Different Productive and Reproductive Aspects in Duck.Advances in Animal and Veterinary Sciences, 5(4):179−184.

Gilbert, A. B.; Reynolds, M. E. and Lorenz, F. W. 1968. Distribution of spermatozoa in the oviduct and fertility in domestic birds. Journal of Reproduction and Fertility, 17:305−310.

Gill, S. P.; Buss, E. G. and Mallis, R. J. 1996. Cryopreservation of rooster semen in thirteen and sixteen percent glycerol. Poultry Science, 75:254−256.

Gillan, L.; Evans, G. and Maxwell, W. M., 2005. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology, 63:445−457.

Glander, H. J. and Schaller, J. 1999. Binding of annexin V to plasma membranes of human spermatozoa: a rapid assay for detection of membrane changes after cryostorage. Molecular Human Reproduction, 5 :109−115.

Graham, E. F.; Nelson, D. S. and Schmehl, M. K. L. 1982. Development of extender and techniques for frozen turkey semen. 2. Fertility trials. Poultry Science, 61:558−563.

Graham, E.F.; Schmehl, M. K. L. and Nelson, D. S. 1980. Problems with laboratory assays. In: Proceedings of Eighth National Association of Animal Breeders Technical Conference. AI Reproduction, 59−66.

Graham, J. K.; Kunze, E. and Hammerstedt, R. H. 1990. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. Biology of Reproduction, 43:55−64.

Graham, J. K. 1996. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. Biology of Reproduction, 43:55−64.

Graham, J. K. and Mocé, E. 2005. Fertility evaluation of frozen/thawed semen. Theriogenology, 64:492−504.

Graham, J. K. 2005. Fertility evaluation of boar semen. Theriogenology, 46:42−50.

Graham, J. K. 2001. Assessment of sperm quality: a flow cytometric approach. Animal Reproduction Science, 68:239−247.

Griffiths, J. B.; Cox, C. S.; Beadle, D. J.; Hunt, C. J. and Reid, D. S. 1979. Changes in cell size during the cooling, warming and post-thaw periods of the freeze-thaw cycle. Cryobiology, 16:141−151.

Grobbelaar, J. A. N.; Sutherland, B. and Molalakgotla, N. M. 2010. Egg production potentials of certain indigenous chicken breeds from South Africa. Animal Genetic Resources, 46: 2532.

Grogan, S. B. and Collins, V. H. 1990. Cryopreservation of sheep sperm and survivability and fertility after insemination. Journal of Andrology,5:137−144.

Gurung, B. S. and Singh, M. 1999. Network project on survey of poultry Aseel genetic sciences. Terminal Report, (1996−1999): 29−30.

Habibullah, M., Hashem, M. A.; Rana, M. S. and Islam, M. H. 2015. Effect of Artificial Insemination on different production parameter in Hubbard classic broiler parent stock. Journal of Bangladesh Agricultural University, 13(1): 71−77.

Hammerstedt, R. H.; Amann, R.P.; Rucinsky, T. Morse, P. D. Jr, Lepock, J.; Snipes, W.; and Keith, A. D. 1976. Use of spin labels and electrol spin resonance spectroscopy to characterize membranes of bovine sperm: effect of butylated hydroxytoluene and cold shock. Biology of Reproduction, 14:381−397.

Hammerstedt, R. H. 1990. Cryopreservation of sheep sperm: fertility and survivability. Journal of Andrology, 11:73−88.

Hammerstedt, R. H.; Graham, J. K. and Nolan, J. P. 1990. Cryopreservation of mammalian sperm: What we ask them to survive. Journal of Andrology, 11:73−88.

Hammerstedt, R. H. and Graham, J. K. 1992. Cryopreservation of poultry sperm: The enigma of glycerol. Cryobiology, 29:26−38.

Hammerstedt, R. H. 1995. Cryopreservation of poultry semen: Current status and economics. In: Bakst MR, Wishart GJ (eds), Proceedings First International Symposium on the Artificial Insemination of Poultry, Poultry Science Association, Savoy, Illinois, 229−250.

Hammerstedt, R. H. 1996. Artificial insemination using extended liquid semen: An old technology of great value to modern industry. USDA 88: 3742− 3752.

Hammerstedt, R. H. and Andrews, J. C. 1997. Metabolic support of normothermia. In: Karrow, AM. and Crister, JK. (eds), Academic Press, New York. Reproductive tissue banking, pp.139−166.

Hanada, A. and Nagase, H. 1980. Cryoprotective effects of some amides on rabbit spermatozoa. Journal of Reproduction and Fertility, 60:247−252.

Hanan, A. H. 2010. Variations in egg performance and plasma constituents at different ages of female Japanese quail. Egyptian Poultry Science Journal, 30:565−581.

Hanawa, S.; Niinomi, T.; Miyata, T.; Tsutsui, M. and Tajima, A. 2010. Cryopreservation of chicken semen using Nmethylacetamide as cryoprotective agent. Japanese Journal of Poultry Science, 47:J27−J32.

Harrison, R. A.; Dott, H. M.; Foster, G. C. 1982. Bovine serum albumin, sperm motility and the "dilution effect." Journal of Experimental Zoology, 222:81−88.

Harrison, R. A. P. and Vickers, S. E. 1990. Use of fluorescent probes to access membrane integrity in mammalian spermatozoa. Journal of Reproduction and Fertility, 88:343−352.

Haunshi. S.; Niranjan, M.; Shanmugam, M.; Padhi, M. K.; Reddy, M. R.; Sunitha, R. and Panda, A. K. 2011. Characterization of two Indian native chicken breeds for production, egg and semen quality, and welfare traits. PoultryScience,90:314−320.

Haunshi, S.; Shanmugam, M.; Padhi, M. K.; Niranjan, M.; Rajkumar, U.; Reddy, M. R. and Panda, A. K. 2012. Evaluation of two Indian native chicken breeds for reproduction traits and heritability of juvenile growth traits. Tropical Animal Health and Production. 44:969−973.

Haunshi, SANTOSH.; Padhi, M. K.; Niranjan, M.; Rajkumar, U.; Shanmugam, M. and Chatterjee, R. N. 2013. Comparative evaluation of native breeds of chicken for persistency of egg production, egg quality and biochemical traits. Indian Journal of Animal Sciences, 83:59−62.

Hocking, M. and Bernared, A. C. 1997. The influence of day length, body weight and age on the production ability of breeder cockerels. World Poultry Science Journal, 63: 1705−1710.

Horst, P. 1988. Native fowl as reservoir for genomes with direct and indirect effects on productive adaptability. 18th World's Poultry Congress. Nagoya, Japan, 99−105.

Howarth, B. K. 1983. Comparison of diluents for holding cock semen six hours at 41 °C. Poultry Science, 62 :1084−1087.

Hu, J.; Chen, J. L.; Wen, J.; Zhao, G. P.; Zheng, M. Q.;

Hughes, R. N. and Dunkin, S. D. B. 1984. Effect of dietary history on selection of prey, and foraging behavior among patches of prey, by the dog whelk, Nucella lapillus (L.). Journal of Experimental Marine Biology and Ecology, 79 :159−172.

Hurwitz, S.; Wax, E.; Nisenbaum, Y.; Ben-Moshe, M. and Plavnik, I. 1998. The response of laying hens to induced molt as affected by strain and age. Poultry Science, 77:22−31.

ICAR. 2004. DARE/ICAR Annual Report 2003−2004.

ICAR. 2009. DARE/ICAR Annual Report 2008−2009. Indian Council of Agricultural Research.

Idziorek, T.; Estaquier, J.; De Bels, F. and Ameisen, J. C. 1995. YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interferring with cell viability. Journal of Immunological Methods, 185 :249−258.

Iqbal, A.; Akram, M.; Sahota, A. W.; Javed, K.; Hussain, J.; Sarfraz, Z. and Mehmood, S. 2012. Laying characteristics and egg geometry of four varieties of indigenous Aseel chicken in Pakistan. Journal of Animal and Plant Science, 22:848−852.

Islam, K. R. and Weil, R. R. 2000. Land use effects on soil quality in a tropical forest ecosystem of Bangladesh. Agriculture Ecosystems and Environment, 79:9−16.

Islam, M. A.; Bulbul, S. M.; Seeland, G. and Islam, A. B. M. M. 2001. Egg quality of different chicken genotypes in summer and winter. Pakistan Journal of Biological Science. 4:1411−1414.

Israelachvili, J. N. 1978. The packing of lipids and proteins in membranes. In Light transducing membranes: Structure, function and evolution, ed. Deamer DW. Academic Press, 91−107: New York, NY.

Jacob, J.; Pescatore, T. and Cantor, A. 2011. Avian female reproductive system. Cooperative Extension Service, University of Kentucky, 1-6: Lexington, KY.

Jacob, J. P.; Miles, R. D. and Mather, F. B. 2000. Egg quality. University of Florida, IFAS extension.

Jayalaxmi, P.; Gupta, R. B.; Chatterjee, R. N.; Sharma, R. P. and Reddy, R. V. 2010. Genetic analysis of growth and production traits in IWK strain of White Leghorn. Indian Journal of Poultry Science, 45(2): 123−126.

Kalita, N.; Pathak, N.; Ahmed, M. and Saikia, K. 2013. Various causes related to dead in shell embryos of cross bred PB-2 x Indigenous chicken egg. Veterinary World, 610:774−777.

Kamble, K. T.; Murthy, B. S.; Pal, V. and Rao, K. S. 1996.External fixation in unstable intertrochanteric fractures of femur. Injury, 27:139−142.

Kemps, B.; Bamelis, F.; De Ketelaere, B.; Mertens, K.; Tona, K.; Decuypere, E. M. and De Baerdemaeker, J. G. 2006. Visible transmission spectroscopy for assessment of egg quality, Journal of the Science of Food and Agriculture, 86:1399−1406.

Khan, R. U. 2011. Antioxidants and poultry semen quality. Worlds Poultry Science Journal, 67, 297−08.

Kohrle J.; Jakob, F. and Contempré, B. 2005. Selenium, the thyroid and the endocrine system. Endocrine Reviews, 26:944−984.

Koohpar, H. K.; Sayyahzadeh, H.and Pirsaraei, Z. A. 2010.Comparing the natural mating with artificial insemination (A.I) atmazandran native hen. International Journal of Poultry Science, 9: 711−715.

Korver, D. R.; Saunders-Blades, J. L. and Nadeau, K. L. 2004. Assessing bone mineral density *in vivo*: Quantitative computed tomography. PoultryScience, 83:222−229.

Kothari, S.; Thompson, A.; Agarwal, A. and du Plessis, S. S. 2010. Free radicals: their beneficial and detrimental effects on sperm function. Indian Journal of Experimental Biology, 48: 425−435.

Lake, P.E. and Ravie, O. 1984. An exploration of cryoprotective compounds for fowl spermatozoa. British Poultry Science, 25:145−150.

Lake, P.E.; Ravie, O. and Stewart, J. M. 1984. An exploration of cryoprotective compounds for fowl spermatozoa. British Poultry Science, 25:151−160.

Lake, P. E. and Stewart, J. M. 1978. Preservation of turkey semen in liquid nitrogen-an improved method. British Poultry Science, 19:187−194.

Lake, P. E.; Stewart, J. M. and Ravie, O. 1978. Preservation of fowl semen in liquid nitrogen-an improved method. British Poultry Science, 10:88−94.

Lake, P. E. 1986. The history and future of the cryopreservation of avian germ plasm. Poultry Science, 65:1−15.

Lake, P. E. 1995. Historical perspective of artificial insemination technology. In: Bakst, M.R. and Wishart, G.J. (eds), Proc. First Int. Symposium Artificial Insemination of Poultry, pp. 1−20. The Poultry Science Association, Savoy, Illinois.

Li, Y.; Zhan, K.; Li, J.; Liu, W.; Ma, R.; Liu, S.; Han, T.; Li, S.; Wang, S. and Hu, Y. 2018. Comparison of Natural mating and artificial insemination on laying performance, egg quality and welfare of fast feathering Huainan partridge chickens. Pakistan Journal of zoology, 50(3):1131−1135.

Lima, L. R.; Junior, J. B. F. S.; Melo, A. S.; Torquato, J. L. and Filho, G. F. 2012. Performance and egg quality of Naked Neck hens in semiarid. World´s Poultry Science Journal. Expanded Abstract - Poster Presentation.

Liu, H. B.; Luo, H. L.; Liu, Z. H. 2005. Effect of dietary vitamin E concentration on semen quality of goat. Chinese Journal of Animal Science, 10:12–14.

Long, J. A.; Purdy, P. H.; Zuidberg, K.; Hiemstra, S. J.; Velleman, S. G. and Woelders, H. 2004. Cryopreservation of turkey semen: Effect of freezing method on spermquality, fertilization, and hatching.Cryobiology, 38:61−68.

Long, J. A.; Bongalhardo, D. C.; Pelaéz, J.; Saxena, S.; Settar, P.; O'Sullivan, N. P.; Fulton, J. E. 2010. Rooster semen cryopreservation: Effect of pedigree line and male age on postthaw sperm function. Poultry Science, 89:966−973.

Long, J. A. 2010. Rooster semen cryopreservation: Effect of maturity and male age on postthaw sperm function. Poultry Science, 89:856−863.

Long, J. A.; Purdy, P. H.; Zuidberg, K.; Hiemstra, S. J.; Velleman, S. G. and Woelders, H. 2014. Cryopreservation of turkey semen: Effect of breeding line and freezing method on post-thaw spermquality, fertilization, and hatching.Cryobiology, 68:371−378.

Lopes, S.; Jurisicova, A. and Sun, J. G. 1998. Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Human Reproduction, 134:896−900

Lukaszewicz, E. 2002. An effective method for freezing white Italian gander semen.Theriogenology, 58:19−27.

Luo, H. L.; Jia, Z. H.; Zhu, S. E. 2004. Effect of vitamin E on the qualities of fresh and frozen thawed ram semen. China Herbivores, 24:14–16.

Malago, J. J. and Baitilwake, M. A. 2009. Egg traits, fertility, hatchability and chick survivability of Rhode Island Red, local and crossbred chickens. Tanzanian Veterinary Journal, 26(1):24−36.

Malo, M. J. 2011. Effect of vitamin E on the quality of frozen ram semen. Asian Journal of Poultry Science, 6(3): 65−69.

Mansour, N.; McNiven, M. A. and Richardson, G. F. 2006. The effect of dietary supplementation with blueberry, alphatocopherol or astaxanthin on oxidative stability of Arctic char (Salvelinus alpinus) semen. Theriogenology, 66:373–82.

Marin-Guzman, J. Mahan, D. C. and Chung, Y. K. 1997. Effects of dietary selenium and vitamin E on boar performance and tissue response, semen quality and subsequent fertilization rates in mature gilts. Journal of Animal Science, 75:2994−3003.

Marin-Guzman, J.; Mahan, D. C.and Pate, J. L. 2000. Effect of dietary selenium and vitamin E on spermatogenic development in boars. Journal of Animal Science, 78:1537−1543.

Martin, G.; Sabido, O.; Durand, P. and Levy, R. 2004. Cryopreservation induces an apoptosis like mechanism in bull sperm. Biology of Reproduction, 71 :28−37.

Maxwell, W. M. C. and Stojanov, T. 1996. Liquid storage of ram semen in the absence or presence of some antioxidants. Reproduction Fertility and Development, 8 :1013−1020.

Mazur, P. and Cole, K. W. 1989. Roles of unfrozen fraction, salt concentration, and changes in cell volume in the survival of frozen human erythrocytes. Cryobiology, 26:1−29.

Mazur, P. 1984. Freezing of living cells: Mechanisms and implications. American Journal of Physiology, 247: C125−142.

Mengesha, M. 2012. Indigenous chicken production and the in-nate characteristics. Asian Journal of Poultry Science, 6(2): 56−64.

Meryman, H. T. 1971. Cryoprotective agents. Cryobiology, 8:173−183.

Michael, A. L.; Alexopoulos, C. and Pontiki, E. A. 2008. Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozenthawed canine spermatozoa. Theriogenology, 68:204−212.

Mitchell, R. L. and Buckland, R. B. 1975. Fertility of frozen chicken semen after intravaginal and intrauterine inseminations using various concentrations and a range of freezing and thawing rates. Poultry Science, 54:219−226.

Mitchell, R. L. and Buckland, R. B. 1976. Fertility of frozen chicken semen after intravaginal and intrauterine inseminations using various concentrations and

equilibration times of dimethlysulphoxide and a range of freezing and thawing rates. Poultry Science, 55:2195−2200.

Mocé, E.; Purdy, P. H. and Graham, J. K. 2010. Treating ram sperm with cholesterol-loaded cyclodextrins improves cryosurvival. Animal Reproduction Science, 118:236−247.

Moench, G.L. and Holt, H. 1931. Sperm morphology in relation to fertility. American Journal of Surgery, 47:586−596.

Mohammed, M. D.; Abdalsalam, Y. I.; Kheir, A. R. M.; Jin-yu, W. and Hussein, M. H. 2005. Growth performance of indigenous x exotic crosses of chicken and evaluation of general and spe-cific combining ability under Sudan condition. International Journal Poultry Science, 4(7): 468−471.

Mohan, J. and Sharma, S. K. 2017. Recent advances in poultry semen diluents. Proceedings of 26th Annual Conference of Society of Animal Physiologists of India, Bidar, India, pp. 103−108.

Mohan, J., Sastry, K. V. H.; Moudgal, R. P. and Tyagi, J. S. 2008. Production and other characteristics of Aseel Peela desi hens under normal rearing system. Indian Journal Poultry Science, 43:217–219.

Mohan, J., Sastry, K. V. H.; Moudgal, R. P. and Tyagi, J. S. 2005. Production and other characteristics of Aseel Peela desi hens under normal rearing system. Indian Journal Poultry Science, 34:17–21.

Mohan, J.; Sharma, S. K.; Kolluri, G.; Singh, R. P.; Tyagi, J. S. and Kataria, J. M. 2016. Semen quality characteristics and seasonality in different varieties of male guinea fowl. Advances in animal and Veterinary Sciences 4 (6): 320−326.

Moore, A. I.; Squires, E. L. and Graham, J. K. 2005. Adding cholesterol to the stallion sperm plasma membrane improves cryosurvival. Cryobiology, 51:241−249.

Morris, G. J.; Acton, E.; Murray, B. J. and Fonseca, F. 2011. Freezing injury: The special case of the sperm cell. Cryobiology, 64:71−80.

Morris, G.J.; Faszer, K.; Green, J.E.; Draper, D.; Grout, B. W. and Fonseca, F. 2007. Rapidly cooled horse spermatozoa: Loss of viability is due to osmotic imbalance during thawing, not intracellular ice formation. Theriogenology, 68:804−812.

Nagase, H.; Tomizuka, T.; Hanada, A. and Hosoda, T. 1972. Cryoprotection of some amide solutes on spermatozoa of domestic animals. I. Effects of formamide, acetamide, and lactamide on the motility of bovine spermatozoa in pellet freezing. Japanese Journal of Animal Reproduction, 18:15−21.

Narushin, V. G. and Romanov, M. N. 2002. Egg physical characteristics and hatchability. World's Poultry Science Journal, 58:297−303.

Narushin, V. G.; Van Kempen, T. A.; Wineland, M. J. and Christensen, V. L. 2004. Comparing infrared spectroscopy and egg size measurements for predicting eggshell quality. Journal of Biosystems Engineering, 87:367−373

Neville, W. J.; Macpherson, J. W. and Reinhart, B. 1971. The contraceptive action of glycerol in chickens. Poultry Science, 50:1411−1415.

Nwagu, B. I.; Olorunju, S. A. S.; Oni, O. O.; Eduvie, L. O.; Adeyinka, I. A.; Sekoni, A. A. and Abeke, F. O. 2007b. Response of egg number to selection in Rhode Island chickens selected for part period egg production. International Journal of Poultry Science 6(1): 18−22.

Nwagu, B. I.; Olorunju, S. A. S.; Oni, O. O.; Eduvie, L. O.; Adeyinka, I. A.; Sekoni, A. A. and Abeke, F. O. 2007a. Inbreeding effect on performance of Rhode Island chickens selected for part-period egg production. International Journal of Poultry Science 6(1):13−17.

Oldenhof, H.; Friedel, K.; Akhoondi, M.; Gojowsky, M.; Wolkers, W.F. and Sieme, H. 2012. Membrane phase behavior during cooling of stallion sperm and its correlation to fertility. Molecular Membrane Biology, 29(3-4): 95−106.

Olson, E.G.; Winfrey, V.P. and Hill, K. E. 2004. Sequential development of flagellar defects in spermatids and epididymal spermatozoa of selenium deficient rat. Reproduction, 127:335−342.

Parker, H. M.; Yeatman, J. B.; Schultz, C. D.; Zumwalt, C. D. and McDaniel, C. D. 2000. Use of a sperm analyser for evaluating broiler breeder males. 2. Selection of young broiler breeder roosters for the sperm quality index increases fertile egg production. Poultry Science, 79 :771−777.

Parks, J. E. and Lynch, D. V. 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology, 29:255−266.

Pena, F. J.; Johannisson, A. and Wallgren, M. 2003. Antioxidunt supplementation *in vitro* improves boar sperm motility and and mitochondrial membrane potential after cryopreservation of different fractions of the ejaculate. Animal Reproduction Science, 78:85−98.

Peters, S. O.; Omidiji, E. A.; Ikeobi, C. O. N.; Ozoje, M. O. and Adebambo, O. A. 2004. Effect of Naked Neck and frizzled genes on egg traits, fertility and hatchability in local chicken. Self sufficiency of animal protein in Nigeria. In: Proc. 9th Ann. Conf. Anim. Sci. Assoc. Nig., Abakaliki, Nig., pp. 262−264.Ebonyi State University, Nigeria.

Peters, S. O.; Shoyebo, O. D.; Ilori, B. M.; Ozoje, M. O.; Ikeobi, C. O. N. and Adebambo, O. A. 2008. Semen quality traits of seven strain of chickens raised in the humid tropics. International Journal of Poultry Science, 7: 949−953.

Pexa, F. J.; Saravia, F.; Johannisson, A.; Walgren, M. and Rodrigues-Martinez, H. 2005. A new and simple method to evaluate early membrane changes in frozen-thawed boar spermatozoa. International Journal of Andrology, 28:107−114.

Phillips, J. J.; Bramwell, R. K. and Graham, J. K. 1996. Cryopreservation of rooster sperm using methyl cellulose. Poultry Science, 75:915−923.

Pickett, B.W. and Komarek, R. J. 1967. Effect of cold show and freezing on loss of lipid from spermatozoa. Journalof Dairy Science, 50:753−757.

Pisenti, J. M.; Delany, M. E.; Taylor, R. L.; Abbott, U. K Jr.; Abplanalp, H.; Arthur, J. A.; Bakst, M. A.; Baxter-Jones, C.; Bitgood, J. J.; Bradley, F. A.; Cheng, K. M.; Dietert, R. R.; Dodgson, J. B.; Donoghue, A. M.; Emsley, A. B.; Etches, R. J.; Frahm, R. R.; Gerrits, R. J.; Goetinck, P. F.; Grunder, A. A.; Harry, D. E.; Lamont, S. J.; Martin, G. R.; McGuire. P. E.; Moberg, G. P.; Pierro, L. J.; Qualset, C. O.; Qureshi, M. A.; Shultz, F. T. and Wilson, B. W. 1999. Avian genetic resources at risk: An assessment and proposal for conservation of genetic stocks in the USA and Canada. 1999; Report No. 20: University of California Division of Agriculture and Natural Resources, Genetic Resources Conservation Program, Davis, CA.

Polge, C.; Smith, A. U. and Parkes, A. S. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature (London), 164:666.

Polge, C. 1951. Functional survival of fowl spermatozoa after freezing at -79°C. Nature (London), 167:949−950.

Purdy, P. H.; Graham, J. K. 2004a, Effect of cholesterol-loaded cyclodextrins on the cryosurvival of bull sperm. Cryobiology, 48:36−45.

Purdy, P. H. and Graham, J. K. 2004b.Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction and fertility. Biology of Reproduction, 71:522−527.

Purdy, P. H.; Mocé, E.; Stobart, R.; Murdoch, W.J.; Moss, G. E.; Larson, B.; Ramsey, S.; Graham, J. K. and Blackburn, H. D. 2010. The fertility of ram sperm held at 24h at 5 degrees C prior to cryopreservation. Animal Reproduction Science, 118:231−235.

Purdy, P. H.; Song, Y.; Silversides, F. G.; Blackburn, H. D. 2009. Evaluation of glycerol removal techniques, cryoprotectants and insemination methods for cryopreserving rooster sperm with implications of regeneration of breed or line or both. Poultry Science, 88:2184−2191.

Qadri, F. S.; Savaliya, F. P.; Patel, A. B.; Joshi, R. S.; Hirani, N. D. and Patil, S. S. 2013. Genetic study on important economic traits in two strains of White Leghorn chicken. Indian Journal of Poultry Science, 48(2): 149−153.

Quinn, P. J. 1989. Principles of membrane stability and phase behavior under extreme conditions. Journal of Bioenergetics andBiomembranes, 21:3−19.

Roberts, J. 2004. Factors affecting egg internal quality and egg shell quality in laying hens. Journal of Poultry Science, 41:161−177.

Robertson, R. N. 1983. The lively membranes. Cambridge University Press, Cambridge, UK.

Robertson R. N. 1984. The lively membranes. Cambridge University Press, Cambridge, UK.

Robinson, R. N. 1996. Effects of short storage conditions and broiler breeder age on hatchability, hatching time, and chick weights. World Poultry Science Journal, 76: 1459−1466.

Saeki, Y. and Nagomi, Y. 1964. Seasonal differences in fertility and hatchability of eggs roduced by the natural mating and artificial insemination methods of chickens. The Japanese Journal of Animal Reproduction 10: 37−43.

Santiago-Moreno, J.; Castaño, C.; Toledano-Díaz, A.; Coloma, M.; López-Sebastián, A.; Prieto, M. and Campo, J. 2011. Semen cryopreservation for the creation of aSpanish poultry breeds cryobank: Optimization of freezing rate and equilibration time.Poultry Science, 90:2047−2053.

Sapp, R. L.; Rekaya, I.; Misztal, C. and Wing, T. 2004. Male and female fertility and hatchability in chickens: A longitudinal mixed model approach. Journal of Poultry Science, 83:1253−1259.

Sarica, S.; Corduk, M.; Suicmez, M. and Cedden. 2007. The effects of dietary Lcarnitine supplementation on semen traits, reproductive parameters, and testicular histology of Japanese Quail breeders. Journal of Applied Poultry Research, 16:178−186.

Sarkar, S.; Pressey, R. L.; Faith, D. P.; Margules, C. R.; Fuller, T.; Stoms, D. M. and Andelman, S. 2006. Biodiversity conservation planning tools: present status and challenges for the future. Annual Review of Environment and Resources. 31.

Sasaki, K.; Tatsumi, T.; Tsutsui, M.; Tatsuya, N.; Imai, Y.; Naito, M.; Tajima, A. and Nishi Y. 2010. A method for cryopreserving semen from Yakido roosters using N-methylacetamide as a cryoprotective agent. Journal of Poultry Science, 47:297−301.

Sayyazadeh, H. and Shahsavarani, H. 2005. Effects of Artificial Insemination on performance of broiler breeders. Department of Animal Science, Faculty of Agriculture, Mazandaran University, Sari, Iran

Schmehl, M. K.; Vazquez, I. A. and Graham, E. F. 1986. The effects of non-penetrating cryoprotectants added to TESTyolk-glycerol extender on the post-thaw motility of ram spermatozoa. Cryobiology, 23:512−517.

Sen, G. R.; Sen, G. E. and Dhakal, B. K. 2004. Vitamin C and Vitamin E protect the rat testes from cadmium-induced reactive oxygen species. Molecules and Cells, 17:132−139.

Sexton, T. J. 1981.Sperm number required for maximum fertility of chicken semen processed for freezing. Reproduction Nutrition Development, 21:1043−1048.

Shaffner, C. S.; Henderson, E. W. and Card, G. C. 1941.Viability of spermatozoa of the chicken under various environmental conditions. Poultry Science, 20:259−265.

Shaffner, C. S. 1964. Observations on freezing chicken semen. Veterinary International Congress of Animal Reproduction, IV:426−429.

Shafiq, M.; Usman, M.; Ahmad, Z.; Akram, M.; Hussain, J. and Rehman, A. 2013. Pre and post-molt egg quality parameters as influenced by age in four varieties of Aseel chicken.33rd Pakistan Congress of Zoology (International). Islamabad, Pakistan. 363.

Shahverdi, A.; Sharafi, M.; Gourabi, H.; Yekta, A. A.; Esmaeili, V.; Sharbatoghli, M.; Janzamin, E.; Hajnasrollahi, M. and Mostafayi, F. 2015. Fertility andflow cytometricevaluations of frozen-thawed rooster semen in cryopreservation medium containing low-density lipoprotein.Theriogenology, 83:78−85.

Shannon, P. 1965. Contribution of seminal plasma, sperm numbers, and gas phase to dilution effects of bovine spermatozoa. Journal of Dairy Science, 48:1357−1367.

Singh, A.; Singh, C. V.; Singh, R. V. and Kumar, D. 2000. Selection index for genetic improvement of broiler traits. Indian Journal of Animal Science, 70:747−750.

Singh, D. P. 2001. Aseel of India. In: Souvenir, National Seminar on Appropriate Poultry for Adverse Environment. Organized by Acharya N G Range Agricultural University and Project Directorate on Poultry, Hyderabad, 11th January 2001.

Singh, V. K.; Mohan, M.; Verma, S. B.; Mandal, K. G. and Singh, D. P. 1999. Analysis of body weights at different ages in pure and crossbred chicken. Indian Journal of Poultry Science, 34:155−160.

Slanina, T.; Petrovičová, L. and Massányi, P. 2011. Factors effecting spermatozoa motility in turkey – temperature and diluent. In: Proc. Int. Ph.D. Students Conf. MendelNet, pp. 793−801. Mendel University, Brno.

Smith, O. B. and Polge, O. O. 1950. Artificial insemination and reproduction in farm animals. Animal Reproduction Science, 51:49−60.

Smith, O. B. and Akinbamijo, O. O. 2000. Micronutrients and reproduction in farm animals. Animal Reproduction Science, 61:549−560.

Sohail, A.; Muhammad, A.; Hussain, J.; Iqbal, A.; Usman, M.; Rehman, A. and Hussnain, F. 2013. Comparative study on production performance, egg quality, egg geometry and hatching traits of three age groups of indigenous Peshawari Aseel chicken. Journal of Veterinary Science, 2. 21−25.

Sonmez, M.; Turk, G. and Yuce, A. 2005. The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of Wistar rats. Theriogenology, 63:2063–2072.

Spizziri, B. E.; Fox, M. H.; Bruemmer, J. E.; Squires, E. L. and Graham, J. K. 2010. Cholesterol-loaded cyclodextrins and fertility potential of stallions spermatozoa. Animal Reproduction Science, 118:255−264.

Squires, E. L.; Keith, S. L. and Graham, J. K. 2004. Evaluation of alternative cryoprotectants for preserving stallion spermatozoa. Theriogenology, 62:1056−1065.

Stromberg, J. 1975. A Guide to Better Hatching Stromberg Publication Co., USA. 8−25.

Sun, J. G.; Jurisicova, A. and Casper, R. F. 1997. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization *in vitro*. Biology and Reproduction, 56 :602−607.

Sundararaian, R.; Haja, N. A. and Venkatesan, K. 2006.Cytisusscoparius link a natural antioxidant. BMC Complementary Medicine and Therapies, 6:8−10.

Surai, P. F. and Wishart, G. J. 1996. Poultry artificial insemination technology in the countries of the former USSR. World Poulty. Science Journal, 52: 29−43.

Tabatabaei, S.; Batavani, R and Ayen, E. 2011. Effects of Vitamin E Addition to Chicken Semen on Sperm Quality During *In vitro* Storage of Semen. Veterinary Research Forum, 2(2):103−111.

Tajima, A.; Graham, E. F. and Hawkins, D. M. 1989. Estimation of the relative fertilizing ability of frozen chicken spermatozoa using a heterospermic competition method. Journal of Reproduction and Fertililty, 85:1−5.

Takanami, Y.; Iwane, H. and Kawai, Y. 2000. Vitamin E supplementation and endurance exercise: are there benefits. Sports Medicine, 29:73–83.

Thurston, R. J. 1995. Storage of poultry semen above freezing for twenty-four to forty-eight hours. In: Bakst, M.R. and Wishart, G.J. (eds), Proc. 1st Int. Sym. on the Artificial Insemination of Poultry, pp. 107−122. The Poultry Science Association, Savoy, Illinois.

Thuwanut, P.; Chatdarong, K. and Techakumphu, M. 2008. The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of frozen-thawed epididymal cat spermatozoa. Theriogenology, 70:233−240.

Trzcinska, M.; Bryla, M. and Smorag, Z. 2008. Effect of liquid storage on membrane integrity and mitochondrial activity: a new diagnostic method of evaluating boar sperm quality. Journal of Animal Feed Science, 17 :372−380.

Tselutin, K.; Seigneurin, F. and Blesbios, E. 1999. Comparison of cryoprotectants and methods of cryopreservation of fowl spermatozoa. Poultry Science, 78:586−590.

Usman, M.,; Ahmad, Z.; Akram, M.; Hussain, J.; Mehmood, S.; Shafiq, M. and Iqbal, A. 2013. Pre and Post moult Productive performance of three age groups in four varieties of Aseel Chicken. In 33rd Pakistan Congress of Zoology (International) Pakistan (Vol. 355).

Usman, M.; Basheer, A.; Akram, M. and Zahoor, I. 2014. A comparative study of production performance and egg quality parameters of Naked-Neck and indigenous Aseel chicken of Pakistan. Journal of Basic and Applied Sciences, 10:160−163.

Verma, R. J. and Nair, A. 2001. Ameliorative effect of vitamin E on aflatoxininduced lipid peroxidation in the testis of mice. Asian Journal of Andrology, 3:217–221.

Watson PF, Kunze E, Cramer P, Hammerstedt RH. 1992. A comparison of critical osmolality and hydraulic conductivity and its activation energy in fowl and bull spermatozoa. Journal of Andrology, 13:131−138.

Watson, P. and Morris, G. 1987. Cold shock injury in animal cells. Symposium. Society for Experimental Biololgy, 41:311−340.

Westfall, F.D. and Harris, G. C. Jr. 1975. The ability of cryopreservatives to prevent motility loss and freeze-thaw damage to the acrosome of chicken spermatozoa. Cryobiology, 12:89−92.

Westfall, F. D. and Howarth, B. 1977. The effects of glycerol removal on cation concentrations and morphology of chicken spermatozoa. Poultry Science, 56:924−925.

White, L. M.; Beal, W. E.; Bame, J. H.; Saacke, R. G. and Marshall, C. E. 1984. Characteristics of bovine spermatozoa after migration through a bovine serum albumin gradient. Journal of Animal Science, 59:454−459.

Williams, W. W. and Savage, A. 1927. Methods of determining the reproductive health and fertility of bulls: a review paper with additional notes. Cornell Veterinary, 17:353−375.

Willoughby, C. E.; Mazur, P.; Peter, A. T. and Critser, J. K. 1966. Osmotic tolerance limits and properties of murine spermatozoa. Biology of Reproduction, 55:715−727.

Wilson, H. R. 1991. Physiological requirements of the developing embryo: Temperature and turning. In: S. G. Tullett, (ed), Avian Incubation. Butterworth-Heinemann, London, UK; :145–156.

Wilson, J. L. 1997. Breakout fertility. In: Bakst MR, Cecil HC (eds), Techniques for Semen Evaluation, Semen Storage, and Fertility Determination, Poultry Science Association, Savoy, Illinois, p.80−84.

Wishart, G. J. 1989. Physiological changes in fowl and turkey spermatozoa during *in vitro* storage. British Poultry Science, 30:443−454.

Wishart, G. J. 1984. Metabolism of fowl and turkey spermatozoa at low temperatures. Journal of Reproduction and Fertility, 70 :145−149.

Woelders, H.; Zuidberg, C. A. and Hiemstra, S. J. 2006. Animal genetic resources conservation in the Netherlands and Europe: Poultry perspective. Poultry Science, 85:216−222.

Yoshimura, Y.; Olira, A. and Heryanto, H. 1997. Reproductive physiology in domestic animals: a basic knowledge to improve poultry production in Asian countries, Journal of International Development and Cooperation, Hiroshima University. 3:27−41.

Yousef, M. I.; Abdallah, G. A.; Kamel, K. I. 2000 of ascorbic acid and vitamin E supplementation on semen quality and biochemical parameters of male rabbits. Animal Reproduction Science, 76:99−111.

Zein El-Dein, A. 1981. Use of some marker genes in improving rate of growth in chickens. Ph.D. Thesis, Ain Shams University, Egypt.

Zhandani, M.; Ansari, M.; Roknabadi, P.; ZareShahneh, A. and Sharafi, M. 2017. Orally administered chrysin improves post-thawed sperm quality and fertility of roaster. Reproduction in Domestic Animals, 52: 004−1010.

Zulkifli, I.; Yamada, Y.; Kadijah, W.; Vidyadaran, M. K. and Dahlan, I. 1992. The effect of sex-linked dwarf, Naked Neck, and frizzle genes on the egg quality traits of laying hens under tropical conditions. Veterinary Malay Journal, 4:33−39.