ABSTRACT

Title of Document: THE EFFECT OF SIALIC ACID ON THE SUCCESS OF POULTRY SPERMATOZOA CRYOPRESERVATION.

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Avian semen cryopreservation is an invaluable reproductive strategy for conserving desirable genetic traits for commercial poultry production and endangered species conservation. Here we provide the effects of including sialic acid (SA) in the cryodiluent for tom and rooster sperm cryopreservation. Tom and rooster (n=6 males/treatment) semen was incubated at 25°C or 4°C with (0, 20, 40, 80, 120, 160, 200, or 240 µg/mL, depending on the species) SA for up to 2 h. At 30 min intervals, sperm viability and SA uptake were evaluated. Turkey and chicken (n=6 females/treatment) hens were inseminated with frozen/thawed semen treated with SA either pre-freeze only or pre-freeze/post-thaw (0, 20, 40, 80, 120, 160, or 240 µg/mL, depending on the species) and percent total fertility was evaluated. Our results demonstrate that the optimal SA incubation conditions for improved fertility with frozen/thawed sperm occurs with 160 µg/mL SA pre-freeze for turkey and 120 µg/mL SA pre-freeze for chicken ($P<0.05$). However, differences were observed between the two species with respect to the optimal temperature for SA incubation.

THE EFFECT OF SIALIC ACID ON THE SUCCESS OF POULTRY SPERMATOZOA CRYOPRESERVATION.

By

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Dedication

This work is dedicated to my children:

Jade Renee

Tess Nicole

Megan Leigh

Robert Patrick

Jeannette Elizabeth

Greeley Anthony III

and

Lia Renee

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Chapter 1: Literature Review

Use of Artificial Insemination for Commercial Poultry Production

The commercial poultry industry began in the 1920's with the development of the broiler chicken line for meat. In its early stages, the industry relied on individually managed farms, feed mills, hatcheries, and processing plants to provide the final product. Shortly after WWII, increased demand for broilers led to the development of vertical integration and subsequently a complex multi-layered poultry breeding system owned and managed by a few large companies (Lasley, 1983). Worldwide, commercial poultry breeding today consists not only of chickens and turkeys, but ducks, geese, quail and ostriches. Poultry provide not only meat and eggs, but feathers, leather and oil as well. Economically speaking, however, the chicken remains the most important. Poultry and other agricultural animal industries rely on the use of a highly complex and classified selective breeding pyramid (Figure 1). At the top level, the pure or elite lines consist of birds with the most favorable traits to achieve the desired products. The general beneficial traits across all livestock selective breeding programs is vigor, general welfare, feed conversion, fertility, ease of birthing/hatchability, and disease resistance. The remaining traits that livestock are selected for depend on the final product desired. In laying hens, longevity, rate of lay, lifetime of egg production, and egg quality are considered the most valuable traits to maintain in the elite population of birds (Table 1). For turkeys, emphasis is placed on body weight and age at slaughter. At the elite level, only pure line birds are bred with each other. There is a back-up stock of pure line birds kept away from a central breeding program to maintain biosecurity. While these pure line birds are considered the source for the commercial lines, other

birds that have the potential to be used for future breeding, or experimental lines are maintained at this level as well. The great grandparent level is used to increase the number of pure line offspring without any additional crossbreeding. Cross breeding of lines begins at the grandparent level (Figure 1). Since chickens and turkeys have the shortest generation intervals among agricultural animals, traits are rapidly distributed to the final product. In this way genetic progress can be distributed very rapidly, however, diseases and other problems can be rapidly disseminated as well.

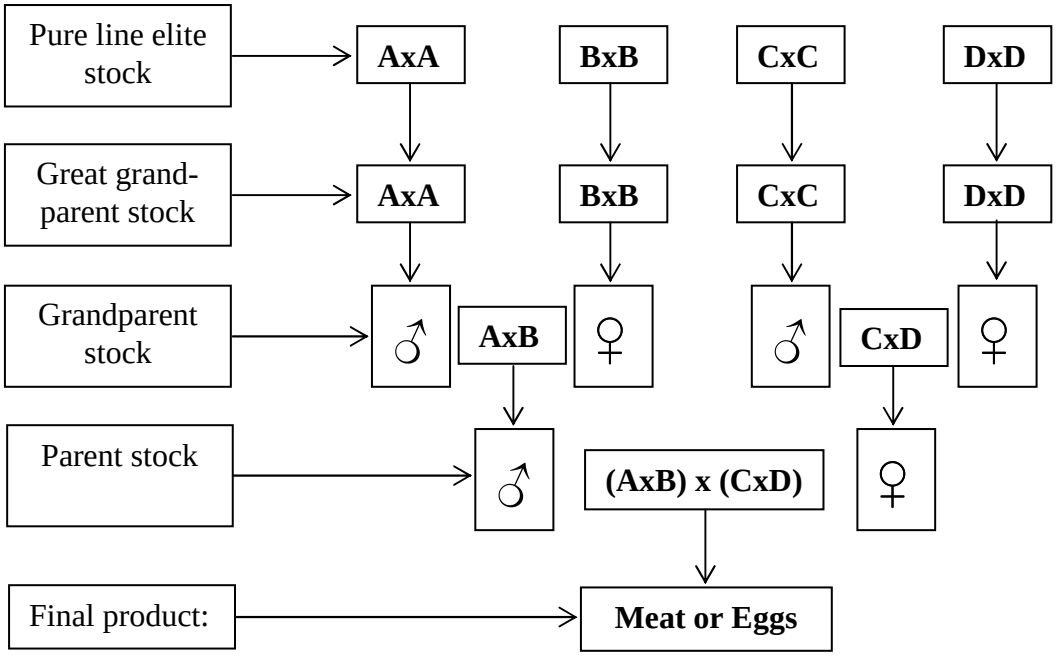


Figure 1. The commercial poultry breeding scheme based on the breeding pyramid for poultry.

Table 1. The most important traits in the breeding goals for commercial poultry species.

Trait Group	Layers	Broilers (Turkeys, Ducks, Geese)
Egg / Meat production	Age 1st egg, hen-day egg production, persistency of production, broodiness, egg size/weight	Growth rate and body weight at slaughter
Production efficiency	Mature body weight, feed conversion	Feed conversion and abdominal fat
Reproductive performance	Female and male fertility, hatchability	Age 1st egg, hen-day hatching egg production, egg weight, female and male fertility, hatchability.
Product quality	Egg deformation, shell strength, thickness, and porosity, fishy odour, albumen and yolk weight	Drip loss, pH, meat colour
Functional traits	Heat tolerance, disease resistance, leg strength, survival, cannibalism, flightiness	Egg deformation, survival/mortality, leg health, infectious diseases
Others	Plumage and egg (shell) colour	Plumage and skin colour

Adapted from Mark et al., 2011.

The improvements in commercial turkeys from genetic selection to produce larger birds with greater breast width and feed conversion performance over the last two decades has been phenomenal (Havenstein et al., 2007). A major disadvantage of this improvement, however, is a loss of the bird's ability to successfully mate and produce fertile eggs without intervention. Today the commercial turkey industry relies exclusively on artificial insemination (AI) for fertile egg production (Morrell, 2011).

Semen collection in poultry is generally a two-person job. In roosters, one person gently restrains the bird exposing the cloaca while massaging the abdomen

below the pelvic bones. The restrainer then holds the bird's tail back while the semen collector holds the bird's legs. When stimulation is successfully achieved, the copulatory organ will protrude and the semen collector can express semen by gently squeezing the bulbous glands. This same method for stimulating ejaculation and expressing the semen applies to toms except that a restraining bench is necessary to assist in restraining a large tom (Burrows and Quinn, 1937).

The technique for AI in poultry was developed in the 1930s and is still used today. It involves applying pressure to the hen's abdomen and everting the vaginal orifice through the cloaca (Quinn and Burrows, 1936). Semen is deposited with straws, syringes or plexi-tubes 2 to 4 cm into the vaginal orifice concurrently with the release of pressure on the hen's abdomen. Commercial operations may employ automated semen dispersal systems equipped with individual pre-set AI dose straws. Several factors that affect semen quality include, age of males, photo-period, season, body weight, and diet (Sexton, 1986, 1987). Timing of AI is another important factor and is best performed in the late afternoon to minimize the number of hens with hard-shelled eggs in the shell gland. In turkeys, Brillard and Bakst (1990) demonstrated that spermatozoa numbers in the sperm storage tubules (SST) of hens inseminated before the onset of lay was twice that of hens inseminated at the beginning of egg production. Therefore, turkey hens are generally inseminated 14 to 17 days after photo-stimulation and chickens are inseminated when they are laying (Bakst and Brillard, 1995).

Semen Cryopreservation as a Tool for Preserving Genetic Diversity

The value of poultry spermatozoa cryopreservation is twofold – first, as a tool for genetic resources conservation to preserve variability in endangered avian and other animal species, and second, to maximize poultry production efficiency to meet the increasing demands for sustainable human food resources.

Spermatozoa cryopreservation is an invaluable strategy for preserving genetic variability in endangered avian species. Globally, over sixty percent of chicken and turkey breeds are listed as either extinct, or in critical or endangered status (FAO, 2007). This dilemma can be attributed to the industrial merging during the vertical integration management transformation when many established chicken lines were displaced to meet industry standards (Silversides et al., 2012). Likewise, 134 of the known species of wild birds are currently extinct and over 1300 species are listed as endangered or critical status (IUCN, 2013). Early efforts to restore endangered avian species, such as turkeys included reintroducing them back into the wild. Although, turkeys were successfully reintroduced to self-stabilizing capacity, this approach disrupted the historical patterns of genetic diversity and gene flow which led to increased homogenization of subspecies and the loss of locally adapted gene complexes (Mosbey et al., 1975; Bailey, 1980; Leberg et al., 1994; Mock et al., 2004; Latch et al., 2005). Furthermore, maintaining live animals as a method of conservation is more labor- and resource-intensive than gamete cryopreservation methods. Several studies have shown that the most feasible method for ex situ management of genetic resources in birds is semen cryopreservation (Gee, 1995; Hammerstedt, 1995; Reedy et al., 1995). In

a more recent cost comparison study for preservation of avian genetic resources, cryopreserved semen and ovaries were found to be approximately ninety percent less expensive than the total costs for maintaining live birds (Silversides et al., 2012).

In 2012, the United States value of all egg production was over $7.0 billion, a seven percent increase from 2011. The value of turkeys produced in 2012 was over $5.0 billion, up ten percent from 2011 (USDA, 2013). With a projected human population of 9 billion by 2050, it is evident that the demand for poultry will continue to rise. One way to maximize production efficiency to meet these demands is through poultry spermatozoa cryopreservation. Since commercial turkeys are unable to mate naturally and have to be artificially inseminated, stockpiling desirable germplasm from elite poultry lines using cryopreservation would prove to be a very cost effective management tool by greatly reducing the risk of spreading epidemic disease and reducing the labor force needed to AI the hens. Unfortunately, even in chickens, semen-cryopreservation is not yet widely used because its success is highly dependent on the fertility of the breed and within-breed variability.

Of all the commercial agricultural animal industries, dairy has achieved the greatest success in the use of frozen/thawed spermatozoa for animal reproduction. Shortly after Polge et al. (1949) discovered the protective properties glycerol had when combined with yolk citrate extender (Salisbury et al., 1941) on frozen poultry spermatozoa, Bratton et al. (1955) showed success with high fertility post-thaw in bull spermatozoa stored at -79°C and packed on

solid carbon dioxide. The lipids found in egg yolk were found to protect bull spermatozoa from cold shock during freezing (Watson and Martin, 1973, 1975; Foote, 1998). Several early studies tested the use of different proteins with glycerol on the success of post-thaw fertility before finding Tris-buffered egg yolk-glycerol provided the best protection and is now the worldwide standard cocktail used for extending dairy bull spermatozoa (Davis et al., 1963; Foote, 1970; Watson and Martin, 1973, 1975). Early investigations also discovered that storing the cells in liquid nitrogen at -196°C prolonged spermatozoa viability significantly better than the -79°C solid carbon dioxide method (Sherman, 1954, 1963).

Another area that has achieved success using sperm cryopreservation is in human assisted reproduction. Following Polge et al. (1949) work with glycerol, Bunge and Sherman (1953) froze human sperm and reported a 67 percent survival rate and three human pregnancies following artificial insemination with frozen-thawed sperm. This led many investigators to pursue work toward using human sperm cryopreservation as a method for the treatment of infertility. All human pregnancies before 1964, however, were accomplished using short-term sperm storage until Perloff et al. (1964) reported pregnancies from frozen-thawed sperm stored for one to five months. Cryopreservation of human spermatozoa has come a long way. Work investigating the addition of polysaccharide hyaluron to the cryoprotectant to improve sperm motility post-thaw was successful (Sbracia et al., 1997). More recently, Horne et al. (2004) reported a live birth following IVF/ICSI using sperm that had been cryopreserved for 21 years.

General Principles of Semen Cryopreservation

Reproductive cell and tissue cryopreservation technology has been developed for a wide range of species over the last sixty years since Polge (1951) discovered that glycerol would maintain the motility of frozen rooster spermatozoa. Despite the fact that rooster semen was used in this scientific advancement, the overall fertility rates using frozen/thawed poultry spermatozoa are too variable to use routinely in commercial poultry production or avian genetic resources conservation (Long, 2006). Commercial poultry industries will only employ standardized cryopreservation methods when the fertility rates of cryopreserved spermatozoa are equal to the success rates of non-treated spermatozoa (>96%). However, success of cryopreserved spermatozoa can be achieved with 60-70% fertility rates as long as the hatchability of fertile eggs remains high (Long, 2006). The greatest success for agricultural standardized use of semen cryopreservation has been achieved in mammalian species, such as dairy and beef cattle (Long, 2006). Fertility rates of cryopreserved poultry spermatozoa retain <2% of the fertilizing capacity of raw semen which is lower than all the domestic mammalian species (Wishart, 1985).

Cryopreservation stores cells and tissues in a state of temperature-induced suspended animation. At temperatures between -140 and -200□C, cryopreserved cells and tissues can be stored for several decades (Leibo and Songsasen, 2002; Fuller and Paynter, 2007), if not, indefinitely. There is a wealth of published reviews summarizing the empirical research involving the types of cryoprotectants, packaging methods, and freezing and thawing rates for avian

spermatozoa (Lake, 1986; Hammerstedt and Graham, 1992; Donoghue and Wishart, 2000; Blesbois et al., 2008).

In general, semen is cooled to around 5□C before dilution or equilibration with a cryoprotectant agent. Samples are then held at this temperature for about ten minutes before cooling further. The two most commonly used methods of avian spermatozoa cryopreservation today are (1) the slow freeze method (7□C/min) using glycerol as the cryoprotectant (Seigneurin and Blesbois, 1995) and French straw packaging or (2) the rapid freeze method (50□C/min) using dimethylacetamide (DMA) and French straw packaging (Blesbois and Grasseau, 2002).

The slow freezing method involves loading 0.5 mL French straws with semen diluted to 2 x 10^9 cells/mL and a final glycerol concentration of 11%. The straws are equilibrated to 5□C for thirty minutes (Tselutin et al., 1999) and then undergo a step-wise programmed temperature reduction from 5 to -35□C at 7□C/min and from -35 to -140□C at 20□C/min and plunged into liquid nitrogen. Donoghue and Wishart (2000) determined that freezing rates of -5□C, -7□C and -10□C per minute appeared to maintain fertility better than -1□C per minute. Straws are thawed by rapid agitation in a 5□C alcohol bath since this temperature is optimal for dilution or dialysis to remove glycerol (Wishart, 1995) and then into a 75□C bath (Lake et al., 1981; Kurbatov et al., 1988; Seignuerin and Blesbois, 1995).

For the rapid freezing method, which was adapted from Tselutin et al. (1999), semen is diluted and packaged in the same manner as the slow freeze method, except that 6% DMA is used as the cryoprotectant. The straws are rapidly frozen at a rate of 50°C/min up to -140°C, then are plunged and stored in liquid nitrogen (Tselutin et al., 1999). The straws are thawed in a 50°C water bath for five seconds. Unlike glycerol, DMA does not need to be removed following thaw. Customized thawing methods for cryopreserved chicken and turkey semen have been described (Buss, 1993; Tselutin et al., 1995). Of these two particular cryoprotectants, glycerol has the least deleterious effects on the viability and membrane integrity of fowl spermatozoa (Tselutin et al., 1999).

Cryoprotectants

One of the most critical steps for successful semen cryopreservation is the choice of the cryoprotectant. The use of a cryoprotectant is necessary to protect spermatozoa viability from the cryoinjury sustained during the freezing and thawing process. Cryoprotectants help prevent enzyme denaturation, protein destabilization, and ice crystal formation during the freeze/thaw process (Chao, 1991). There is a wide range of chicken and turkey spermatozoa cryoprotectants available, some of the most commonly used include glycerol and DMA. In addition to the aforementioned glycerol and DMA, several other cryoprotectants have been used such as dimethylsulphoxide, ethyleneglycol, dimethylformamide, and propyleneglycol (Sexton, 1977; Lake et al., 1981; Lake and Ravie, 1982, Hammerstedt and Graham, 1992; Surai and Wishart, 1996). Because glycerol and DMA have been used most extensively, the other cryoprotectants will not be

discussed further (Donoghue and Wishart, 2000; Blesbois, 2007; Woelders et al., 2006). In comparing the efficacy of different compounds for their cryoprotective ability or toxicity towards chicken and turkey spermatozoa, the combination of cryoprotectant type, equilibration time and temperature, and freeze/thaw method should be chosen carefully.

Glycerol is the least toxic and most effective cryoprotectant, however, it has a contraceptive effect on intravaginally inseminated chicken and turkey spermatozoa (Hammerstedt and Graham, 1992). This contraceptive effect of glycerol has been discussed at length in earlier reviews (Hammerstedt and Graham, 1992; Bellagamba et al., 1993) and remains somewhat ambiguous. Evidence that glycerolated spermatozoa retain motility in vitro at room temperature and that their fertilizing ability when intravaginally inseminated is poor compared to intrauterine or intramagnal routes, suggests an interaction between the glycerolated spermatozoa and the vaginal mucosa (Bellagamba et al., 1993). Since transport and storage of spermatozoa is much more efficient from intrauterine or intramagnal sites (Bakst et al., 1994), and because glycerol damages spermatozoa at physiological temperatures (Lake, 1968), then the 'contraceptive' effect may be the result of an interaction of glycerol and spermatozoa at temperatures near 40□C. Additionally, this deleterious interaction may be osmotic damage and/or may involve modifications of the surface-associated spermatozoa glycocalyx proteins (Hammerstedt and Graham, 1992; Steele, 1992). To successfully reduce the glycerol contraceptive effect and improve post-thaw fertility of spermatozoa, removal of glycerol from the frozen-

thawed spermatozoa at 5□C is performed through dialysis or by centrifugation and resuspension. However, dialysis is time-consuming, and centrifugation causes further injury to the spermatozoa (Steele, 1992).

DMA is known to be toxic to spermatozoa (Blesbois and Brillard, 2007; Chalah et al., 1999). However, it does not induce osmotic shock as seen with glycerol (Mocé et. al., 2010). In another study comparing the effects of glycerol and DMA on the success of American kestrel spermatozoa cryopreservation, fresh semen and semen containing DMA yielded the highest fertility rates (56.5% and 52.2%, respectively). An intermediate fertility rate of 30.4% was achieved with semen frozen with DMA, whereas freshly diluted or frozen semen containing glycerol produced the poorest fertility (13.6% and 11.8%, respectively). Unlike glycerol, DMA does not require removal from frozen-thawed spermatozoa before AI, therefore, it may be useful as an alternative spermatozoa cryoprotectant for a variety of birds (Brock and Bird, 1991).

There have been major improvements in spermatozoa cryosurvival in a variety of mammalian species by combining permeating cryoprotectants, such as DMA, with natural osmoprotectants that do not penetrate the plasma membrane, such as sucrose or trehalose (Woelders et al., 1997; Dalimata and Graham, 1997; Aboagla and Terada, 2003; Yamashiro et al., 2007; Gutiérrez-Pérez et al., 2009). Permeating cryoprotectants increase membrane fluidity preventing dehydration and reduced intracellular ice formation at lower temperatures (Holt, 2000). Non-permeating cryoprotectants create an osmotic pressure that allows cells to leak solutes, thereby causing dehydration which lowers the freezing temperature of the

medium and decreases extracellular ice formation (Aisen et al., 2002). Combining the two provides an additive protective effect.

Cryodiluents

Semen cryodiluents are buffered salt solutions used to make semen less concentrated and viscous. The use of diluents in semen extension helps to maintain spermatozoa viability *in vitro* and to maximize the number of hens that can be inseminated with one ejaculate. Semen dilution with an extender for poultry is important because poultry semen is viscous and highly concentrated, containing approximately six to twelve billion spermatozoa per mL for roosters and toms, respectively (Donoghue and Wishart, 2000). Semen diluents were derived over 30 years ago from the biochemical composition of chicken and turkey semen and have since been only slightly modified (Lake, 1995; Long and Conn, 2012). It was found that the amino acid, glutamic acid is the main anionic component of poultry seminal plasma, and therefore, became a standard ingredient in diluents (Lake 1958, 1960; Lake and McIndoe, 1959). There are a variety of poultry semen extenders available in published reports and sold commercially. Many researchers have summarized the composition and fertility results comparisons among of various diluents (see Howarth, 1983; Bootswala and Miles, 1992). What becomes apparent from these reviews is that there is still no standard diluent for poultry semen (Donoghue and Wishart, 2000). One major problem is the amount of variation among the studies regarding the AI dose, time and frequency, vaginal depth, and the amount and type of fertility data analyzed such that the benefits of one diluent over another isn't readily apparent

(Donoghue and Wishart, 2000). The basic requirements for a successful diluent are to maintain pH and osmolarity, and provide a continuous energy supply for the spermatozoa (Christensen, 1995; Long and Conn, 2012). Since the pH of a diluent can affect the metabolic rate and motility of spermatozoa, buffering agents composed of an acid and its conjugate base are used in formulating a diluent to prevent changes in pH. For this reason, poultry diluents are composed of phosphates, citrates and/or organic zwitterionic molecules such as *N*,*N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) and *N*-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES) (Donoghue and Wishart, 2000). Lactic acid buildup occurs in chicken semen after increased storage time, which can lower the diluent pH. Therefore, zwitterionic molecules are an important component in diluents as well (Christensen, 1995). Both chicken and turkey spermatozoa can survive a pH range of 6.0-8.0. However, their motility and metabolic rate can be detrimentally altered by the diluent pH (Bogdonoff and Schaffner, 1954; Van Wambeke, 1967). A low pH reduces motility, lactic acid production, and oxygen uptake in chicken spermatozoa, whereas a high pH increases metabolic rate *in vitro* (Bogdonoff and Schaffner, 1954). In a comparative study of three commercial poultry diluents: Minnesota, I.M.V.-French, and Beltsville Poultry Semen Extender (BPSE), Giesen and Sexton (1983) found that turkey spermatozoa extended with BPSE at a pH of 6.0 after six hours had significantly improved fertility and hatchability. For 24 hour storage of chicken spermatozoa, Lake and Ravie (1979) demonstrated that a pH between 6.8 and 7.1 was optimum for maintaining spermatozoa fertilizing ability.

Poultry spermatozoa can remain viable in diluents with osmolarities ranging from 250 to 460 mosM/kg H_2O (Donoghue and Wishart, 2000). When placed in a solution of low osmolarity, the net movement of water into the spermatozoa causes them to swell and burst, whereas in a hyperosmotic solution, spermatozoa lose water and shrink (Bakst, 1980). In hypoosmotic solutions, spermatozoa displayed increased incidence of bent necks, which has been found to reduce fertility (Bajpai and Brown, 1964; Clark et al., 1984). Sexton and Geisen (1982) observed a reduction in turkey spermatozoa numbers over an 18 hour storage period also suggesting that spermatozoa were swelling and hypothesized that the use of hypertonic diluents could reverse the swelling and improve survival *in vitro*.

Important to the development of diluents and cryopreservation methods for poultry semen is the understanding of the physiological differences and metabolic requirements of spermatozoa among different avian species. Chicken spermatozoa are capable of generating ATP in both aerobic and anaerobic environments *in vitro,* whereas turkey spermatozoa require high levels of oxygen for energy metabolism (Sexton, 1974; Wishart, 1981). Aerobic metabolism necessitates the aeration of turkey semen which is generally accomplished by placing the diluted semen in a well plate on a gentle agitator to maximize surface to volume ratio. To date, the most effective BPSE has been developed (Table 2) and published by the Sexton's animal physiology laboratory at the Beltsville Agricultural Research Service in Beltsville, Maryland (Sexton, 1977).

Table 2. The composition of Beltsville Poultry Semen Extender.

Component	**Concentration Grams/liter**
Potassium diphosphate • $3H_2O$	12.70
Sodium glutamate	8.67
Fructose (anhydrous)	5.00
Sodium acetate • $3H_2O$	4.30
TES*	1.95
Potassium citrate	0.64
Potassium monophosphate	0.65
Magnesium chloride • $6H_2O$	0.34
pH	7.50
Osmotic pressure (m.Osm./kg. H_2O)	333

*N-tris [Hydroxymethyl]methyl-2-Aminoethane Sulfonic Acid.

Biological Challenges of Avian Semen Cryopreservation

It is well known that the challenges circumventing successful poultry spermatozoa cryopreservation are due to species differences in at least two sources of variability: 1) the physiology and morphology of the spermatozoa in the male reproductive tract, and 2) the variations in the anatomy and physiology of spermatozoa transport in the female reproductive tract (Holt, 2000).

Species Differences in the Male Reproductive Tract

In mammals, secretions from the testis, epididymis, and ductus deferens make up the seminal fluid prior to ejaculation. Immediately following secretion from the testis, mammalian sperm are not capable of fertilizing an oocyte. This ability is progressively gained as the sperm traverse the epididymis. The exact location will vary by species, but generally, fertilization-competence is gained in the body or proximal tail of the epididymis (Yanagimachi, 1994). Secretions from the accessory sex glands are incorporated into the semen which raise the pH and add nutrients critical for spermatozoa maturation in a process known as capacitation/decapacitation (Schwarz et al., 2013). Mammalian spermatozoa can survive for many months in the male genital ducts, however, once they are ejaculated, they can live for only 24-72 hours at body temperatures. In avian species, capacitation/decapacitation of spermatozoa is not necessary for fertilization to occur. Avian spermatozoa taken directly from the ductus deferens have fertilizing ability. Seminal fluids in avian species are composed of secretions originating only from the testis, epididymis, and ductus deferens,

because they have no accessory sex glands (al-Aghbari, 1992). In addition, a fluid secreted during erection which stimulates the engorgement of the phallic structure in the cloaca combines with the seminal fluid to produce transparent fluid. The significance of transparent fluid as a component of seminal plasma is not fully understood (Lake, 1966). Unlike mammals, avian spermatozoa, once delivered into the hen's oviduct, can retain their fertilizing capacity for prolonged periods of time (Bakst, 2011).

Another major difference between mammalian and avian reproductive biology is the semen volume obtained per collection. The average ejaculate volume of a bull, for example ranges from 5 to 8 mL while semen volumes collected from poultry are considerably less, ranging from 0.1 to 0.3 mL (Long, 2006). The average concentration of poultry semen is much higher (6 to 10×10^9 sperm/mL) than bull semen (1 to 2×10^6 sperm/mL) (Long, 2006). Excessive dilution adversely effects poultry spermatozoa function by lowering the overall fertilizing ability of frozen-thawed poultry spermatozoa (Sexton, 1977; Duplaix and Sexton, 1983).

Further differences among avian and bull spermatozoa physiology contribute several unique features of poultry that likely influence the outcome of semen cryopreservation. One prominent difference is the morphology of the avian spermatozoon cell compared with the mammalian spermatozoa. Unlike the paddle-shaped bovine spermatozoa, avian gametes are thread-like or filiform shaped with a smaller surface area-to-volume ratio and a more condensed nucleus. These unique morphological differences explain some of the differing responses

to stresses imposed by the cryopreservation process, such as the critical osmolality at which 50% of the spermatozoa cells are lysed. The critical osmolality for rooster spermatozoa is considerably lower (17 mOsm) than for bull spermatozoa (36 mOsm) therefore poultry spermatozoa have a smaller capacity for increased volume than bull spermatozoa (Watson et al., 1992). Moreover, it has been shown that poultry spermatozoa seem to be highly susceptible to morphological disruptions during the freeze/thaw process, as frozen-thawed rooster spermatozoa were found to have higher incidences of mitochondrial, midpiece, and perforatorium abnormalities than fresh spermatozoa (Xia et al., 1988), and electron microscopy also revealed that 60% of turkey spermatozoa exhibited swollen midpieces following cryopreservation (Bakst & Sexton, 1979; Marquez and Ogasawara, 1977).

Another interesting feature unique to avian spermatozoa is they are considerably longer (80 to 90µm) than bull spermatozoa (50 to 60µm). The avian spermatozoa tail is nearly eight times the length of the spermatozoa head which renders them more susceptible to mechanical injury such as pipetting, centrifugation, (Agca and Critser, 2002) and freezing damage (Donoghue and Wishart, 2000), common during cryopreservation.

The process of cryopreservation places not only many physical stresses on the spermatozoa, but also interferes with the metabolic pathways essential for generating ATP energy to support motility. Both poultry and bovine spermatozoa are unable to maintain sufficient ATP content after cryopreservation (Wishart and Palmer, 1986; Soderquist et al., 1991). Additionally, there are species-specific

differences in turkey and chicken spermatozoa metabolism which are likely to contribute to the differences in their ability to survive the freeze/thaw process. While chicken spermatozoa are equally capable of aerobic or anaerobic metabolism (Sexton, 1974), turkey spermatozoa derive their energy requirements by the aerobic process of oxidative phosphorylation only (Sexton, 1974; Wishart, 1981; Sexton and Giesen, 1982).

Turkey and chicken spermatozoa also respond differently when subjected to lower temperatures. When held at temperatures below 15ºC, the fertilizing ability of turkey spermatozoa was compromised, whereas rooster semen held at 5ºC did not have reduced fertilizing ability (Sexton, 1981). Due to their varying responses to cryopreservation, it is evident that the success of turkey and chicken spermatozoa cryopreservation require different strategies. This rationale can be extended to the success of many unique genotypic strains of poultry research stocks and all endangered avian species (Bacon et al., 1986; Froman and Bernier, 1987; Tajima et al., 1990; Alexander et al., 1993). Narrowing the scope of the discussion from the basic reproductive differences to comparative spermatozoa physiology, we find more properties that are unique to poultry that likely influence the outcome of semen cryopreservation.

Species Differences in the Female Reproductive Tract

In mammals, few viable spermatozoa are required for successful fertilization because many females only express one ovum during the estrous cycle. Even litter-bearing species only ovulate approximately ten or less oocytes per cycle. In contrast, poultry hens are managed to produce eggs daily for either a

5 to 7 month period (turkey) or a 12 to 14 month period (chicken layer). Artificial insemination in mammals is usually timed to coincide within 24 to 48 hours of ovulation; whereas, hens are inseminated once per week during the egg production period, weekly insemination requires that a relatively high number of poultry spermatozoa remain viable for a minimum of 7 days within the hen's SST to ensure fertilization between inseminations.

From a broad perspective, the species-specific difference between avian and mammalian reproductive tracts begins with the anatomy. Although, birds have many of the same anatomical reproductive features as mammals, their function is considerably different. At coitus, mammalian spermatozoa are generally deposited into the anterior vagina and migrate to the protection of the cervical mucus barrier in the cervix to avoid vaginal acid and immune response before entering the uterus (Suarez and Pacey, 2006). The cervical mucus barrier acts as a filter to prevent spermatozoa with poor morphology and motility from entering the cervix (Suarez & Pacey, 2006). Following spermatozoa deposit in the vagina of hens, however, spermatozoa ascend to the SST located only on the uterovaginal junction (UVJ) surface epithelium. The avian female possesses the unique ability to store viable spermatozoa for up to five weeks (chicken) or twelve weeks (turkey) in the SST caudal to the avian uterus (Bakst, 1989). To ensure that an ample population of spermatozoa is present at the site of fertilization at the infundibulum, spermatozoa continually egress the SST while the hen is in egg production (Bakst, 1993, 1998, 2002). The avian molecular and cellular mechanisms surrounding spermatozoa selection in the vagina,

spermatozoa entrance and survival with the SST and their migration from the SST are not well understood (Bakst, 2011).

Fertilization in mammals occurs when spermatozoa in the female reproductive tract migrate from the cervix through the uterine cavity to the ampullary-isthmic junction of the oviduct to meet the oocyte migrating from the infundibulum. After fertilization, the egg enters the uterine cavity and implants as a blastocyst. In avian species, spermatozoa must migrate farther up the oviduct to the infundibulum for fertilization to occur. After fertilization, the ovum travels through the magnum (production of albumen), isthmus (formation of the shell membrane), and the uterus (formation of the egg shell) before exiting the cloaca.

In mammals spermatozoa are guided to the oocyte by two mechanisms, thermotaxis and chemotaxis (Eisenbach and Giojalas, 2006). Studies involving rabbits (David et al., 1972) and pigs (Hunter and Nichol, 1986) have shown that at ovulation there is a 1-2□C temperature difference between the spermatozoa storage site in the cervical crypts and the warmer ampullary-isthmic junction fertilization site. In the rabbit's oviduct, the temperature difference is due to a decrease in temperature at the spermatozoa storage site (Bahat et al., 2005). Bahat et al. (2003) further showed this to be an ovulation-dependent difference beginning 10.5 to 11 hours post-mating rising from 0.8±0.2□C before ovulation to 1.6±0.1□C after ovulation. These findings suggest that mammalian spermatozoa are thermotactically responsive to an ovulation-dependent temperature gradient by migrating towards the warmer fertilization site (Bahat et al., 2003). Furthermore, only capacitated spermatozoa are thermotactically responsive (Bahat et al., 2003).

Capacitated spermatozoa may first be guided by thermotaxis (Bahat et al., 2003; Bahat and Eisenbach, 2006), then chemotactically via progesterone and other unknown chemoattractant(s) secreted from the oocyte cumulus cells as the spermatozoa near the oocyte (Sun et al., 2005; Teves et al., 2006; Oren-Benaroya et al., 2008). This is consistent with the observation that oocyte cumulus cells secrete a substance that alters the pattern of spermatozoa mobility (Bronson and Hamada, 1977) and that cumulus secretions improve the fertilizing ability of spermatozoa penetrating into the oocyte (Tanghe et al., 2002). It is known that Ca^{2+} signaling in mammalian spermatozoa is critical for spermatozoa motility, hyperactivation, capacitation, and the acrosome reaction (Carleson et al., 2003; Suarez and Ho, 2003; Publicover et al., 2008; Kirkman-Brown et al., 2002). It also plays an important role in mammalian and non-mammalian spermatozoa chemotaxis, chemotactic and thermotactic signal-transduction pathways in many unicellular species, and thermotaxis in a variety of eukaryotic cells (Cook et al., 1994; Eisenbach, 1999; Spehr et al., 2003; Kaupp et al., 2003; Böhmer et al., 2005; Kaupp et al., 2006).

Unlike a wealth of published literature regarding the thermo and chemotactic mechanisms of mammalian spermatozoa mobility through the female reproductive tract, there is only evidence of these mechanisms reported in marine invertebrates (Kaupp et al., 2003, 2006) We do know, however, that avian spermatozoa harness energy by Ca^{2+} cycling to activate phospholipase A_2, which generates palmitic and stearic acid for β-oxidation within the mitochondrial matrix for spermatozoa motility (Froman and Feltmann, 2005). However, while

all mobile spermatozoa are motile, not all motile spermatozoa are mobile, thus spermatozoa mobility is critical for fertility and not motility (Froman, 2003; Froman and Feltmann, 2005). Immobile spermatozoa contain dysfunctional mitochondria (Froman and Kirby, 2005). It has been suggested that the success of long-term commercial cryopreservation may depend not only on preserving mitochondrial integrity and Ca^{2+} cycling, but the spermatozoa mobility phenotype (Froman and Feltmann, 2010) This would explain the high variation in cryopreservation efficacy among lines and among males within lines due to variation in spermatozoa phenotype (Fulton, 2006; Froman and Feltmann, 2010).

The Role of Carbohydrates in Poultry Sperm Function

The glycocalyx is a dense carbohydrate- rich coating on the external surface of all eukaryotic cells. This coat is composed of oligosaccharide chains covalently bound to glycolipids or glycoproteins as well as polysaccharide chains covalently linked to proteoglycans. Interestingly, not all the glycoconjugates are tightly bound to the cell surface membrane; some are absorbed by the outer cell membrane to assist in forming the glycocalyx (Alberts et al., 1994).

The composition of the spermatozoa glycocalyx has been investigated and identified through the use of specific lectins by many investigators (Koehler, 1981; DeCerezo et al., 1996; Peláez and Long, 2007). Specific lectins bind to different types of carbohydrate chains, and thereby provide the distribution of the sugar moieties forming the glycocalyx (Cummings, 1994). These studies have shown that the spermatozoa glycocalyx is highly complex. For comparison, the

glycocalyx of mammalian oocytes is composed of only three glycoproteins (Yanagimachi, 1994), whereas the spermatozoa glycocalyx is estimated to contain 50 to 150 distinct glycoconjugates (Schröter et al, 1999). Additionally, the glycoconjugates are segregated to form different functional domains rather than homogenously distributed on the surface of the spermatozoa (Schröter et al, 1999). The mammalian spermatozoa glycocalyx is continuously and extensively modified during maturation and transit through the epididymis and the female reproductive tract, which makes exact characterization extremely difficult (Diekman, 2003).

The glycocalyx of spermatozoa serves as the primary interface between the male gamete and a hostile female reproductive environment. Not all of the specific functions have been delineated, however we know that the spermatozoa glycocalyx assists in spermatozoa maturation, motility, binding and penetration of the oocyte, and provides immunoprotection for the spermatozoa as it traverses the female genital tract (Schröter et al, 1999; Peláez and Long, 2007). In the mammalian reproductive tract, glycoconjugates play a critical role in the mechanisms that control spermatozoa maturation and transport, and gamete interactions (Diekman, 2003).

The majority of the research pertaining to the composition, distribution and function of the spermatozoa glycocalyx has been obtained from mammalian species. There have been a few studies documenting the distribution, composition, and functional implications of the spermatozoa outer membrane glycoconjugates of other taxa, such as invertebrates (Dallai et al., 2011), and

fishes (Walter et al., 2005). Research documenting the composition, distribution and function of the avian spermatozoa glycocalyx, by contrast, is limited. Characterization of the poultry spermatozoa glycocalyx is the first step in determining the precise physiological impact of semen storage on spermatozoa function (Peláez and Long, 2007). Early research has shown that the glycocalyx of chicken spermatozoa contains sialic acid (SA) residues (Froman and Thurston, 1984). A more recent investigation by Peláez and Long (2007) identified the specific glycoconjugates associated with the turkey and chicken spermatozoa and their distribution. The glycocalyx of turkey and chicken spermatozoa is composed mainly of glycoconjugates containing β-galactose, α-mannose/α-glucose, and terminal SA residues (Peláez and Long, 2007).

Poultry Sperm Sialic Acid

Sialic acids are nine-carbon backbone amino sugars found abundantly on the glycocalyx (Schauer, 1982, 2004). The entire glycocalyx of both turkey and chicken spermatozoa is coated by SA residues (Long, 2006). The general biological functions of SA are to modulate immune recognition during fertilization and embryonic development (Ma, 2012). In the rooster, SA plays a role in spermatozoa mobility through the hen's vagina. This was demonstrated in poultry spermatozoa treated with neuraminidase to cleave terminal SA residues prior to insemination (Steele and Wishart, 1996). This suggests that removal of spermatozoa surface SA residues may decrease immunoprotection, resulting in cell death by an immunologically-based spermatozoa-selection mechanism (Steele and Wishart, 1996). Thus, the SA coating of the poultry spermatozoa

glycocalyx enables spermatozoa to survive as allogeneic cells in the female reproductive tract despite female immunity. SA have also been implicated for spermatozoa agglutination during storage in the hen's SST (Froman and Thursam, 1994).

SA has been found to mask antigens and receptor sites on the cell membrane, which therefore makes it difficult to determine their specific distribution (Schauer, 1985). Masking of a large majority of the carbohydrate residues in poultry spermatozoa glycocalyx was demonstrated following the enzymatic removal of terminal SA residues with neuraminidase from the glycocalyx (Peláez and Long, 2007). To date, the only way to quantitatively measure SA residues is indirectly through fluorescence intensity following incubation with fluorescein-labeled lectin binding proteins (Miller et al., 1982).

Limax flavus (LFA) Lectin

Lectins are carbohydrate-binding proteins with a high binding affinity for specific glyconjugates (Lis and Sharon, 1973; Sharon & Lis, 1975; Pereira and Kabat, 1979; Goldstein and Hayes, 1978). Due to the high degree of specificity exhibited by individual lectin binding of glycolipid and glycoprotein carbohydrate residues, lectins have been used extensively in characterization and distribution studies of eukaryotic cell membrane components (Miller et al., 1982). Lectins are ubiquitous in nature and found in almost every plant and animal. Garden slug *Limax flavus* (LFA) lectin exhibits specificity for SA and is, therefore, the lectin of choice for measuring SA-containing glycoproteins (Miller et al., 1982).

Chapter 2: The Effect of Sialic Acid on the Success of Poultry Spermatozoa Cryopreservation

Abstract

There is significant interest in developing methods for avian semen cryopreservation for conserving desirable genetic traits for both elite lines for commercial poultry production and endangered species conservation. Presently, cryopreserved poultry sperm retains < 2% of the fertilizing ability of fresh semen. Turkey and chicken hens can maintain viable sperm in sperm storage tubules (SST) for up to 10 weeks (turkey) and 3 weeks (chicken) post-insemination. The glycocalyx of poultry sperm contains large amounts of terminal sialic acid (SA) residues. Poultry sperm treated with the enzyme neuraminidase to remove SA do not successfully reach the hen's SST. Hypothermic storage of poultry sperm reduces the amount of SA detectable on the surface of the sperm. Ongoing studies suggest that turkey sperm held at 4°C for 24 h with different concentrations of SA have improved fertility. We hypothesized that poultry sperm can incorporate exogenous SA and that this restoration will improve the fertility of frozen/thawed sperm. The objective of our first experiment was to determine the optimal time, dose, and temperature, for SA uptake by tom and rooster sperm. Semen samples were collected from toms and roosters, transported to the laboratory at 25°C, and pooled. Pools were diluted to 2.5 x 10^9 sperm/mL with extender and aliquoted as control (no SA treatment) or 1 of 5 SA treatments: 1) 40, 80, 120, 160, or 240 µg/mL (toms) or 2) 40, 80, 120, 160, or 200µg/mL (roosters). Control and SA-treatment semen samples were incubated at 25°C or 4°C for 2 h. At 30 min intervals, aliquots were removed and incubated for 30 min at 37°C with Limax flavus lectin conjugated to a fluorochrome (to detect SA residues) and propidium

iodide (PI) (to detect and exclude dead sperm). Following incubation, samples were centrifuged (1800 rpm; 5 min), and the fluorescence of the two probes were determined by flow cytometry. Data (n=6 toms; n=6 roosters) were analyzed using mixed model Proc Mixed of the least square means, and significant effects ($P<0.05$) were observed for both sperm viability and SA content as a function of SA incubation temperature, incubation time, and dose. For both turkey and rooster, the viability of the control and SA-treated sperm over two hours was significantly higher at 25°C (turkey: 64.2 to 72.0% ± 2.4% SEM; rooster: 67.9 to 78.6% ±2.0% SEM) than spermatozoa that incubated at 4°C (turkey: 63.0 to 65.7% ±2.4% SEM; rooster: 69.4 to 70.9% ±2.0% SEM) ($p=0.0040$ and $p=0.0093$ respectively). Interestingly however, the percentage of spermatozoa exhibiting the highest levels of SA was different in toms than in roosters. The percentage of high SA content in toms was significantly higher at 4°C (8.2 to 14.1% ±1.7%) than at 25°C (2.4 to 8% ±1.7%) ($p<.0001$), whereas in roosters the percentage of high SA content was significantly higher in sperm incubated at 25°C (12.6 to 17.1% ±3.5%) than at 4°C (2.1 to 6.9%±3.5%) ($p=0.0001$). Our second experiment, to evaluate pre-freeze vs. pre-freeze/post-thaw as the ideal time-point for SA incubation, was designed based on the results from experiment one. For toms, we incubated the control and 80, 120, 160, and 240µg/mL SA-treated spermatozoa for 30 min prior to freezing (pre-freeze) and again for 15 min post-thaw (pre-freeze/post-thaw). For roosters, we incubated the control and 20, 40, 80, and 120 µg/mL SA-treated spermatozoa for 30 min prior to freezing (pre-freeze) and again for 5 min post-thaw (pre-freeze/post-thaw). Data (n=6

hens/treatment) were analyzed using a repeated measures Proc Glimmix program for binomial distribution of data of the least square means. Significant effects ($P<0.05$) were observed for the percentage of total fertility in turkeys and chickens. In turkeys, the 160 µg/mL SA pre-freeze treatment improved the fertility of frozen/thawed spermatozoa 40% vs. 7% (control) ($p=0.0513$). In chickens, the 120 µg/mL SA pre-freeze treatment improved the fertility of frozen/thawed spermatozoa 10% vs 3% (control) ($p=0.0040$).

We conclude that poultry spermatozoa can incorporate exogenous SA and both turkey and chicken SA-treated spermatozoa had improved total fertility (40%, 10% respectively).

Introduction

The importance of avian semen cryopreservation has long been received as an invaluable reproductive strategy for conserving desirable genetic traits for both commercial meat and egg production and endangered species conservation (Long, 2006). Spermatozoa cryopreservation technology has been around for more than 50 years following the discovery of glycerol and its cryoprotectant qualities (Agca and Critser, 2002). This scientific breakthrough is widely used in the commercial dairy and beef industries. However, despite the fact that it was the rooster that led to this achievement (Polge, 1951), cryopreserving poultry semen still is not effective. Cryopreserved poultry spermatozoa retains < 2% of the fertilizing ability of fresh semen (Wishart, 1985). This rate of success is obviously too low for use in commercial poultry management applications as a method for long-term storage of genetic stocks (Long, 2006). In addition, there are significant differences in spermatozoa viability and functionality following cryopreservation among commercial species (turkey, broiler-type chicken and layer-type chicken) (Long, 2006).

Turkey and chicken hens have the ability to store spermatozoa in SST for up to 10 weeks (turkey) and 3 weeks (chicken) and maintain the fertilizing capacity of the spermatozoa in the absence of a rooster (Blesbois and Brillard, 2007). This enables the hen to lay fertile eggs for a prolonged period of time from one insemination (Blesbois and Brillard, 2007). This phenomenon is made possible by sperm sequestration within the SST, providing a reservoir of viable spermatozoa (Froman and Thursam, 1994). Although the precise mechanism of

prolonged fertility from spermatozoa stored within SST is not understood, cell surface SA has been found to assist in spermatozoa sequestration (Froman and Thursam, 1994). The glycocalyx of poultry spermatozoa contains large amounts of terminal sialic acid (SA) residues attached to proteins and lipids within the plasma membrane (Pelaez and Long, 2007). It has been shown that poultry spermatozoa that have been treated with the enzyme neuraminidase to remove SA do not successfully reach the SST in the hen's oviduct (Froman and Engel, 1989). More recently, it has been shown that hypothermic storage of turkey (Pelaez and Long, 2008) and chicken (Frohman and Thursam, 1994) spermatozoa reduces the amount of SA detectable on the surface of the sperm. Ongoing studies suggest that turkey spermatozoa held at 4°C for 24 hours with different concentrations of SA have improved fertility. This project will test the hypothesis that SA lost under hypothermic conditions can be replaced by including SA in the cryodiluent, and that the fertility of frozen/thawed poultry sperm treated with SA will be improved. Accordingly, the specific objectives for this study were: 1) to verify that poultry sperm can incorporate exogenous SA *in vitro*; 2) to determine the optimal temperature and length of time for SA uptake by poultry sperm; 3) to determine optimal dose of SA for cryopreservation of poultry sperm; and 4) to evaluate pre-freeze vs. pre-freeze/post-thaw as the ideal time-point for SA incubation.

Materials and Methods

Research Facilities

The animal research for this project was conducted at the Beltsville Agricultural Research Center (BARC) in Beltsville, Maryland. The coursework and statistical analysis for this project were conducted at the Animal and Avian Sciences Department at the University of Maryland, College Park.

Animals

Data were collected from 144 turkeys (84 male and 60 female Hybrid Grade Converter Turkeys; Kitchener, Canada) and 204 chickens (84 male and 120 female chickens Hy-Line W-36; Hy-Line International, Elizabethtown, Pa) maintained using standard management practices in the BARC poultry facilities under lighting conditions (14L:10D light cycle for turkey; 16L:8D light cycle for chicken) for spermatozoa and egg production.

Semen Collection and Evaluation

For maintenance, semen was routinely manually collected (Quinn and Burrows, 1936) from each tom twice per week and three times per week for roosters. On the day of an experiment, collected semen was immediately transported in a covered Styrofoam box to the testing laboratory to avoid contact with the variable environmental conditions. In the laboratory, the neat semen was pooled after being found to have normal color and consistency. To determine the initial spermatozoa concentration, 10µL of neat semen was added to 1.99 mL of 3% sodium citrate solution and measured on a photometer. Spermatozoa were then diluted to a concentration of 2.5×10^9 sperm/mL with the appropriate

extender, depending on the species dilution factors and the type of experiment performed. Only spermatozoa samples having >50% baseline viability following flow cytometry evaluation of Sybr-14/Propidium iodide (PI) Live/Dead stained cells (L-7011; Molecular Probes, Eugene, OR) were included in further analysis.

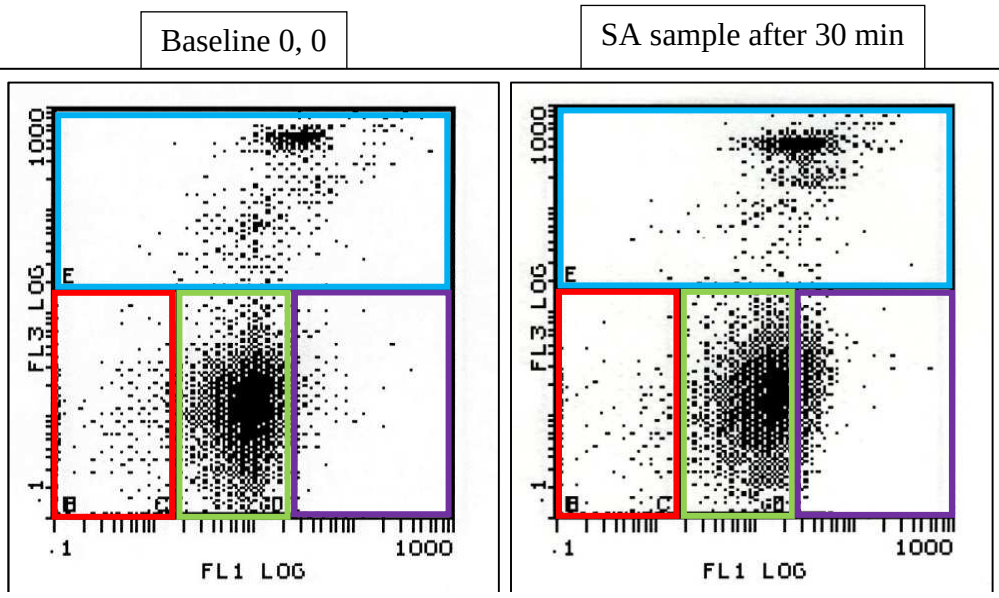
Detection of Terminal SA on Spermatozoa Membranes

On the day of an experiment, SA stock concentrations were prepared using ≥95% synthetic N-Acetylneuraminic acid (sialic acid) (Sigma-Aldrich, St. Louis, MO) stored at 20 ºC and Beltsville Poultry Semen Extender (BPSE) or other cryodiluent, depending on the experiment performed. Verification that poultry spermatozoa can incorporate exogenous SA *in vitro* was determined using SA-specific lectin *Limax flavus* lectin (LFA) with added PI. For all experiments, LFA was used at a concentration of 100 µg/mL in Tris-HCl buffer (TBS; 0.05 M Tris HCl, 0.3M NaCl [pH 7.5]) (EY, Laboratories, Inc., San Mateo, CA) (Pelaez and Long, 2008). On the day of use, 50 µL of stock LFA (1mg/mL) was diluted with 450µL of Tris-HCl buffered solution. Each 0.05µL semen sample analyzed was first incubated in 120µL LFA with 0.05µL PI.

Flow Cytometry

A Coulter Epics XL-MCL Flow Cytometry (Coulter Corporation, Miami, Fla) equipped with a single 488-nm excitation source was used for all analyses. Forward and side scatter gating was employed to select single spermatozoa from clumps and debris. The fluorescence from FITC-stained spermatozoa was collected in FL1 (525nm band pass) fluorescence detector. The percentage of the viable sperm population and high uptake of SA by spermatozoa based on

fluorescence intensity/cell was recorded from the FL1 detector output to determine lectin-binding related changes in the population (Figure 1). Only the fluorescence of the live sperm cells (PI negative) was used in the final analysis.



B. Weakly positive LFA cells indicating sperm population with low levels of SA.
C. Positive LFA cells indicating sperm population with normal levels of SA.
D. Strongly positive LFA cells indicating sperm population with high levels of SA.
E. Black dots above the line represent PI+ or non-viable cells and were not included in the analysis. Black dots below the line represent PI- or viable cells and were included in the analysis.

Figure 2. Flow cytometer gating showing the shifting of the spermatozoa over time.

General Cryopreservation Procedures

The general poultry spermatozoa cryopreservation procedures are outlined in Table 3. Raw tom semen was diluted 1:1 with 325mOs/Kg ASG ("solution 7.1," Lake and Ravie, 1979) plus SA cryodiluent. Diluted semen was equilibrated for 30 minutes at 4^0C, and then further diluted 1:2 with ASG containing 18% dimethyl acetimide (DMA) for a final concentration of 6% DMA. Semen was immediately loaded into 0.50mL French straws and sealed with a bead or powder. The straws were placed on a Styrofoam raft and floated 1.25cm above the liquid nitrogen (LN_2) for two minutes, then straws were plunged into the LN_2 and placed in a LN_2 canister for -196ºC storage. Raw rooster semen was diluted 1:1 with 360mOs/Kg, 6.8 pH Hanzawa (Hanzawa et al., 2006) cryodiluent containing none or 4X concentration of SA, equilibrated for 30 minutes at 5^0C, then further diluted 1:1 with 18% methyl acetimide (MA) to yield a final concentration of 9% MA. Semen was immediately loaded into 0.50mL French straws and sealed with a bead or powder. The straws were placed on a Styrofoam raft and floated 4.5cm above the LN_2 for two minutes, and then straws were plunged into the LN_2 and placed in a LN_2 canister for -196ºC storage. Tom and rooster straws removed from LN_2 storage were thawed by vigorous agitation for 30 seconds in 5^0C water bath. The excess water was removed from each straw and straw ends were cut and allowed to flow directly into an Eppendorf tube.

Table 3. General Cryopreservation Procedures

Procedure		
Treatment	Toms	Roosters
Cryodiluent	ASG	Hanzawa
Semen:diluent *	1:1	1:1
Cooling**	5°C for 30 min	5°C for 30 min
Cryoprotectant	Dimethyl acetimide (DMA) Initial [18%] Final [6%]	Methyl acetimide (MA) Initial [18%] Final [9%]
Package	French straw (0.5mL)	French straw (0.5mL)
Vapor freezing	1.25cm above LN_2 for 2 min; then plunge	4.5cm above LN_2 for 2 min; then plunge
Thawing	Vigorous agitation at 5°C for 30 sec	Vigorous agitation at 5°C for 30 sec

* For turkey, diluted 1:1 with 0, 160, 240, 360, or 240 µg/mL SA; for chicken, diluted 1:1 with 0, 40, 80, 160, or 240 µg/mL SA. .

**For chicken, separate aliquots of diluted semen were incubated with SA at 25°C for 30 min, then cooled to 5°C for another 30 min.

Artificial Insemination and Fertility Assessment

A total of 54 turkey and 108 chicken hens that were in egg laying production for one month were selected for use. Each turkey hen was inseminated with 300 x 10^6 sperm, and each chicken hen received 200 x 10^6 sperm per dose, per day, for two consecutive days. Following the insemination of the

hens, eggs were collected daily and set on day seven. A total of 1776 (10 weeks) turkey and a total of 1529 (2 weeks) eggs were individually candled after seven days of incubation and categorized as fertile or infertile. Fertile eggs were placed back in the incubator to hatch while infertile eggs were broken out to verify infertility or early (<48h) or late (>48h) embryonic death. All eggs that candled fertile and either died early or late during embryonic development were included in the statistical analysis.

Experiment 1: Determining the Optimum Temperature, Time, and Dose for Tom and Rooster Spermatozoa Sialic Acid Uptake.

For both turkey toms and chicken roosters a baseline time 0 for the pooled semen samples was diluted with BPSE (1:3 for toms and 1:1 for roosters), and 5 µL was placed in a 1.5mL Eppendorf tube containing 120 µL LFA and 5 µL of PI counterstain, and then incubated at 37 ºC for 30 minutes. The baseline samples were used to establish the flow cytometer gating (Figure 1) for the viability and SA uptake measurements for each experimental replication. For turkey, the remaining pooled semen was first diluted 1:2 with BPSE, and then divided equally among six treatment wells. An equal volume of 2x concentration of SA was added to the appropriate well to obtain the desired final concentrations of either 40, 80, 120, 160, or 240µg/mL, and samples were incubated in the dark at either 25 ºC or 4 ºC for two hours. For the turkey control (no SA) 1:2 BPSE was added and incubated in the dark at either 25 ºC or 4 ºC for two hours. At 30 min intervals, 5uL of each treatment and control were incubated in LFA/PI stains for 30 min at 37 ºC. Following incubation, the samples were centrifuged at 1800 rpm

for 5 minutes, supernatant removed, and the sperm pellet washed and re-suspended with 1mL Tris-HCl buffer and read on the flow cytometer. For roosters, the remaining pooled semen was divided equally among six Eppendorf tubes and diluted 1:1 with BPSE (control 0 SA) or 1:1 with a 2x concentration of SA plus BPSE to obtain the desired final concentrations of 40, 80, 120, 160, or 200µg/mL SA. The remaining procedures for the semen samples are outlined in Figure 3.

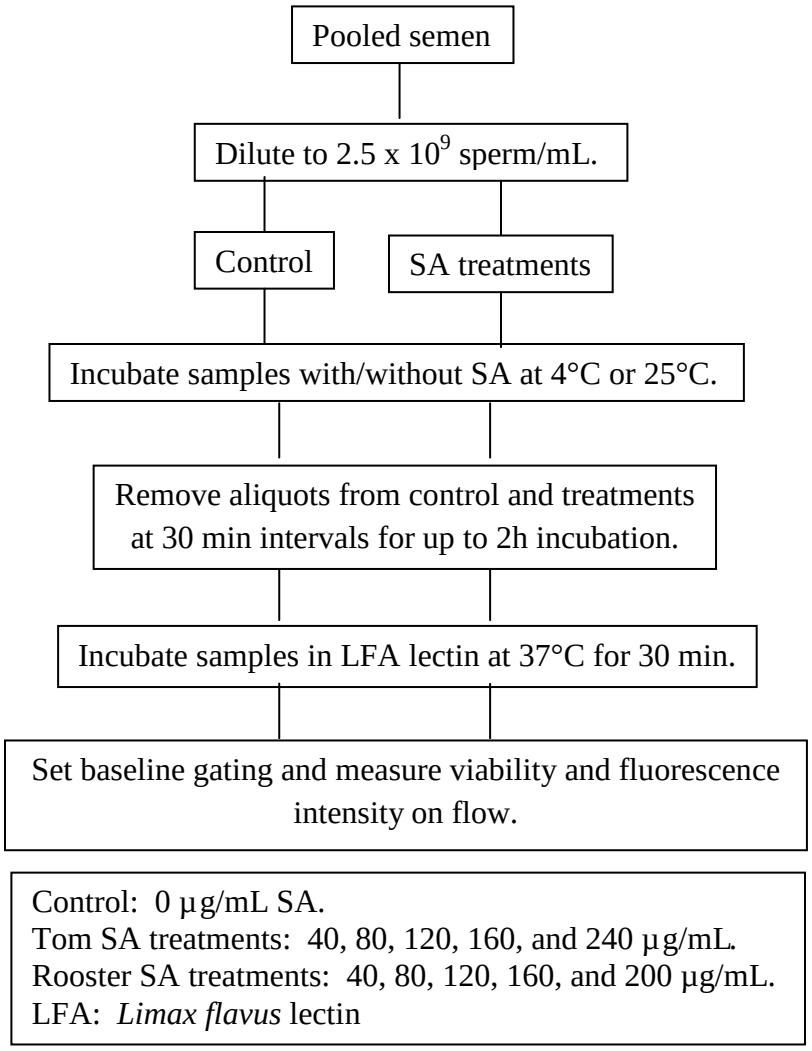


Figure 3. General experimental design of experiment 1: Determining the optimal temperature, time, and dose for tom and rooster sperm SA uptake.

Experiment 2: Determining the Optimum Pre-freeze or Pre-freeze/Post-thaw conditions for SA Uptake by Turkey Spermatozoa.

The experimental design for turkey experiment two (Figure 4) was based on the results from experiment one for turkeys. For turkeys, a total of ten treatments (n=6 hens/treatment) were used to assess fertility.

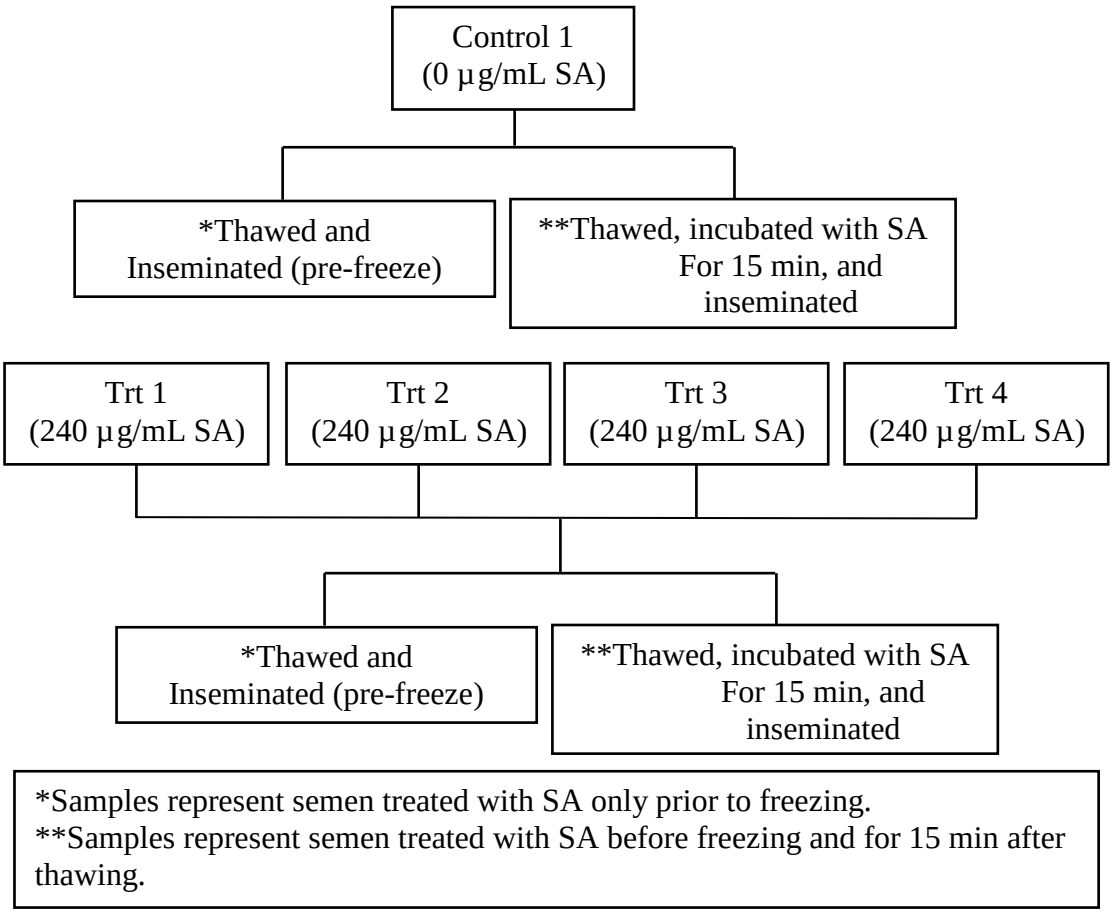


Figure 4. General experimental design for turkey experiment 2: thawing and inseminating.

First, the frozen turkey semen samples were thawed as two groups: 1) samples treated with SA (80, 120, 160 or 240 µg/mL) at 4°C for 30min before freezing (pre-freeze), then administered to hens; and 2) samples treated with the

aforementioned SA concentrations before freezing and again after thawing at 4°C for 15min (pre-freeze/post-thaw), then administered to hens. The control groups (no SA) were treated the same, but without the added SA, then administered to hens.

Experiment 2: Determining the Optimum Pre-freeze or Pre-freeze/Post-thaw conditions for SA Uptake by Rooster Spermatozoa.

The experimental design for rooster experiment two (Figure 5) was based on the results from experiment one for roosters. For roosters, a total of twenty treatments (n=6 hens/treatment) were used to assess fertility. We divided semen into four groups: 1a) semen treated with SA (20, 40, 80, or 120 µg/mL) for 30min at 5°C (pre-freeze), then administered to hens; 1b) pre-freeze treated semen plus additional SA (20, 40, 80, or 120 µg/mL) for 5min at 5°C after thawing (pre-freeze/post-thaw), then administered to hens; 2a) semen treated with SA (20, 40, 80, or 120 µg/mL) for 30 min at 25°C, then cooled to 5°C and administered to hens (pre-freeze); and 2b) pre-freeze treated semen plus additional SA (20, 40, 80, or 120 µg/mL) for 5min at 25°C after thawing (pre-freeze/post-thaw), then administered to hens. The controls (no SA) were treated the same, but without the added SA, then administered to hens.

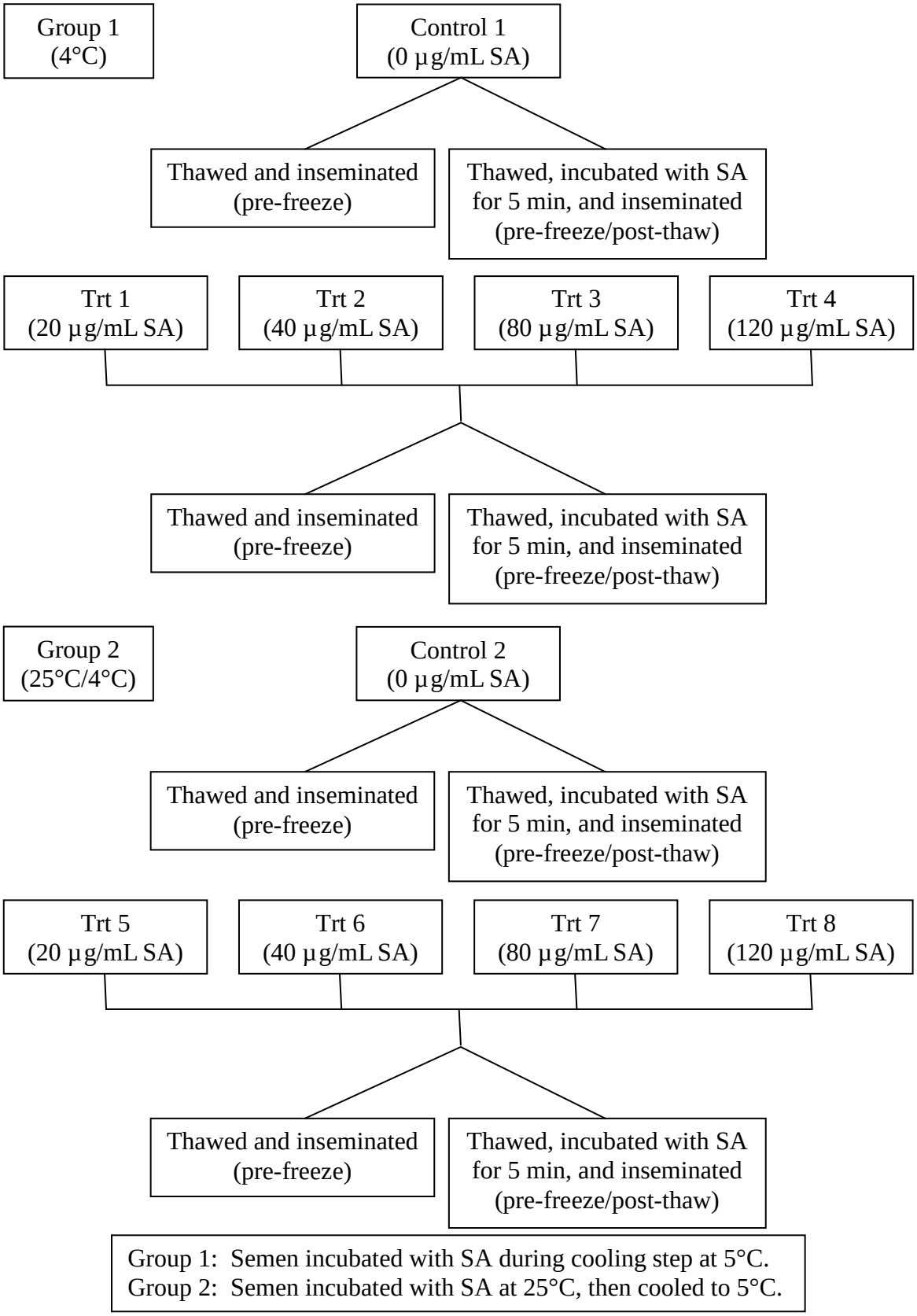


Figure 5. General experimental design for chicken experiment 2: thawing and inseminating.

Statistical Analysis

Treatment and fertility data analysis were performed using SAS 9.2 (SAS Institute, Cary, NC). A repeated measure, mixed-model analysis of variance was utilized, with the p value for significance set at 0.05 (Little et al., 1996). For treated spermatozoa data, six replications with minimum baseline viability of 50% were analyzed after raw data was 100% normalized to the percentage of live at each dose, time and temperature. Homogeneity of treatment variance and Shapiro-Wilk normality of distribution of residual errors were both examined prior to the selection of covariance structure. An LSMEANS statement using the DIFF option was used for comparison of least squares means. For the statistical model, assay and dose rate were considered a fixed effects, while the sialic acid incubation time in minutes was considered a random effect. For the fertility data analysis, PROC GLIMMIX was performed for binomial distribution, with hen as the fixed effect and week in laying production considered a random effect.

Results

Experiment 1: Determining the Optimum Temperature, Time, and Dose for Tom Spermatozoa Sialic Acid Uptake.

Overall, the mean percentage of membrane-intact or viable turkey spermatozoa (Figure 6) incubated at 25°C (64.2 to 72.0% ± 2.4%) was significantly higher than spermatozoa that was incubated at 4°C (63.0 to 65.7% ±2.4%) ($p=0.0040$).

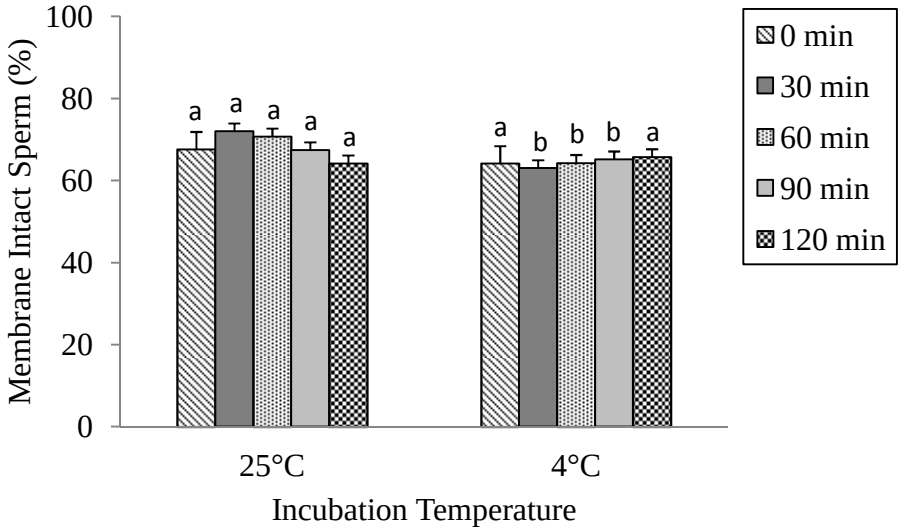


Figure 6. The percentage of membrane intact turkey spermatozoa (viability) at 25°C and 4°C incubation temperature with/without SA up to 2hr. The different superscripts between the two incubation temperatures indicates significant differences at those time points ($p<0.05$).

The mean percentage of membrane intact turkey spermatozoa with strongly LFA-positive fluorescence or high levels of SA uptake (Figure 7) was significantly higher at 4°C (8.2 to 14.1% ±1.7%) ($p<.0001$) than at 25°C (2.4 to 8.0% ±1.7%) incubation temperature with/without SA up to 2hr.

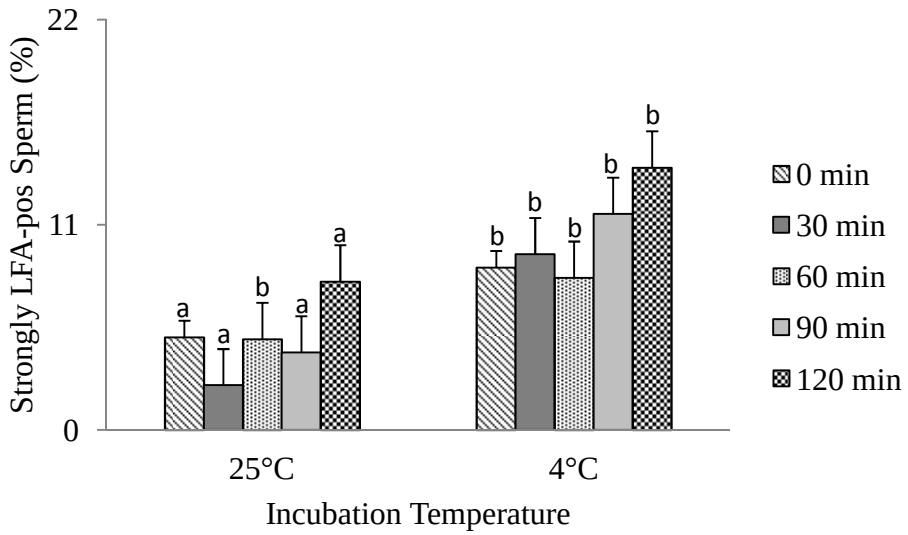


Figure 7. The percentage of membrane intact turkey spermatozoa with strongly LFA-pos fluorescence at 4°C and 25°C incubation temperature with/without SA up to 2hr. The different superscripts between the two incubation temperatures indicates significant differences at those time points ($p<0.05$). Incorporation of SA as assessed by LFA was greater at 4°C than 25°C ($p<0.05$).

When the mean fluorescence intensity (MnFl) of the strongly LFA-positive turkey spermatozoa at 4°C (Figure 8) was analyzed, none of the time points were different from the control value of 26.0 ±1.6 MnFl ($p>0.05$), therefore, we elected to incubate sperm for the shortest duration of time in experiment 2.

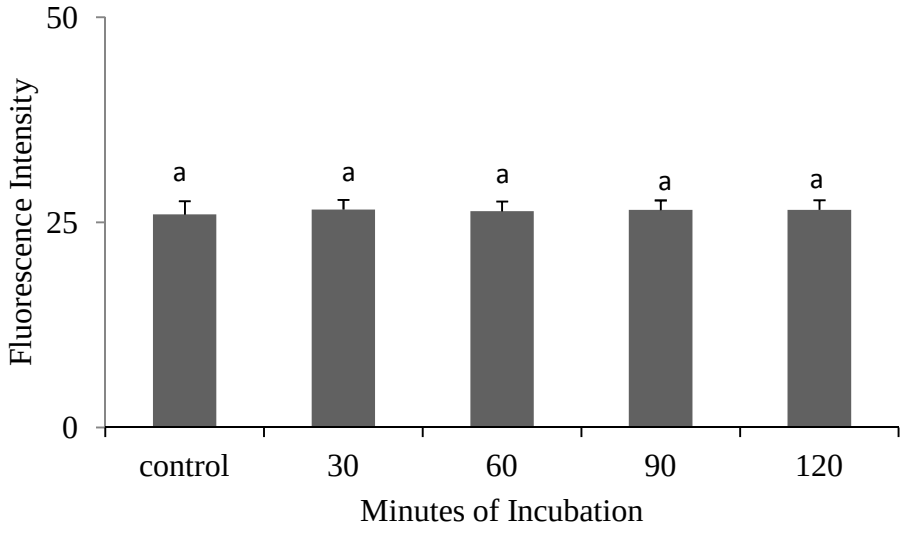


Figure 8. The MnFl of strongly LFA-positive turkey sperm at 4°C over time.

When the percentages of strongly LFA-positive turkey spermatozoa in control and SA treatments at 4°C for 30min (3.7 to 11.4% ±3.5%) (Figure 9) were analyzed, none of the SA doses were significantly different from each other ($p>0.05$). Therefore, we elected to treat the sperm with the four highest SA doses: 80, 120, 160, or 240 µg/mL in experiment 2.

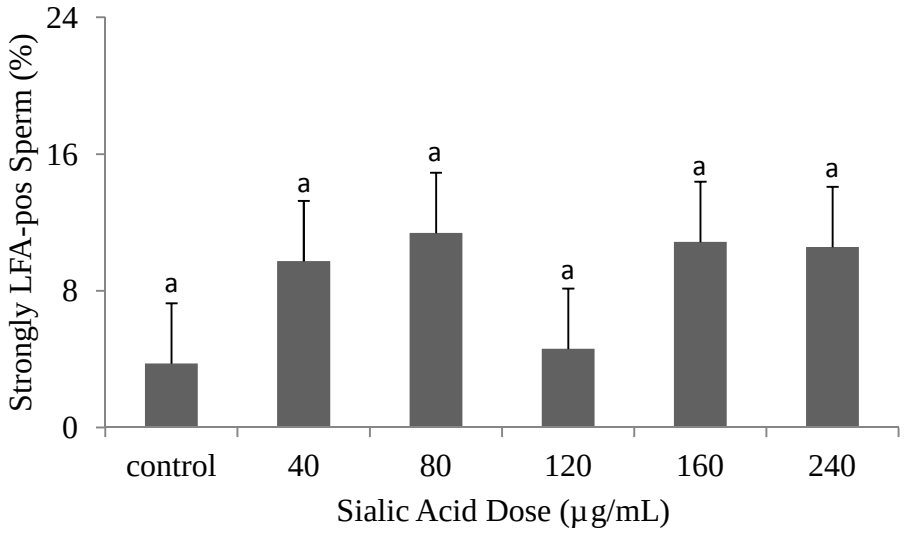


Figure 9. The percentage of strongly LFA-positive turkey spermatozoa at 4°C for 30min with 0 to 240 µg/mL SA.

Experiment 2: Determining the Optimum Pre-freeze or Pre-freeze/Post-thaw Time-point for SA Uptake by Turkey Spermatozoa.

The mean percentage of total fertility (Figure 10) for eggs (n=1776) laid over a 10-week period from turkey hens inseminated with 160 µg/mL, pre-freeze SA-treatment than the control (p=0.0164), 80 and 120 µg/mL SA treatments (p=0.0057, p=0.0118 respectively).

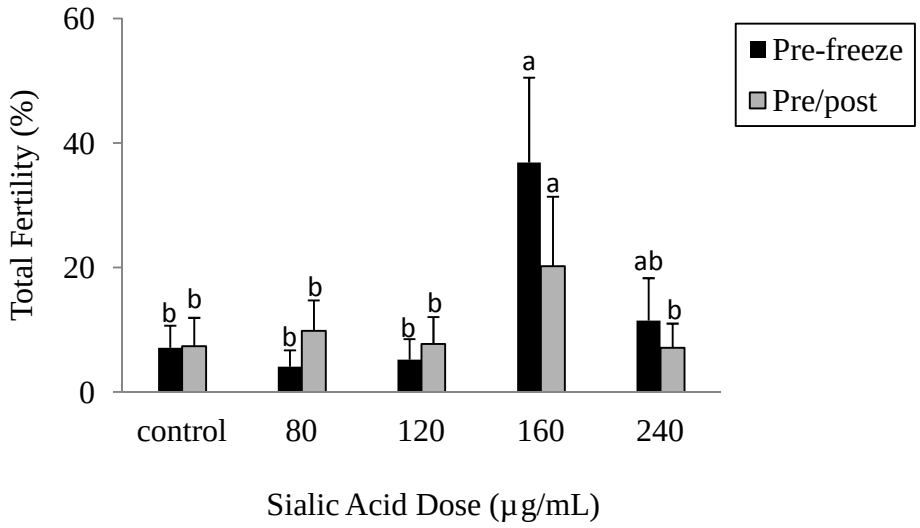


Figure 10. Total turkey fertility (%) from pre-freeze and pre-freeze/post-thaw control and SA treated sperm. The different superscripts between the control and SA doses indicates significant differences ($p<0.05$).

Although the 160 µg/mL SA pre-freeze treatment (Figure 10) exhibited the highest percent of total fertility, the same success did not hold true for the pre-freeze/post-thaw 160 µg/mL SA treated sperm.

Experiment 1: Determining the Optimum Temperature, Time, and Dose for Rooster Spermatozoa Sialic Acid Uptake.

Similar to turkeys, the mean percentage of membrane-intact or viable rooster spermatozoa (Figure 11) incubated at 25°C (67.9 to 78.6% ±2.0%) was significantly higher than spermatozoa that was incubated at 4°C (69.4 to 70.9% ±2.0%).

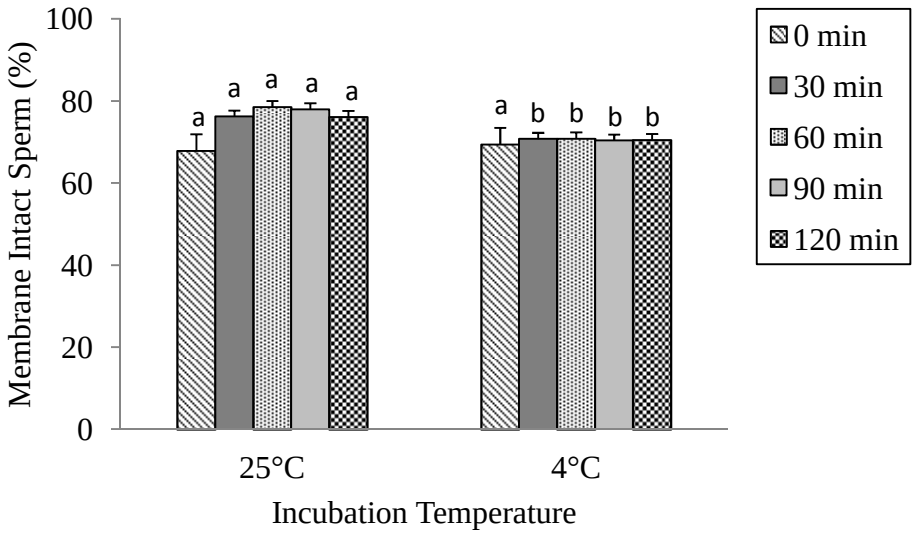


Figure 11. The percentage of membrane intact turkey spermatozoa at 25°C and 4°C incubation temperature with/without SA up to 2hr. The different superscripts between the two incubation temperatures indicates significant differences at those time points ($p<0.05$).

Contrary to the turkey results, the mean percentage of membrane-intact rooster spermatozoa with strongly LFA-positive fluorescence or high levels of SA uptake, (Figure 12) was significantly higher at 25°C (12.6 to 17.1% ±3.5%) rather than at 4°C (2.1 to 6.9% ±3.5%) incubation temperature with/without SA up to 2hr.

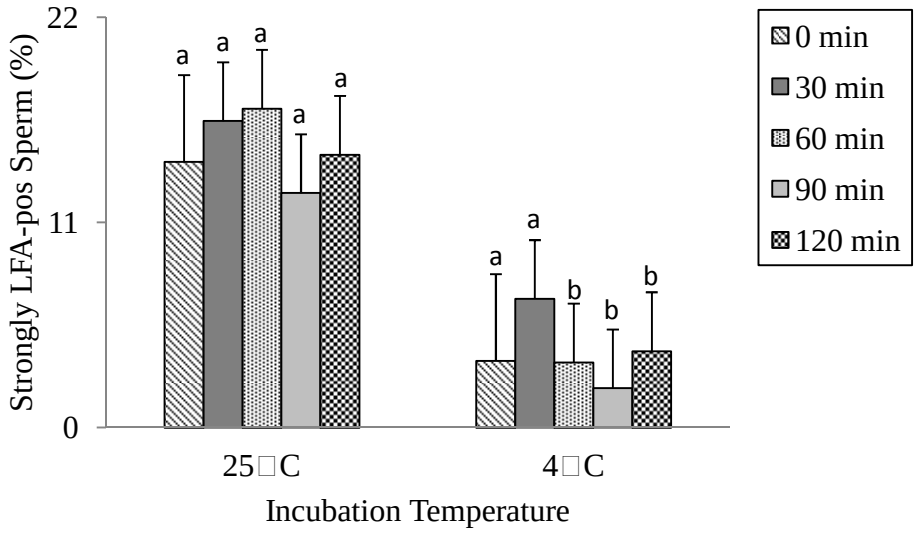


Figure 12. The percentage of membrane intact rooster spermatozoa with strongly LFA-pos fluorescence at 4°C and 25°C incubation temperature with/without SA up to 2hr. The different superscripts between the two incubation temperatures indicates significant differences at those time points ($p<0.05$). Incorporation of SA as assessed by LFA was greater at 25°C than 4°C ($p<0.05$).

Similar to turkey, when the MnFl of the strongly LFA-positive rooster spermatozoa at 25°C (Figure 13) was analyzed, none of the time points were different from 44.1 ±3.1 MnFl ($p>0.05$). Therefore, we elected to incubate sperm for the shortest duration of time (30 min) in experiment 2.

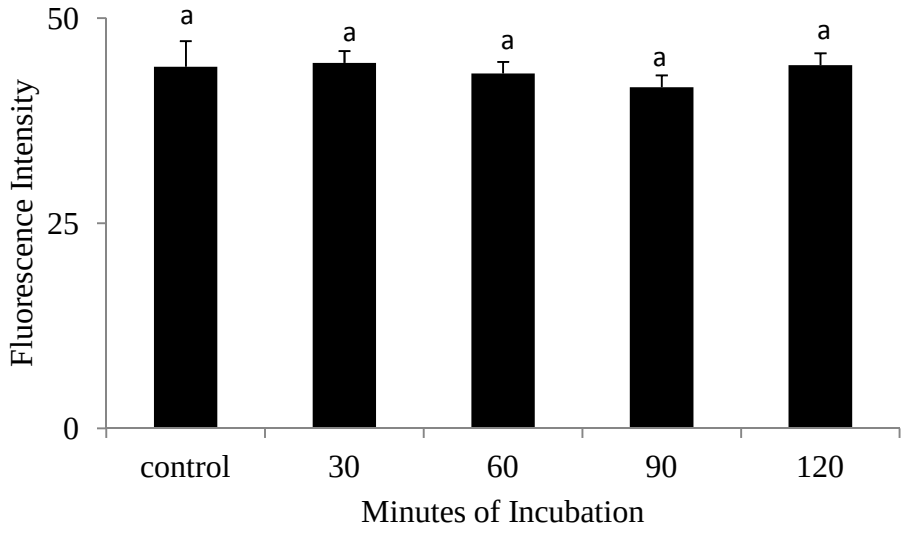


Figure 13. The mean fluorescence intensity of strongly LFA-positive rooster sperm at 25°C over time.

When the percentages of the strongly LFA-positive rooster spermatozoa in the control and SA treatments at 25°C for 30min (9.3 to 18.9% ±4.6%) (Figure 14) were analyzed, none of the SA doses were significantly different from each other ($p>0.05$). Therefore, we elected to treat the sperm with the three lowest SA doses: 40, 80, or 120 µg/mL in experiment 2.

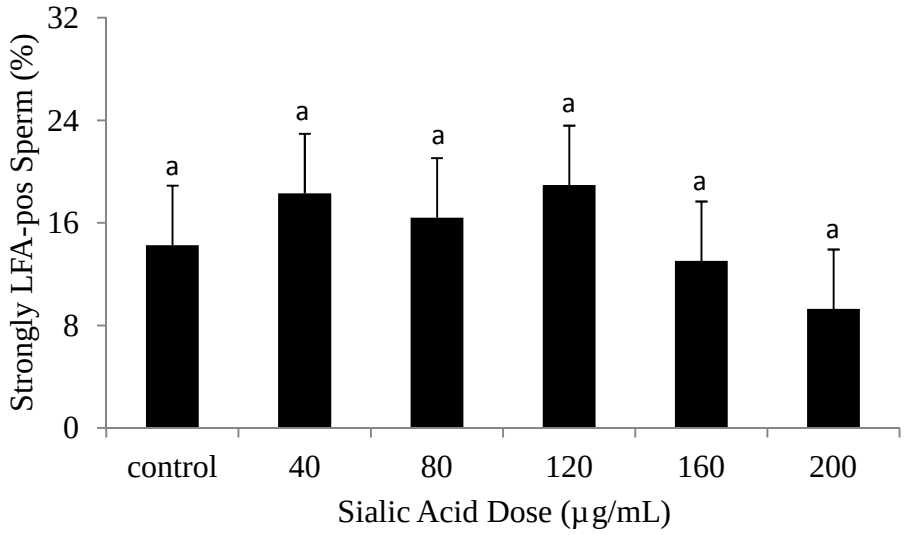


Figure 14. The percentage of strongly LFA-positive rooster spermatozoa at 25°C for 30 min with 0 to 200 µg/mL SA.

The mean percentage of total fertility (Figure 9) for eggs (n=1776) laid over a 10-week period from turkey hens inseminated with frozen/thawed spermatozoa was significantly higher for the 160 µg/mL, pre-freeze treatment than the control (p=0.0164), 80 and 120 µg/mL SA treatments (p=0.0057, p=0.0118 respectively).

Experiment 2: Determining the Optimum Pre-freeze or Pre-freeze/Post-thaw SA Dose for Chicken Fertility.

The mean percentage of total fertility (Figure 15) for eggs (n=825) laid over a two-week period from chicken hens inseminated with frozen/thawed spermatozoa was significantly for the 120 µg/mL, pre-freeze SA-treatment than the controls for the pre-freeze treatments at 5□C, 25□C, and the pre-freeze/post-thaw treatment at 25□C (p=0.0246, p=0.0184, and p=0.0312 respectively). The 120 µg/mL pre-freeze SA-treatment was also significantly better than the 40 µg/mL SA pre-freeze treatment at 25□C (p=0.0511) and the 80 µg/mL SA pre-freeze/post-thaw treatment at 25□C (p=0.0467).

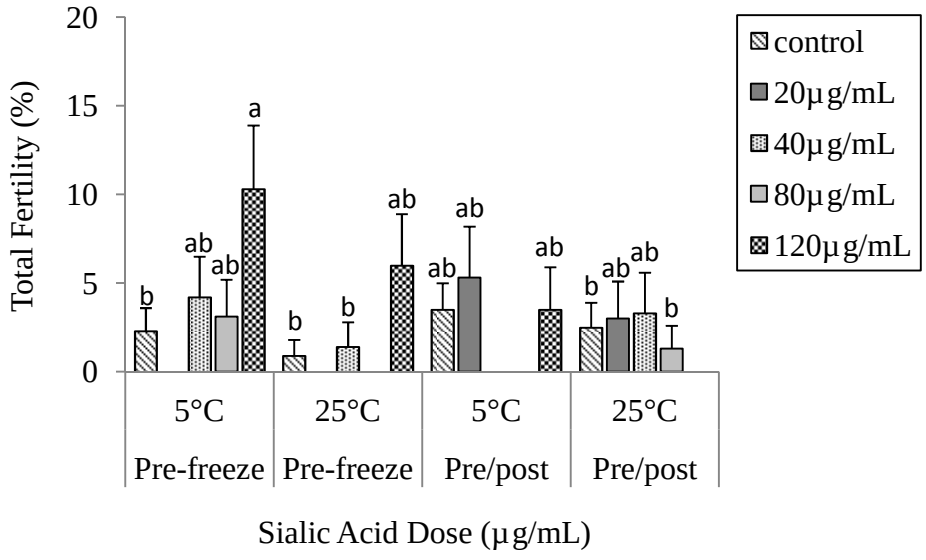


Figure 15. Total percent of chicken fertility with pre-freeze and pre-freeze/post-thaw SA treated sperm. The different superscripts between the four treatment groups indicates significant differences ($p<0.05$).

Discussion

At present, poultry spermatozoa cryopreservation procedures decrease the fertilizing capacity of frozen/thawed sperm. Froman and Engel (1989) observed that modifications in the carbohydrate content of rooster spermatozoa corresponded to a decreased fertility in hens. More recently, it has been shown that the glycoconjugates of the poultry sperm glycocalyx are extensively modified over time during hypothermic storage. Turkey spermatozoa stored for 24 hours at 4□C lose significant amounts of SA residues (Pelaez and Long, 2008). Similarly, Pelaez and Long (2011) demonstrated that the SA content is significantly lower in frozen/thawed rooster spermatozoa. We are the first to provide evidence that the glycocalyx of poultry spermatozoa can incorporate exogenous SA *in vitro*.

SA has been found to mask antigens and receptor sites on the cell membrane (Schauer, 1985). The fact that a large majority of the carbohydrate residues in the poultry spermatozoa glycocalyx are masked by SA was demonstrated following the enzymatic removal of terminal SA residues with neuraminidase (Peláez and Long, 2007). In the hen, spermatozoal SA is necessary for sperm transport to the SST, storage in the SST, transport to the site of fertilization at the infundibulum, and gamete recognition (Steele and Wishart, 1996). Sperm mobility is directly correlated with poultry fertility (Froman et al, 1999; King et al, 2000). Furthermore, immunologic recognition and masking of sugar residues is essential for sperm selection (Steele and Wishart, 1996).

We used flow cytometry to measure the percentage of membrane-intact spermatozoa and the fluorescence intensity produced by spermatozoa incubated

with LFA. Since LFA specifically recognizes and binds to SA residues (Miller et al., 1983), fluorescence intensity is considered to be equivalent to the amount of SA available for binding. Therefore, an increase in fluorescence intensity indicates an increase in SA. As we expected, the percentage of membrane-intact or viable spermatozoa for both toms and roosters were significantly higher at 25ºC than at 4ºC as has been previously shown (Nelson et al., 1980; Kurbatov et al., 1986; Schramm and Hubner, 1988; Wishart, 1989). This study adds to this knowledge by clearly demonstrating the temperature effects on the viability of poultry sperm.

Interestingly, the mean percentage of strongly LFA-positive membrane-intact sperm, indicative of high levels of SA uptake, was different between the species. Turkey sperm SA uptake was significantly higher at 4°C while rooster SA uptake was significantly higher at 25°C. For turkeys, this is evidence that substantial SA shedding is occurring at lower temperatures and therefore more SA residues are available for binding. It has been noted in previous work (Pelaez and Long, 2008, 2011) that when temperatures are reduced there is a decrease in the fluorescence intensity of LFA-positive sperm. It makes sense that if SA acts, in part, as a protective barrier for the sperm as it travels through the hen's reproductive tract, and fertility is adversely affected by hypothermic conditions that one possible reason why we have poor fertility in frozen/thawed poultry sperm is due to high amounts of SA shedding. The question then becomes when to add the SA during the cryopreservation process to allow for optimum uptake of the exogenous SA by sperm to provide added protection.

Because we saw the opposite effects of temperature on the mean percentage of strongly LFA-positive membrane-intact rooster sperm compared to turkey sperm, we speculate that this is due to the species-specific differences in turkey and chicken spermatozoa metabolism, which are likely to contribute to the differences in their ability to survive the freeze/thaw process. While chicken spermatozoa are equally capable of aerobic or anaerobic metabolism (Sexton, 1974), turkey spermatozoa derive their energy requirements by the aerobic process of oxidative phosphorylation only (Sexton, 1974; Wishart, 1981; Sexton & Giesen, 1982).

When we looked at the effect of incubation time on the MnFl of the strongly LFA-positive turkey spermatozoa for each species at their optimum uptake temperature (4°C or 25°C) and found no significant differences in the incubation time, we elected to incubate the sperm in SA for the shortest duration. It is well known that the longer sperm is held *in vitro* with or without extenders, the lower the viability. Donoghue and Donoghue (1997) reported that turkey sperm viability was reduced by almost 50% between 0 and 48 h. Decreased viability in rooster sperm extended with Lake's, the EK extender described by Łukaszewicz, and an extender described by Tselutin et al. and stored for 3, 6, and 24 h at 4°C was demonstrated in four chicken breeds by Siudzińska and Łukaszewicz (2008).

The effect of the control and SA-treated sperm on the percent of strongly LFA-positive sperm at 4°C and 30 min were not significantly different from each other. However, all SA-treated sperm except 120 µg/mL appeared to be better

than the control. Since we did not see a dose response, we decided to use the four highest doses: 80, 120, 160, or 240 µg/mL SA. We speculate that the decrease in the 120 µg/mL might be due to the binding and shedding phenomenon that has been suggested before. We have seen bi-phasic trends with the doses over time. This may be SA shedding and binding mechanisms that we still don't fully understand.

Similar to turkeys, in roosters, the effect of the control and SA-treated sperm on the percent of strongly LFA-positive sperm at 25°C and 30 min were not significantly different from each other. However, we did see a trend of decreased strongly LFA-positive sperm at the 120 µg/mL SA dose. Since we also noticed that the 40 µg/mL (lowest) to 120 µg/mL SA doses appeared to be better than the control, we decided to incorporate a 20 µg/mL SA dose in experiment 2.

When we designed the experiment to assess the fertility of frozen/thawed turkey sperm treated with SA, there was the question of when to add the SA to the cryopreservation process. Since the incubation temperature for the uptake of SA by turkey sperm was best at 4°C, it conformed to the 5°C equilibration step of the established cryopreservation procedures. However, we did not know if we should incubate the sperm before freezing only, after thawing, or at both time points. In our pilot sperm freezing experiments, we looked at pre-freeze and post-thaw incubations for 30 min and none of the post-thaw treatments were successful. We then decided to look further at the pre-freeze for 30 minutes and pre-freeze plus post-thaw for 15 minutes (pre-freeze/post-thaw). The total percent of turkey fertility for 1776 eggs over a ten week period was calculated based on the

number of eggs candled fertile that hatched, or sustained early/late embryonic death. Of all the treatments, the 160 µg/mL SA pre-freeze was significantly the best dose. The 160 µg/mL pre-freeze SA-treated sperm improved total fertility of frozen/thawed sperm (40%) vs. control (7%).

Since 25°C was the best incubation temperature for the uptake of SA by rooster sperm and did not concur with the 5°C equilibration step of established cryopreservation procedures, we had to include a 4°C (pre-freeze and pre-freeze/post-thaw) control group to compare against a 25°C/4°C (pre-freeze and pre-freeze/post-thaw) SA treatment group. Because we found no benefit to extending the post-thaw incubation time (15 min) in turkeys, we opted to use 5 min for the post-thaw incubation time for rooster sperm. Of all the treatments, the 120 µg/mL SA pre-freeze was significantly the best dose. However, this proved to be only slightly successful since, overall, the total fertility from the 120 µg/mL pre-freeze SA-treated sperm (10%) was a marginal improvement over the fertility of control frozen/thawed sperm (3%).

In conclusion, both tom and rooster spermatozoa can incorporate exogenous SA *in vitro*. In toms, the maximum uptake of strongly LFA-positive membrane-intact sperm occurred by 30 min at 4°C. In roosters, the maximum uptake of strongly LFA-positive membrane-intact sperm occurred by 30 min at 25°C. Turkey hens inseminated with 160 µg/mL SA-treated sperm (pre-freeze) had improved total fertility by 40%. Chickens hens inseminated with 120 µg/mL SA-treated sperm (pre-freeze) had slightly improved total fertility (10%). Our findings are still too low for use in commercial poultry management applications

as a method for storage of genetic stocks. However, potential implications for these findings do help to support re-generation of endangered poultry lines.

Chapter 3: Concluding Summary

Many investigators have attempted to improve the fertility rates of frozen/thawed avian sperm by using a variety of cryodiluents, cryoprotectants, packaging methods, and freezing/thawing rates with minimal success. Our goal was to lay the foundation for the development of a standard poultry sperm cryopreservation protocol for turkeys and chickens using those same methodologies, but adding an integral carbohydrate known to provide immunologic protection and assist in sperm transport. We hypothesized that adding protection in the form of SA might be a step forward in achieving a standardized cryopreservation protocol to improve the fertility of frozen/thawed poultry sperm.

We first had to verify that tom and rooster sperm could incorporate exogenous SA and then determine the optimal incubation temperature to maximize the SA uptake by sperm. In order to coincide with the established poultry cryopreservation protocol, we tested the effects of 25□C and 4□C. We are the first to demonstrate that both species of poultry sperm can incorporate exogenous SA *in vitro* and that the viability of the SA-treated sperm is not compromised. In fact, for both species the SA uptake occurred at both 25□C and 4□C. What we found particularly noteworthy early on in our work was the clear differences in the incubation temperature effect on the two species. While the viability of both tom and rooster sperm was nearly a mirror image of each other at 25□C, the temperature effect on the SA uptake by species was the complete opposite. To our surprise, at the 4□C incubation temperature, tom sperm SA

uptake was substantially higher than the uptake observed at 25□C, whereas, the rooster sperm SA uptake was significantly higher at 25□C.

Once we determined the optimal incubation temperature for the SA uptake by both species, we had to determine if there would be a benefit to extending the incubation time beyond 30 min. The results for incubation time clearly demonstrated that optimal SA uptake by sperm occurs by 30 min and there is no advantage to be gained by extending the incubation time period to 120 min.

The next obstacle to overcome was to determine the appropriate SA dose to maximize the SA uptake without compromising the sperm viability. We chose to use 40, 80, 120, 160, or 200 µg/mL SA (roosters), or 240 µg/mL SA (toms). While we did not see a dose response among the control and SA-treated sperm due to the high SEM, we did notice that the SA uptake of the SA-treated sperm appeared to be equally higher than the control (except 120 µg/mL SA) in toms and (except 160, or 200 µg/mL SA) in roosters. Taking this into account, we elected to use the four highest SA doses (80, 120, 160, or 240 µg/mL) in experiment 2 for toms and the four lowest SA doses (20, 40, 80, or 120 µg/mL) in experiment 2 for roosters.

In summary from our first experiment for toms: incubate sperm at 4□C for 30 min with 0, 80, 120, 160, or 240 µg/mL SA prior to cryopreservation; for roosters: incubate sperm at 25□C for 30 min with 0, 20, 40, 80, or 120 µg/mL SA prior to cryopreservation.

For evaluating the optimum pre-freeze or pre-freeze/post-thaw SA dose (experiment 2) for toms, we first diluted raw semen 1:1 with ASG (325mOs/Kg,

7.1 pH) plus SA cryodiluent. Diluted semen was equilibrated for 30 minutes at 4^0C, and then further diluted 1:2 with ASG containing 18% DMA for a final concentration of 6% DMA. Semen was immediately loaded into 0.5 mL French straws and contained with sealing powder. The straws were placed on a Styrofoam raft and floated 1.25 cm above the liquid nitrogen (LN_2) for two minutes, then straws were plunged into the LN_2 and placed in a LN_2 canister for -196ºC storage.

For roosters, raw semen was diluted 1:1 with Hanzawa (360mOs/Kg, 6.8 pH) cryodiluent containing none or 4X concentration of SA, equilibrated for 30 minutes at 5^0C, then further diluted 1:1 with 18% methyl acetimide (MA) to yield a final concentration of 9% MA. Semen was immediately loaded into 0.5 mL French straws and contained with a sealing bead. The straws were placed on a Styrofoam raft and floated 4.5 cm above the LN_2 for 30 minutes, and then straws were plunged into the LN_2 and placed in a LN_2 canister for -196ºC storage.

Prior to inseminating the hens with the pre-freeze treatments, tom and rooster straws were removed from LN_2 storage and thawed by vigorous agitation for 30 seconds in 5^0C water bath. The excess water was removed from each straw and straw ends were cut and allowed to flow directly into an Eppendorf tube. Each turkey hen was inseminated with 300 x 10^6 sperm, and each chicken hen received 200 x 10^6 sperm per dose, per day, for two consecutive days.

Before inseminating the turkey hens with the pre-freeze/post-thaw treatments, thawed tom sperm was incubated a second time with either 0, 80, 120, 160, or 240 µg/mL SA at 4□C for 15 min. Likewise, rooster sperm was incubated

a second time prior to inseminating chicken hens, however, unlike the toms, we had to incorporate a 4°C equilibration control with the 25°C/4°C pre-freeze/post-thaw rooster treatments. Therefore, thawed rooster sperm was incubated a second time with either 0, 20, 40, 80 or 120 µg/mL SA at 4°C for 5 min or at 25°C/4°C for 5 min before inseminating the chicken hens.

Following the insemination of the hens, eggs were collected daily and set on day seven. A total of 1776 (10 weeks) turkey and a total of 825 (2 weeks) eggs were individually candled after seven days of incubation and categorized as fertile or infertile. Fertile eggs were placed back in the incubator to hatch while infertile eggs were broken out to verify infertility or early (<48h) or late (>48h) embryonic death. All fertile eggs were included in the statistical analysis.

In the turkey hens, the 160 µg/mL SA pre-freeze sperm treatment clearly yielded the highest percent of total fertility. However, the same success did not hold true for the percent of total fertility in chicken hens. Although the pre-freeze 120 µg/mL SA treated sperm proved to be significantly better than the pre-freeze controls, it was only a 10% improvement, whereas, the turkey fertility improved by 40%.

We have achieved some success in obtaining our goals for this project. Since previous work over the last 60 years has seen slow progress in improving the fertility rates of frozen/thawed poultry sperm, we certainly did not expect to solve the problem from these experiments alone. However, we have some noteworthy findings that can be further explored in future efforts to develop a standard poultry sperm cryopreservation protocol to improve the fertility rates of

frozen/thawed tom and rooster spermatozoa. We feel strongly that the fertility of frozen/thawed poultry spermatozoa can be improved not only by maintaining the proper pH, osmolarity, and providing a continuous energy supply, but also through the addition of enhanced protection in the form of SA. We propose that inclusion of pre-freeze SA-treated poultry spermatozoa cryopreservation should be a starting point for future studies aimed at improved fertility rates of frozen/thawed avian sperm.

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