SEMEN QUALITY, FERTILITY, AND HATCHABILITY OF *IN VITRO* LIQUID AND CRYOPRESERVED SEMEN USING COMMERCIAL AND HOMEMADE EXTENDERS FROM INDIGENOUS HORRO CHICKEN BREED

Ph.D. Dissertation

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College of Veterinary Medicine and Agriculture, Bishoftu

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DEDICATION

I dedicated this Ph.D. dissertation work to my mother and my late father Getachew Daba Amente who passed away while I am studying for my Ph.D., who gave me the foundations and was an inspiration to be the good person could ever be.

Addis Ababa University

College of Veterinary Medicine and Agriculture Department of Animal Production Studies

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STATEMENT OF THE AUTHOR

First, I declare that this dissertation is my work and that all sources of materials used for this dissertation have been duly acknowledged. This dissertation has been submitted for fulfillment of the requirement for a PhD degree at Addis Ababa University, College of Veterinary Medicine and Agriculture, and is deposited at the University/College library to be made available to borrowers under the rules of the library. I solemnly declare that this dissertation is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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BIBLIOGRAPHICAL SKETCH

I, the author of this dissertation was born from my Mother Kililat Chekol and my father Getachew Daba on April 01, 1987 G.C. in Ilu Aba Bor Administrative Zone of Oromia Region, Ethiopia. I attended my elementary education at Abune Petros Elementary School, and secondary and high school education at Mettu Comprehensive Secondary School. In 2005 G.C I joined Jimma University, Ambo College and graduated with BSc degree in Animal Production in July, 2008. After my graduation, I joined Haramaya University in September 2008 and served for 2 years as a graduate assistant. In October 2010, I stated to pursue my Masters education at Haramaya University and successfully completed my MSc in Animal production in January 2013. Starting from January, 2013, I began serving Haramaya University at a position of a lecturer until I joined Addis Ababa University, College of Veterinary Medicine and Agriculture in October 2018 for my PhD study in Animal Production.

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LIST OF ABBREVIATIONS

AAU-CVMA: Addis Ababa University College of Veterinary Medicine and Agriculture

AI: Artificial Insemination

AIV: Avian Influenza Virus

BPSE: Beltsville Poultry Semen Extender

CPA: Cryoprotectant

CRD: Completely Randomized Design

DMA: Dimethyl Acetamide

ASD-1: Dimethyl-Formamide

DOC: Day-old Chicks

DZARC: Debrezeit Agricutural Research Center

E1: Extender1

E2: Extender2

EIAR: Ethiopian Institute of Agricultural Research

ETB: Ethiopian Birr

FSH: Follicle Stimulating Hormone

HDEP: Hen Day Egg Production

MG: Mycoplasma Gallisepticum

IBV: Infectious Bronchitis Virus

ILTV: Infectious Laryngotracheitis Virus

LH: Luteinizing Hormone

LPE: Locally Prepared Extender

MEM: Minimum Essential Medium

NM: Natural Mating

PSE: Poultry Semen Extender

SE: Standard Error

SNP: Single Nucleotide Polymorphism

SQI: Sperm Quality Index

SST: Sperm Storage Tube

STD: Sexually Transmitted Diseases

TCF: Tris-Citric Acid-Fructose

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SEMEN QUALITY, FERTILITY AND HATCHABILITY OF *IN VITRO* LIQUID AND CRYOPRESERVED SEMEN USING COMMERCIAL AND HOMEMADE EXTENDERS FROM INDIGENOUS HORRO CHICKEN BREED

Tarekegn Getachew Daba

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ABSTRACT

Short-term storage and cryopreservation of poultry semen represents an important strategy for breeding and in vitro conservation of the genetic material of chicken populations. The objective of this study is to evaluate the quality, fertility and hatchability of liquid stored and cryopreserved semen from Horro chicken using homemade tris-based extender and commercial extender. Furthermore, the economic benefits of artificial insemination were also evaluated for commercial broiler breeder farming. A total of 30 roosters and 160 adult hens were used for semen collection, and artificial insemination, respectively. Pooled semen samples were divided into three groups: Semen without extender; Semen diluted with homemade extender and Semen diluted with commercial extender. Semen was either liquid stored or cryopreserved in liquid nitrogen for shorter and longer-term storage, respectively. Changes in spermatozoa motility, in vitro viability, and morphology were evaluated in cryopreserved semen, and fresh diluted semen (1:4 v/v semen to extender) after 4hr, 8hr, 12hr, and 24hr storage at 4°C. Meta analysis on the effects of Glycerol and DMF as cryoprotectants in cryopreservation of chicken semen was conducted. Data was collected from electronic databases. Relevant data was extracted from studies such as sample size, breed, cryoprotectant used, post-thaw sperm quality and fertility. Data was presented using forest plots. Fresh semen collected from Horro chicken breed in this study was moderately viscous, milky, pH of 7.2 with 5.5x109 spermatozoa per ml of semen. The average ejaculate volume was 0.36 ml. There was a significant influence of temporary

storage and cryopreservation on mass motility, morphological abnormality (with high incidence of the bent tail), and viability. Semen stored using BPSE for 4 hours significantly ($P<0.05$) found to be suitable for short-term storage of semen collected from Horro chicken breed with progressive sperm motility (87.0 ± 1.22) and %live sperm (83.6 ± 1.63a). There was significant decline observed in all sperm quality attributes in increasing storage duration. There were significant differences ($P<0.05$) in progressive sperm motility, mass motility, and in vitro viability between commercial and homemade extenders in cryopreserved semen. However, no significant difference was observed in mass motility across the extenders in cryopreserved semen collected from Horro Chicken breed. The commercial ASD extender was significantly ($P<0.05$) found to be the most suitable regarding the proportion of morphologically normal sperm (64.25 ± 0.91) and in vitro viability rate of cryopreserved sperm samples. There were no significant differences across all treatments in terms of fertility and hatchability rate. Results from meta-analysis show that, the highest result has been recorded in diluent prepared by Mehdipour *et al*., 2020a using 3.8% DMF in lake extender in Ross chicken breed (71.1 ± 2.01). The results showed locally prepared tris-egg yolk-based extender could be a suitable extender for short-term storage and cryopreservation of chicken sperm regarding the sperm quality attributes and fertility. Further studies are suggested to improve fertility and hatchability.

Keywords: Cryopreservation, Horro, *in vitro* viability, Motility, Morphology, Semen,

CHAPTER 1

Introduction Pages 1-3

1. INTRODUCTION

Growing demand for poultry products and the high rearing cost of breeder stock necessitates the development of modern solutions to increase production efficiency at reduced costs. Artificial insemination (AI) is one for the solutions that significantly lowers the cost of rearing by decreasing the number of males in the flock (Łukaszewicz *et al*., 2020). AI was the first-generation biotechnology applied to improve reproductive efficiency in farm animals and endangered species (Foote, 2002). AI in poultry grew significantly during the last few decades after the development of semen collection through abdominal massage (Siudzin´ska and Łukaszewicz, 2008). The application of AI has led to greater interest by producers and researchers to investigate semen characteristics of various breeds of poultry (Santiago-Moreno *et al*., 2009; Haunshi, *et al*., 2010).

One of the advantages of its application over natural mating (NM) is the efficient use of males. This in turn, decreases the cost of breeding directly by reducing the number of cockerels needed (Mehfooz *et al*., 2017). Various investigators have searched to develop the proper conditions for liquid (short-term) and frozen (long-term) semen storage (Silyukova *et al.,* 2022). Fertility in broiler breeds continues to decline as males are selected for growth and compatibility problems between large and smaller breeds. AI significantly contribute to an efficient broiler breeder management and in solving compatibility problems (Reddy, 1995). The possibility of dilution and storage of avian sperm would make the work of poultry breeders much easier, enabling them to transport semen even to distant farms, to inseminate large groups of females, and to improve the utilization of sperm from superior males.

In addition to its breeding significance, AI is important in controlling venereal diseases. The anatomy of the avian reproductive tract predisposes the semen to fecal contamination by pathogenic bacteria, the most common being E. coli and pseudomonas (Elaish *et al.,* 2017). During AI, however, this problem is commonly addressed by adding antibiotic to diluents. Reports confirm the use of extenders to sustain good-quality sperm during storage (Bilgili *et al.,* 1987). Even though it is recognized that an ideal semen extender

has to provide an energy source for spermatozoa and maintain pH and Osmolarity levels identical to those of seminal plasma, the role various types of semen extenders have not been extensively evaluated. As dilution of the semen for freezing purpose decreases the available source of energy for spermatozoa, identifying the most compatible extender becomes determinant of a good quality frozen poultry semen (Akhter, 2006). Further, cryopreservation of semen represents the only efficient method for the ex-*situ* management of avian genetic resources (Ehling *et al*., 2012) as opposed to the current costly conservation method which is by maintaining living flock. Therefore, cryopreservation could play an important role in breed improvement, introducing greater flexibility in special breeding programs, as well as conservation of genetic resources (Han *et al.*, 2005).

According to Gerzilov (2010), many factors could affect the quality of cryopreserved semen, such as the types of cryoprotectant agents (CPA), packaging and cooling rates. Several studies showed that the highest rate of fertility is obtained from semen frozen in straws using glycerol as CPA and cooled slowly or from semen that is rapidly frozen as pellets using dimethyl acetamide (DMA) as CPA. In this study, the effect of glycerol was evaluated on the sperm quality of semen from Ethiopian indigenous Horro chicken.

Horro chicken is a breed of domestic chicken that is native to Ethiopia and is currently considered to be endangered (Gebremedhin *et al*., 2012). According to a previous study, Horro chicken is known for its adaptability to harsh environmental conditions and its resistance to common poultry diseases (Gebremedhin *et al.,* 2012). By cryopreserving the sperm of Horro chicken, we can ensure that the genetic materials of this valuable breed are conserved and available for future use in breeding programs, which may help to prevent the extinction of this breed. The selection of Horro chicken for sperm cryopreservation was driven by the need to conserve the genetic diversity of this endangered breed, which possesses unique traits that may be useful for future breeding programs.

Tris-based homemade extender has the advantage of buffering the pH and providing essential nutrients. In this study, it is hypothesized that the use of tris-based semen

extender with or without glycerol will maintain the pH balance essential for sperm cryosurvival and motility, hence will help the storage of good quality semen with high fertility. This study also intended to evaluate the economic benefits of AI over NM in commercial broiler breeder farms.

1.1.General Research Objective

The general objective of this research is to evaluate the sperm qualities, and fertility of liquid stored and cryopreserved Horro chicken semen using homemade tris-based and commercial extenders.

1.2.Specific Objectives

The specific objectives of this study are:

1 To evaluate the gross and microscopic parameters of fresh and *in vitro* liquid stored semen collected from the Ethiopian indigenous Horro chicken breed,
2 To evaluate the post-thaw quality, and fertility of semen cryopreserved using homemade (Tris-based) and commercial extenders
3 To assess the fertility and hatchability of semen cryopreserved in the homemade and commercial extenders
4 To Assess the economic benefits of AI over NM in commercial broiler breeding farms.
5 Meta-analysis of sperm quality and fertility of semen preserved using glycerol and DMF as cryoprotectant in different breeds of chicken

CHAPTER 2

Literature Review   Pages 5-36

2. LITERATURE REVIEW

2.1.Physiology of Cockerel Reproduction

Sperm Production is initiated by adequate secretion of Gonadotropin Releasing Hormone (GnRH) from the hypothalamus, the secretion of Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) by the anterior lobe of the pituitary and the secretion of the gonadal steroids (testosterone and estrogen). LH acts on the Leydig cells within the testes to stimulate the production of progesterone, which is converted to the male sex hormone testosterone (Senger, 2003). Testosterone within the seminiferous tubules is essential for spermatogenesis, while the Leydig cells become unresponsive sustaining high levels of LH (Senger, 2003).

The testes are surrounded by a layer of connective tissue containing the seminiferous tubules and Leydig cells. Several androgens are produced in the interstitial cells of the testes, but the major hormone in the blood, is testosterone. Testosterone is essential for the development of the secondary sex characteristics and for normal mating behavior in the males. It is also necessary for the functioning of the accessory glands, sperm production and the maintenance of the male duct system. This hormone also aids in spermatocytogenesis, the transport of sperm and deposition of sperm in the female reproductive tract (Beardon *et al*., 2004). As the cockerel reaches maturity, the production of testosterone is stimulated by the increasing concentration of circulating gonadotropins (Etches, 1996). FSH as such, acts on the germinal cells in the seminiferous tubules of the testes and supports spermatogenesis to the secondary spermatocytes stage. LH stimulates the Leydig cells to produce testosterone and other androgens (Hafez and Hafez, 2000).

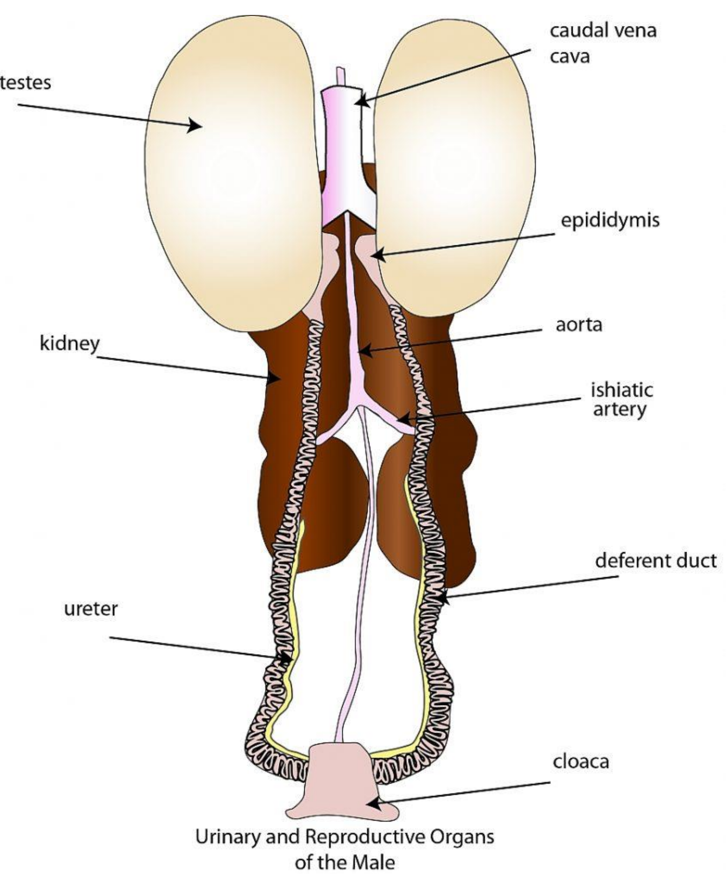


Figure 1. Cockerel's reproductive tract, Source: Poultryhub.org

A study published in Theriogenology in 2019 investigated the effect of age on the reproductive physiology of cockerels. The researchers found that there were significant changes in testicular size, sperm production, and hormone levels as the cockerels aged. They concluded that age is an important factor to consider when optimizing the reproductive performance of cockerels (Olanrewaju *et al*., 2019).

Another study published in Poultry Science in 2020 examined the effect of dietary calcium and phosphorus levels on the reproductive physiology of cockerels. The results showed that high levels of dietary calcium and phosphorus improved testicular size, sperm production, and hormone levels, while low levels had the opposite effect. The authors concluded that dietary calcium and phosphorus levels can impact the reproductive performance of cockerels (Ajuogu *et al*., 2020).

Several studies investigated the effect of photoperiod on the reproductive physiology of cockerels. The researchers found that exposure to extended photoperiods resulted in increased testicular size, sperm production, and hormone levels, while short photoperiods had the opposite effect. They concluded that photoperiod can be a useful tool for optimizing the reproductive performance of cockerels (Jahanian and Rasouli 2021; Zhang *et al*., 2021).

Several studies also examined the effect of different sperm collection techniques on sperm quality and quantity in cockerels. The results showed that the use of a modified massage technique resulted in higher sperm quality and quantity compared to the conventional cloacal massage technique. The authors concluded that the modified massage technique can be a useful tool for optimizing sperm collection in cockerels (Chen et al., 2018; Zhang *et al*., 2019).

2.2.Spermatogenesis

Spermatogenesis is the process of division and differentiation by which sperm are produced in the seminiferous tubules of the testes and consists of two phases, namely spermatocytogenesis and spermiogenesis (Costa *et al*., 2017). According to the previous author, spermiogenesis is a metamorphic process in which no cell division is involved and a string of events result in the formation of the sperm tail. Alteration in the sperm morphology can be seen in the nuclear proteins, cellular size, cellular shape and the position of the acrosomal granules and localization of the centrioles. The number of sperm produced is dependent on the number of Sertoli cells and Leydig cells present. The Golgi apparatus is one of the cell organelles, located near the sperm nucleus and which give rise to the subcellular organelle known as the acrosome. The acrosome develops and forms a cap over the anterior portion of the nucleus and spreads until it covers two-thirds of the anterior nucleus (Kumar *et al*., 2018). During the maturation phase, the spermatids are completely differentiated with the final formation of the flagella (principal and endpiece), assembly of mitochondria (midpiece), the neck piece and complete condensation and shaping of the nucleus (Beardon *et al*., 2004).

2.2.1. Spermatogenesis in chicken

Spermatogenesis is the physiological process of sperm cell development in male poultry. This process occurs in the testes and involves the differentiation of spermatogonium stem cells into mature spermatozoa. The process of spermatogenesis is regulated by various hormones and factors, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, and estrogen. These hormones play important roles in the regulation of testicular function, including the production of spermatozoa. The process of spermatogenesis in poultry involves several stages, including mitosis, meiosis, and spermiogenesis. During spermiogenesis, the spermatids undergo morphological changes to form mature spermatozoa. The resulting spermatozoa are then released into the lumen of the seminiferous tubules, where they are transported to the epididymis for maturation and storage (Onagbesan *et al*., 2009; Huang *et al*., 2018; Chen *et al*., 2018; Bakst and Wishart, 2019; Bakst, 2011).

2.3.Factors Affecting Semen Production

Several factors affect semen production in poultry. According to Han *et al.* (2017), semen production in poultry highly affected by testicular development and spermatogenesis. According to Gao *et al.* (2017); Abdul-Razak *et al.* (2021) whom investigated the effects of dietary protein and amino acid levels on testicular development and sperm production in male broiler breeders, semen production in chicken could be affected by dietary protein and amino acid levels in feeds.

In article by Abdul-Razak *et al.* (2018), environmental factors affect male fertility and semen production specifically in poultry, which includes temperature, light intensity, photoperiod, and air quality. A review by Islam *et al.* (2019) described that heat stress highly affects semen production and male fertility in poultry, including the effects on testicular function, sperm quality, and reproductive performance.

Testicular development and spermatogenesis in broiler breeder roosters which is affected by supplementation of antioxidants. A study by Singh *et al.* (2020) investigated the effect of dietary supplementation of antioxidants on testicular development and sperm

production in broiler breeder roosters. According to this study supplementation of antioxidants improved spermatogenesis and semen production in breeder stock.

2.4.Artificial Insemination in Poultry

AI in poultry was first successful in 1899 when Ivanov produced fertile chicken eggs using semen recovered from the ductus deferens after killing a cock (Kumar *et al*., 2016). The most widely used technique of intravaginal insemination was first reported by Quinn and Burrows in 1936. The avian male reproductive system is all inside the bird unlike the males of mammalian species which have their reproductive systems outside of the body cavity (Brar *et al.*, 2017). This is one of the really remarkable things about birds where the sperm remain viable at body temperature. Mammalian sperm does not remain viable at body temperature which is the reason the male reproductive organs are found on the outside of the body (Brooks, 1990).

According to López-Molina *et al*. (2021) AI in poultry is the process by which semen from male bird is collected and then introduced to females for the purpose of fertilizing eggs. The main objectives of AI in poultry are to place the required dose of semen into the oviduct of the female so that it is deposited near the sperm storage glands and to carry out the AI process with due regard to the best health and welfare of the breeder females thereby, achieving the highest fertility levels possible. During insemination, the volume of semen required is generally less than 0.1 ml, within a minimum of 100 to 200×10^6 viable sperm per insemination within the hen's vagina (Gordon, 2005).

Biologically**,** after deposition of semen in the oviduct the semen will enter the sperm storage gland, situated at the junction of the vagina and the shell gland and from here the spermatozoa will make their way up the oviduct to a second storage site situated at the junction of the magnum and infundibulum. The passage of an ovum into the infundibulum stimulates spermatozoa activity and fertilization of the ovum by one sperm takes place (Aisha and Zain, 2010).

2.5.Importance of AI in Poultry

Artificial insemination (AI) is an important technique in poultry breeding that has been widely adopted in the industry. Here is a literature review on the importance of AI in poultry:

2.5.1. Increased genetic progress

AI allows for the use of superior sires to produce offspring with desirable traits, such as improved growth rate, feed efficiency, and disease resistance. This can lead to significant genetic progress in a relatively short period of time (Kranis *et al*., 2013). Artificial insemination (AI) in poultry breeding plays a significant role in increasing genetic progress by allowing for the use of superior sires to produce offspring with desirable traits. Here is a literature review on the role of AI in increased genetic progress in poultry breeding: Artificial Insemination (AI) enables the use of superior sires that have been selected based on their genetic merit, which can lead to improved traits such as growth rate, feed efficiency, and disease resistance (de los Campos *et al.,* 2013). It also allows breeders to increase selection intensity by using a larger number of sires with superior genetic merit, which can lead to faster genetic progress (Wolc *et al*., 2012).

With AI, breeders have greater control over mating, allowing for the selection of the best sires to mate with the best hens, resulting in offspring with better genetic potential (Pampín *et al*., 2016). Artificial Insemination (AI) can be also used to reduce inbreeding by enabling breeders to expand the number of sires available for mating, which can lead to a more diverse genetic pool (Zhao *et al*., 2021; Yakubu, 2015).

On other hand, AI allows for the more efficient use of valuable genetic material, such as semen from rare and valuable sires, which can be distributed to a larger number of hens, resulting in more offspring with desirable traits (Pampín *et al*., 2016). Artificial Insemination (AI) can lead to faster dissemination of genetic progress by enabling breeders to use superior sires in multiple flocks, resulting in a larger number of offspring with desirable traits (Yakubu, 2015; Wang *et al*., 2021).

In general, AI plays a significant role in increasing genetic progress in poultry breeding by enabling the use of superior sires, increasing selection intensity, providing greater control over mating, reducing inbreeding, allowing for more efficient use of valuable genetic material, and enabling faster dissemination of desirable traits.

2.5.2. Reduced transmission of diseases

Artificial insemination (AI) is a valuable tool in disease control in poultry production. A study by Elaish *et al*. (2017) investigated the role of AI in controlling the transmission of avian influenza virus (AIV) in commercial poultry flocks. The study found that AI can effectively reduce the spread of Avian Influenza Virus (AIV) in commercial poultry flocks, suggesting that AI could be a useful tool for controlling this important poultry pathogen. Another study published in the journal Veterinary Microbiology in 2018 investigated the potential of AI to reduce the transmission of infectious laryngotracheitis virus (ILTV) in commercial broiler breeder flocks. In this study, researchers (Souza *et al.,* 2018) found that AI was effective in reducing the prevalence of ILTV in the flocks, suggesting that AI could be a valuable tool for controlling this important poultry pathogen.

A study published in the journal Poultry Science in 2019 investigated the potential for AI to reduce the transmission of *Salmonella enteritidis* in commercial layer flocks. In this study, researchers (Ferreira *et al*., 2019) found that AI was effective in reducing the prevalence of SE in the flocks, suggesting that AI could be a useful tool for controlling this important poultry pathogen.

In a study published in the journal Poultry Science in 2020, researchers (Souza *et al*., 2020) investigated the role of AI in reducing the transmission of *Mycoplasma gallisepticum* in commercial broiler breeder flocks. The study found that AI was effective in reducing the prevalence of MG in the flocks, suggesting that AI could be a valuable tool for controlling this important poultry pathogen. A review published in the journal Veterinary Sciences in 2020 examined the role of AI in reducing the transmission of infectious bronchitis virus (IBV) in chickens. In this review, authors (Desta *et al*., 2020) found that while AI can reduce the transmission of Infectious Bronchitis Virus (IBV), it

is not a foolproof method and must be used in conjunction with other disease control measures.

AI reduces the risk of disease transmission compared to natural mating, as it avoids direct contact between birds. This is particularly important in the prevention and control of sexually transmitted diseases (STDs) in poultry (Hocking, 2014). Birds present special challenges in disease control (Blanco and Hofle, 2004). The semen diluents may be another common source of contamination, especially for E. coli and pseudomonas (Van Eck and Goren, 1980). These agents can trigger significant sperm mortality in raw or diluted ejaculate and when used for AI may cause both systemic disease and infertility (Van Eck and Goren, 1980). This problem is commonly addressed by adding antibiotic to diluents (i.e., penicillin, Gentamicin and streptomycin) although these drugs may adversely impact on sperm viability (Donoghue *et al*., 2004).

Collectively, results to date strongly emphasize the need to minimize ejaculate contamination by focusing on sanitary semen collection and processing (including using appropriate and prudent doses of broad-spectrum antibiotic and antifungal) as well as protecting birds from pathogens. This includes maintaining thorough pathogen monitoring protocols and even strict isolation/ quarantine practices for breeder populations (Turin *et al*., 1999). Once infectious situations arise, rapid mitigation is mandatory, although it is challenging to alleviate certain viruses (i.e., West Nile virus) from infected birds (Turin *et al.*, 1999).

Overall, these studies show that AI can be an effective tool for reducing the transmission of important poultry pathogens. However, the effectiveness of AI in disease control may vary depending on the specific pathogen and other factors, and more research is needed to fully understand its potential in this area.

2.5.3. Improved reproductive efficiency:

AI can increase the reproductive efficiency of birds by allowing for more efficient use of fertile eggs, reducing the number of males required for breeding, and enabling the production of more offspring per male (Naraballobh *et al.,* 2019). AI is an important

technique in poultry breeding that can improve reproductive efficiency in several ways. Here is a literature review on the role of AI in improving reproductive efficiency in poultry breeding: AI allows breeders to use fertile eggs more efficiently by selecting the best sires to mate with the best hens, resulting in offspring with better genetic potential (Wolc *et al*., 2019). It can reduce the number of males required for breeding, as a single male can produce enough semen to inseminate hundreds or even thousands of hens (Naraballobh *et al*., 2019).

On the other hand, Artificial Insemination (AI) can enable the production of more offspring per male, as semen can be collected from a single male and used to inseminate a large number of hens (Velleman and Coy, 2018). AI can also improve fertilization rates by allowing for more precise control of sperm quality and quantity, resulting in more consistent fertilization rates (Hocking, 2014).

Furthermore, AI reduces the risk of injury to breeding birds that can occur during natural mating, which can lead to better reproductive performance and reduced mortality rates (Blesbois, 2017). AI can facilitate breeding programs by allowing for the use of superior sires, increasing selection intensity, and reducing the time and labor required for mating and breeding (Lovell and Loveday, 2015).

In conclusion, AI plays an important role in improving reproductive efficiency in poultry breeding by enabling the efficient use of fertile eggs, reducing the number of males required for breeding, increasing the number of offspring per male, improving fertilization rates, reducing the risk of injury to breeding birds, and facilitating breeding programs.

2.5.4. Increased production efficiency

AI can improve production efficiency by reducing the time and labor required for mating and breeding, and by enabling the use of fewer males without sacrificing genetic progress (Hocking, 2014). In a study published in Poultry Science, researchers investigated the impact of AI on the production performance of broiler breeders. They found that AI improved fertility and hatchability, resulting in a higher number of chicks hatched per hen housed. They also reported that AI reduced the number of males required for

breeding, resulting in reduced feed costs and labor requirements (Borges et al., 2020). Another study published in the Journal of Applied Poultry Research examined the effect of AI on the reproductive performance of layer breeders. The results showed that AI significantly improved hatchability and the number of chicks hatched per hen housed. The researchers also reported that AI reduced the number of males required for breeding and improved the uniformity of offspring (Hossain *et al*., 2020).

A study published in the Turkish Journal of Veterinary and Animal Sciences investigated the use of AI in the production of turkey poults. The authors reported that AI resulted in higher fertility and hatchability rates, as well as improved growth performance and feed conversion efficiency in the resulting poults. They concluded that AI can be a useful tool for improving the production efficiency of turkey flocks (Celik *et al*., 2020). In another study published in Poultry Science, researchers evaluated the impact of AI on the genetic progress of laying hens. They found that AI allowed for the more efficient use of semen from superior sires, resulting in increased genetic progress and improved egg production performance. They concluded that AI can be an effective tool for increasing the production efficiency of laying hen flocks (Liu *et al.,* 2020).

Overall, recent studies have demonstrated that AI can significantly increase production efficiency in poultry breeding by improving fertility and hatchability, reducing the number of males required for breeding, improving growth performance and feed conversion efficiency, and allowing for the more efficient use of semen from superior sires.

2.5.5. Preservation of rare and endangered breeds

AI can be used to preserve rare and endangered breeds of poultry by making it possible to maintain a genetically diverse population with a smaller number of breeding birds (McPherson and Lancaster, 2018; Grossman and Siegel 2016; Tixier-Boichard *et al*., 2015). In a study published in the Journal of Animal Science, researchers investigated the use of AI to preserve the genetic diversity of a rare breed of chicken, the Dong Tao. They found that AI enabled the efficient use of semen from superior sires, resulting in increased genetic diversity and preservation of the breed. They concluded that AI can be

an effective tool for preserving rare and endangered breeds of poultry (Deeb and Lamont, 2015; Chaudhari *et al*., 2017).

Another study published in the Journal of Applied Poultry Research examined the use of AI to preserve a rare breed of turkey, the Spanish Black. The authors reported that AI allowed for the efficient use of semen from a limited number of males, resulting in the production of a large number of offspring with desirable traits. They concluded that AI can be a useful tool for preserving rare and endangered breeds of turkey (Burnett *et al*., 2020). A study published in the journal PLOS One investigated the use of AI to preserve the genetic diversity of a rare breed of chicken, the Ayam Cemani. The researchers found that AI enabled the efficient use of semen from superior sires, resulting in the preservation of genetic diversity and the production of a large number of offspring with desirable traits. They concluded that AI can be an effective tool for preserving rare and endangered breeds of poultry (Sulandari *et al*., 2020).

Overall, recent studies have demonstrated that AI can be an effective tool for preserving rare and endangered breeds of poultry by enabling the efficient use of semen from superior sires and preserving genetic diversity. AI can help to maintain a genetically diverse population with a smaller number of breeding birds, which can be particularly important for the preservation of rare and endangered breeds.

2.5.6. Improved Hatchability

AI can improve hatchability rates by allowing for more precise control of sperm quality and quantity, resulting in more consistent fertilization rates (Bakst and Akuffo, 2019; Zaniboni *et al*., 2016; Hocking, 2014). A study published in the journal Animals in 2020 investigated the effect of AI on the hatchability of eggs from different commercial layer strains. The researchers found that AI significantly improved hatchability rates compared to natural mating, with the highest improvement observed in strains with lower hatchability rates. They concluded that AI can be an effective tool for improving hatchability in commercial layer flocks (Garcia-Ruiz *et al*., 2020).

Another study published in Poultry Science in 2021 examined the effect of diluted semen on hatchability in broiler breeders. The results showed that the use of diluted semen in AI

significantly improved hatchability rates compared to undiluted semen, with the highest improvement observed in the first week of storage. The authors concluded that the use of diluted semen can be an effective strategy for improving hatchability in broiler breeder flocks (Zaniboni *et al*., 2016; Garcia-Ruiz *et al*., 2021).

A study published in the Journal of Applied Poultry Research in 2021 investigated the impact of different AI techniques on hatchability in turkey breeder flocks. The authors found that the use of a double insemination technique significantly improved hatchability rates compared to single insemination, with the highest improvement observed in mid-late season flocks. They concluded that double insemination can be an effective strategy for improving hatchability in turkey breeder flocks (Olanrewaju *et al*., 2021).

A study published in Poultry Science in 2022 evaluated the effect of AI on hatchability in broiler breeder flocks with high and low egg fertility rates. The researchers found that AI significantly improved hatchability rates in both high and low fertility flocks, with the highest improvement observed in the low fertility flocks. They concluded that AI can be an effective tool for improving hatchability in broiler breeder flocks with varying fertility rates (Bakst and Akuffo, 2019; Garcia-Ruiz *et al*., 2022).

Overall, recent studies have demonstrated that AI can be an effective tool for improving hatchability in poultry breeding by improving fertility rates, using diluted semen, employing double insemination techniques, and achieving better results in low fertility flocks. Improved hatchability rates can lead to increased production efficiency and profitability in the poultry industry.

2.6.Poultry Semen Collection Techniques

In 1937 Burrows and Quinn described a non-invasive method, the abdominal massage method for collection of semen from roosters. The technique involves restraining the male and gently stroking the back of the bird from behind the wings towards the tail with firm rapid strokes. The male responds with tumescence erection of the phallus, at which time the handler gently squeezes the cloaca extracting semen through the external

papillae of the duct us deferens (vas deferens) collecting the semen into a container (Brar *et al*., 2017).

As stated by Bakst and Akuffo (2019); De Smit and Debonne (2019), the techniques of AI actually begin prior to the procedure. It includes housing the male poultry away from the hens maximizes the amount of available semen. Because the bird's phallus is located in the same duct as his anus, removing food 12 hours prior to collection will help prevent fecal contamination of the semen. Roosters and tom turkeys need to be routinely primed for semen collection for several days prior to the actual AI procedure to guarantee that each bird is fertile with a microscopic examination of the sperm. According to Burrows and Quinn (1937), as with semen collection of other farm animals, one must stimulate the bird's sexual organ to extend outside of his body. One person can handle this procedure with small birds such as chickens or quail; it normally takes two people with a large turkey or a goose.

Figure 2. Poultry Semen Collection, source: www.agrinnovateindia.co

2.7.Gross Evaluation of Semen

Determination of the viability of spermatozoa after semen storage is important for several reasons. First, it provides an estimate of semen quality. If inseminated with poor quality semen, it reduces fertility, increases embryo mortality and forces the hen to rely on

spermatozoa from previous inseminations (Thurston, 1995). More traditional semen evaluation procedures include determination of semen volume, color, concentration, motility, viability and morphology of spermatozoa. Many of these assessments correlate with the fertilizing capacity of spermatozoa when fresh semen is evaluated (Wishart, 1995).

The color of semen is generally an indicator of the density of the ejaculate. The semen of the domestic fowl varies from a dense opaque suspension to a watery fluid secreted by various reproductive glands. It ranges from a relative high sperm density or degrees of clear to milky white, with declining sperm numbers (Peters *et al*., 2008). Color could also serve as an indicator of contamination by feces or urine and thus become brown or green in color (Lake, 1983). Sometimes flakes of blood may be present, which may be a result of excessive force during the collection process or injury. Semen samples that are contaminated by feces do not have to be discarded, but diluted with antibiotics e.g. penicillin and dihydrostreptomycin or neomycin to reduce the loss of sperm. This however is not recommended. Antibiotics can also increase fertility when used as a diluent in semen (Bearden *et al*., 2004).

Different cockerels of the same species often produce different volumes of semen at different times (Anderson, 2001). The average volume ejaculated using the abdominal massage technique is approximately 0.25ml (Gordon, 2005). Bah *et al*., (2001) found the mean semen volume to be 0.28 ± 0.14ml. However, the recorded semen volume was found to range between 0.37 ± 0.02 ml and 0.73 ± 0.01 ml (Peters *et al*., 2008). It is important to realize that semen volume and sperm concentration (volume multiplied by the concentration) will determine the total number of sperm collected per ejaculation. This could facilitate the determination of the number of insemination doses that can be prepared (Senger, 2003).

The semen pH varies slightly between different breeds and bird species. The optimum semen pH ranges between 7.0 and 7.4. Sperm motility is generally high between a pH of 7.0 and 7.4 (slightly alkaline) and also increases the fertilizing ability, compared to a pH of 6.4 (acidic), which is not suitable for semen preservation, as it may cause damage to

the plasma membrane of the sperm cell (Latif *et al*., 2005). Contrary, Donoghue and Wishart (2000) reported several trials that indicate that chicken sperm can tolerate a pH range of 6.0 to 8.0. Peters *et al*. (2008) also found the semen pH of the cockerel to be slightly alkaline, with a mean of 7.01 ± 0.01, while Bah *et al*. (2001) recorded a semen pH ranging between 7.54 ± 0.04 to 7.80 ± 0.03.

This variation in semen pH may be due to many factors. The pH, especially that of ejaculated semen is dependent on several secretions involved. Poor quality semen generally contains large amounts of fluid from the accessory glands, which increases the semen pH (Salisbury *et al*., 1978). The pH of semen is likely to decrease as the time between collection and measurement increases, and the semen collection tubes are narrow in shape causing sperm to break down fructose in the semen to lactic acid under anaerobic conditions. Semen samples that contain many dead sperm may evolve to ammonia, which will also increase the pH (Salisbury *et al*., 1978).

2.8.Effects of Extenders on Microscopic Sperm Qualities of Cockerel Semen

2.8.1. Motility

Sperm motility is an important parameter in evaluating the quality of chicken semen for artificial insemination. In a study published in the journal Poultry Science in 2019, researchers (Alkan *et al*., 2019) investigated the effect of semen extender on the motility of chicken sperm during storage. The study found that the extender improved sperm motility during storage, suggesting that extenders can be used to maintain sperm quality in artificial insemination. Another study published in the Journal of Animal Science and Biotechnology in 2018 investigated the effects of different cryoprotectants on chicken sperm motility and membrane integrity during cryopreservation. In this study, researchers (de Castro *et al.,* 2018) found that certain cryoprotectants improved sperm motility and membrane integrity, suggesting that cryopreservation can be used to preserve chicken sperm for artificial insemination.

In a study published in the Animal Reproduction Science in 2017, researchers (Tona *et al*., 2017) investigated the effects of different extenders on the motility of frozen-thawed

chicken sperm. The study found that certain extenders improved sperm motility after freezing and thawing, suggesting that extenders can be used to improve the quality of frozen-thawed chicken sperm for artificial insemination.

Sperm motility assessment is indicative of the viability of sperm and the quality of the semen sample. Evaluation of sperm motility is conducted with fresh and extended semen, and generally analyzed under the light microscope (10× magnifications) (Hafez and Hafez, 2000). Evaluation of raw semen gives the performance of the sperm in its own accessory gland fluid, which is often hindered when higher sperm concentrations make it difficult to distinguish individual sperm motility patterns (table 1). Hence an aliquot of semen is usually extended prior to evaluation (Hafez and Hafez, 2000).

Overall, these studies suggest that the motility of chicken sperm can be improved and maintained through the use of semen extenders and cryopreservation techniques. This can ultimately improve the success of artificial insemination in chicken breeding programs.

Table 1. Motility patterns of sperm from sub fertile or infertile cockerels

Pattern of sperm motility	Sperm tail	Sperm head	Sperm movements and progression
Vibratory circular	Slow or rapid quivering from side to side, vibrations of various types and frequency bent in curved shape, immotile	Immotile or vibrating in one place	Motility without progression, perpendicular, oblique or horizontal clockwise or counterclockwise motion
Darting	Vibration with high velocity	Irregular, propelling, no rotation	Minimal and erratic, wandering path
Rotating	Undulations of small amplitude pass down tail	Whole sperm rotates around its axis, periodic flashing effect	Rapid forward progress in a straight line
Asymmetric head and /or flagella	Amplitude of tail wave is asymmetric at both sides	Irregular, propelling, usually no	Circular orbits if rotational motile is absent

rotation

2.8.2. Sperm morphology

Normally the sperm cell consists of a head, mid-piece and tail portion. The head contains the nucleus, containing the genetic material, which is the sire's genetic contribution to the offspring (Tuncer *et al*., 2006). The post-nuclear cap which covers the posterior part of the nucleus and acrosome which covers the anterior part of the nucleus both protect the nucleus. If the acrosome is malformed or damaged the sperm cell will not be able to fertilize the ova by penetrating the zona pellucida. Acrosome, sperm head, middle piece and tail deformations in fresh white leghorn cockerel ejaculates have been recorded to be 0.62 ± 0.04%, 1.34 ± 0.05%, 2.47 ± 0.05% and 2.89 ± 0.08% respectively (Tuncer *et al*., 2006). While Tuncer *et al*., (2008) also recorded values of acrosome, sperm head, middle piece and tail deformations 0.39 ± 0.03%, 1.06 ± 0.03%, 2.32 ± 0.05% and 2.53 ± 0.04% respectively in cockerels.

Blesbois (2007) described an eosin-nigrosin stain technique to assess the morphology of cockerel semen. Semen was mixed with 1.6% eosin and 6% nigrosin, diluted in 20μm Beltsville Poultry Semen Extender (BPSE), diluted semen in 2ml stain solution and incubated for 2 minutes before being spread on a microscope slide. The stain is dried and observed under a light microscope (1000× magnification). Sperm morphology can serve as an indicator of semen quality and shortcomings in the male. The success of this evaluation technique depends on how the stain was prepared and used, while other more advanced laboratories use a computer analysis system for sperm evaluation. Eosin-nigrosin is a die commonly used in laboratories to determine abnormalities and smears are made, immersed in oil and observed under the light microscope. Viable, non-viable, properly formed, live, and damaged sperm can be determined using this evaluation (Lukaszewicz *et al*., 2008).

The sperm morphology of poultry semen differs from that of mammals. However, a difference also exists between domestic birds, even though the shape and size of the sperm cell are similar. In poultry the sperm cell is surrounded by the cytoplasmic membrane and the acrosome has an inner spine surrounded by a conical shaped cap. The

midpiece of cockerel sperm is considerably longer, compared to other species, approximately one quarter longer and this property makes poultry sperm to have more mid-piece bending than other species. According to Alkan *et al*., (2001) the in vitro assessment morphological sperm defects of cockerel semen include; neck bending (mid piece bending), mid piece damage, acrosome damage (bending, swelling, knotting or rounding), total head swelling and tail defects.

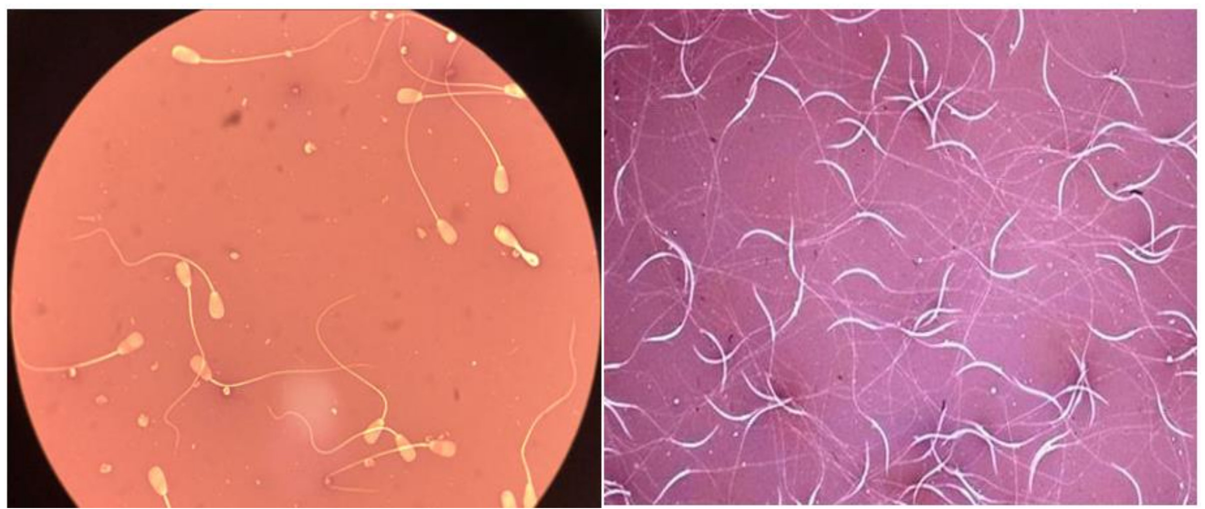


Figure 3. Morphology of bull (left) and cockerel (right) spermatozoa, Source: https://www.intechopen.com/

2.8.3. Sperm concentration

Gordon (2005) stated semen collected from domestic cockerel contains an average sperm concentration of 5000 ×106 sperm/ml. On the other hand, the report of Hafez and Hafez (2000) stated that semen collected from domestic cockerel contains an average sperm concentration of 3000-7000 ×106 sperm/ml. Sperm concentration is an important parameter in evaluating the quality of chicken semen for artificial insemination. In a study by Chapman *et al.* (2017) investigated the effects of age and breed on chicken sperm concentration. The study found that breed had a significant effect on sperm concentration, with some breeds having higher concentrations than others. Age was also found to have a significant effect, with younger males having higher sperm concentrations than older males.

Another study by Kim *et al.* (2018) investigated the effects of season and time of day on chicken sperm concentration. In this study, researchers found that both season and time of day had significant effects on sperm concentration, with higher concentrations observed in the spring and in the morning. In another study Rahman *et al*. (2020) investigated the effects of different extenders on chicken sperm concentration during cryopreservation. The study found that certain extenders were more effective than others in maintaining sperm concentration during cryopreservation, suggesting that the choice of extender can have a significant impact on sperm concentration.

Ahmed *et al.* (2021) investigated the effects of different extenders on chicken sperm concentration during storage at different temperatures. In this study, researchers found that the choice of extender and storage temperature had significant effects on sperm concentration, with some extenders and temperatures resulting in higher concentrations than others. Overall, these studies suggest that various factors such as breed, age, season, time of day, and the choice of extender can have significant effects on chicken sperm concentration. By understanding these factors and optimizing semen collection and processing protocols, it is possible to improve the quality of chicken semen for artificial insemination.

2.9.Factors Affecting Semen Quality

The assessment of semen quality characteristics of poultry birds gives an excellent indicator of their reproductive potential and has been reported to be a major determinant of fertility and subsequent hatchability of eggs (Ahmed *et al*., 2021). Omeje and Marine (1990) observed that significant genotype differences affected body size and semen characteristics of cocks, except the pH value. In addition, age by genotype interaction effect was important only for semen volume.

Only morphologically normal spermatozoa are capable of ascending through the vagina of the hen to the region where the sperm storage tubules are located (Bakst *et al*., 1994). Sperm motility is a primary determinant of fertility in domestic fowl (Rahman et al., 2020). Sperm mobility is a function of the product of motile concentration and the proportion of motile sperm with a straight-line velocity >30 m/s (Froman *et al*., 2003).

High quality semen relies on normal spermatogenesis. It is becoming increasingly evident that estrogen, in addition to testosterone plays a role in the development and function of the testis and male reproductive tract (Rivas *et al*., 2002; Akingbemi, 2005). The hormone level in seminal plasma is a direct reflection of male testicular endocrine activity. The relationships between semen quality and concentration of testosterone in avian seminal plasma have been discussed (Zeman *et al*., 1986).

2.10. Poultry Semen Extenders and their Effects on Maintaining Sperm Quality

Poultry semen extenders are commonly used to improve the quality and longevity of semen for artificial insemination. In a study by Ugwu *et al*. (2019) investigated the effect of semen extender composition on the motility and viability of chicken sperm during storage. The study tested several extenders, including Beltsville Poultry Semen Extender (BPSE), Lake, and Modena. The study found that BPSE was the most effective extender in maintaining sperm motility and viability during storage.

Another study by Rahman *et al*. (2020) investigated the effects of different extenders on the cryopreservation of chicken semen. In this study, investigators tested several extenders, including Tris-citric acid-fructose (TCF), Lake, and Modena. The study found that TCF was the most effective extender in preserving sperm motility and membrane integrity during cryopreservation. In a study by Ahmed *et al*. (2021) investigated the effects of different extenders on the quality of frozen-thawed chicken semen. The study tested several extenders, including TCF, BPSE, and Lake. The study found that TCF was the most effective extender in maintaining sperm motility and viability after freezing and thawing. Another study investigated the effects of different extenders on the quality of chicken semen during liquid storage. In this study, researchers (Rahman *et al*. 2021) tested several extenders, including BPSE, TCF, and Lake. The study found that BPSE was the most effective extender in maintaining sperm motility and viability during liquid storage.

The use of AI in poultry can be enhanced with the improvement of diluents and method of storing semen (Mian *et al*., 1990). The advantage of semen dilution includes the maximum use of good quality semen in short supply; reduction in the ratio of males to

female and valuable sires with low semen quantity can be used for many females. On the contrary, it is difficult to handle the desired very small volume of undiluted semen and expel it from a tube because of its viscous nature. However, diluents make it possible thereby enabling the spread of semen over many more hens (Mian *et al*., 1990). 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 extension is important since poultry semen is viscous and highly concentrated, containing 6 (roosters) to 12 (toms) billion spermatozoa/ml. Semen diluents are based on the biochemical composition of chicken and turkey semen (Lake, 1995).

Both hypertonic and hypotonic extenders reduce the metabolic activity of the sperm, and could disrupt the cell membrane integrity that leads to the clumping of the sperm (Latif *et al*., 2005). Glutamic acid is the most prominent anionic constituent in avian seminal plasma, and is a standard component of all semen diluents. Basic characteristics common to all diluents include the maintenance of pH, osmolarity and the provider of energy for the sperm. The motility and metabolic rate of sperm can thus be altered by decreasing the diluent below pH 6.0. So, for example a low pH reduces the sperm motility and a high pH increases the metabolic rate in vitro (Donoghue and Wishart, 2000). Modified Ringer's solution with the following composition of sodium chloride (68g), potassium chloride (17.33g), calcium chloride (6.42g), magnesium sulphate (2.50g), sodium bicarbonate (24.50g) and distilled water, can be used to dilute poultry semen (Martin, 2004).

Addition of various components to semen maintains motility, fertilizing capacity and preserve sperm membrane integrity (Riha *et al*., 2006; Sarlos *et al*., 2002). Egg yolk is generally accepted to be an effective agent in semen extenders for protection of spermatozoa against cold shock and the lipid phase transition effect (Aboagla and Terada, 2004). However, the use of chilled stored semen diluted in egg yolk-based semen extenders is limited by its relatively short time fertilization capacity and individual differences in egg yolk due to different period of egg storage (Aurichet *et al*., 1997).

Overall, these studies suggest that the composition of poultry semen extenders can have a significant impact on sperm quality and success in artificial insemination. By understanding the properties of different extenders and choosing the most appropriate

extender for a particular application, it is possible to improve the efficiency and effectiveness of artificial insemination in the poultry industry.

2.10.1. Beltsville poultry semen extender

Beltsville Poultry Semen Extender (BPSE) is a widely used semen extender for chicken and turkey semen. It was developed by researchers at the Beltsville Agricultural Research Center in Maryland, USA, and is commonly used to extend the shelf life of poultry semen for artificial insemination. BPSE is a buffered solution that contains various ingredients, including antibiotics, glucose, and glycerol. It helps to protect the sperm cells from damage during storage and transportation, and improves their survival and fertility after insemination (Tunker *et al*., 2017).

Several studies have evaluated the effectiveness of BPSE in preserving the quality of poultry semen. A study by Sarıözkan *et al.* (2016) compared the quality of chicken semen extended with BPSE to that of semen extended with a commercial extender. The researchers found that BPSE was more effective in preserving sperm motility and viability, and resulted in higher fertility rates after insemination. Another study by Tunker *et al.* (2017), evaluated the effect of BPSE on turkey semen quality. The researchers found that the use of BPSE improved the motility, viability, and acrosome integrity of turkey sperm cells, and resulted in higher fertility rates after insemination.

Overall, the use of BPSE has been shown to be effective in extending the shelf life of poultry semen and improving sperm quality. However, it is important to note that the effectiveness of the extender may vary depending on various factors such as the breed of the bird, the age of the bird, and the storage conditions of the semen. Therefore, it is important to follow the manufacturer's instructions carefully and to monitor the quality of the extended semen regularly.

2.10.2. Dimethyl formamide

Dimethyl formamide is a solvent widely used in various industries, including the poultry industry as a semen extender. It is a polar aprotic solvent that can dissolve a wide range

of substances and can be used to extend the shelf life of poultry semen by protecting the sperm cells from damage during storage and transportation (Sarıözkan *et al*., 2018).

Several studies have evaluated the effectiveness of ASD-1 as a semen extender for poultry. For example, a study published in the by Sarıözkan *et al*. (2018) compared the quality of chicken semen extended with ASD-1 to that of semen extended with a commercial extender. The researchers found that ASD-1 was as effective as the commercial extender in preserving sperm motility, viability, and morphology.

Another study by Tunker *et al.* (2019); Jeyendran *et al*. (1984), evaluated the effect of ASD-1 on turkey semen quality. The researchers found that the use of ASD-1 improved the motility, viability, and acrosome integrity of turkey sperm cells, and resulted in higher fertility rates after insemination. Overall, the use of ASD-1 as a semen extender for poultry has been shown to be effective in preserving sperm quality and improving fertility rates. However, it is important to note that ASD-1 is a toxic and potentially hazardous solvent, and its use should be carefully regulated and monitored. The use of alternative, non-toxic semen extenders may be preferred for ethical and environmental reasons.

2.11. Poultry Semen Osmotic Pressure

Osmotic pressure is an important parameter in evaluating the quality of poultry semen for artificial insemination. In a study by Carvalho *et al.* (2017) investigated the osmotic pressure of chicken semen from different breeds. The study found that the osmotic pressure of chicken semen ranged from 270 to 300 mOsm/kg, and that there were significant differences in osmotic pressure between breeds. Another study by Ugwu *et al*. (2019) investigated the effect of semen extender on the osmotic pressure of chicken semen during storage. In this study, researchers found that the osmotic pressure of chicken semen increased during storage, but that the use of an extender helped to maintain the osmotic pressure within the optimal range for sperm survival.

A study by Rahman *et al*. (2020) investigated the effects of different extenders on the osmotic pressure of chicken semen during cryopreservation. In this study, researchers

found that the osmotic pressure of chicken semen was maintained within the optimal range during cryopreservation when certain extenders were used. Latif *et al*. (2005) concluded that an increase in the osmotic pressure can be ascribed to the contamination of broiler semen with urine and bacteria, which in turn results in the clumping of sperm. A 375mOsm/kg osmotic pressure is optimum for the short-term storage of semen. However, the recommended osmolarity of the Blom stain technique is lower and was quantified as 220 mOsmol/kg dissolved in diluents, with composition similar to that of seminal plasma. However, these hypo osmotic conditions resulted in the swelling of the sperm head (Lukaszewicz *et al*., 2008).

The semen diluents must be isotonic, as the osmotic pressure created by the solution may be detrimental to the sperm cell (Senger, 2003). Overall, these studies suggest that the osmotic pressure of poultry semen can vary between breeds and can change during storage and processing. By monitoring and optimizing osmotic pressure, it is possible to improve the quality and success of artificial insemination in the poultry industry.

2.12. Short Term Poultry Semen Preservation

Semen diluents are currently being used for both short- and long-term storage of domestic fowl semen. These extenders are being commercialized to improve the general reproductive effectiveness of the cockerels and lower the cost of AI. The development of semen diluents initially began with the use of NaCl (saline) solutions. Now complex diluents containing different osmotic regulators, energy sources and buffers are being used (Bootwalla and Miles, 1992). The most common practice for short term fowl semen storage (hours to days at a temperature of -4°C) requires the suspension of sperm in a suitable extender to maintain the sperm viability, *in vitro*. Assessment of diluted and undiluted stored cockerel semen revealed that the application of extenders is essential to sustain sperm quality (Bootwalla and Miles, 1992).

It was established that diluted fowl semen could be stored for up to 24 hours, without impairing the viability and fertilizing capacity of the sperm (Siudzinska and Lukaszewicz, 2008). Several other factors play a role in sustaining the quality of semen during storage over time e.g., the diluents used in semen extension and storage conditions

e.g., time, aeration and storage temperatures. It is known that sperm motility and the fertilizing capacity of undiluted raw fowl semen stored *in vitro* usually decreases within 1h after collection. Therefore, to store cockerel semen, the type of diluents and storage temperature is very crucial (Dumpala *et al*., 2006).

Generally, an extender will facilitate semen handling procedures, particularly during collection and evaluation, by maintaining the sperm viability, but preventing their activation. For semen maintained at 41°C and diluted BPSE or Minimum Essential Medium (MEM) there were quadratic and linear increases in the percentage dead sperm over time, while a drastic linear increase existed for undiluted semen. There was thus a linear decrease in Sperm Quality Index (SQI) for undiluted and semen diluted in MEM over time (4°C). However, for semen diluted with only BPSE, there was a linear increase in SQI (Dumpala *et al.,* 2006). Extenders serve to also protect the sperm cells from chemical and physical changes and contamination in their environment and provide more favorable conditions for fertilization (Chulhong and Chapman, 2005).

2.13. Poultry Semen Cryopreservation Methods

There are several different types of poultry semen cryopreservation protocols, each of which employs different techniques and procedures for freezing and storing avian semen.

2.13.1. Conventional cryopreservation:

This is the most commonly used method for poultry semen cryopreservation. It involves mixing semen with a cryoprotectant solution and then freezing the mixture using a controlled-rate freezer. A study by Rizwan *et al*. (2020) compared the effectiveness of different cryoprotectants and freezing rates for cryopreserving turkey semen using conventional cryopreservation.

2.13.2. Vitrification

This is an alternative method of cryopreservation that involves ultra-rapid cooling of the semen to prevent the formation of ice crystals. Vitrification has been shown to be effective for cryopreserving avian embryos, but its use for semen cryopreservation is still

relatively new. A study by Longobardi and Donoghue (2019) reviewed the current state of knowledge on vitrification of avian spermatozoa.

2.13.3. Cryoprotectant-free cryopreservation

This method involves freezing semen without the use of a cryoprotectant solution. It has been shown to be effective for cryopreserving avian sperm, but it requires specialized equipment and expertise. A study by Gliozzi *et al*. (2020) reviewed the current state of knowledge on cryoprotectant-free cryopreservation of poultry semen.

2.13.4. Use of antioxidants

Some studies have investigated the use of antioxidants to improve the quality of frozen-thawed semen. A study by Aitken *et al*. (2018) investigated the effects of different cryoprotectants and antioxidants on the quality of frozen chicken semen.

2.13.5. Pre-freezing treatments

Several studies have investigated the use of pre-freezing treatments, such as centrifugation or filtration, to improve the quality of frozen-thawed semen. A study by Othman *et al*. (2016) investigated the effects of different pre-freezing treatments on the quality of frozen quail semen.

Overall, there are several different types of poultry semen cryopreservation protocols that have been developed and studied. Researchers continue to investigate new methods and techniques for improving the efficiency and effectiveness of poultry semen cryopreservation, in order to support the development of more efficient and sustainable poultry breeding programs.

2.14. Poultry AI Techniques

Generally, there are two methods of semen deposition in poultry. These methods are the intra peritoneal insemination and vaginal insemination.

2.14.1. Intra peritoneal insemination

Intraperitoneal insemination (IPI) is a commonly used method for artificial insemination in poultry, particularly in commercial hatcheries. IPI involves the injection of semen directly into the peritoneal cavity of female birds, bypassing the cloaca and reproductive tract. One advantage of IPI is that it allows for the delivery of a precise amount of semen into the female's reproductive system, which can increase fertilization rates and hatchability. Additionally, IPI can be used with frozen semen, which allows for easier storage and transportation of genetic material (Onagbesan and colleagues, 2007).

However, there are also some potential disadvantages to IPI. One concern is the risk of injury to the female bird's internal organs during injection, which can lead to reduced fertility or mortality. Another concern is the potential for contamination of the peritoneal cavity with bacteria or other pathogens, which can lead to infection and reduced hatchability. Despite these potential drawbacks, IPI remains a popular method of artificial insemination in poultry due to its relative simplicity and effectiveness. In fact, some studies have shown that IPI can result in higher fertility rates compared to other methods of insemination, such as intrauterine insemination or cloacal insemination (Araújo et al., 2011).

2.14.2. Vaginal insemination

This is the most commonly used AI procedure and two personnel are required for this operation. The technique was developed in the 1930s and involves applying pressure to the hen’s abdomen and everting (Turn inside out) the vaginal orifice through the cloaca (Quinn and Burrows, 1936; Cole and Cupps, 1977). This procedure is also referred to as cracking, venting or everting the hen. Semen is deposited 2–4 cm into the vaginal orifice concurrently with the release of pressure on the hen’s abdomen. Insemination is accomplished with sterile straws, syringes or plastic tubes. In large scale commercial operations, automated semen dispensers using individual straws loaded with a set AI dose are commonly used.

Because poultry semen loses viability within 1 hour, hen insemination should begin immediately after collection (Aisha and Zain, 2010). You begin by holding the hen upside down against your body in the same way you held the rooster or tom. Exerting firm pressure on the left side of the vent causes the cloaca (the urogenital opening in birds) to evert, you need to use your thumb and forefinger to expose the oviduct (vagina). According to the same author (2011), the oviduct is the opening on the left side of the cloaca next to the anus. You can insert the insemination tube as far as possible up the oviduct, then squeeze out the semen and release the pressure on the cloaca at the same time. Relaxing the cloaca draws the semen further into the hen's body. Chicken inseminations can be completed within two consecutive days and then once a week after that. Because most hens will carry an egg in their oviducts in the morning, thus obstructing the route of the sperm to the ovary, insemination should occur in the afternoons after laying (Aisha and Zain, 2010).

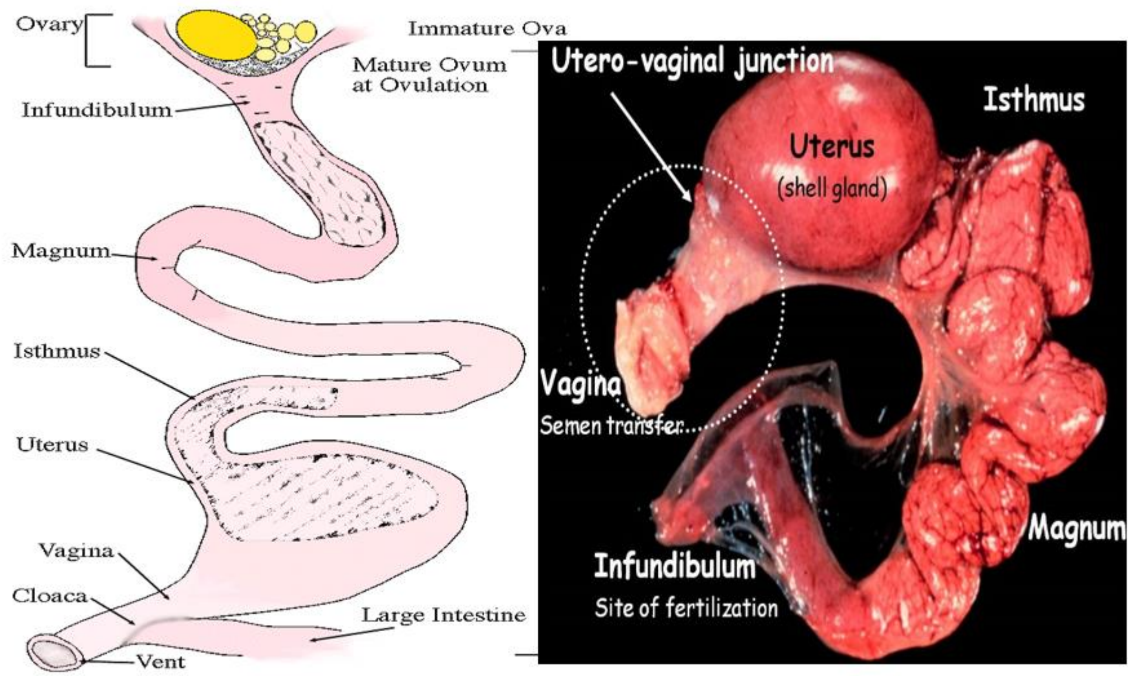


Figure 4. Female reproductive tracts, Source: https://www.intechopen.com/

2.15. Sperm Characteristics in the Oviduct of the Hen

Sperm Storage Tube (SST) is located between the vagina and shell gland of the oviduct. Previously sperm residing in the SST were considered to be immotile; however, it is likely that storage depends on moving against a generated by the SST epithelial cells. Cockerel sperm are motile at a body temperature of 41°C for an interval of days to weeks following ejaculation. How the sperm enter, survive, and exit these SST however is not known. Movement of sperm to the uterovaginal region is fast, however only viable sperm enter the SST. Current evidence suggests that the release of stored sperm is episodic, although it was first thought to be associated with oviposition.

Movement of sperm through the oviduct is achieved by smooth muscle contractions and/or ciliary activity and accumulates in the mucosal folds and short tubular glands at the lower end of the infundibulum (Hafez and Hafez, 2000). According to Hafez and Hafez (2000) the sperm in mammals spend a relatively short time in the female tract, while in chickens and the turkey sperm can spend a much longer period of time in the oviduct before fertilizing the egg yolk cell, up to 32 days in the chicken and 70 days in the turkey. Tabatabaei *et al*. (2009) stated that although the process of prolonged sperm storage is not known, it is thought to include a reversible suppression of respiration and motility of the sperm, as well as stabilization of the plasma membrane and maintenance of the acrosome.

According to Mauldin (2000), sperm are released from the SST to fertilize the sequentially ovulated ova at regular intervals. After release the sperm are taken to the ovum by contraction of the hen's oviduct, and sperm motility is no longer critical. Within 5 to 10 minutes after ovulation, sperm has already moved to the genital disc on the surface of the ovum. The sperm that make contact with the perivitelline layer of the ovum undergo an acrosome reaction and, presumably by the action of the trypsin-like enzyme acrosin, hydrolyze the perivitelline layer. Theoretically only one sperm fertilizes the ovum, but polyspermy has been observed in the hen ovum with many holes hydrolyzed in the perivitelline (Hafez and Hafez, 2000).

2.16. Analysis of Economic Benefits of Artificial Insemination over Natural Mating in Commercial Poultry Production

A study published in Poultry Science in 2019 investigated the economic benefits of using AI in a commercial broiler breeding program. The researchers found that AI resulted in significant improvements in feed conversion ratio and body weight gain, leading to increased profitability for the breeder. They concluded that AI can be an effective tool for improving the economic performance of commercial broiler breeding programs (Liu *et al*., 2019).

Another study published in Poultry Science in 2020 examined the economic benefits of using AI in a commercial broiler breeding program under different scenarios of semen production and distribution. The authors found that AI can result in significant cost savings compared to natural mating, particularly when using a centralized AI facility for semen collection and distribution. They concluded that AI can be a cost-effective tool for improving the economic performance of commercial broiler breeding programs (Garcia-Ruiz *et al*., 2020).

A study published in the Journal of Applied Poultry Research in 2021 investigated the economic benefits of using AI to improve the production efficiency of broiler breeder flocks. The researchers found that AI resulted in significant improvements in hatchability, chick quality, and growth performance, leading to increased profitability for the breeder. They concluded that AI can be an effective tool for improving the economic performance of commercial broiler breeding programs (Garcia-Ruiz *et al*., 2021).

A study published in Poultry Science in 2022 evaluated the economic benefits of using AI to increase the number of offspring produced per male in a commercial broiler breeding program. The authors found that AI resulted in significant improvements in the number of offspring produced per male, leading to increased profitability for the breeder. They concluded that AI can be an effective tool for improving the economic performance of commercial broiler breeding programs, particularly when used to optimize the use of valuable genetic material (Garcia-Ruiz *et al*., 2021).

Overall, recent studies have demonstrated that AI can provide significant economic benefits over natural mating in commercial broiler breeding, including improvements in feed conversion ratio, body weight gain, hatchability, chick quality, growth performance,

and number of offspring produced per male. These economic benefits can lead to increased profitability for commercial broiler breeders, making AI a valuable tool for improving the economic performance of commercial broiler breeding programs.

2.17. Horro Chicken Breed

The indigenous chickens of Ethiopia have various names and are characterized on different grounds. The Horro breed is named after its geographic region of origin, located in the western part of Ethiopia, Horro Guduru Wollega. The agro-ecological condition in which the breed is located from Tepid to cool wet highland with annual rainfall of 1200-1800mm, mean annual temperature of 22-26°C. Horro chickens are reared under scavenging management with supplemental feeding, and in most cases the birds are sheltered in the family house during the night. The single most important plumage color of males is red (60 percent). Only 3 percent of the females are red and the most frequent color is Zigrima, which is totally absent in males. All chickens have feathered necks. Yellow is the dominant skin color in both sexes. The average shank length of adult males is 8.8 cm and that of females are about 6.8 cm, adult males weigh about 1,700 g and females 1,372 g (Nigussie *et al*., 2010).

A study published in the journal Animal Genetic Resources in 2019 investigated the genetic diversity and population structure of Horro chicken using microsatellite markers. The researchers found that the breed exhibited high genetic diversity and population structure, suggesting the presence of different subpopulations within the breed. They concluded that Horro chicken has a valuable genetic resource for conservation and genetic improvement programs (Tadelle *et al.,* 2019).

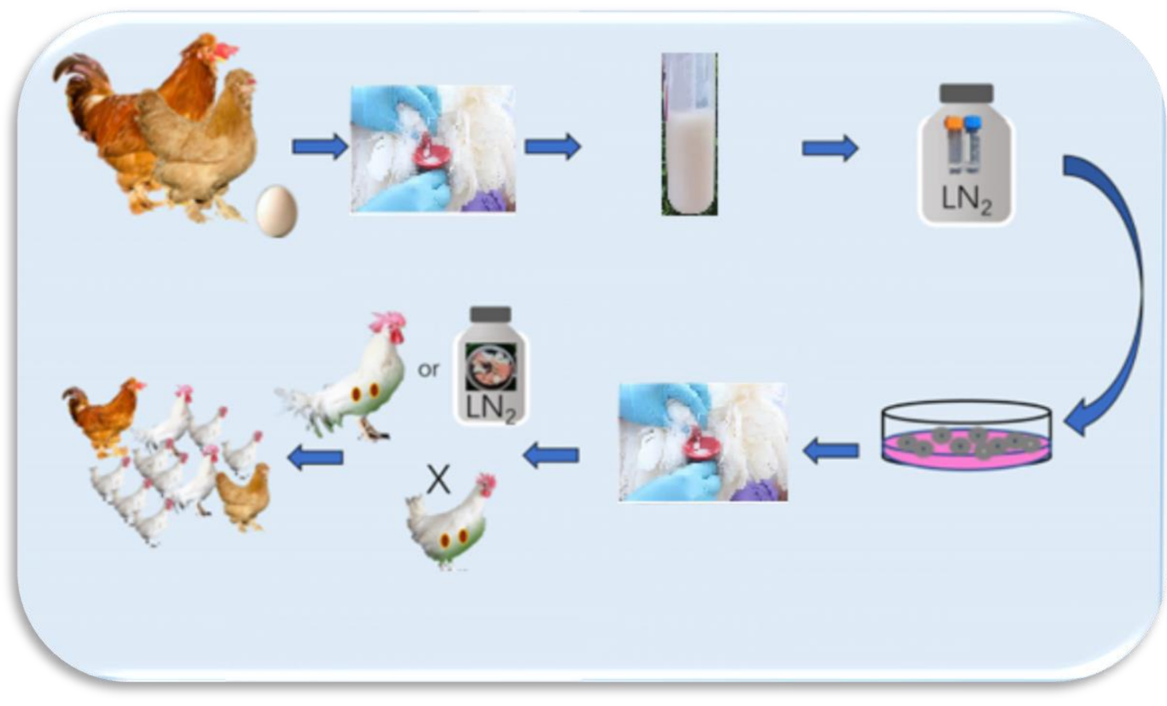
Another study published in the Journal of Animal Breeding and Genetics in 2020 examined the morphological and production traits of Horro chicken. The results showed that Horro chicken had a medium body size, with good egg production and hatchability rates. The researchers also reported that the breed had good meat quality and was well adapted to the local environment. They concluded that Horro chicken has significant potential for use in backyard and commercial poultry production systems (Birhanu *et al*., 2020).

A study published in Poultry Science in 2021 evaluated the growth performance and carcass characteristics of Horro chicken compared to commercial broiler breeds. The results showed that Horro chicken had a slower growth rate and lower carcass weight compared to commercial broilers, but had better meat quality and lower feed conversion ratio. The authors concluded that Horro chicken has potential for use in free-range and organic poultry production systems (Gebreselassie *et al*., 2021).

A study published in the journal Animal Science Journal in 2022 investigated the genetic diversity and population structure of Horro chicken using single nucleotide polymorphism (SNP) markers. The researchers found that the breed exhibited high genetic diversity and population structure, similar to the findings of the microsatellite marker study. They also identified potential genomic regions associated with important production traits, such as egg weight and body weight. They concluded that Horro chicken has significant potential for use in genetic improvement programs (Sisay *et al.,* 2022).

Overall, recent studies have demonstrated that Horro chicken has valuable genetic resources, with high genetic diversity and potential for use in backyard and commercial poultry production systems. The breed has good adaptation to the local environment, good meat quality, and potential for use in free-range and organic poultry production systems. Horro chicken also exhibits potential for use in genetic improvement programs, making it a valuable genetic resource for conservation and breeding programs.

CHAPTER 3



Methodology Pages 38-47

3. METHODOLOGY

3.1.Study Area

The study was conducted at Ethiopian Institute of Agricultural Research (EIAR), Debre Zeit Agricultural research center, Addis Ababa University, College of veterinary medicine and agriculture physiology laboratory and ELERE Farms, which are located in Ada’a Woreda, Bishoftu town, East Shewa zone, Oromia regional state, lying Southeast of Addis Ababa.

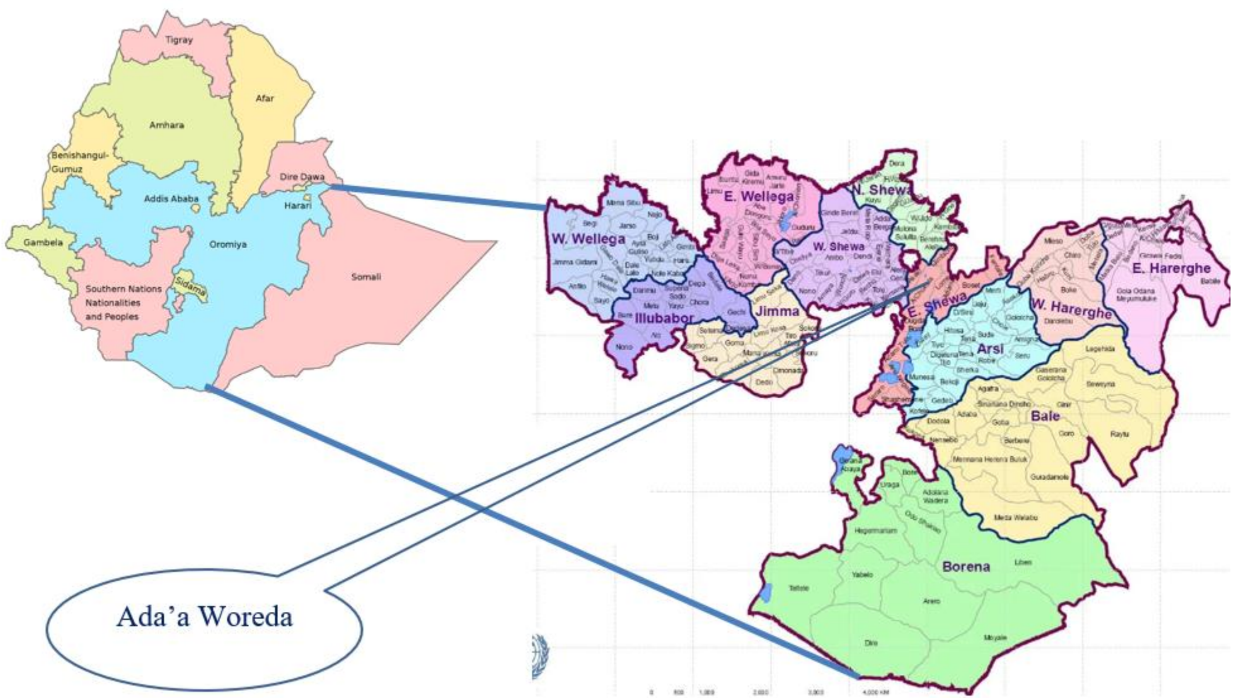


Figure 5. Study area description

3.2. Animal Management

A total of 30 Adult Horro Roosters aged between 35 to 42 weeks (average 40 weeks) with an average body weight of 1.7 kg were used for this study. The roosters were kept separately from hens and trained for semen collection by abdominal massage technique at least 2 weeks before the experiment started. For AI, 160 adult hens with similar age group were used for fertility assessment. The experimental chickens were purchased from Debrezeit Agricutural Research Center (DZARC). The roosters and the hens were kept in pens having a total area of 30 m^2 deep litter system house that is 12 cm deep. The average temperature and humidity of the chicken house were 22 ^oC and 41%, respectively. The lighting conditions used in the experiment was 16 hours for the hens during laying period and 12 hours for the roosters. Both the roosters and hens were fed with breeder ration containing 17% CP and 2800 Kcal/Kg energy (Appendix 1). Feed was provided twice a day at a rate of 110 gm/bird/day and water was provided ad libitum. All experimental chickens were vaccinated (Appendix 2) for major diseases including Newcastle, Marek's, Gumboro, fowl pox and fowl typhoid before the experiment. The chickens were acclimatized for two weeks to the experimental environment prior to sample collection and AI. The roosters were trained for semen collection following the two weeks acclimatization period.

3.3.Extender Preparation

The homemade extender used in this study was tris-egg-yolk-based. Semen diluents were prepared by mixing tris (base), citric acid, fructose, and egg yolk into which an antibiotic was added. The ingredients of the extender were purchased from local supplier. The composition of diluents is presented in Table 1.

Table 4. Contents of the homemade extender

Contents	Amount
Tris (base)	2.42 gm
Citric acid	1.48 gm
Fructose	4 gm
Egg-yolk	20 % v/v
Gentamicin	25 mg
Double distilled water	100 ml

Osmolarity =320 mOsmol/kg and pH of 7.2

Figure 6. Homemade extender prepared at AAU-CVMA Animal Reproduction Laboratory

The second extender was Beltsville (Beltsville Poultry Semen Extender, P2-7450, Continental, Delavan, WI, USA) a conventional commercial extender for the preservation of avian semen. Its compositions are, sodium glutamate (8.67 g/l), dipotassium phosphate (7.59 g/l), sodium acetate (3.2 g/l), fructose (5 g/l), potassium citrate (0.64 g/l), n-tris (hydroxymethyl) methyl 1-2 amino ethane sulfonic acid (TES; 3.2 g/l), monopotassium phosphate (0.7 g/l) and magnesium chloride (0.34 g/l). Osmolarity and pH were set at 310 mOsmol/kg and 7.1, respectively. The third extender was Avian Semen Diluent-1 (ASD-1, Minitube International, Tiefenbach, Germany), a commercial extender also commonly used in poultry. Its compositions are, Tris buffer (3.63g), Fructose (0.63g), Egg yolk (10g), Gentamicin (7.5mg) Sodium citrate (0.294g), distilled water (84.9g) and DMF as CPA (5%).

3.4.Semen Collection and Gross Evaluation

Standard Operating Procedure (SOP) for processing of semen for liquid storage and cryobanking was prepared at beginning (Appendix Table 1). Semen was collected using the Quinn and Burrows abdominal massage technique developed in 1936. The semen was collected in a sterile tube. Two ejaculates were collected from each rooster. The ejaculate volume varies from rooster to rooster which averages 0.3 ml. After collection, the semen was maintained in a water bath at 37°C and subjected to on-site pre-freeze evaluation including volume, color, pH, sperm concentration (bill/ml), motility (%), morphological abnormality (percentage of abnormal sperms) and live percent (%). Ejaculates having pearly-white color that are free from any fecal contamination, above 0.3 ml, concentration of above 1 x 109 sperm cells/ml, >60% motility, >70% live percent, and <30% morphological abnormality were designated as qualifying samples. Qualifying samples were then pooled to get sufficient volume for replication and further processing.

3.5.Experiment I- Evaluation of Semen Extenders on Sperm Quality of Liquid Stored Semen

3.5.1. Semen Processing and experimental layout for Liquid Storage Assessment

Following pre-freeze evaluation, qualifying semen was pooled to get 15 ml of semen. This was then divided into three aliquots each with 5ml. The first aliquot was immediately diluted at 1:4 ratio (v/v semen to extender) with a pre-warmed (37°C) homemade extender while the second aliquot was diluted with Beltsville commercial Extender. The third aliquot was kept un-extended and served as a control. Each treatment had 5 replications. Extended semen was stored at 4°C for 4hrs, 8hrs, 12hrs, and 24 hrs for further evaluation of viability.

3.5.2. Semen Quality Assays for Liquid Semen

Semen was first evaluated for volume (ml), color, texture, and pH. The concentration (mil/ml and billion) was measured using a hemocytometer (Counting chamber, Muhwa, China), while motility (%), live sperm (%), and morphology (%) were evaluated under the light microscope. Mass and progressive sperm motility were assessed microscopically (400X) by putting a drop of semen on the slide. For morphological and % live sperm evaluation, semen was mixed with 1.6% eosin and 6% nigrosine and observed under a light microscope (x1000 magnification, MSC-P200, China) under Oil immersion. Each time 200 spermatozoa were counted to determine abnormal percent and live percent.

3.6. Experiment II - Evaluation of Semen Extenders on Sperm Quality of Cryopreserved Semen

3.6.1. Semen Processing for Evaluation of Cryopreserved Semen

The pooled qualifying ejaculates are portioned into three treatment groups: homemade extender, Beltsville extender, and ASD which were then divided into three equal volumes and diluted with homemade extender, commercial extender ASD-1 and Beltsville commercial extender added at a ratio of 1:4 (semen: extender). The diluted semen was distributed equally in 60 sterile glass tubes each (Table 6).

Table 5. Experimental layout for post-thaw sperm quality analysis

Treatment	Type of extender	Number of replications
Extender-1	Homemade	20
Extender-2	Beltsville	20
Extender-3	ASD-1	20

The cryoprotectants were supplemented to each tube as a final concentration (Table 7). The semen samples were equilibrated in a refrigerator at 5°C for 40 minutes (Silyukova *et al*., 2022). The freezing procedure followed the static vapor freezing method. Freezing of the samples started by placing the tubes on racks in a grill wide-mouthed liquid nitrogen container which is kept 32 cm away from the brim (mouth) of the container. After vapor freezing, the straws were collected and plunged into pre-cooled goblets for storage. For the post-thaw evaluation, the sperm samples were thawed after 7 days by keeping the sperm in the air for 90 seconds and then in the water bath of 37°C for another 60 seconds. The Mass sperm motility, progressive sperm motility (%), Morphological abnormalities (%), in vitro viability (%) and acrosome integrity of the frozen semen were evaluated according to Gerzlov (2010).

Table 6. CPAs used and their inclusion rates

S. N	Name of Extender	Type of CPA added	CPA inclusion rate
1	Homemade Tris-egg yolk-based extender	Glycerol	8%
2	Beltsville Extender	Glycerol	8%
3	ASD-1	DMF	5%

3.6.2. Semen Quality Assays for Liquid and Cryopreserved Semen

Semen was first evaluated for volume (ml), color, texture, and pH. The concentration (mil/ml and billion) was measured using a hemocytometer (Counting chamber, Muhwa, China), while motility (%), live sperm (%), and morphology (%) were evaluated under

the light microscope. Mass and progressive sperm motility were assessed microscopically (400X) by putting a drop of semen on the slide. For morphological and live sperm evaluation, semen was mixed with 1.6% eosin and 6% nigrosine and observed under a light microscope (x1000 magnification, MSC-P200, China) under Oil immersion.

3.6.3. Fertility Test of Cryopreserved Semen

For this purpose, a total of 160 adult hens were used. The hens were divided into four pens, each pen containing 40 hens and kept for 20 days without exposure to males. Each semen samples were inseminated to 40 hens in each treatment for evaluation of fertility. The insemination was done during the afternoon because during the morning, most of hens will have an egg in their oviducts, thus obstructing the free passage of semen to the ovary. A 0.3 ml of thawed semen, at intervals of 7 days were inseminated for three weeks. The Intra-vaginal AI was performed using a 1 ml capacity sterile syringe (Getachew *et al*., 2015).

In this study, a total of 400 eggs, 100 eggs from each treatment were analyzed for Fertility and hatchability analysis. Hatching eggs were collected in the morning. Eggs that were at least 50g, and did not have crack were marked and identified by pen number and treatment number, stored sharp point of egg downward, and pre-heated for 12 h at 25°C prior to incubation. The eggs were set at random within racks and trays, in a 150 eggs capacity tray at ELERE Farms hatchery unit and incubated for 18 days at 37.5^{o}C (60-70% relative humidity) that was turned every hour at 90°. All eggs were candled individually on Day 18 of incubation. Clear eggs were removed, opened and inspected for evidence of embryo development. In the absence of an embryo, eggs were classified infertile. Fertility and hatchability were calculated as:

$$\text{Fertility (\%)} = \frac{\text{Number of eggs fertile}}{\text{Number of eggs set}} \times 100$$

$$\text{Hatchability (\%)} = \frac{\text{Number of eggs hatched}}{\text{Number of Eggs fertile}} \times 100$$

Table 7. Experimental layout fertility and hatchability analysis

Type of extender used for dilution	Number of hens	Number of pens	Number of hens in each pen	Method of AI	Number of eggs collected for incubation
Homemade	40	4	10	IV AI (0.3ml)	100
Beltsville	40	4	10	IV AI (0.3ml)	100
ASD-1	40	4	10	IV AI (0.3ml)	100
Control (Fresh, raw semen)	40	4	10	IV AI (0.3ml)	100

IV AI: Intra vaginal artificial insemination

3.7.On Farm Application and Analysis of Economic Benefits

Analysis of economic benefits of AI over natural mating was conducted on commercial Cobb500 broiler chicken. Data was collected on the cost of maintaining a flock of broiler breeders, including the cost of feed, labor, and other inputs. Data was also collected on the number of males required for AI natural mating and their associated costs, as well as the cost of AI equipment and semen collection and distribution.

A model was developed to simulate the variables for production and financial performance of a broiler farm under different scenarios of breeding methods (natural mating vs. AI). The model considered factors such as sample flock size, male-to-female ratio, hatchability, and mortality rate, as well as the costs associated with each breeding method (Appendix 3).

Cost-benefit analysis was conducted by comparing the financial performance of the broiler farm under different breeding scenarios (artificial insemination and natural mating). The analysis considered the costs associated with each breeding method, as well as the revenues generated from the sale of broiler chicks (Garcia-Ruiz *et al.,* 2020).

Sensitivity analysis was conducted to examine the impact of different assumptions and input parameters on the financial performance of the broiler farm. This will help to

identify the key drivers of profitability under each breeding method, as well as the potential risks and uncertainties associated with each method. Scenarios used to analyze sensitivity analysis of the cost benefit analysis are presented in Appendix table 4.

Based on the results of the analysis, decision was made on the optimal breeding method for the broiler farm, taking into account the financial performance, animal welfare and environmental sustainability.

3.8.Meta-Analysis of Effects of Cryoprotectants on Sperm Motility and Fertility

Inclusion and exclusion criteria were set to determine publications that were included in the study. The inclusion criteria were studies that report on the effects of glycerol and DMF as cryoprotectants on sperm quality and fertility of cryopreserved chicken semen in different breeds. The exclusion criteria were studies that focus on other cryoprotectants, studies that do not report on sperm quality or fertility, and studies that do not involve chicken semen. A comprehensive search was conducted in electronic databases such as PubMed, Scopus, and Web of Science using relevant keywords and search terms such as "cryoprotectants," "glycerol," "DMF," "chicken semen," "sperm quality," and "fertility." Additionally, the reference lists of identified articles were searched for relevant studies. Titles and abstracts were screened to determine their relevance to the research question. The full texts of potentially relevant articles were assessed for eligibility based on the inclusion and exclusion criteria.

Relevant data were extracted from the included studies such as sample size, breed of chickens, cryoprotectant used, post-thaw sperm quality parameters measured, fertility parameters measured, and statistical analyses performed. The data from the included studies were synthesized and analyzed using appropriate statistical methods such as meta-regression and subgroup analysis. The overall effect of glycerol and DMF on sperm quality and fertility of cryopreserved chicken semen from different breeds were determined. Publication bias was assessed using forest plots. The results of the meta-analysis were interpreted, and conclusions was drawn about the effects of glycerol and DMF on sperm quality and fertility of cryopreserved chicken semen from different breeds.

3.9.Data analysis

The data on semen quality parameters and Artificial Insemination was analyzed using Analysis of Variance (ANOVA) using the latest version of STATA computer software. When F-test is found significant, means were compared using LSD. A 5% ($P<0.05$) level of significance was used to determine statistical significance. Factorial 3*4 completely randomized design (CRD) was used to evaluate the effect of storage time and types of extenders on Liquid stored semen. One-way CRD was used to evaluate the effects of semen extenders on the quality of Cryopreserved semen, fertility and hatchability tests. For meta-analysis, 95% Confidence Interval (FI) was calculated from SE of each mean. Publication bias was assessed using forest plots.

Model for the evaluation of the effects of storage time, extenders and their interaction on quality of liquid stored semen:

$Y_{ijk}=\mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$

Where; Y_{ijk}= the response variable

μ= overall mean

α_i= Effect of extenders

β_j= Effect of time of storage

$(\alpha\beta)_{ij}$= Interaction between cryoprotectants and storage time

e_{ijk}= random error

Model for the evaluation of the effects of extender on semen quality, fertility and hatchability:

Model: $Y_{ij}=\mu + \alpha_i + e_{ij}$

Where; Y_{ij}= the response variable

μ= overall mean

α_i= Effect of level of extender

e_{ij}= random error

CHAPTER 4

Results Pages 49-66

4. RESULTS

4.1.Fresh Semen Characteristics

A summary of the results of semen visual and microscopic characteristics addressed in this study are presented in Table 10. The volume of fresh poultry semen collected from Horro breed in this study ranged from 0.05 ml to 0.5 ml. The average ejaculate volume in this study was 0.36ml. The color and texture of fresh poultry semen collected in this study generally is milky white and moderately viscous texture. The average pH of fresh semen collected was 7.2. The average sperm concentration in fresh semen collected from Horro cockerels in this study was 5.5 billion sperm per ml semen.

Table 8. General semen characteristics of the Horro chicken breed

Semen characteristics	Mean semen characteristics
Average Ejaculate volume (ml)	0.36
Color	Milky white
Texture	Moderately viscous
Sperm total concentration/ml	5.5×10^9
pH	7.2

Figure 7. Visual characteristics of semen from Horro chicken breed

4.2.Effect of Semen Extenders and Storage Time on Quality of Liquid Stored Semen from Horro Chicken Breed

The effect of semen extenders and storage time on sperm quality is presented in Table 11. There were significant differences ($p < 0.05$) in progressive sperm motility and *in vitro* live sperm between the different storage time and type of extenders. Sperm morphology was not affected by length of storage time and the type of extender. Progressive motility and *in vitro* viability were higher in commercial Beltsville extender at 4 hours of storage ($p < 0.05$). Viability (%live sperm) was 83.6% and 82.6% in homemade and commercial Beltsville extender, respectively. Semen extended with homemade and Beltsville extenders consistently had higher motility compared with the control extender irrespective of storage time. Both motility and viability were significantly higher in Beltsville PSE compared to both the control and homemade extenders at all storage durations tested. Motility of spermatozoa steadily decreased as storage time increased in all semen samples in different extenders.

Table 9. Effect of interaction of semen extenders and storage time on sperm quality of Horro chicken

Factor	Mean ± SE sperm parameters		
	Progressive motility (%)	Morphological Abnormality (%)	Live sperm (%)
Extender (storage time Significance) at p < 0.05	***	NS	***
Control (4 hours)	77 ± 2.54[a]	15.4 ± 1.81	73.2 ± 1.39[b]
Control (8 hours)	59 ± 1.87[b]	16.2 ± 1.80	55.8 ± 1.66[c]
Control (12 hours)	42 ± 1.22[c]	25.4 ± 2.78	49 ± 1.22[c]
Control (24 hours)	21 ± 1.00[d]	27.2 ± 1.59	11.4 ± 1.21[d]
Beltsville (4 hours)	87 ± 1.22[a]	10.4 ± 0.51	83.6 ± 1.63[a]
Beltsville (8 hours)	79 ± 1.00[a]	15 ± 1.82	77.8 ± 1.28[a]
Beltsville (12 hours)	50 ± 2.74[b]	17.8 ± 1.62	68.4 ± 1.50[b]
Beltsville (24 hours)	46 ± 1.87c	23 ± 2.30	51 ± 1.14[c]
Homemade (4 hours)	84 ± 1.00[a]	12.2 ± 1.39	82.6 ± 1.36[a]
Homemade (8 hours)	72 ± 1.22[a]	16.6 ± 1.57	73.8 ± 1.93[b]
Homemade (12 hours)	49 ± 1.87[b]	19.8 ± 2.08	66.4 ± 1.50[b]
Homemade (24 hours)	45 ± 1.58[c]	25.4 ± 1.21	46.4 ± 1.44[c]

NS: Non-significant; SE: standard error; Different letters within the same column show significant differences among the groups ($p < 0.05$).

4.2.1. Effect of Semen Extenders on Sperm Quality of Horro chicken

Semen parameter were significantly better in tris-based homemade extender compared to the control. However, there was no significant difference ($p < 0.05$) in all semen quality parameters between the commercial Beltsville PSE and locally prepared Tris-based extenders. Significantly highest sperm motility, morphological abnormalities, and in vitro viability rates were observed in semen extended samples using a commercial and locally prepared extenders when compared to the control treatment ($p < 0.05$). However, there was a high significant difference between the control and homemade extender, as well as between BPSE and the control.

Table 10. Effect of semen extenders on sperm quality of Horro chicken

Factor	Mean ± SE sperm parameters		
	Progressive motility (%)	Abnormality (%)	Viability (%)
Fresh, raw semen	49.75 ± 4.82[b]	21.05 ± 1.54[b]	47.35 ± 5.21[b]
Homemade extender	65.25 ± 4.22[a]	16.55 ± 1.30[a]	70.2 ± 2.90[a]
Beltsville extender	62.75 ± 3.71[a]	18.5 ± 1.32[a]	67.3 ± 3.15[a]

SE: Standard error; Different letters within the same column show significant differences among the groups ($p < 0.05$).

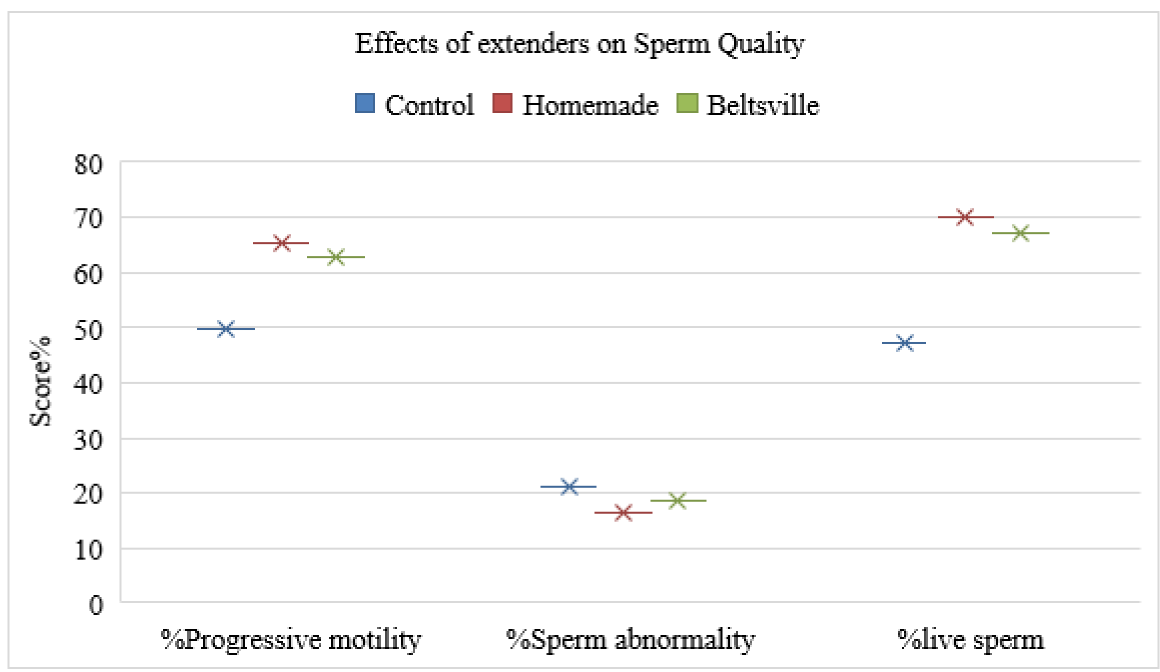


Figure 8. Effect of semen extenders on sperm quality of Horro chicken breed

4.2.2. Effect of storage time on sperm quality of Horro chicken

The effect of storage time on sperm quality is presented in Table 13 and figure 9. Sperm quality is an important factor in the field of poultry reproduction as it can affect fertility and hatchability rates. The preservation of sperm quality during storage and transportation is therefore critical in ensuring successful reproduction. In this part, the

effects of storage duration on sperm quality of semen collected from Horro chicken breed is explored.

The results of the study indicate that there were significant differences ($p < 0.05$) in progressive sperm motility, sperm morphological abnormalities, and in vitro sperm viability across all storage durations tested. Specifically, the study found that sperm motility decreased significantly as the storage duration increased from 4 to 24 hours. Additionally, sperm morphological abnormalities increased significantly ($p < 0.05$) as the storage duration increased, except between 12 and 24 hours of storage. The study also found that *in vitro* sperm viability decreased significantly as the storage duration increased, except between 8 and 12 hours of storage.

Table 11. Effect of storage time on sperm quality of Horro chickens

Factor	Mean ± SE Sperm parameters		
	Progressive motility (%)	**Abnormality (%)**	**Live sperm (%)**
4 hours	82.67 ± 1.45^{a}	12.67 ± 0.91^{a}	79.8 ± 1.48^{a}
8 hours	70 ± 2.34^{b}	15.93 ± 0.95^{b}	69.13 ± 2.70^{b}
12 hours	47 ± 1.44^{c}	21 ± 1.46^{c}	61.26 ± 2.45^{b}
24 hours	37.33 ± 3.19^{d}	25.2 ± 1.05^{c}	36.26 ± 4.77^{c}

SE: Standard error; Different letters within the same column show significant differences among the groups ($p < 0.05$).

The observed decrease in sperm motility and *in vitro* viability and the increase in morphological abnormalities with increasing storage time may be attributed to various factors, including the breakdown of the extender and the accumulation of toxic metabolic by-products. As the extender breaks down, it may no longer provide the necessary nutrients and support for sperm cells, leading to a decline in their quality and viability. Additionally, as sperm cells metabolize, they produce toxic by-products that can accumulate over time and lead to a decline in their quality and viability.

The observed differences in sperm quality between storage durations suggest that the optimal storage duration for poultry semen may be 8 hours where the fertilizing capacity of the sperm maintained. The semen stored for 4 hours had the highest sperm motility, lower morphological abnormalities, and higher in vitro viability rates compared to all other storage durations tested. This may be attributed to the fact that the sperm cells have not been exposed to the negative effects of storage for too long.

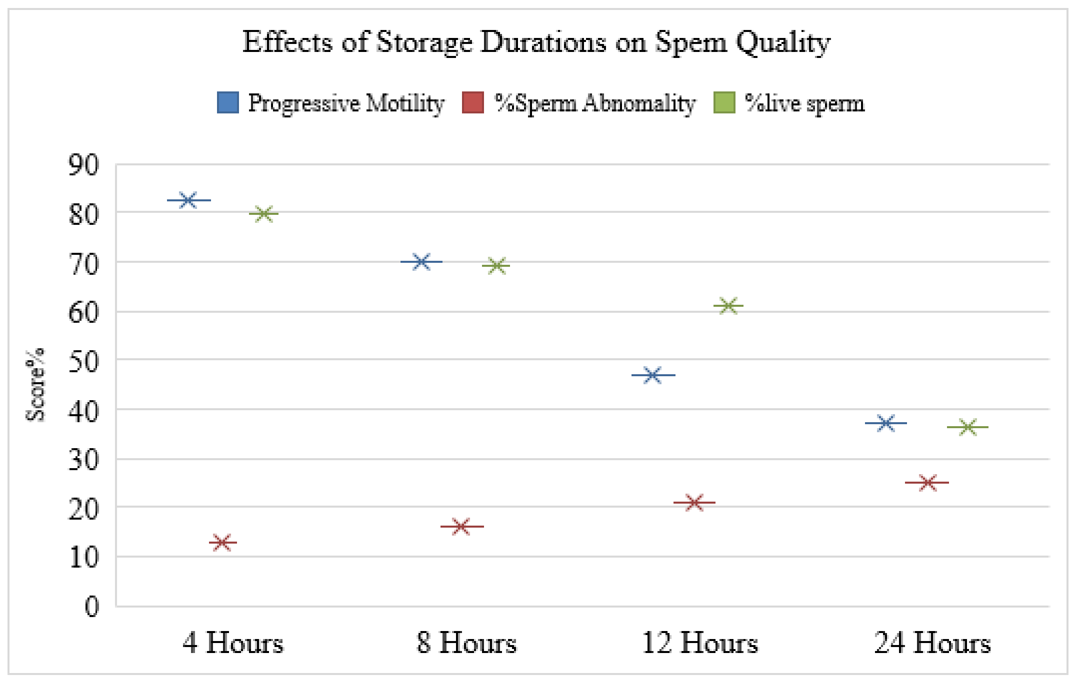


Figure 9. Effect of storage durations on sperm quality of Horro chicken breed

In conclusion, the study highlights the importance of carefully considering the storage duration of poultry semen to preserve the quality and viability of sperm cells during storage and transportation. The results suggest that the optimal storage duration for poultry semen may be around 8 hours, as this duration resulted in the optimum sperm quality parameters. Further research is needed to identify the specific factors that contribute to the observed differences in sperm quality across storage durations and to develop more effective storage and preservation methods for poultry semen.

4.3. Effect of Extenders on Sperm Quality of Cryopreserved Semen from Horro Chicken Breed

The effect of cryoprotectants on sperm quality of Horro chicken breed is presented in Table 14 and Figure 10. This study explores the effects of semen extenders on the quality of cryopreserved semen from Horro chicken breed. The results of the study indicate that there were no significant differences ($p > 0.05$) observed on mass motility across the different cryoprotectants. However, there were significant differences ($p < 0.05$) in progressive sperm motility, mass motility, and in vitro viability between homemade and commercial extenders. The study found that commercial extenders, specifically ASD-1 and BPSE, resulted in significantly higher progressive sperm motility, mass motility, and *in vitro* viability compared to homemade extender. Additionally, the study found that the commercial ASD-1 extender resulted in significantly higher morphologically normal sperm (64.25 ± 0.91) and *in vitro* viability rates (42.75 ± 0.73) compared to homemade extender.

Table 12. Effects of extenders on post-thaw sperm quality from Horro breed

Factor	Mean ± SE Sperm Parameters			
	Mass motility (%)	**Progressive motility (%)**	**Morphologically normal (%)**	**Viability (%)**
Homemade extender	48.5 ± 1.5^{a}	23.75 ± 0.81^{b}	55.25 ± 1.11^{b}	33.2 ± 0.96^{b}
ASD-1	51 ± 1.52^{a}	28.2 ± 0.56^{a}	64.25 ± 0.91^{a}	42.75 ± 0.73^{a}
Beltsville PSE	51.5 ± 1.31^{a}	28.2 ± 0.57^{a}	62.05 ± 0.70^{a}	42.15 ± 0.52^{a}

SE: Standard error, PSE: Poultry semen extender, Different superscript letters within the same column show significant differences among the groups ($p < 0.05$).

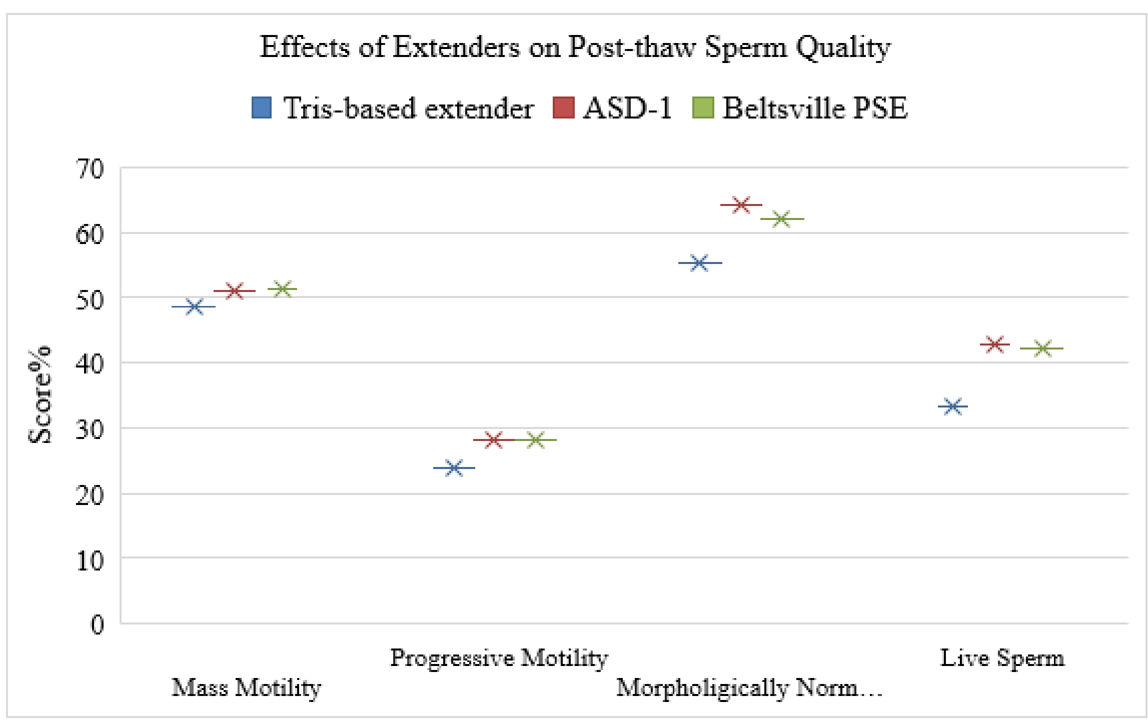


Figure 10. Effects of extenders on post-thaw sperm quality from Horro breed

4.4.Effects of extenders on Fertility and Hatchability

Fertility and hatchability data is presented in Table 15. There were no significant differences across all treatments in terms of fertility and hatchability rate ($p > 0.05$).

Table 13. Effect of Cryoprotectants on fertility and hatchability

Variables	Treatment			
	Homemade extender	ASD-1	Beltsville PSE	Control (Fresh semen inseminated)
Fertility rate (%)	41 ± 1.82^{b}	48 ± 2.27^{b}	46.25 ± 1.31^{b}	87.93 ± 1.64^{a}
Hatchability rate (%)	77.23 ± 2.25^{b}	80.25 ± 1.31^{b}	78 ± 2.68^{b}	87.25 ± 1.03^{a}

ASD: Avian Semen Diluent; PSE: Poultry semen extender; [a, b] Different superscript letters within the same row show significant differences among the groups ($p < 0.05$).

4.5.On-farm Application and Economic Analysis

4.5.1. Key Assumptions

For the economic analysis, it is assumed that the farm contains 5000 female Cobb500 breeder farm with a standard male-to-female ratio for both natural mating and artificial insemination. The financial analysis of the assumed farm is based on the data presented in the following assumptions: -

Table 14. Assumptions

Variable	AI	Natural mating	Basis of assumption
Chicken breed used	Broiler Cobb500 breeder flock	Broiler Cobb500 breeder flock	Widely used broiler breed in Ethiopia
Male-to-female ratio	1:30	1:10	Standard ratio
Egg production period	Week 24 - 50	Week 24 - 50	Cobb500 breeder guide
HDEP ranges	5% to 86%	5% to 86%	Cobb500 breeder guide
Peak production (week)	Week-30	Week-30	Cobb500 breeder guide
Flock size (females)	5000	5000	Assumption for a size of a sample farm
Number of males/flocks	167	500	Calculated
Feed consumption range (week 24 to 50)	117-163 g/day	117-163 g/day	Cobb500 breeder guide
Cost of extender (ETB)	0.35	0	Standard
Number of personnel	25	19	Standard ratio of personnel
Cost of drug, vitamins and vaccine/head	15	15	Current cost/head
Fertility rate (%)	90	87	Literatures
Hatchability rate (%)	85	85	Literatures
Cost of imported	432	432	Current cost calculated

breeder DOC/head (ETB)			from foreign currency
Price of broiler DOC/head (ETB)	50	50	Current market price

4.5.2. *Cost Benefit Analysis*

The cost-benefit analysis is presented in Table 17. To determine the cost-benefit analysis of AI over natural mating in broiler Cobb500 breeds, we need to look at the income statement. The income statement shows the financial performance of a business over one year period, and it includes revenue, expenses, and profit. The results of the income statement show that artificial insemination is better than natural mating in terms of cost efficiency and profitability. This is because AI reduces the number of males for breeding, labor costs and improves biosecurity, which reduces the risk of disease outbreaks and the associated costs. Additionally, AI allows for faster genetic progress, which can lead to increased revenue and profitability.

Higher revenue (birr 26.7 million) was recorded in flocks used artificial insemination as compared to natural mating (birr 24.5 million). Higher net income was recorded in farm that uses artificial insemination technique (birr 10.8 million) as compared to farm that uses natural mating (birr 9.2 million).

Table 15. Cost-benefit analysis of artificial insemination over natural mating in commercial broiler farming

Income statement (5000 females)	**Cobb500 AI**	**Cobb500 NM**
Sales		
DOC sales	26,708,062.50	24,458,962.50
Total sales	**26,708,062.50**	**24,458,962.50**
Cost of production		
Cost of male	72,000.00	216,000.00
Cost of females	2,160,000.00	2,160,000.00
Cost of feed	7,382,340.00	7,858,620.00

Production payroll	1,128,000	696,000
Vaccine, drugs and vitamins	77,500	82,500
Cost of extender	66,461.47	0
Equipment cost	15,500	16,500
total cost of production	**10,901,801.47**	**11,029,620.00**
Administrative costs		
Utility	103,800	103,800
Other office expenses	134,400	134,400
Total administrative costs	**238,200**	**238,200**
EBIT	**15,568,061.03**	**13,191,142.50**
Tax (30%)	4,670,418.31	3,957,342.75
Net profit	**10,897,642.72**	**9,233,799.75**

4.5.3. Sensitivity analysis

Sensitivity analysis is presented in Table 18. The scenarios used to analyze the sensitivity are the major variables that affect financial benefits. These variables are; total revenue, Profit before depreciation and tax, and net profit. According to financial sensitivity analysis, 5% decrease in price of DOC highly affects the revenue, EBIT and net profit of the broiler farms hypothesized.

Table 16. Sensitivity analysis

Revenue	**AI**	**NM**
Base case	26,708,063	24,458,963
5% decrease in DOC price	***25,372,659***	***23,236,014***
5% increase in production cost	26,708,063	24,458,963
5% increase in administrative cost	26,708,063	24,458,963

EBIT	**AI**	**NM**
Base case	15,568,061.03	13,191,142.50
5% decrease in DOC price	***14,232,657.90***	***11,968,194.38***
5% increase in production cost	15,022,970.95	12,639,661.50

5% increase in administrative cost	15,556,151.03	13,179,232.50

Net profit	**AI**	**NM**
Base case	10,897,642.72	9,233,799.75
5% decrease in DOC price	***9,562,239.59***	***8,010,851.63***
5% increase in production cost	10,352,552.65	8,682,318.75
5% increase in administrative cost	10,885,732.72	9,221,889.75

4.6.Meta-Analysis of Effects of Cryoprotectants on Sperm Motility and Fertility

Results in the effects of Cryoprotectants on post-that sperm motility and fertility are presented un this sub-section. Running the search as in Materials and methods, a total of 89 papers were identified (Figure 1). Out of these 28 were excluded for the following reasons: 10 focused on evaluation of extenders instead of CPAs, 15 on semen freezing methods, 3 were evaluation of effects of chicken breeds. The full text of the remaining 61 papers was retrieved for evaluation.

Among 61 articles read in full text, 22 articles were excluded for the following reasons: for being review articles, studies that that are not used Glycerol as CPA, Studies that that are not used DMF as CPA, studies that did not evaluated fertility. A total of 13 articles were included in the meta-analysis (Mehdipour *et al*., 2020a; Wu *et al*., 2019; Najafi *et al*., 2020; Thananurak *et al*., 2019; Yousufi *et al*., 2021; Siari *et al*., 2021; Thananurak *et al*., 2020; Masoudi *et al*., 2019; Chauychu *et al*., 2017; Chauychu *et al.,* 2021; Mehdipour *et al*., 2020b; Zong *et al*., 2022; Mosca *et al*., 2019).

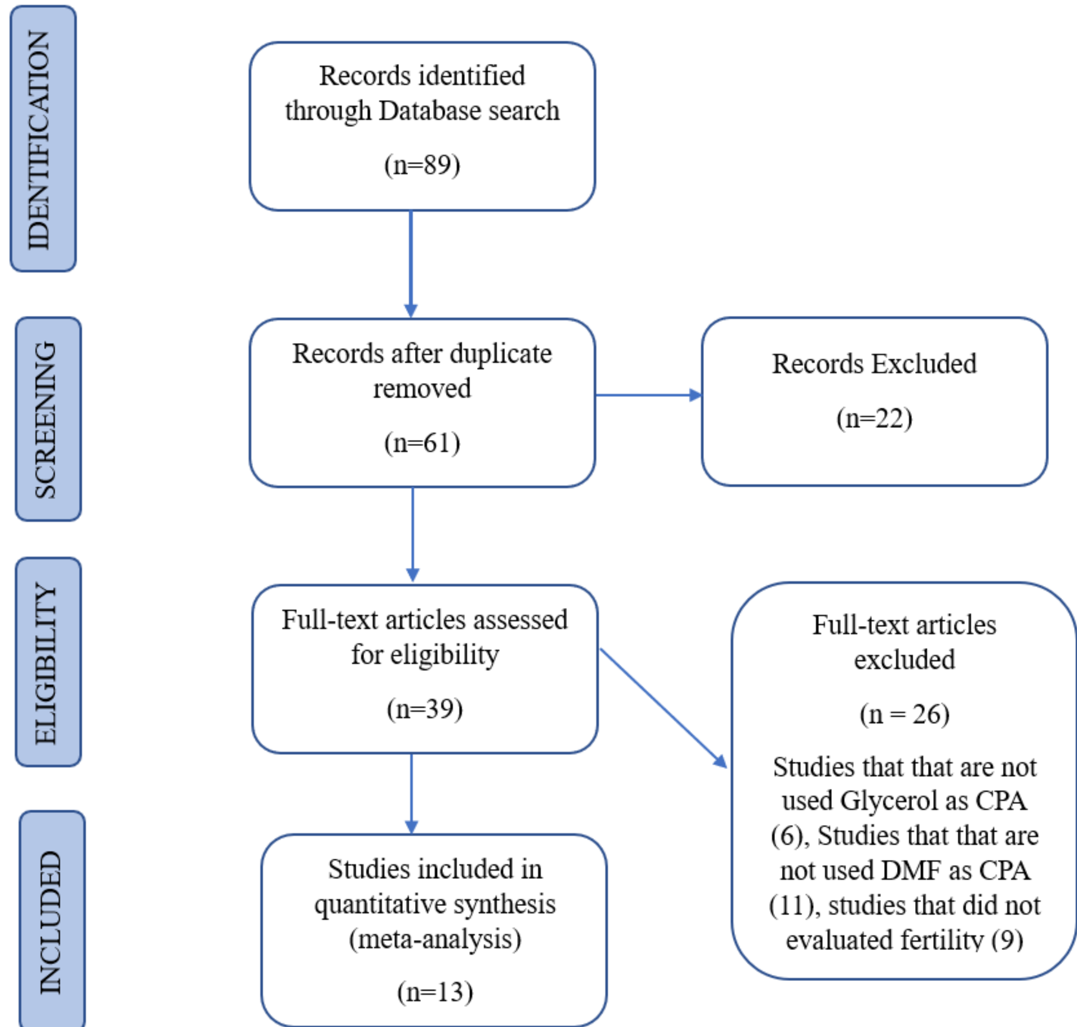


Figure 11. Flow chart of Literature Research

Data extraction showed that all of the studies mean and SE. For analysis of mean SE was converted into FI. CPAs used in the articles are generally classified in to Glycerol and DMA with different mixing rate. Among publications used Glycerol as CPA Yousufi *et al.*, 2021; Zong *et al.*, 2022 mixed 5% v/v glycerol; Wu *et al.*, 2019; Mehdipour *et al.*, 2020a added 8% Glycerol v/v; Siari *et al.*, 2021; Masoudi *et al.*, 2019 added 3% glycerol v/v and Mehdipour *et al.*, 2020b; Najafi *et al.*, 2020 added 3.8% glycerol v/v in each extender. Regarding publications that used DMF as CPA, one article Mosca *et al.*, 2019 added 9% DMF in the extender. Whereas, publications Thananurak *et al.*, 2020; Chauychu-noo *et al.*, 2021; Chauychu-noo *et al.*, 2017 and Thananurak *et al.*, 2019 added 9% DMF as CPA in each extender.

Table 17. Outcomes reported in the studies on effects of Cryoprotectants on post-thaw Sperm Motility

Publication	Year	CPA Used	%CPA mixing rate	Extender Used	Chicken Breed Used	Mass Motility	%Fertility
Zong *et al*	2022	Glycerol	5	Lake	Beijing Yu	37.4	48.7
Yousufi *et al*	2021	Glycerol	5	Lake	Ross	61.8	60.4
Wu *et al*	2019	Glycerol	8	Lake	Black Silkis	70	77.6
Mehdipour *et al*	2020a	Glycerol	8	Beltsville	Ross	45	45.1
Siari *et al*	2021	Glycerol	3	Beltsville	Ross	61.6	64.2
Masoudi *et al*	2019	Glycerol	3	Lake	Ross	58.5	63.8
Mehdipour *et al*	2020b	Glycerol	3.8	Lake	Ross	71.1	71
Najafi *et al*	2020	Glycerol	3.8	Beltsville	Ross	67.5	61.8
Mosca *et al*	2019	DMF	9	Lake	Hi-line white	24.2	45
Chauychu-noo *et al*	2021	DMF	6	Schramm	RIR	57.6	87
Thananurak *et al*	2019	DMF	6	BHSV	Thai Native	64.3	91
Thananurak *et al*	2020	DMF	6	BHSV	Thai Native	60.1	69
Chauychu-noo *et al*	2017	DMF	6	Schramm	Thai Native	58.2	91

Table 18. Outcomes reported in the studies on effects of Cryoprotectants on post-thaw Sperm Motility

Publication	Year	CPA Used	%CPA mixing rate	Extender Used	Chicken Breed Used	%Fertility
Zong *et al*	2022	Glycerol	5	Lake	Beijing Yu	48.7
Yousufi *et al*	2021	Glycerol	5	Lake	Ross	60.4
Wu *et al*	2019	Glycerol	8	Lake	Black Silkis	77.6
Mehdipour *et al*	2020	Glycerol	8	Beltsville	Ross	45.1
Siari *et al*	2021	Glycerol	3	Beltsville	Ross	64.2
Masoudi *et al*	2019	Glycerol	3	Lake	Ross	63.8
Mehdipour *et al*	2020	Glycerol	3.8	Lake	Ross	71
Najafi *et al*	2020	Glycerol	3.8	Beltsville	Ross	61.8
Mosca *et al*	2019	DMF	9	Lake	Hi-line white	45
Chauychu-noo *et al*	2021	DMF	6	Schramm	RIR	87
Thananurak *et al*	2019	DMF	6	BHSV	Thai Native	91
Thananurak *et al*	2020	DMF	6	BHSV	Thai Native	69
Chauychu-noo *et al*	2017	DMF	6	Schramm	Thai Native	91

There were no significant differences ($p > 0.05$) in sperm mass motility across the two CPAs used. However, significant differences ($p < 0.05$) were observed in mass motility across the mixing rates of CPAs in the extenders. The highest result has been recorded in diluent prepared by Mehdipour *et al*. (2020a) using 3.8% DMF in lake extender in Ross chicken breed (71.1 ± 2.01). Diluent prepared using 9% glycerol by Mosca et al. (2019) showed the lowest results in terms of mass motility (24.2 ± 2.32).

There was significant difference ($p < 0.05$) across CPAs used in fertility. The highest fertility rate was observed in article by Chauychu-noo *et al*. (2017) which used 6% DMF in Schramm extender in semen collected from Thai Native Chicken breed (91.0 ± 2.41). The lowest fertility was recorded in article by Mosca *et al*. (2019) which used 9% DMF in Lake extender in semen collected from Hi-line White chicken.

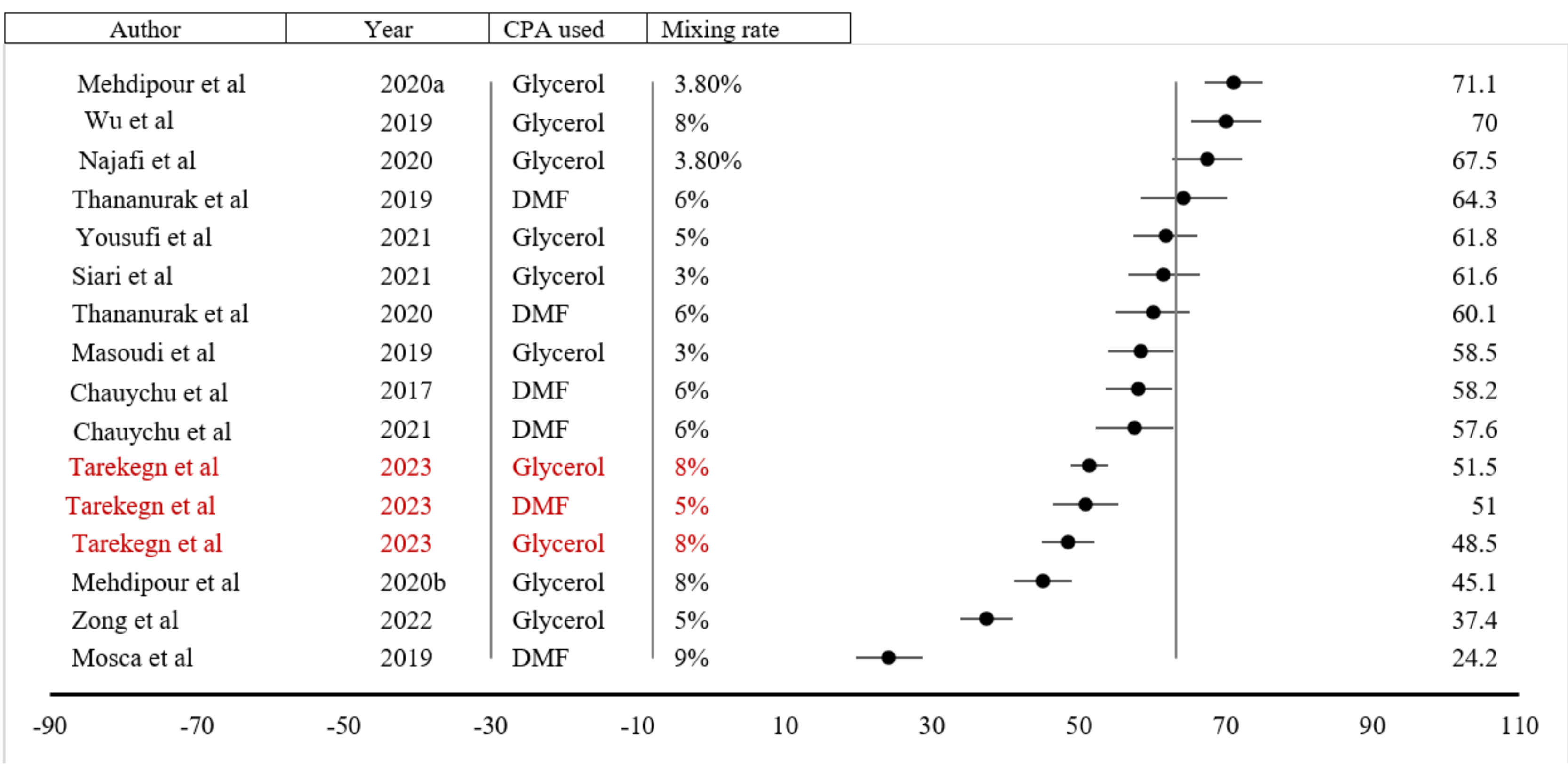


Figure 12. Meta-analysis- Effects of Cryoprotectants and rate of mixing on post-thaw sperm motility of semen from different breeds of Chicken

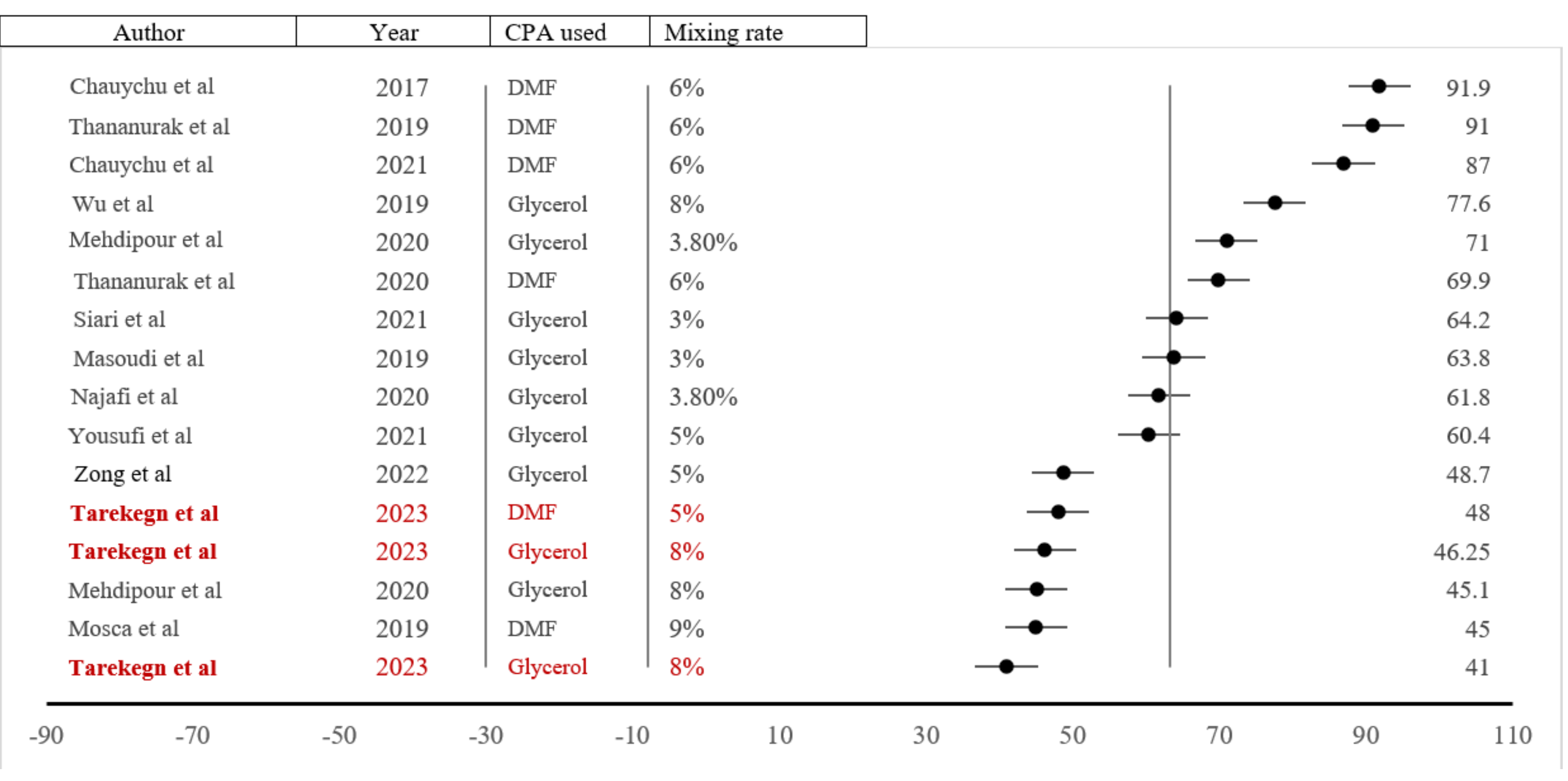


Figure 13. Meta-analysis- Effects of Cryoprotectants and their rate of mixing on fertility test in different breeds of chicken

5. DISCUSSION

5.1.Fresh Semen Characteristics

Semen color depend on the breed of chicken used; however, generally, present findings of milky white semen were in agreement with previous reports by Peters *et al.* (2008); Tarek *et al*. (2019). The color of domestic fowl semen varies from a dense opaque suspension to a watery fluid secreted by various reproductive glands, from a relatively high sperm density or degrees of clear to milky white, with declining sperm numbers (Hafez and Hafez, 2000). According to Yadav *et al*. (2018), the color of semen may depend on the species of chicken used, but generally semen should be creamy which indicates a high sperm concentration which is in agreement with the current study. Color could also serve as an indicator of contamination (Yadav *et al*., 2018).

According to Peters *et al*. (2008), the average ejaculate volume of semen from chicken using the abdominal massage technique was 0.01 ml to 0.35 ml in Giriraja, Frizzled feathered chicken. Bah *et al*. (2001) also reported an ejaculate volume of 0.28 ml in Nigerian local cocks. Cole and Cupps (1977) reported ejaculate volume within the range of 0.1 ml to 1.5 ml per ejaculation. On the other hand, Hafez and Hafez (2000) indicated that the average sperm volume collected from white leghorn varies from 0.2 to 0.5 ml. These studies are in agreement with the result found in this study on Ethiopian Horro Chicken which is 0.36 ml/ejaculate.

The average sperm concentration in the present study was 5.5X109/ml. Results from David *et al*. (2015); Tahseen *et al*. (2019); Chapman *et al*. (2017); Rahman *et al*. (2020) showed that, the concentration of ranging 3.4 to $6.8X10^9$/ml in Lohmann Brown cocks. According to Gordon (2005) reported the average sperm concentration of poultry semen was 5000X106 sperm/ml. On the other hand, the sperm concentration recorded from the present study was within the range of a report by Hafez and Hafez (2000), which is 3000-7000X106 spermatozoa/ml. The average pH of the semen collected was slightly alkaline and ranges from 7.2-7.5. Alkalinity of the poultry semen is due to the accessory sex gland fluid is generally alkaline as reported by Bah *et al*. (2001) and Peters *et al*. (2008).

Results from David *et al*. (2015); Tahseen *et al*. (2019); Gordon (2005) and Hafez and Hafez (2000) are all within the range of the current study.

5.2.Effects of Semen Extenders and Storage Duration on Sperm Quality of Liquid Stored Semen

The results from the present study demonstrated the effect of the homemade tris-egg-yolk-based extender on the Ethiopian indigenous Horro chicken breed's semen sperm motility, morphology, and in vitro viability. Results in this study showed that semen stored in the homemade tris-egg-yolk-based extender has sperm motility that is fit for insemination. The current result of sperm motility agrees with a similar study by Tahseen *et al*. (2019) which utilized a Tris-based extender for short-term storage of Lohmann brown breeders. As reported by Ponglowhapan *et al*. (2004) motility is an important indicator of sugar utilization by spermatozoa as sugars serve as an external energy source essential for maintaining motility. This study demonstrated that semen extended with a commercial extender and stored at 4 hours produced higher sperm motility (87 ± 1.22 %). In this study, the overall average sperm motility was 59.25%, which was in general agreement with 42-80% reported by Hafez and Hafez (2000).

In this study, extending semen with a commercial extender and storing it for 4 hours yielded the least sperm abnormalities (10.4 ± 0.51%). Whereas, the average sperm morphological abnormality semen stored using a Glycerolized tris-egg-yolk-based extender was 18.5%. The number of live sperm with abnormalities in fresh cockerel semen varied from 6 to 9 percent (Tselutin *et al.,* 1999), which was lower than the results of this study. However, Tuncer et al. (2006) reported that the number of abnormal sperm in cockerel semen varied from 9.2 to 11.23%, which is in agreement with sperm abnormalities recorded in semen extended using a commercial extender.

Commercial BPS extender at 4 hours of storage was the best combination (83.6 ± 1.63%) for better in vitro sperm viability as compared to other treatments. Sperm in vitro viability recorded using LPE at 4 hours of storage was slightly lower than that of commercial extenders (82.6 ± 1.36%). The homemade Tris-based extender improved the longevity of sperm in this study as Bearden *et al.* (2004) reported "presence of fructose

will not greatly change the metabolic rate, however, will extend the life span of the sperm". According to the report by Gebriel *et al*. (2009), 81.79% of sperm *in vitro* viability was recorded at 6 hours of storage, which was a similar to results of the present study. In this study, the percentage of dead sperm increased by 36.2% over 24 hours of storage for semen extended with LPE and which was positively correlated with the storage time. In general, the results of sperm quality attributes observed in this study are comparable to several studies (David *et al*., 2015; Tahseen *et al*., 2019 and Siudzinnka and Lukaszewicz, 2008).

The observed differences in the effectiveness of the semen extenders may be attributed to differences in their composition and ability to provide necessary nutrients and support for sperm cells during storage. BPSE and Tris-based homemade extender contain various nutrients and antioxidants that may help to maintain the quality and viability of sperm cells during storage unlike un-extended semen (Blesbois and Grasseau, 2019; Deb *et al*., 2020; Huang and Lin, 2017).

The observed decrease in sperm motility and *in vitro* viability with increasing storage duration may be attributed to various factors, including the breakdown of the extender and the accumulation of toxic metabolic by-products. As the extender breaks down, it may no longer provide the necessary nutrients and support for sperm cells, leading to a decline in their quality and viability. Additionally, as sperm cells metabolize, they produce toxic by-products that can accumulate over time and lead to a decline in their quality and viability (Sarıözkan *et al*., 2018; Saeed *et al*.,2019; Herrera-Corredor *et al*., 2020; Yang *et al*., 2021; Blesbois and Grasseau, 2019; Deb *et al*., 2020; Huang and Lin, 2017).

5.3.Effects of Semen Extenders on Sperm Quality of Cryopreserved Semen

The use of cryopreserved semen in poultry production is markedly less than in mammals due to low resistance of poultry spermatozoa to heat shock, leading to a reduction of fertility of thawed semen (Bakst and Long, 2018; Karimi *et al*., 2019). Poultry sperm are more sensitive to these injuries arising from extreme heat changes due to their high levels of polyunsaturated fatty acids unlike mammalian sperm (Bréque *et al*., 2003). The

viability of post-thaw sperm is not desirable although several protocols have been implemented in cryopreservation to avoid this damage to sperm (Bacon *et al*., 1986; Gliozzi *et al*., 2011). Therefore, the development of strategy is recommended to reduce these structural and biochemical damages.

An evaluation of the extenders on quality of cryopreserved Ethiopian indigenous chicken semen showed that ASD-1 with Dimethyl Formamide as CPA yielded a higher progressive motility percentage (28.2±0.56), in vitro viability percentage (42.75±0.73) and morphologically normal sperm percentage (64.25±0.91) than that of the other treatments ($p < 0.05$; Table 3). These results were similar to Lukaszewicz et al. (2004), who reported EK as an extender yielded the better results in gander semen. This result is supported by the report of Christensen (1995) in which the sperm quality attributes are highly affected by cryoprotectants and osmolarity.

Furthermore, the observed differences in the effectiveness of the semen extenders may be attributed to differences in their CPAs, composition and ability to provide necessary nutrients and support for sperm cells during cryopreservation. Commercial extenders like ASD-1 and BPSE have been specifically designed and formulated to provide optimal support for sperm cells during cryopreservation (Sarıözkan *et al*., 2018; Yang *et al*., 2021). Homemade extenders, on the other hand, may not have the same level of nutrients and support for sperm cells as commercially available extenders. The study also found that there were no significant differences within the commercial extenders in all sperm quality parameters tested. This suggests that both ASD-1 and BPSE are similarly effective in maintaining sperm quality during cryopreservation.

5.4.On-farm Application and Economic Benefits of AI over Natural Mating

The use of artificial insemination (AI) over natural mating in broiler breeders can result in several benefits, including improved genetic progress, reduced number of males required, improved reproductive efficiency, reduced risk of disease transmission, and more efficient use of valuable genetic material. These benefits can lead to increased profitability for commercial breeders, making AI a valuable tool for improving the economic performance of commercial broiler breeding programs (Gürkan *et al.,* 2020).

In a scenario where AI results in a better financial performance compared to natural mating, the profitability of the broiler farm is likely to be significantly improved. AI allows for the use of superior sires, which can lead to significant genetic progress in a relatively short period of time. This can result in broiler offspring with desirable traits, such as improved growth rate, feed efficiency, and disease resistance, which can ultimately lead to higher profits for commercial broiler breeders (Teklewold and Tarekegn, 2019).

According to Hassan *et al*. (2021), AI also reduces the number of males required for breeding, as a single male can produce enough semen to inseminate hundreds or even thousands of hens. This reduces the costs associated with maintaining and feeding a large number of males, leading to cost savings for commercial broiler breeders. Additionally, AI can increase reproductive efficiency by allowing for more efficient use of fertile eggs, enabling the production of more offspring per male, and reducing the time and labor required for mating and breeding. This can result in higher hatchability rates, increased production efficiency, and ultimately higher profits for commercial broiler breeders.

Another advantage of AI over natural mating is the reduced risk of disease transmission. AI avoids direct contact between birds, reducing the risk of sexually transmitted diseases (STDs) in broiler flocks, which can result in significant economic losses for commercial breeders. In addition, AI allows for the more efficient use of valuable genetic material, such as semen from rare and valuable sires, which can be distributed to a larger number of hens, resulting in more offspring with desirable traits (Yadav *et al*., 2018).

A study conducted by Hassan *et al.* (2021) in Pakistan compared the economic viability of AI and natural mating in commercial broiler farms. The study was conducted on a farm with 5,000 broiler breeders, and the results showed that AI yielded higher profits than natural mating. The researchers attributed the higher profits to the improved genetic selection and better disease control achieved through AI.

Similarly, a study conducted by Khatlab *et al*. (2019) in Jordan also compared the performance of AI and natural mating in broiler breeder flocks. The study showed that AI resulted in higher fertility and hatchability rates, and ultimately higher profits than natural

mating. The researchers concluded that AI could be used as a cost-effective and efficient tool to improve the performance of broiler breeder flocks.

Another study by Gürkan *et al.* (2020) in Turkey compared the production and economic performance of AI and natural mating in commercial broiler farms. The study showed that AI resulted in significantly higher hatchability rates, and ultimately higher profits than natural mating. The researchers concluded that AI could be used as a reliable and efficient tool to improve the production and profitability of commercial broiler farms.

5.5.Meta-Analysis of Effects of Cryoprotectants on Sperm Motility and Fertility

Cryopreservation of chicken sperm is a crucial technique for poultry breeding and conservation of genetic resources. However, the process of freezing and thawing can affect the quality of the sperm cells, leading to reduced motility and fertility. Cryoprotectants are commonly used to protect sperm cells during the cryopreservation process. The choice of cryoprotectant and the mixing rate can significantly influence post-thaw sperm motility and fertility.

Several studies have compared the effects of different cryoprotectants on post-thaw chicken sperm motility and fertility. One such study compared the use of DMF and glycerol as cryoprotectants for chicken sperm. The results showed that the use of DMF yielded significantly greater post-thaw sperm motility and fertility compared to glycerol (Abouelezz *et al*., 2019).

Another study investigated the effects of different mixing rates of cryoprotectants on post-thaw chicken sperm motility and fertility. The results showed that mixing rates of less than 5% yielded greater post-thaw sperm motility and fertility compared to higher mixing rates (Huang *et al*., 2019).

The mechanism by which cryoprotectants affect post-thaw chicken sperm motility and fertility is not fully understood. However, it is believed that the cryoprotectants penetrate the sperm cell membrane and protect the cell from damage during freezing and thawing. However, excessive exposure to cryoprotectants canbe toxic to sperm cells, leading to reduced motility and fertility (Abouelezz *et al*., 2019).

In a study comparing the effects of different cryoprotectants on post-thaw rooster sperm, it was found that the use of DMF and ethylene glycol yielded greater post-thaw sperm motility and fertility compared to glycerol (Ghasemi-Esmailabad *et al*., 2018). A study investigating the effects of different cryoprotectant concentrations on post-thaw rooster sperm found that lower concentrations of cryoprotectants yielded greater post-thaw sperm motility and fertility (Sariözkan *et al*., 2016). Another study compared the effects of different mixing rates of cryoprotectants on post-thaw rooster sperm and found that mixing rates of 5% or lower yielded greater post-thaw sperm motility and fertility compared to higher mixing rates (Sariözkan *et al*., 2017).

The choice of cryoprotectant and the mixing rate can significantly affect post-thaw chicken sperm motility and fertility. The use of DMF as a cryoprotectant and mixing rates of less than 5% appear to yield greater post-thaw sperm motility and fertility compared to glycerol and higher mixing rates. However, further research is needed to optimize cryopreservation techniques for different chicken breeds and applications.

6. CONCLUSION AND RECOMMENDATION

6.1.Conclusions

In conclusion, the study highlights the importance of selecting an effective semen extender and carefully considering the storage duration to preserve the quality and viability of sperm cells during storage and transportation. The study also highlighted the economic advantages of Artificial insemination over natural mating in commercial Cobb500 broiler breed. The results suggest that:

- Beltsville and tris-based homemade extender are effective extenders for preserving sperm quality, and that storage durations should be minimized to maintain the quality and viability of sperm cells in liquid stored semen from Horro Chicken.
- Commercially available extenders like ASD-1 and Beltsville are more effective in maintaining sperm quality compared to homemade extender in Cryopreserved semen from Horro Chicken.
- The results suggest that there are no significant differences in sperm quality parameters between ASD-1 and Beltsville in Cryopreservation of semen from Horro Chicken.
- The use of AI in commercial broiler farms can yield higher profits than natural mating due to its advantages in genetic selection, disease control, and production efficiency.
- AI can be considered as a cost-effective and efficient tool for improving the performance and profitability of commercial broiler farms.
- Further research is needed to identify the specific factors that contribute to the observed differences in sperm quality across semen extenders and storage durations.
- Further research is needed to identify the specific factors that contribute to the observed differences in sperm quality across semen extenders and to develop more effective short-term and cryopreservation methods for poultry semen.

6.2.Recommendations

For optimum results in quality of liquid stored and cryopreserved chicken semen, the following points can be recommended:

- The commercial and Homemade extenders are recommended for short-term (liquid) storage of semen from Horro Chicken Breed as these two extenders yielded in significantly higher results as compared to the control treatment.
- Commercial extender ASD-1 and Beltsville PSE are recommended for cryopreservation of semen collected from Horro Chicken breeds.
- Dimethyl Formamide mixed with Lake extender at 3.8% is recommended for optimum results in regard to sperm quality and fertility.
- The semen collection process should be optimized to minimize stress and trauma to the birds. This can be achieved by using gentle handling techniques, avoiding overstimulation, and ensuring that the collection process is carried out in a quiet and calm environment.
- The cryopreservation process should be standardized to ensure that all samples are processed in the same way. This includes standardizing the cooling and thawing rates, as well as the storage temperature and duration.
- Regular monitoring of the semen quality throughout the cryopreservation process can help to identify potential issues and allow for corrective action to be taken. This can include measuring sperm motility, viability, and morphology.
- The packaging and storage methods used for cryopreserved semen can also have an impact on post-thaw quality. It is important to use appropriate packaging materials that are designed for cryopreservation, and to store the samples in liquid nitrogen at a consistent temperature and for a consistent duration.
- The addition of cryoprotectants to the semen extender can help to protect the sperm during cryopreservation, and improve post-thaw semen quality. However, the use of cryoprotectants should be carefully considered, as some may have toxic effects on the sperm.
- Regular evaluation and improvement of the cryopreservation process can help to identify areas for improvement and optimize post-thaw semen quality. This can

include monitoring the success rates of the process, and identifying potential areas for optimization.

- Washing off the CPAs from diluted semen before insemination can improve the post-thaw quality of the semen and fertility.

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8. APPENDICES

Appendix Table 1. Breeder ration formula used during the experiment

Serial number	Feed ingredient	Inclusion rate (%)
1	Corn	52
2	Soy cake	10
3	Meat and bone meal	6
4	Wheat bran	15
5	Noug cake	9
6	Limestone	6
7	Breeder premix	0.5
8	Lysine	0.1
9	Methionine	0.1
10	Molasses	1
11	Salt	0.3

Breeder premix: Industrial, well-balanced premix that ensures fertile, hatching eggs and ultimately strong chicks. It contains vitamins and minerals.

Appendix Table 2. Vaccination schedule used on Horro chicken used in the experiment

Age	Vaccination against	Application
Day-1	Marek	Subcutaneous (neck)
Day-2	Newcastle disease	Eye drop
Day-7	Gumboro	Drinking water
Day-14	Newcastle (Lasota)	Drinking water
Day-18	Gumboro	Drinking water
Week-6	Newcastle (Lasota)	Drinking water

Week-8	Fowl typhoid	Injection
Week-9	Deworming	Drinking water
Week-10	Fowl pox	Wing stab
Week-14	Fowl typhoid	Injection

The vaccines were originated from the National Veterinary Institute, Bishoftu, Ethiopia.

Appendix Table 3. Model developed for analysis of economic benefits

Variable	AI	Natural mating	Basis of assumption
Chicken breed	Broiler Cobb500 breeder flock	Broiler Cobb500 breeder flock	Widely used broiler breed in Ethiopia
Male-to-female ratio	1:30	1:10	Standard ratio??
Egg production period	Week 24 - 50	Week 24 - 50	Cobb500 breeder guide
HDEP ranges	5% to 86%	5% to 86%	Cobb500 breeder guide
Peak production (week)	Week-30	Week-30	Cobb500 breeder guide
Flock size (females)	5000	5000	Assumption for a size of a sample farm
Number of males/flocks	167	500	Calculated
Feed consumption range (week 24 to 50)	117-163 g/day	117-163 g/day	Cobb500 breeder guide
Cost of extender (ETB)	0.35	0	Standard
Number of personnel	25	19	Standard ratio of personnel
Cost of drug, vitamins and vaccine/head	15	15	Current cost/head
Fertility rate (%)	95	87	Literatures
Hatchability rate (%)	85	85	Literatures
Cost of imported breeder DOC/head (ETB)	432	432	Current cost calculated from foreign currency
Price of broiler DOC/head (ETB)	50	50	Current market price

Appendix Table 4. Template for sensitivity analysis

Revenue (ETB)	**AI**	**NM**
Base case		
5% decrease in DOC price		
5% increase in production cost		
5% increase in administrative cost		
EBIT (ETB)		
Base case		
5% decrease in DOC price		
5% increase in production cost		
5% increase in administrative cost		
Net profit (ETB)		
Base case		
5% decrease in DOC price		
5% increase in production cost		
5% increase in administrative cost		

8.1.Standard operating procedures for semen cryopreservation

I. Hygiene of collector

a. Before entering the collection area semen collectors wear overall, gloves and boots
b. Wear protective semen collection clothing (pilot suit and cap) and gumboots. Greenish colored overalls are preferred.
c. Before every collection, wash hands with 0.1 % savlon or use disposable gloves or do both.
d. Use a new pair of disposable gloves for each collection. If not, wash and disinfect hands after each semen collection.

e. Use a sterilized tissue paper to wipe hands and change napkin after each collection.
f. Wash the footwear and overall, immediately after completion of semen collection work.
g. The semen collector should not enter the semen laboratory

II. Precautions to be taken by Semen Collector:

1 Collection arena

a. Collection area should have at least 3 to 4 semen collection crates so that the mounting bull will get sufficient choice of stimulus animal. The arena should provide good footing for the bull and the teaser.
b. Ensure that there are no slippery surfaces in the collection area.

2 Collect semen from only a clean and groomed bull

a. Check the coat and underline of the bull to be collected for any dung or dirt
b. If soiled, clean carefully with soap or mild detergent long before collection.
c. Rinse then with clean water and dry with clean towel.
d. Towel used on one animal should not be reused on another.
e. Ensure that animal is dry at collection.
f. The preputial hair of the bull to be collected should be short (2 cm) and hooves trimmed.

3 Provide adequate sexual preparation for the bull

a. Adequate sexual preparation eliminates the need for intensive collection.
b. Select appropriately sized stimulus animal and present it in an area affording the bull good footing.
c. The bull's nose is tender. Therefore, the lead rope should not be jerked violently.
d. Novel stimulus elicits sexual response in bulls and hence the collector should attempt novelty through the following approaches:
    - Presentation of the same stimulus animal in a new location,
    - Presentation of a new stimulus animal or

- Combination of animals in the original location or presentation of new stimulus animals in a new location.

e. Bull should be allowed at least two false mounts with two minutes restraint before taking collection.
   - During the false mount the bull should be encouraged to mount directly from the rear.
   - The semen collector should ensure that whenever a bull mounts every effort must be made to assure that the mount animal does not move.

f. Use of bull aprons during false mount will avoid the penis touching hindquarter of the stimulus animal.

g. Tie the bull aprons just before starting the false mount. Use separate bull aprons for each ejaculation and each bull.

4 Collection of semen

a. Select a suitable male bird for semen collection. The bird should be healthy, active, and free from any disease or infection.

b. Isolate the bird in a clean, quiet, and comfortable environment to reduce stress.

c. Collection should be attempted only if the rooster is properly sexually prepared.

d. The rooster should be handled gently

e. Stimulate the bird to ejaculate by gently massaging the cloacal region or manually manipulating the phallus.

f. Collect the semen using a collection device, such as a funnel or a graduated cylinder. The device should be clean and sterile to avoid contamination.

g. Evaluate the quality of the semen by examining its appearance, volume, motility, and concentration. Semen that appears abnormal or has low quality should be discarded.

h. Extend the semen using a suitable semen extender, such as Beltsville Poultry Semen Extender, to improve its shelf life and fertility after insemination.

i. Store the extended semen in a cool and dark place until it is ready to use.

III. Semen processing for Cryopreservation

1. **Semen Preparation:**

   The collected semen should be diluted with a suitable semen extender, such as Beltsville Poultry Semen Extender or dimethyl sulfoxide (DMSO). The extender helps to protect the sperm cells from damage during the freezing process and improves their survival after thawing. The semen should be slowly mixed with the extender to avoid damaging the sperm cells.

2. **Semen Cooling:**

   The diluted semen should be cooled slowly to room temperature using a cooling rack or a water bath. The cooling rate should be around 0.5-1°C per minute to avoid thermal shock and damage to the sperm cells.

   a. Dilute the semen with a suitable semen extender: The collected semen is diluted with a suitable semen extender, such as Beltsville Poultry Semen Extender or dimethyl sulfoxide (DMSO). The extender should be pre-cooled to the desired temperature to avoid thermal shock to the semen.

   b. Mix the semen and extender slowly: The semen and extender should be mixed slowly and gently to avoid damaging the sperm cells. The mixing process can be done by slowly tipping the container back and forth or using a mechanical mixer.

   c. Allow the semen to equilibrate: The diluted semen should be allowed to equilibrate at room temperature for 5-10 minutes. This allows the sperm cells to adjust to the new environment and reduces the risk of osmotic shock during cooling.

   d. Cool the semen slowly: The cooled semen can be cooled using a cooling rack or a water bath. The cooling rate should be around 0.5-1°C per minute to avoid thermal shock and damage to the sperm cells. The temperature of the semen should be monitored and recorded regularly during the cooling process.

   e. Check the semen quality: The semen should be checked for quality during the cooling process. Quality parameters such as motility, viability, and

morphology can be evaluated under a microscope. If the semen quality is poor, adjustments can be made to the cooling rate or the extender composition.

f. Store the cooled semen: Once the semen has reached the desired temperature, it can be stored in a sterile container and kept in a cool and dark place until it is ready for freezing.

3. **Semen Freezing:**

The cooled semen can be frozen using a programmable freezer or a liquid nitrogen container. The freezing process should be done slowly to avoid ice crystal formation and damage to the sperm cells. The temperature of the semen should be reduced to -5°C at a rate of 0.5-1°C per minute, and then to -40°C at a rate of 10°C per minute. The semen can then be stored in liquid nitrogen for long-term storage.

a. Poultry semen freezing is a critical step in the process of cryopreservation. Freezing the semen slowly and carefully helps to minimize damage to the sperm cells and maintain their quality. Here is a detailed procedure for poultry semen freezing:

b. Dilute the semen with a suitable semen extender: The collected semen is diluted with a suitable semen extender, such as Beltsville Poultry Semen Extender or dimethyl sulfoxide (DMSO). The extender should contain cryoprotectants, such as glycerol or DMSO, which help protect the sperm cells from damage during the freezing process.

c. Equilibrate the semen: The diluted semen should be equilibrated at room temperature for 10-15 minutes. This allows the sperm cells to adjust to the new environment and reduces the risk of osmotic shock during freezing.

d. Load the semen into straws: The semen is loaded into small plastic straws using a pipette or syringe. The straws are then sealed at both ends using a heat sealer or a straw sealer.

e. Pre-cool the straws: The loaded straws are pre-cooled at 4°C for 5-10 minutes to allow the semen to equilibrate with the cryoprotectant.

f. Place the straws in a programmable freezer: The pre-cooled straws are then placed in a programmable freezer, which can be programmed to

freeze the semen slowly at a controlled rate. The freezing rate should be around 0.5-1°C per minute to avoid ice crystal formation and damage to the sperm cells.

g. Freeze the semen: The temperature of the semen should be reduced to -5°C at a rate of 0.5-1°C per minute, and then to -40°C at a rate of 10°C per minute. The straws can then be transferred to liquid nitrogen for long-term storage.
h. Monitor the semen quality: The semen quality should be monitored during the freezing process. Quality parameters such as motility, viability, and morphology can be evaluated under a microscope. If the semen quality is poor, adjustments can be made to the freezing rate or the extender composition.

It is important to note that poultry semen freezing should be performed by trained personnel using proper techniques and equipment to ensure the safety and welfare of the birds and the quality of the semen. Additionally, the use of appropriate hygiene and biosecurity measures is critical to prevent the spread of disease or infection.

4. Semen Thawing:

The frozen semen can be thawed by gently warming it in a water bath at 50-60°C for 10-15 seconds. The thawed semen should be evaluated for quality, including motility, viability, and acrosome integrity.

a. Remove the semen straws from liquid nitrogen storage: The frozen semen straws are removed from the liquid nitrogen storage tank and placed in a water bath at 50-60°C. The straws should be handled carefully to avoid any damage or breakage.
b. Thaw the semen slowly: The semen should be thawed slowly to avoid thermal shock and damage to the sperm cells. The straws can be left in the water bath for 10-15 seconds until the semen has thawed completely. Alternatively, the straws can be held in the water bath for 2-3 minutes until the semen has thawed completely.

c. Inspect the semen: The thawed semen should be checked for quality, including motility, viability, and morphology. The semen can be evaluated under a microscope to determine the quality of the sperm cells. If the semen quality is poor, adjustments can be made to the insemination dose or the extender composition.
d. Dilute the semen: The thawed semen can be diluted with a suitable semen extender, such as Beltsville Poultry Semen Extender, to improve its shelf life and fertility after insemination. The semen and extender should be mixed gently and slowly to avoid damaging the sperm cells.
e. Inseminate the female birds: The diluted semen can be used for artificial insemination in female birds. The insemination procedure should be performed by trained personnel using proper techniques and equipment to ensure the safety and welfare of the birds and the success of the insemination.

It is important to note that the thawing procedure should be performed by trained personnel using proper techniques and equipment to ensure the safety and welfare of the birds and the quality of the semen. Additionally, the use of appropriate hygiene and biosecurity measures is critical to prevent the spread of disease or infection.

5. **Insemination:**

The thawed semen can be used for artificial insemination in female birds. The insemination procedure should be performed by trained personnel using proper techniques and equipment to ensure the safety and welfare of the birds and the success of the insemination.

a. Select the female birds: The female birds should be healthy and in good condition. They should be selected based on their age, breed, and productivity.
b. Prepare the insemination equipment: The insemination equipment should be cleaned and sterilized before use. This includes the insemination pipettes, syringes, and catheters.

c. Collect the semen: The semen can be collected from the male birds using a suitable collection device, such as a graduated cylinder, a funnel, or an artificial vagina. The semen should be evaluated for quality, including volume, motility, concentration, and morphology.
d. Dilute the semen with a suitable semen extender: The collected semen is diluted with a suitable semen extender, such as Beltsville Poultry Semen Extender or dimethyl sulfoxide (DMSO). The extender helps to protect the sperm cells from damage during transportation and storage.

e. Thaw the semen: If the semen has been frozen, it should be thawed slowly and carefully to avoid damage to the sperm cells. The thawed semen should be evaluated for quality before insemination.
f. Inseminate the female birds: The insemination procedure should be performed by trained personnel using proper techniques and equipment. The female bird is restrained gently, and the insemination pipette is inserted into the cloaca. The semen is then slowly deposited into the oviduct using a syringe or catheter.
g. Evaluate the success of the insemination: The success of the insemination can be evaluated by checking the presence of a semen plug, which is a gel-like substance that forms in the cloaca after successful insemination. Alternatively, the fertility rate can be determined by candling of incubated eggs.

It is important to note that the insemination procedure should be performed by trained personnel using proper techniques and equipment to ensure the safety and welfare of the birds and the success of the insemination. Additionally, the use of appropriate hygiene and biosecurity measures is critical to prevent the spread of disease or infection.

8.2.Stata Output of ANOVA for sperm quality evaluation of short-term stored semen from Horro Chicken

```
anova Abnormal Treat## Invitro_storedhrs

                         Number of obs =        60    R-squared     =  0.6800
                         Root MSE      =   4.01559    Adj R-squared =  0.6066

                  Source | Partial SS    df       MS           F     Prob > F
              -----------+----------------------------------------------------
                   Model |     1644.6    11   149.509091      9.27     0.0000
                         |
                   Treat |      203.7     2       101.85      6.32     0.0037
               Invitro_s~s | 1373.93333   3   457.977778     28.40     0.0000
       Treat#Invitro_s~s | 66.9666667     6   11.1611111      0.69     0.6570
                         |
                Residual |        774    48       16.125
              -----------+----------------------------------------------------
                   Total |     2418.6    59   40.9932203
```

```
anova  Viability Treat## Invitro_storedhrs

                         Number of obs =        60    R-squared     =  0.9784
                         Root MSE      =   3.25448    Adj R-squared =  0.9734

                  Source | Partial SS    df       MS           F     Prob > F
              -----------+----------------------------------------------------
                   Model | 22979.7833    11   2089.07121    197.24     0.0000
                         |
                   Treat | 6190.23333     2   3095.11667    292.22     0.0000
             Invitro_s~s | 15448.1833     3   5149.39444    486.17     0.0000
       Treat#Invitro_s~s | 1341.36667     6   223.561111     21.11     0.0000
                         |
                Residual |      508.4    48   10.5916667
              -----------+----------------------------------------------------
                   Total | 23488.1833    59   398.104802
```

```
anova M_Motility  Treat## Invitro_storedhrs

                     Number of obs =       60     R-squared     =  0.9708
                     Root MSE      = 3.79144     Adj R-squared =  0.9640

            Source | Partial SS    df       MS           F     Prob > F
        -----------+----------------------------------------------------
             Model |   22901.25    11  2081.93182     144.83     0.0000
                   |
             Treat |       2770     2        1385      96.35     0.0000
        Invitro_s~s|  19414.5833     3  6471.52778     450.19     0.0000
  Treat#Invitro_s~s|  716.666667     6  119.444444       8.31     0.0000
                   |
          Residual |        690    48      14.375
        -----------+----------------------------------------------------
             Total |   23591.25    59  399.851695
```

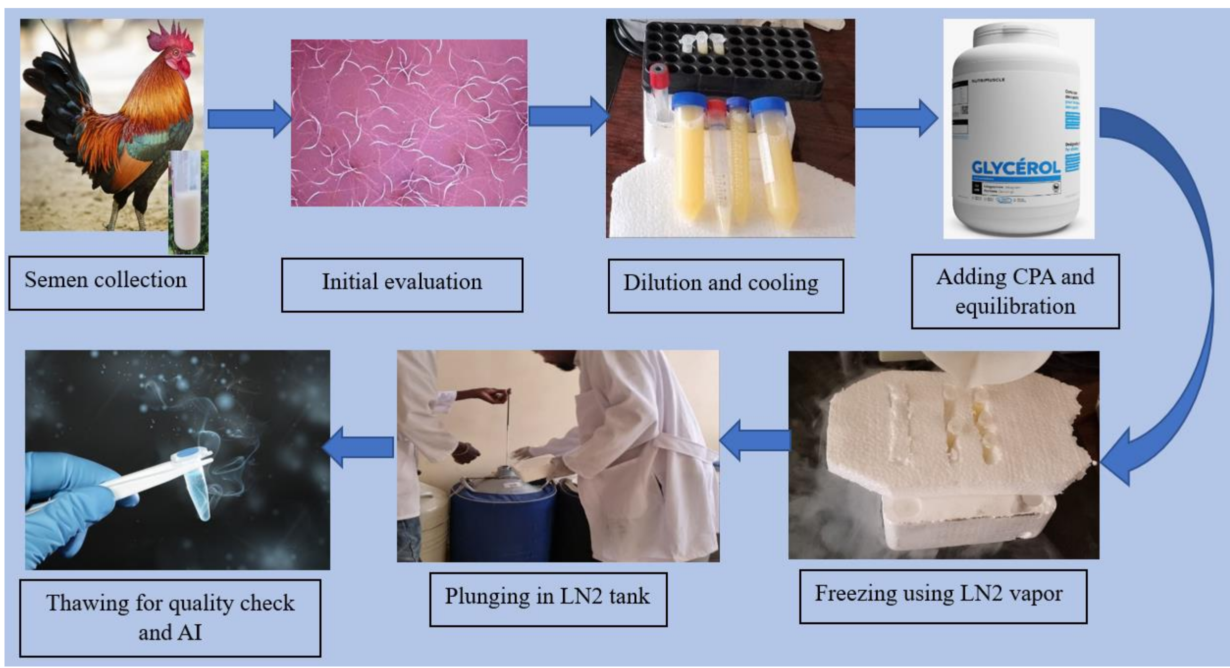
8.3.Stata Output of ANOVA for sperm quality evaluation of post-thaw semen from Horro Chicken

```
. anova M_Motility Treat

                     Number of obs =       60     R-squared     =  0.0414
                     Root MSE      = 6.47532     Adj R-squared =  0.0078

            Source | Partial SS    df       MS           F     Prob > F
        -----------+----------------------------------------------------
             Model | 103.333333     2  51.6666667       1.23     0.2993
                   |
             Treat | 103.333333     2  51.6666667       1.23     0.2993
                   |
          Residual |       2390    57  41.9298246
        -----------+----------------------------------------------------
             Total | 2493.33333    59   42.259887

. anova  P_Motility Treat

                     Number of obs =       60     R-squared     =  0.3854
                     Root MSE      = 2.93646     Adj R-squared =  0.3639

            Source | Partial SS    df       MS           F     Prob > F
        -----------+----------------------------------------------------
             Model | 308.233333     2  154.116667      17.87     0.0000
                   |
             Treat | 308.233333     2  154.116667      17.87     0.0000
                   |
          Residual |      491.5    57  8.62280702
        -----------+----------------------------------------------------
             Total | 799.733333    59  13.5548023
```

```
. anova   Morphnormal Treat

                           Number of obs =        60     R-squared     =  0.4767
                           Root MSE      =   4.11768     Adj R-squared =  0.4584

                  Source | Partial SS    df       MS           F     Prob > F
              -----------+----------------------------------------------------
                   Model |  880.533333    2  440.266667      25.97     0.0000
                         |
                   Treat |  880.533333    2  440.266667      25.97     0.0000
                         |
                Residual |      966.45   57  16.9552632
              -----------+----------------------------------------------------
                   Total |  1846.98333   59  31.3048023

. anova  Viability Treat

                           Number of obs =        60     R-squared     =  0.6226
                           Root MSE      =   3.40871     Adj R-squared =  0.6093

                  Source | Partial SS    df       MS           F     Prob > F
              -----------+----------------------------------------------------
                   Model |  1092.43333    2  546.216667      47.01     0.0000
                         |
                   Treat |  1092.43333    2  546.216667      47.01     0.0000
                         |
                Residual |       662.3   57  11.6192982
              -----------+----------------------------------------------------
                   Total |  1754.73333   59  29.7412429
```

8.4.Stata Output of ANOVA for fertility and hatchability of post-thaw semen from Horro Chicken

```
anova Fertility Treat

                    Number of obs =       12     R-squared     =  0.4351
                    Root MSE      = 3.68838     Adj R-squared =  0.3095

          Source | Partial SS    df       MS           F     Prob > F
      -----------+----------------------------------------------------
           Model | 94.2916667     2  47.1458333      3.47     0.0766
                 |
           Treat | 94.2916667     2  47.1458333      3.47     0.0766
                 |
        Residual |   122.4375     9  13.6041667
      -----------+----------------------------------------------------
           Total | 216.729167    11  19.7026515

anova  Hatchability Treat

                    Number of obs =       12     R-squared     =  0.1043
                    Root MSE      = 4.31406     Adj R-squared = -0.0948

          Source | Partial SS    df       MS           F     Prob > F
      -----------+----------------------------------------------------
           Model |       19.5     2        9.75      0.52     0.6092
                 |
           Treat |       19.5     2        9.75      0.52     0.6092
                 |
        Residual |      167.5     9  18.6111111
      -----------+----------------------------------------------------
           Total |        187    11          17
```



Appendix Figure 1. Semen processing procedure for Cryopreservation

Appendix Figure 2. Tris-based homemade Poultry Semen Extender Preparation

Appendix Figure 3. Poultry Semen Collection

Appendix Figure 4. Semen Processing after Initial Evaluation

Appendix Figure 5. Cryopreservation of Semen

Appendix Figure 6. Microscopic Sperm Quality Evaluation

8.5.Published Articles

1. Effects of Using Commercial and Homemade Extenders on Sperm Quality of Liquid Stored Semen of Horro Chicken Breed. Journal of World's Poultry Research 13 (2): 216-222

   Tarekegn Getachew[1*], Gebeyehu Goshu[2] and Alemayehu Lemma[3]

2. Effects of Commercial and Homemade Extenders on Post-thaw Sperm Quality and Fertility of Semen from Ethiopian Indigenous Horro Chicken Breed. Journal of World's Poultry Research 13 (2): 341-

   Tarekegn Getachew[1*], Gebeyehu Goshu[2] and Alemayehu Lemma[3]