Artificial Insemination in Poultry

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Additional information is available at the end of the chapter

1. Introduction

Artificial insemination (AI) is the manual transfer of semen into the female's vagina. Basically it is a two step procedure: first, collecting semen from the male [1]; and second, inseminating the semen into the female [2]. In poultry, depending on the objectives and goals of the farm or laboratory, there may be intervening steps such as semen dilution, storage, and evaluation.

Artificial insemination is practiced extensively with commercial turkeys. This is primarily the result of selective breeding for a heavier and broader-breasted commercial turkey and the consequent inability of toms to consistently transfer semen to the hen at copulation. The broiler industry has not adapted AI to the extent of the turkey industry but it is occasionally used in pedigree lines and in regions where labor is relatively cheap.

To grasp the magnitude of AI in the turkey industry compared to that of livestock, a hypothetical flock of 500 breeder hens inseminated with 100 μL of diluted semen (1:1) twice the week before the onset of egg production and once weekly thereafter for the 24 wk of egg production would entail 13,000 inseminations using 650 mL of semen. It should be apparent with these numbers, semen collection and hen inseminations are labor intensive as each male and female must be handled each week.

Looking back over the use of AI in the turkey industry one can safely say that in the 1960s, weekly inseminations were based on semen volume per dose using undiluted semen. In the 1970s and early 1980s, breeder farms began to dilute semen and inseminate a known number of sperm per dose. In the mid-1980s through the 1990s, hens were initially inseminated a week before the onset of lay and inseminations were performed with a known number of 'viable' sperm. Currently, while inseminating before the onset of egg production remains widely practiced, most companies, but not all [3], have gone back to inseminating a known volume of semen or number of sperm per dose, in the 1970s and 1980s.

In the following chapter we will review the basics of AI and fertility evaluation in poultry. To better appreciate the biological basis of these techniques, an overview of the reproductive biology of poultry is provided. Detailed descriptions of techniques for the collection, evaluation, dilution, and storage of poultry semen are available in a recent publication by Bakst and Long [4]. Earlier comprehensive reviews include Lake and Stewart [5], Bakst and Wishart [6], and Bakst and Cecil [7].

2. Reproductive biology of poultry

This section will introduce to some and review for others the strategy of avian reproduction with emphasis on the hen. For more comprehensive reviews on reproduction in the avian male and female see Jamieson [8].

2.1. Overview

The goal of AI is to produce a succession of fertilized eggs between successive inseminations. To accomplish this, weekly inseminations must replenish the sperm population in the uterovaginal junction (UVJ) sperm storage tubules (SSTs). Birds do not have an estrous cycle that synchronizes copulation with ovulation. Alternatively, about 7-10 days before their first ovulation, hens mate, sperm ascend the vagina and then enter the SSTs. At the onset of egg production, individual sperm are slowly released from the SSTs, transported to the anterior end of the oviduct, and interact with the surface of the ovum (see [9-10] for recent reviews). Whether fertilized or not, over the next 24-26 hr the ovum is transported though the oviduct, accruing the outer perivitelline layer (PL) in the infundibulum, the albumen in the magnum, the shell membrane in the isthmus, and the hard shell in the uterus (also referred to as the shell gland) before oviposition. If fertilized, the blastoderm in the first laid egg consists of 40,000-60,000 cells in the turkey and 80,000-100,000 cells in the chicken.

Ovary: In the hen only the left ovary and oviduct become functional organs. About 2-3 wk before the onset of lay, small (less than 1 mm in diameter) white-yolk follicles begin to accumulate yellow yolk with some being recruited into a hierarchy of maturing yellow-yolk follicles (Figure 1). At the time of ovulation, the largest follicle, designated as F1, is ovulated. About 17 days were necessary for the 1 mm diameter white yolk follicle to mature to a pre-ovulatory 40 mm diameter yellow yolk follicle [11]. After the F1 follicle is ovulated, the next largest follicle, formerly designated F2, becomes the F1 follicle and will ovulate at the beginning of the next daily "ovulatory cycle" in 24-26 hr.

The follicular sheath surrounding the maturing oocyte consists of histologically distinct concentric layers of cells: the outer serosa (germinal epithelium); the theca externa, which forms the greatest portion of the follicle wall, provides structural support to the follicle and has steriodogenic cells; the theca interna, a highly vascularized layer, which like the theca externa has steroid-producing cells (both thecal layers synthesize androgens and estrogens); and, the granulosa cell layer, enveloping the oocyte, which is responsible for progesterone secretion and the synthesis of the inner PL. The inner PL is homologous to the mammalian zona pellucida and

is a fibrous reticulum about 2 μm thick. At ovulation, only the inner PL envelops the ovum. While there is no corpus luteum formation in birds, the thecal layers and the granulosa of the post-ovulatory follicle (POF) produce prostaglandins [12] and progesterone, respectively [13-16] then regress over the next 72 hr. The POF has a pocket like appearance after ovulation (Figure 1). On the surface of the inner PL overlying the germinal disc (GD), which is a 3.5 mm diameter disc of white yolk containing the haploid pronucleus and associated organelles, are sperm receptors. Sperm bind to the receptors overlying the GD, hydrolyze a path through the inner PL, and are incorporated into the ovum. Polyspermy is normal in birds but only one sperm in apposition to the female pronucleus undergoes nuclear decondensation and initiates syngamy, the reconstitution of the diploid number of chromosomes.
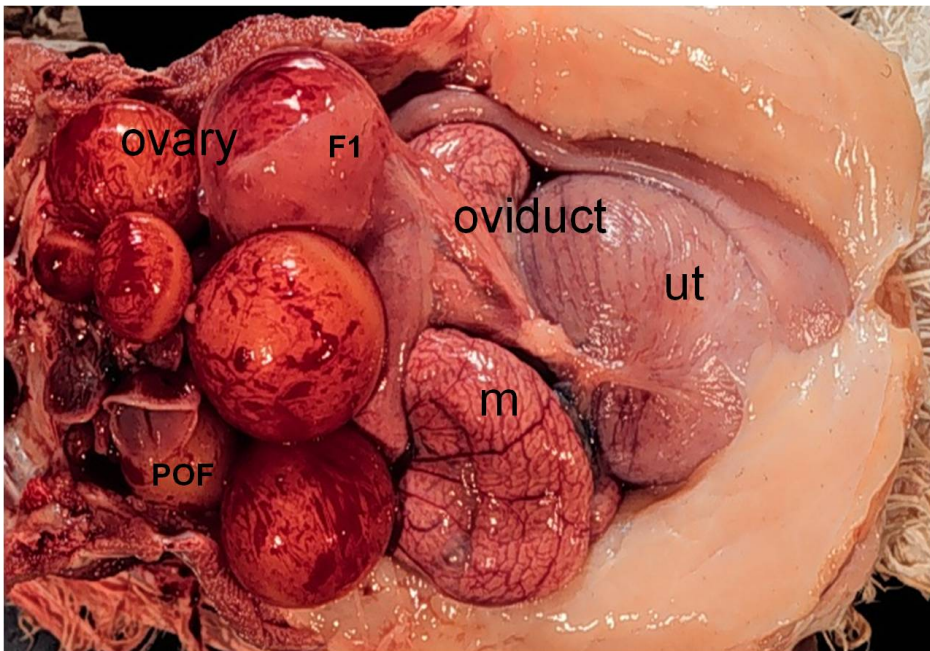

Figure 1. The ovary and oviduct of a turkey hen in egg production occupy much of the abdominal cavity. The ovarian follicular hierarchy consisting of ovarian follicles at various stages of develop (7 maturing follicles visible in this photograph) is observed. The largest follicle, F1 follicle is the next to ovulate. The ovum ovulated about 10 hr earlier has accrued albumen in the magnum (m), a shell membrane in the isthmus, and is observed in the uterus (ut) undergoing shell formation. Its post-ovulatory follicular sheath (POF) appears as an open pocket. The vagina (distal to the uterus and not visible) is embedded in connective tissue and enveloped by the abdominal fat pad.

2.2. Oviduct

The mature oviduct consists of five anatomically and functionally distinct segments (Figures 1 and 2): the infundibulum, which secretes an albumen-like product that forms the outer PL and prevents pathological polyspermy; the magnum, responsible for deposition of the

albumen proteins; the isthmus, which forms the shell membrane; the uterus (also referred to as the shell gland), a pocket-like structure that elaborates the hard-shell; and, the vagina, which is a conduit between the uterus and cloaca for the egg-mass at oviposition and is responsible for sperm selection and storage following semen transfer. Interestingly, when the vagina and uterus are excised and fixed *in toto* and the connective tissue surrounding the vagina subsequently removed, the vagina appears as a coiled segment (Figure 3) [10]. This anatomy explains the resistance one feels when performing a vaginal insemination with a straw regardless of the presence or absence of an egg mass in the uterus. If inseminating a hen within 30 min after oviposition, the connective tissue around the vagina and the smooth muscle composing the vaginal wall are flaccid. Venting (exteriorizing the vagina for placement of the inseminating straw) at this time may induce a partial prolapse leading to a deep insemination (closer to the UVJ) and the forfeiture of sperm selection by the vagina. Such deep inseminations are associated with high embryo mortality, possibly due to pathological polyspermy.

The surface mucosa of each segment of the oviduct is lined with parallel, gently spiraling folds along the longitudinal axis. The surface epithelium lining the luminal mucosa contains varying proportions of secretory and ciliated cells. All segments except the fimbriated region of the infundibulum and the vagina possess sub-epithelial tubular glands that secrete components used in egg formation [17]. However, the anterior 2-3 cm of the vagina, an area referred to as the UVJ (Figure 3), contains the SSTs, the primary sites of sperm storage [10] (Figure 4).

At ovulation, the ovum is grasped by the fimbriated region of the infundibulum and, if sperm are present, the ovum may be fertilized within a 10-15 min interval [18]. Thereafter, infundibular secretions accrue around the ovum, forming the outer PL, which acts as a barrier to further sperm penetration. Birkhead [19] observed that the number of sperm trapped in the outer PL is positively correlated with the size of the ovum and is likewise correlated with the number of sperm that have penetrated the inner PL. Interestingly, the sperm trapped in the outer PL retain an intact acrosome [20-21]. If fertilized, the first cleavage furrow in the GD appears 7-8 hr post-ovulation, while the egg-mass is in the isthmus.

2.3. Oviductal sperm selection, transport, and storage

Following deposition in the oviduct, sperm are transported to UVJ by a combination of their intrinsic motility and cilia beat activity [9-10, 22-23]. Within the SST lumen, sperm are either widely spaced or oriented parallel with their heads toward the distal end of the SST (Figure 4). Sperm are apposed to, but not directly contacting the apical microvilli of the SST epithelial cells. This spatial relationship may facilitate lipid transfer between the resident sperm and the SST epithelial cells [24-25]. Interestingly, alkaline phosphatase, known to play a role in lipid transfer, has been histochemically localized in the apical region of the SST epithelium [26].

The duration of sperm storage in the SSTs is species-dependent. Chickens can store sperm for up to three weeks, whereas turkeys can maintain sperm for 10 weeks in the SST and still lay a fertilized ovum [27-28]. This may be related to number of SSTs present in the UVJ; turkeys have been reported to have 20,000-30,000 SSTs, while chickens have been estimated to have

only 5,000-13,500 [29-30]. Additionally, after several generations of selection for high fertility, chicken hens possessed increased numbers of SSTs when compared to non-selected control hens, suggesting the number of SSTs may be positively correlated with fertility [31]. In contrast, under commercial conditions, different broiler strains exhibiting different fertility levels revealed similar numbers of SSTs [29].
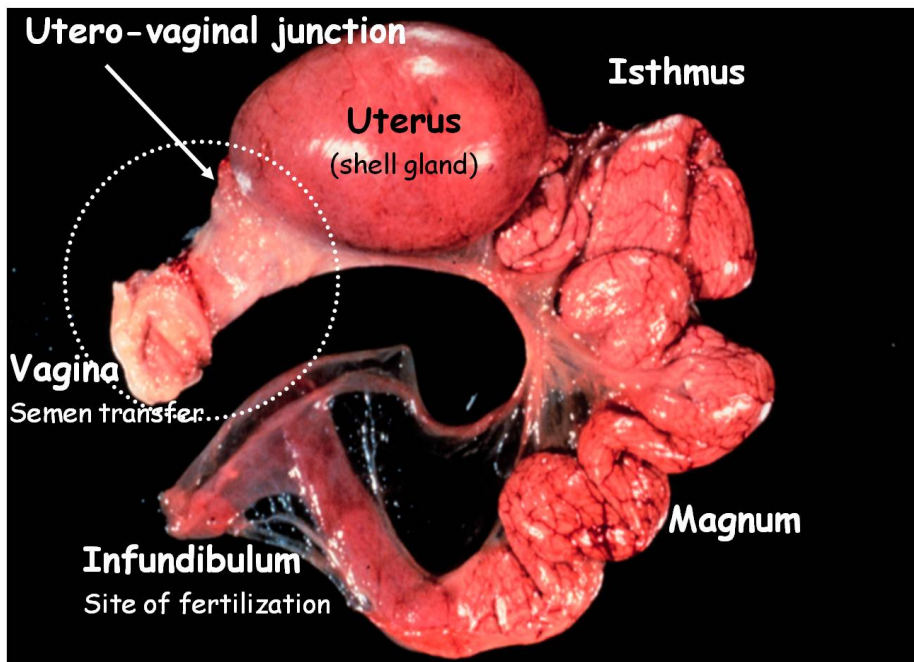

Figure 2. The segments of the turkey oviduct with a hard-shelled egg in the uterus are observed. Sperm transferred into the vagina undergo an intense selection process before reaching the sperm storage tubules (SSTs) localized in the utero-vaginal junction. Sperm are slowly released from the SSTs and ascend to the infundibulum, the site of fertilization. In this photograph, the vagina is enveloped by connective tissue.
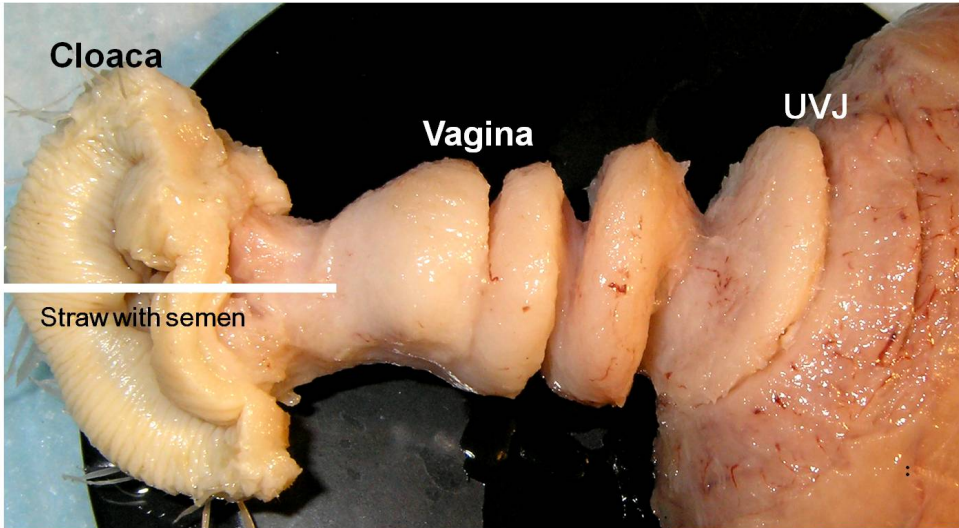
Figure 3. Following fixation in neutral-buffered formalin and the removal of the surrounding connective tissue, the coiled morphology of the turkey vagina is revealed. When inseminating a hen, one should insert the straw with the semen until resistance is felt, then release the semen as the straw is withdrawn. As observed here the resistance is due to the coiled vaginal and not an egg mass in the uterus.

Little is known concerning the cellular and molecular mechanisms that sustain sperm within the SST lumen for prolonged periods of storage. These mechanisms likely involve the reversible suppression of sperm motility and metabolism, protection and repair of the sperm plasma membrane, uptake and storage of molecules to sustain sperm metabolism, and maintenance of the SST lumen by removing by-products of sperm metabolism and degraded sperm [32-33]. It is clear the SSTs generate a discrete environment to maintain sperm viability via the influx and efflux of compounds critical for sperm survival [25, 34]. While ultrastructural analysis has revealed only limited evidence of secretory activity [25], the identification of membrane-bound vesicles released from the apical tips of the SST epithelial cell microvilli suggests a role in the maintenance of resident sperm through lipid transfer [22, 25, 26, 32, 35, 36]. A large proportion of the sperm plasma membrane is composed of polyunsaturated fatty acids [37] that are highly susceptible to damage induced by lipid peroxidation [37]. The peroxidation of these fatty acids results in increased damage to and permeability of the sperm plasma membrane [39, 40]. A complex system of anti-oxidation enzymes are present in the SST epithelial cells and presumably interact with luminal sperm to minimize damage due to lipid peroxidation and maintain sperm membrane integrity [41]. While many metabolites required by sperm in the SSTs have yet to be identified, increased avidin expression is apparent in SSTs relative to surrounding UVJ epithelial tissue possibly providing a means of sequestering biotin and other vitamins for use by the SSTs or resident sperm [42-43]. Interestingly, progesterone has been shown to induce expression of avidin in the oviduct, providing a potential link between progesterone fluctuation and sperm storage in and release from the SSTs [42, 44, 45].
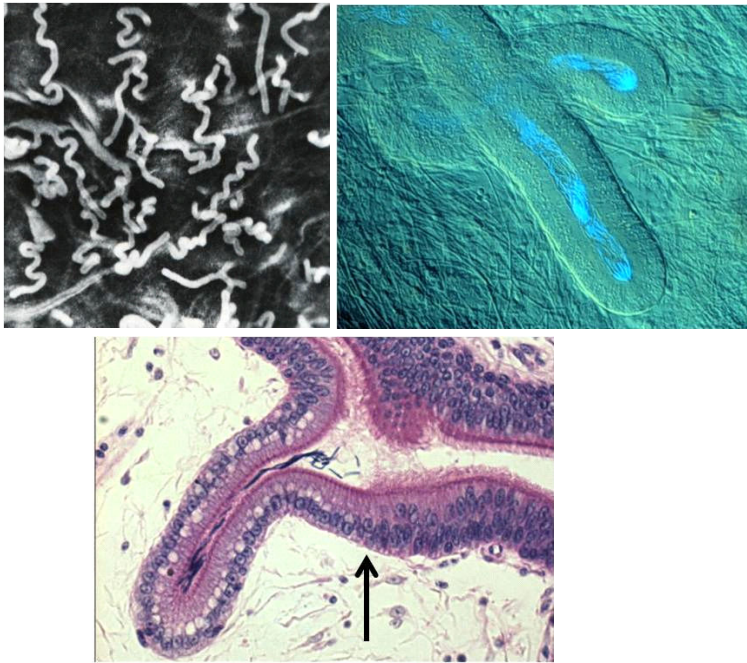
Figure 4. Three views of the turkey's sperm-storage tubules (SSTs) are observed. The left panel is a stereoscope image showing the pleomorphic appearance of the SSTs. The length of the SST can be as long as 300μm. In the right panel a hen was inseminated with sperm stained with Hoechst 33342, a nuclear fluorescent dye, the UVJ mucosa containing SSTs was isolated, and an unfixed squash preparation was observed by dual interference contrast and fluorescence microscopy. Sperm with fluorescing nuclei are observed in the two SST lumina. The lower-middle panel shows a histological section of a portion of a SST containing sperm (the dense rod-like structures in the lumen are sperm nuclei. The arrow indicates the transition between the pseudo-stratified columnar ciliated epithelium of the uterovaginal junction and the simple columnar epithelium of the SST that is characterized in histological preparations by the supra-nuclear vacuole.

Sperm exit the SSTs in a slow, continuous stream [46-49]; however, a stimulus cuing the egress of resident sperm from the SSTs has yet to be identified. The observations that receptors for estrogen and progesterone exist in the SSTs has led to the suggestion that these compounds may trigger release of resident sperm, possibly in response to hormonal cues over the course of the ovulatory cycle[50-52]. However, an alternate theory suggests the inherent mobility of the sperm plays a larger role than hormonal induction in egress of sperm from SSTs [9]. Resident sperm exhibit a slow, synchronized oscillatory movement in the lumen of SSTs, suggesting the presence of a fluid current through the SST lumen [23-24]. The identification of water channels, known as aquaporins, in the apical epithelium of SSTs lends credence to a model wherein motile sperm maintain their residence in the SST lumen by swimming against the fluid current generated via the aquaporins [53-56]. In the SST lumen, sperm retain their motility by fatty acid oxidation. It has been suggested the sperm membrane is the source of this fatty acid and that as the quality of the sperm membrane gradually decreases there is a

reduction of available ATP and sperm motility decreases [56]. Sperm are then swept out of the SST lumen into the UVJ, where they encounter various stimuli enhancing their motility. These sperm are then transported to the infundibulum, the site of fertilization [57]. Such motility-enhancing factors may include changes in environmental pH and neuroendocrine factors such as serotonin [58-62]. Further oxidation of sperm fatty acids, possibly sequestered from the surround milieu, generates the energy required for sperm to respond to such motility-enhancing factors and transcend the oviduct [9, 22, 55, 63].

Once sperm are deposited in the oviduct, several selection barriers must be overcome prior to ascending to the infundibulum and fertilizing an ovum. This selection occurs initially in the vagina: only highly mobile (defined as progressive movement in a viscous medium at 40°C) sperm traverse the vagina [9]. While sperm mobility is a major factor in sperm selection in the vagina, sperm selection is also dependent upon the glycoprotein composition of the sperm plasma membrane. The sperm glycocalyx is highly complex and heavily sialylated and modification of the glycocalyx results in reduced fertility and failure of the sperm to enter the SSTs [64-67]. Interestingly, removal of membrane-associated carbohydrates did not affect sperm entry into SSTs if sperm were inseminated directly into the UVJ or when co-incubated with UVJ explants, suggesting the glycocalyx plays a central role in sperm transport and selection through the vagina [64, 66, 68]. Further barriers to sperm prior participating in the process of fertilization include sperm release from the SST and subsequent transport to the infundibulum, and their interaction with the ovum (reviewed in [69]).

2.4. Sperm: Ovum interaction and fertilization

Given the voluminous nature of the hen's ovum and the GD relative to mammalian ova, one must assume that yet-to-be identified factors "attract" sperm to the GD. Examination of the electrophoretic profile of the GD and non-GD regions of the PL revealed no variation in protein composition [70]. Furthermore, the abrogation of the preferential interaction of sperm and the inner PL overlying the GD *in vitro* suggests the factors underlying the preferential binding of sperm are not necessarily associated with the inner PL [70]. It is clear, however, glycoproteins play a large role in the interaction between the sperm and ova, even if not directly involved in targeting of sperm to the GD *in vivo* [71]. Pre-treatment of either the PL or sperm with N-glycanases resulted in significantly decreased sperm-ovum interaction *in vitro* [68, 71]. Conversely, N-linked oligosaccharides released from the inner PL by N-glycosidase treatment could induce the acrosome reaction in sperm *in vitro* [72]. These findings strongly suggest N-linked glycans, most likely terminal N-acetyl glucosamine residues, have an essential role in the sperm-ovum interaction in avian species, specifically in induction of the acrosome reaction [68, 72].

Interaction between the sperm and inner PL results in induction of the acrosome reaction [73]. During the acrosome reaction, the inner and outer acrosomal membranes dehisce resulting in the release of acrosin (a trypsin-like enzyme) [21, 74]. As the result of the acrosome reaction, sperm hydrolyze a small hole in the inner PL (Figure 5), enabling sperm to reach the microvilli-studded surface of the ovum [21, 74]. The capacity of sperm to hydrolyze and penetrate the inner PL is the biological basis for the sperm penetration assay discussed below and next section.
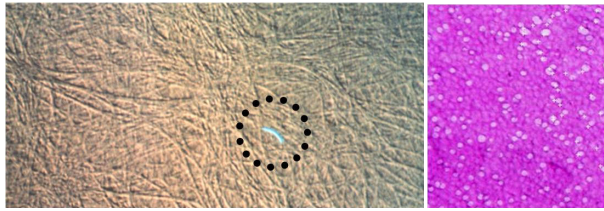
Figure 5. In the left panel, a turkey sperm stained with Hoechst 33342 prior to insemination is observed on the surface of the inner perivitelline layer (PL). The sperm's acrosome will release a trypsin like enzyme, acrosin, and digest a hole through the inner PL. The right panel shows multiple sperm holes (white perforations) in the inner PL overlying the germinal disc (GD) of a duck ovum (polyspermy is normal in birds). Sperm hole numbers can be used to assess true fertility and the duration of the fertile period..

Unlike mammals, polyspermy is the norm in avian fertilization. The GD (3.5 mm in diameter) provides a relatively small target for fertilization in the large megalecithal ova (yolk-filled ova) of chickens and turkeys (3.5 – 4.0 mm in diameter); thus polyspermy may be an evolutionary adaptation to ensure higher rates of fertilization in such species [74]. The inner PL may be penetrated by many sperm, although only one male pronucleus will ultimately fuse (syngamy) with the female pronucleus to form the nascent embryo (reviewed in [75-77]. A single sperm hole in the inner PL does not ensure fertilization. Although turkeys show a lower number of sperm interacting with ova relative to chickens, the presence of three sperm holes in the inner PL predicts a 50% probability of fertilization, whereas, six sperm holes suggest a probability greater than 95% fertilization [78]. The outer PL is rapidly deposited around the ovum in the posterior infundibulum and proximal magnum and is impenetrable by sperm [21, 78-79] thus preventing pathological polyspermy.

Given the volume of the GD relative to a single sperm, another possible function of polyspermy may be to activate specific molecular factors in the GD cytoplasm thereby initiating the process of embryogenesis. Yet, polyspermy also results in the presence of multiple male pronuclei in the GD. To cope with this potentially harmful scenario, the mature ovum has been found to have DNase I and II endonuclease activities, both of which will degrade sperm DNA [76]. In contrast, no such DNase activity has been detected in mammalian ova that engage in mono-spermic fertilization, further suggesting the role of these enzymes in the avian embryo is to protect against detrimental genetic consequences of polyspermy [76].

The number of holes in the inner PL is highly positively correlated with fertility. Correlations exist between the number of sperm inseminated, the number undergoing the acrosome reaction at the inner PL [80], and the number of sperm embedded in the outer PL [81]. The number of sperm holes in the inner PL and the number sperm trapped in the outer PL may be used to estimate the duration of fertility ('fertile period') in hens. While the number of sperm penetrating the inner PL shows a decreasing logarithmic relationship over time [81-82], a positive correlation between the total number of sperm penetrating the inner PL and the number of sperm stored in the SSTs was observed [83]. Given these observations, it should not be surprising there is also a positive correlation between the number of SSTs containing sperm and the proportion of sperm that have undergone the acrosome reaction at the inner PL [82].

3. Techniques in artificial insemination and fertility evaluation in poultry

For non-domestic birds, chapters in Bakst and Long [4], Lake and Stewart [5] and Bakst and Wishart [6] provide overviews of semen evaluation and AI techniques. Artificial insemination technology and reproductive biology for ratites were reviewed by Malecki et al. [84].

3.1. Semen collection

Primarily due to the anatomical variation of the phallic region in different birds, semen collection techniques will vary. In contrast to ratites and water-fowl with an intromittent phallus, Galliformes (chicken, turkey, and quail) do not have an intermittent organ. Their non-intromittent organ consists of folds and bulges that make contact with the female's cloaca at mating. From an anatomical perspective, there are considerable differences between the non-intromittent organs of the chicken and turkey (Figure 6). The rooster has a prominent medial phallic body and relatively small lateral phallic bodies and lymph folds. Conversely, the turkey tom has no medial phallic body but prominent lateral phallic bodies and lymph folds. Sex sorting at hatch by cloacal examination is based on the relative differences in size of these structures between the males and females.
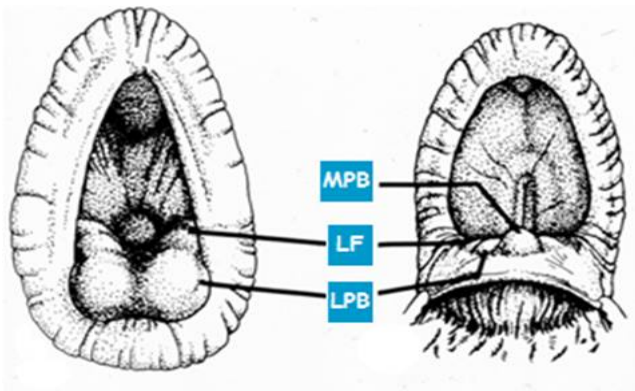

Figure 6. The turkey (left) and chicken (right) cloacae are viewed with the dorsal lips of the cloacae pulled back to expose each species phallus non-protrudens. Unlike the turkey, the chicken has a central protuberance, the medial phallic body (MPB) and regressed lateral phallic bodies (LPB) and lymph folds (LF). The turkey phallus non-protrudens is characterized by dominant LPB and LF and the conspicuous absence of the MPB.

The goal of semen collector is to obtain the maximum volume of clean, high quality semen with the minimal amount of handling. In chickens and turkeys, the abdominal massage technique [1, 4] involves massaging the cloacal region to achieve phallic tumescence. This is followed by a 'cloacal stroke', a squeezing of the region surrounding the sides of the cloaca to express the semen. Little additional semen can be expressed after two cloacal strokes; additional cloacal strokes may cause damage to the phallic and cloacal regions and contribute to semen contamination [85].

Semen should be pearly white, viscous, and clean. With each male collected, the semen collector should perform a visual examination of the semen at the time of ejaculation. This is easier with the turkey because the ejaculate accumulates on the phallus before it is collected by the 'milker' (semen collector). Off-color or watery semen, and semen contaminated with blood or fecal/ urates debris should not be used for insemination. Due to the increased volume of transparent fluid in rooster semen, which is a transudate derived from the phallus at the time of ejaculation, chicken semen is less viscous and sperm concentration lower than that of turkey semen.

3.2. Sperm concentration

If semen is to be diluted, it is best .to have a known volume of semen diluent (a tissue culture-like medium formulated to sustain sperm viability) at ambient temperature in the semen receptacle before collection begins. For routine AI of turkey hens, semen from 10-12 toms are pooled in a single receptacle, mixing the semen gently after each male is collected. Semen volume is determined and if the AI dose is based on numbers of sperm (generally 250-350 million sperm per dose) sperm concentration is determined. The most popular techniques for determining sperm concentration are the packed cell volume (PCV; also referred to as a spermatocrit) and optical density (OD; photometry).

Determining sperm concentration using PCVs is nearly identical to that of determining blood hematocrit values. Semen aspirated into micro-hematocrit tubes are centrifuged in a hematocrit centrifuge until the sperm are tightly packed (10 min); the percentage of packed sperm cells relative to the original semen volume in the micro-tube is determined. Sperm concentration is derived using a conversion factor or standard curve previously derived by comparing and graphically plotting varying ascending sperm concentrations from hemocytometer counts to corresponding spermatocrit readings. (See [4] for detailed protocols to determine sperm concentration and the derivation of standard curves.)

The optical density (OD) is determined using a photometer. The OD of highly diluted semen is directly proportional to the concentration of sperm, thus providing an indirect estimate of the sperm concentration. Like the PCV method, sperm concentration is derived using a conversion factor or previously derived standard curve by comparing and graphically plotting varying sperm concentrations from hemocytometer counts to corresponding OD readings [4].

The PVC and OD methods are two *indirect* methods of determining sperm concentration, that is, the final concentration is calculated from a regression equation or standard curve derived, in part, from *direct* sperm counts with a hemocytometer [4]. Briefly, to derive a regression equation and standard curve, serial dilutions (n=5) covering a wide range of sperm concentrations are prepared and sperm concentrations are determined with a hemocytometer and the instrument or method that requires the standard curve (at least 4 replicates with 4 different semen samples). This is a tedious procedure but if reliable and repeatable sperm numbers are to be inseminated it is best to establish standard curves for each instrument every 12-18 months. The reason for this is that the rotational speed of different centrifuges and the intensity of a photometer's light source may differ as a result of manufacturer's variation, age of the instrument, and/or repeated use of the instrument, thereby producing variations in the respective final readings and subsequent calculations of sperm concentrations.

Another concern when using any semen evaluation method is variation in the operator's techniques. Consistency is the key to repeatable data. The technical staff all must follow the same standard operating procedures (SOPs). For example, when counting sperm with a hemocytometer, all individuals in a lab should following the same SOP for how long the sperm are permitted to settle on the grid and which sperm to count or omit from the count. Also, is the photometer zeroed with the same buffer? If a procedure calls for an incubation period, such as in a live-dead stain, are the samples being incubated for the same duration each time using the same stain concentrations? A lack of consistency in following the SOPs within a laboratory will lead to unwarranted variation and non-reproducible and inaccurate data.

3.3. Sperm viability

In the context of semen evaluation, reference to 'viable' sperm simply implies that such sperm possess an intact plasmalemma and are assumed to be functional. Plasmalemma integrity is frequently determined using either a dead-cell or a live-cell stain alone or simultaneously. The dead-cell stains are excluded by sperm with an intact plasmalemma but stain dead sperm possessing a permeable plasmalemma. Live-cell stains permeate the intact sperm plasmalemma and become visible only after reacting with cytosolic enzymes or interacting with sperm nuclear proteins. Both eosin and propidium iodide are popular dead-cell stains while calcein AM and SYBR-14 are frequently used live-cell stains (see [86] for extended discussion and availability for the live-cell probes). On a commercial breeder farm, the nigrosin/eosin (N/E) technique is most likely the procedure to be used to determine sperm viability [4]. Briefly, sperm are stained with N/E and a smear of the stained sperm is made on a slide (Figure 7). Under a bright field microscope the viable sperm remain pearly white, while eosin will stain non-viable sperm a pink to magenta color. The nigrosin serves as a background to enhance differentiation between the non-viable and viable sperm. In contrast to the N/E technique, a more sophisticated laboratory may use flow cytometry that sorts viable from non-viable sperm after staining with calcein AM or SYBR-14 and propidium iodide.

Figure 7. The left panel shows a nigrosin eosin preparation of turkey sperm with nearly 100% viable sperm (unstained) white nuclei and midpieces. The sperm head is clearly visible as the white arcing segment; the acrosome and midpiece are difficult to differentiate from the nucleus. The upper right panel reveals a normal sperm and a second sperm with an abnormally curved and swollen midpiece. Observed in the lower right panel is a nonviable sperm stained with eosin throughout the nucleus and midpiece. Barely visible at the anterior end of the nucleus is the unstained, conical shaped acrosome.

3.4. Sperm motility and mobility

Sperm motility can be progressive (forward direction) or non-progressive (random movement or oscillations) movement. Generally, progressive motility is determined subjectively at ambient temperature using a microscope at low magnification (hanging-drop technique) or objectively using a computer-assisted semen analysis system. These techniques are reviewed by Bakst and Long [4]. Motility evaluated by microscopy has been shown to have little correlation with fertility and simply reveals that the sperm are motile. First described by Froman and McLean [87] and further elaborated for commercial use by Froman [88], the sperm mobility assay has gained popularity as a measure of an individual male's ability to produce highly mobile sperm [mobility defines the ability of sperm to move progressively against a viscous medium (Accudenz) at 41°C] that are more likely to fertilize an ovum than males producing less mobile sperm. While the sperm mobility assay is a powerful tool for the selection of the most fecund males to be used in AI, it necessitates attention to details and accurate and consistent preparation of the reagents.

3.5. Evaluation of fertility

The measure of a successful AI program is sustained hen fertility. While candling-fertility is useful, there is an eight or more day lag between the last AI and candling-fertility determination, which overlaps with the next insemination (hen insemination is generally at 7-day intervals). With AI programs, it is often desirable to determine the fertility status of a flock before the next weekly insemination. There are several options available: breaking-out fresh eggs and examining the GD to differentiate a fertilized from an unfertilized or early dead embryo; setting normal but culled eggs (checked, hairline cracked, or dirty eggs) in a spare incubator for 24-36 hr before breaking-out [89]; counting sperm in the outer PL; and counting sperm holes in the inner PL. The above procedures are reviewed in Bakst and Long [4].

As noted previously, the sperm penetration assay is not only used to determine true fertility, but also to estimate the number of sperm residing in the use SSTs at the time of ovulation [90]. The isolation of the inner PL and staining procedure, initially developed for chicken eggs by Bramwell et al. [91], was quickly adapted to turkey eggs by Donoghue [92]. The major drawback to the sperm penetration assay as originally described is that it is time consuming, particularly with respect to isolating, washing, and positioning the PL wrinkle-free on the slide. Spasojevic [10] and colleagues at Willmar Poultry Company (Willmar, MN) significantly increased the efficiency of preparing the PL slides from turkey eggs in the following manner: the albumen is removed from the ovum as in the original procedure [4]; a square is outlined on a slide using super glue; the slide is placed firmly on the ovum's surface with the GD centered in the square; after the glue is set, the PL is cut and washed to remove adhering yolk. The advantages here are speed and the PL remains wrinkle free.

A different modification of the sperm penetration assay was suggested by I.A. Malecki (personal communication) and entails placing a filter ring over the GD (inside diameter slightly larger than the GD), cutting around the outside diameter of the filter ring (about 2 mm between the inside and outside perimeter of the ring), and lifting the filter ring off the ovum. The filter ring with the adhering PL is washed gently with saline to remove the yolk and GD material

until transparent, placed on a slide, and then fixed and stained with saline washes after each step. Our laboratory has used the filter ring technique with eggs from broilers, turkeys, ducks, and quail and it is now our preferred method for the performing the sperm penetration assay.

4. Conclusion

Artificial insemination is a common practice in the poultry industry with the turkey industry in North America and Europe using it almost exclusively for the production of hatching eggs. The broiler industry has not adapted AI for several reasons: because of sheer numbers of broiler breeders that need to be inseminated weekly, the labor cost would be very significantly; the initial investment in special housing for the males; an efficient, cost effective means of actually performing the inseminations (housing and catching the hens) would need to be developed; and finally, the concern that after a few generations of breeding broilers by AI, the behaviors associated with natural mating may be less dominant. Notwithstanding these concerns, the benefits of AI for broilers would include the following: the male:female ratio would be increase from 1:10 for natural mating to 1:25 with AI; with fewer males needed, there would be greater selection pressure on the male traits of economic importance and subsequently greater genetic advancement per generation; biosecurity concerns associated with "spiking" aging hen flocks with new and/or younger males to augment mating frequency and fertility would be eliminated; and, differences in body conformation between males and females that impact semen transfer at mating would no longer be a consideration.

In 1995, Sir Peter Lake wrote an excellent review of the history of AI, its impact on the poultry industry, and what is needed to advance the practice of AI with poultry [93]. Unfortunately, AI technology has not advanced significant since this review article. More than 15 years later, the only significant advance is in the evaluation of sperm mobility and the impact that males producing highly mobile sperm have on paternity [9]. Notwithstanding, it is foreseeable that sometime in the future, research addressing poultry sperm biology and the cellular and molecular basis of oviductal sperm transport, selection, and storage will lead to the following innovations in AI technology: insemination intervals increased to 10-14 days (versus 7-day) with fewer sperm per insemination; *in vitro* sperm storage for 24-36 hr at ambient temperature with minimal loss of sperm viability; and, the possibility of transgenic progeny following the insemination of sperm carrying transgenes.

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References

[1] Burrows WA, Quinn JP. The method of obtaining spermatoza from the domestic fowl. Poultry Science 1935;14(4) 251-4.

[2] Quinn JP, Burrows WA. Artificial insemination in fowls. The Journal of Heredity 1936;27(1) 31-7.

[3] Spasojevic R. Two hundred million sperm cells per hen? No Way! *Proceedings – Midwest Poultry Federation Convention*, Turkey Breeder Workshop, March 16-18, 2010. CD available at MPF. St. Paul, Minnesota. www.midwestpoultry.com

[4] Bakst MR, Long JA. Techniques for Semen Evaluation, Semen Storage, and Fertility Determination. 2, 1-113. 2010. St. Paul, Minnesota, The Midwest Poultry Federation.

[5] Lake PE, Stewart JM. Artificial insemination in poultry. Bulletin 213. 1978. London, UK, Ministry of Agriculture, Fish, and Food. Her Majesty's Stationary Office.

[6] Bakst MR, Wishart GJ. Proceedings, First Internation Symposium on the Artificial Insemination of Poultry. 1, 1-297. 1995. Champaign, IL, Poultry Science Association, Inc.

[7] Bakst MR, Cecil H. Techniques for Semen Evaluation, Semen Storage, and Fertility Determination. 1-97. 1997. Savoy, IL, Poultry Science Association.

[8] Jamieson BGM. Reproductive Biology and Phylogeny of Birds. Enfield, New Hampshire: Science Publishers; 2007.

[9] Froman DP, Feltmann AJ, Pendarvis K, Cooksey AM, Burgess SC, Rhoads DD. A proteome-based model for sperm mobility phenotype. Journal of Animal Science 2011;89(5) 1330-7.

[10] Bakst MR. Physiology and Endocrinology Symposium: Role of the oviduct in maintaining sustained fertility in hens. Journal of Animal Science 2011;89(5)1323-9.

[11] Perry MM, Waddington D, Gilbert AB, Hardie MA. Growth rates of the small yolky follicles in the ovary of the domestic fowl. IRCS Medical Science: Experimental Animals 1983;11(11/12) 979-80.

[12] Saito N, Sato K, Shimada K. Prostaglandin levels in peripheral and follicular plasma, the isolated theca and granulosa layers of pre- and postovulatory follicles, and the myometrium and mucosa of the shell gland (uterus) during a midsequence-oviposition of the hen (Gallus domesticus). Biology of Reproduction 1987;36(1) 89-96.

[13] Dick HR, Culbert J, Wells JW, Gilbert AB, Davidson MF. Steroid hormones in the postovulatory follicle of the domestic fowl (*Gallus domesticus*). Journal of Reproduction and Fertility 1978;53(1) 103-8.

[14] Hammond RW, Olson DM, Frenkel RB, Biellier HV, Hertenlendy F. Prostaglandins and steroid hormones in plasma and ovarian follicles during the ovulation cycle of

the domestic hen (*Gallus domesticus*). General and Comparative Endocrinology 1980;42(2) 195-202.

[15] Hertenlendy F, Biellier HV, Todd H. Effects of the egg cycle and the route of administration on prostaglandin-induced oviposition of hens and Japanese quail. Journal of Reproduction and Fertility 1975;44(3) 579-82.

[16] Johnson AL, Woods DC. Ovarian dynamics and follicle development. In: Jamieson BGM, editor. Reproductive Biology and Phylogeny of Birds.Enfield, New Hampshire: Science Publishers; 2007. p. 243-77.

[17] Wyburn GM, Johnston HS, Draper MH, Davidson MF. The fine structure of the infundibulum and magnum of the oviduct of *Gallus domesticus*. Quarterly Journal of Experimental Physiology 1970;55(3):213-32.

[18] Perry MM. Nuclear events from fertilisation to the early cleavage stages in the domestic fowl (*Gallus domesticus*). Journal of Anatomy 1987;150(1) 99-109.

[19] Birkhead TR, Sheldon BC, Fletcher F. A comparative study of sperm–egg interactions in birds. Journal of Reproduction and Fertility 1994;101(2) 353-61.

[20] Bakst MR, Howarth B, Jr. Hydrolysis of the hen's perivitelline layer by cock sperm in vitro. Biology of Reproduction 1977;17(3) 370-9.

[21] Okamura F, Nishiyama H. The passage of spermatozoa through the vitelline membrane in the domestic fowl, Gallus gallus. Cell and Tissue Research 1978;188(3) 497-508.

[22] Bakst MR, Wishart G, Brillard JP. Oviductal sperm selection, transport, and storage in poultry. Poultry Science Reviews 1994;5 117-43.

[23] Froman DP, Feltmann AJ, McLean DJ. Increased fecundity resulting from semen donor selection based upon in vitro sperm motility. Poultry Science 1997;77(1) 73-7.

[24] Bakst MR. Observations on the turkey oviductal sperm-storage tubule using differential interference contrast microscopy. Journal of Reproduction and Fertility 1992;95(3) 877-83.

[25] Schuppin GT, Van Krey HP, Denbow DM, Bakst MR, Meyer GB. Ultrastructural analyses of uterovaginal sperm storage glands in fertile and infertile turkey breeder hens. Poultry Science 1984;63(9) 1872-82.

[26] Bakst MR, Akuffo V. Alkaline phosphatase reactivity in the vagina and uterovaginal junction sperm-storage tubules of turkeys in egg production: Implication for sperm storage. British Poultry Science 2007;48(4) 515-8.

[27] Brillard JP. Sperm storage and transport following natural mating and artificial insemination. Poultry Science 1993;72(5) 923-8.

[28] Christensen VL, Bagley LG. Efficacy of fertilization in artificially inseminated turkey hens. Poultry Science 1989;68(5) 724-9.

[29] Bakst MR, Donoghue AM, Yoho DE, Moyle JR, Whipple SM, Camp MJ, et al. Comparisons of sperm storage tubule distribution and number in 4 strains of mature broiler breeders and in turkey hens before and after the onset of photostimulation. Poultry Science 2010;89(5) 986-92.

[30] Birkhead TR, Moller AP. Numbers and size of sperm storage tubules and the duration of sperm storage in birds: A comparative study. Biological Journal of the Linnean Society 1992;45(4):363-72.

[31] Brillard JP, Beaumont C, Scheller MF. Physiological responses of hens divergently selected on the number of chicks obtained from a single insemination. Journal of Reproduction and Fertility 1998;114(1) 111-7.

[32] Bakst MR. Oviducal sperm selection, transport, and storage in poultry: A review. Reproduction Fertility and Development 1993;5(6) 595-9.

[33] Blesbois E, Brillard JP. Specific features of *in vivo* and *in vitro* sperm storage in birds. Animal 2007;1(10):1472-81.

[34] Van Krey HP, Ogasawara FX, Pangborn J. Light and electron microscopic studies of possible sperm gland emptying mechanisms. Poultry Science 1967;46(1) 69-78.

[35] Fujii S. Histological and histochemical studies on the oviduct of the domestic fowl with special reference to the region of utero-vaginal junction. Archives of Histology and Cytology 1963;23(5) 447-9.

[36] Renden JA, May EB, Benoff FH. Histochemistry of uterovaginal sperm-host glands in Japanese quail (*Coturnix coturnix japonica*) with reference to the period of oviposition. Poultry Sci 1981;60(11) 2529-35.

[37] Blesbois E, Lessire M, Grasseau J, Hallouis JM, Hermier D. Effect of dietary fat on the fatty acid composition and fertilizing ability of fowl semen. Biology of Reproduction 1997;56(5) 1216-20.

[38] Surai PF. Natural Antioxidants in Avian Nutrition and Reproduction. Nottingham, UK: Nottingham University Press; 2002.

[39] Williams K, Frayne J, Hall L. Expression of extracellular glutathione peroxidase type 5 (GPX5) in the rat male reproductive tract. Molecular Human Reproduction 1998;4(9) 841-8.

[40] Catalá A. Lipid peroxidation modifies the picture of membranes from the "Fluid Mosaic Model" to the "lipid whisker model". Biochimie 2012;94(1) 101-9.

[41] Breque C, Surai P, Brillard JP. Roles of antioxidants on prolonged storage of avian spermatoza *in vivo* and *in vitro*. Molecular Reproduction and Development 2003;66(3) 314-23.

[42] Long C, Sonstegard TS, Long JA, van Tassell CP, Zuelke KA. Serial analysis of gene expression in turkey sperm storage tubules in the presence and absence of resident sperm. Biology of Reproduction 2003;69(2) 469-74.

[43] Foye-Jackson OT, Long JA, Bakst MR, Blomberg LA, Akuffo V, Silva VB, et al. Oviductal expression of avidin, avidin-related protein-2, and progesterone receptor in turkey hens in relation to sperm storage: Effects of oviduct tissue type, sperm presence, and turkey line. Poultry Science 2011;90(7) 1539-47.

[44] O'Malley BW, McGuire WL. Studies on the mechanism of action of progesterone in regulation of the synthesis of specific protein. Journal of Clinical Investigation 1968;47(3) 654-64.

[45] Korpela JK, Elo HA, Tuohimaa PJ. Aviadin induction by estrogen and progesterone in te immature oviduct of chicken, Japanese quail, duck, and gull. General and Comparative Endocrinology 1981;44(2) 230-2.

[46] Burke WH, Ogasawara FX. Presence of spermatozoa in uterovaginal fluids of the hen at various stages of the ovulatory cycle. Poultry Science 1969;48(2) 408-13.

[47] Compton MM, Van Krey HP, Siegel PB. The filling and emptying of the uterovaginal sperm-host glands in the domestic hen. Poultry Science 1978;57(6) 1696-700.

[48] Bushman AF, Van Krey HP, Denbow DM, Siegel PB. Effect of the ovulatory cycle on oviductal sperm storage in the domestic fowl. Theriogenology 1985;23(3) 473-9.

[49] Bakst MR. Duration of fertility of turkeys inseminated at different times after the onset of photostimulation. Journal of Reproduction and Fertility 1988;84(2) 531-7.

[50] Das SC, Nagasaka N, Yoshimura Y. Changes in the expression of estrogen receptor mRNA in the utero-vaginal junction containing sperm storage tubules in laying hens after repeated artifical insemination. Theriogenology 2006;64(4) 893-900.

[51] Yoshimura Y, Koike K, Okamoto T Immunolocalization of progesterone and estrogen receptors in the sperm storage tubules of laying and diethylstilbestrol-injected immature hens. Poultry Science 2000;79(1) 94-8.

[52] Ito T, Yoshizaki N, Tokumoto T, Ono H, Yoshimura Y, Tsukada A, et al. Progesterone is a sperm-releasing factor from the sperm-storage tubules in birds. Endocrinology 2011;152(10) 3952-62.

[53] Agre P, Sasaki S, Chrispeels MJ. Aquaporins: A family of water channel proteins. American Journal of Physiology Renal Physiology 1993;265(3) F461.

[54] Zaniboni L, Bakst MR. Localization of aquaporins in the sperm storage tubules in the turkey oviduct. Poultry Science 2004;83(7) 1209-12.

[55] Froman DP, Bowling ER, Wilson JL. Sperm mobility phenotype not determined by sperm quality index. Poultry Science 2003;82(3) 496-502.

[56] Froman DP. Effect of sperm mobility phenotype on fertility, sperm competition, and in vivo sperm storage in the domestic fowl. Journal of Dairy Science 93[Suppl. 1], 504. 2010.

[57] Brillard JP, Galut O, Nys Y. Possible causes of subfertility in hens following insemination near the time of oviposition. British Poultry Science 1987;28(2) 307-18.

[58] Stephens RE, Prior G. Dynein from serotonin-activated cilia and flagella: Extraction characteristics and distinct sites for cAMP-dependent protein phosphorylation. Journal of Cell Science 1992;103(4):999-1012.

[59] Holm L, Ridderstrale Y, Knutsson PG. Localisation of carbonic anhydrase in the sperm storing regions of the domestic hen. Cell Tissues Organs 1996;156(4):253-60.

[60] Holm L, Wishart GJ. The effect of pH on the motility of spermatoza from chicken, turkey and quail. Animal Reproduction Science 1998;54(1):45-54.

[61] Freedman SL, Akuffo VG, Bakst MR. Evidence for the innervations of sperm storage tubules in the oviduct of the turkey (*Meleagris gallopavo*). Reproduction 2001;121(5): 809-14.

[62] Bakst MR, Akuffo V. Serotonin localization in the turkey vaginal but not sperm storage tubule epithelia. Journal of Avian Biology 2008;39(3):348-54.

[63] Froman DP, Bowling ER, Wilson JL. Sperm mobility phenotype not determined by sperm quality index. Poultry Science 2003;82(3):496-502.

[64] Froman DP, Engel HN. Alteration of the spermatozoal glycocalyx and its effect on duration of fertility in the fowl (*Gallus domesticus*). Biology of Reproduction 1989;40(3):615-22.

[65] Froman DP, Thursam KA. Desialylation of the rooster sperm's glycocalyx decreases sperm sequestration following intravaginal insemination of the hen. Biology of Reproduction 1994;50(5):1094-9.

[66] Steele MG, Wishart GJ. Demonstration that the removal of sialic acid from the surface of chicken spermatozoa impedes their transvaginal migration. Theriogenology 1996;46(6):1037-44.

[67] Pelaez J, Long JA. Characterizing the glycocalyx of poultry spermatozoa: I. Identification and distribution of carbohydrate residues using flow cytometry and epifluorescence microscopy. Journal of Andrology 2007;28(2):342-52.

[68] Robertson L, Wishart GJ, Horrocks AJ. Identification of perivitelline N-linked glycans as mediators of sperm-egg interaction in chickens. Journal of Reproduction and Fertility 2000;120(2):397-403.

[69] Birkhead TR, Brillard JP. Reproductive isolation in birds: postcopulatory prezygotic barriers. Trends in Ecology & Evolution 2007;22(5):266-72.

[70] Steele MG, Meldrum W, Brillard JP, Wishart GJ. The interaction of avian spermatozoa with the perivitelline layer in vitro and in vivo. Journal of Reproduction and Fertility 1994;101(3):599-603.

[71] Howarth B. Carbohydrate involvement in sperm-egg interaction in the chicken. Journal of Receptor Research 1992;12(2):255-65.

[72] Horrocks AJ, Stewart JM, Jackson L, Wishart GJ. Induction of acrosomal exocytosis in chicken spermatozoa by inner perivitelline-derived N-linked glycans. Biochemical and Biophysical Research Communications 2000;278(1):84-9.

[73] Koyanagi F, Masuda S, Nishiyama H. Acrosome reaction to cock spermatoza incubated with the perivitelline layer of the hen's ovum. Poultry Science 1988;67(12): 1770-4.

[74] Bakst MR, Howarth B, Jr. The fine structure of the hen's ovum at ovulation. Biology of Reproduction 1977;17(3):361-9.

[75] Wasserman PM, Jovine L, Litscher ES. A profile of fertilization in mammals. Nature Cell Biology 2001;3(2):E59-64.

[76] Stepinska U, Olszanska B. DNase I and II present in avian oocytes: A possible involvement in sperm degradation at polyspermic fertilisation. Zygote 2003;11(1): 35-42.

[77] Stepinska U, Bakst MR. Fertilization. In: Jamieson BGM, editor. Reproductive Biology and Phylogeny of Birds. Enfield, New Hampshire: Science Publishers; 2007. p. 553-87.

[78] Wishart GJ. Quantitative aspects of sperm:egg interaction in chickens and turkeys. Animal Reproduction Science 1997;48(1):81-92.

[79] Bellaris R, Harkness M, Harkness RD. The vitelline membrane of the hen's egg: A chemical and electron microscopal study. Journal of Ultrastructure Research 1963;8(3):339-59.

[80] Bramwell RK, Howarth B, Jr. Preferential attachment of cock spermatozoa to the perivitelline layer directly over the germinal disc of the hen's ovum. Biology of Reproduction 1992;47(5):1113-7.

[81] Wishart GJ. Regulation of the length of the fertile period in the domestic fowl by numbers of oviducal spermatozoa, as reflected by those trapped in laid eggs. Journal of Reproduction and Fertility 1987;81(2):495-9.

[82] Brillard JP, Antoine H. Storage of sperm in the uterovaginal junction and its incidence on the numbers of spermatoza present in the perivitelline layer of hen's eggs. British Poultry Science 1990;31(3):635-44.

[83] Wishart GJ. Quantitative aspects of sperm:egg interaction in chickens and turkeys. Animal Reproduction Science 1997;48(1):81-92.

[84] Malecki IA, Rybnik PK, Martin GB, Malecki I, Glatz P. Artificial insemination tech-
nology for ratites: A review. Australian Journal of Experimental Biology 2008;48(10):
1284-92.

[85] Bakst MR, Cecil HC. Gross appearance of turkey cloacae before and after single or
multiple manual semen collections. Poultry Science 1983;62(4):683-9.

[86] Haugland RP. Handbook of Fluorescent Probes and Research Products. 9th ed. Eu-
gene, Oregon: Molecular Probes, Inc.; 2002.

[87] Froman DP, McLean DJ. Objective measurement of sperm motility based upon
sperm penetration of Accudenz®. Poultry Science 1996;75(6):776-84.

[88] Froman DP. Application of the sperm mobility assay to primary broiler breeder
stock. Journal of Applied Poultry Research 2006;15(2):280-6.

[89] Bakst MR, McGary S, Estevez I, Knapp T. Use of nonsettable eggs to evaluate turkey
hen fertility. Journal of Applied Poultry Research 2002;11(4):402-5.

[90] Brillard JP, Bakst MR. Quantification of spermatozoa in the sperm-storage tubules of
turkey hens and the relation to sperm numbers in the perivitelline layer of eggs. Biol-
ogy of Reproduction 1990;43(2):271-5.

[91] Bramwell RK, Marks HL, Howarth B. Quantitative determination of spermatozoa
penetration of the perivitelline layer of the hen's ovum as assessed on oviposited
eggs. Poultry Science 1995;74(11):1875-83.

[92] Donoghue AM. The effect of twenty-four hour *in vitro* storage on sperm hydrolysis
through the perivitelline layer of ovipositioned turkey eggs. Poultry Science
1996;75(8):1035-8.

[93] Lake, PE. Historical perspective of artificial insemination technology. In: Bakst MR
and Wishart GJ, editors. Proceedings - First International Symposium on the Artifical
Insemination of Poultry. Savoy, Illinois: Poultry Science Association; 1995. p. 1-20