ABSTRACT

FAIRCHILD, BRIAN DAVID. Turkey Hen Age, Fertility and Sperm Penetration of the Inner Perivitelline Layer Affects Embryonic Mortality. (Under the direction of Vern L. Christensen.)

When examining hatchability of eggs, the two main factors that contribute are fertility and embryonic mortality (EM). A common commercial observation across different turkey lines was that early embryonic mortality (EEM) appeared to be greater in young hens than in older hens. One point that complicates this issue is the ability to discriminate between EEM and infertile eggs. Therefore, there has always been a question about the accuracy of the reported number of infertile eggs.

A study was designed to examine the hatch residue from turkey flocks of the same strain at two different ages. One flock was in its first two weeks of egg production and the second flock had been in production for at least 12 weeks. Six hatches with approximately 56,000 eggs total for all hatches combined were examined by macroscopic breakout. There were no differences in fertility or hatchability between the two different hen ages. EEM was greater in younger hens than in older hens. EM occurring during the last week of incubation was greater in older hens than in younger hens. A significant negative correlation was found between fertility and EEM in young hens suggesting that practices that could improve fertility may also minimize EEM. The difference between the two hen ages provided a model to further examine embryonic mortality in turkeys.

Preliminary results from commercial resources indicated lower EM after hens were inseminated with higher numbers of sperm cells. It was hypothesized that sperm binding might differ between hen ages and that increasing the number of

sperm in the insemination might compensate for lower binding. A study was designed to examine the sperm penetration (SP) of hens at two stages of egg production: the beginning of egg production and wk 12 of egg production. Using both in vivo and in vitro SP assays, the results indicated a larger number of SP holes in the perivitelline layer of the young hens when compared to older hens. The similarity between the in vivo and in vitro assays indicated a sperm binding effect independent of oviduct influences such as sperm storage and release rate.

Another study was conducted to examine the female influence on SP and EEM in turkeys. Eggs from females of two different lines were examined at the two different ages examined in the previous studies. Females were mated to a single sire for the duration of the study to examine IPVL SP in the absence of sperm competition among different males. Eggs were used for either in vivo SP analysis or incubated for fertility, hatchability and EM data. Hens inseminated by a singe sire performed differently between the two age periods as demonstrated by a hen by period interaction. The response was inconsistent whereas some hens had increased SP holes, some decreased and some did not change between the two different age periods. Hens in the upper (HI) and lower (LO) third of the flock population based on SP holes were pooled in analyzing sperm hole effects on embryo viability. No differences in fertility or wk 1 mortality were detected between HI and LO hens. However, HI hens had higher hatchability and lower wk 4 EM compared to LO hens.

In conclusion, the results of the current study demonstrated a significant female effect on SP. Furthermore, this effect appears to be due to unknown

differences in IPVL properties as well as differences due to sperm storage and release rate from the sperm storage tubules. More research is needed to understand how the number of sperm cells relates to both early and late EM. These studies indicate that neither a high nor a low SP number, but that an intermediate number might result in optimal embryo survival.

TURKEY HEN AGE, FERTILITY AND SPERM PENETRATION OF THE INNER PERIVITELLINE LAYER AFFECTS EMBRYONIC MORTALITY

by

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BIOGRAPHY

Brian David Fairchild was born in Boone, NC on October 26, 1971. He was raised in Fleetwood, NC where he graduated with honors from Beaver Creek High School. Brian began his studies at North Carolina State University in 1990 and double majored in Animal Science and Poultry Science. He received a Southern States Cooperative Scholarship each year of his undergraduate studies, and was inducted into the Alpha Lambda Delta Honor Society and the Gamma Beta Phi Honor Society. He received the M.B. “Dutch” Gardner Poultry Scholarship in 1992 and the Tom Hester Scholarship/Fellowship Endowment in 1993. Brian graduated with a Bachelor of Science degree in Animal Science and Poultry Science in May 1994.

In August 1994, the author was admitted to the Graduate School at NCSU majoring in Physiology. In May of 1996, he married Ms. Amanda Claudine Smith. He graduated with a Master of Science Degree in 1997 with a major in Physiology and a minor in Poultry Science under the direction of Dr. Vern Christensen. Brian was one of the winners of the 2000 Cliff D. Carpenter Essay contest sponsored by the USA Branch of the World’s Poultry Science Association for his essay “Perspectives on the Future of the Poultry Industry in the United States”.

In May 2001, he completed the requirements for a Ph.D. in Physiology with a minor in Biotechnology. In July 2001, Brian was awarded the Nicholas Communications Award at the first International Animal Agriculture and Food Science Conference, held in Indianapolis, Indiana. In August 2001, Brian accepted

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When not hard at work in the lab, Brian enjoys a good round of golf with friends and family, tweaking computers for maximum efficiency, and vegetable gardening with his wife, Amanda. For relaxation, he likes to read science fiction novels and spend time at the Outerbanks of North Carolina.

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ABBREVIATION KEY

SST	**Sperm Storage Tubules**
UVJ	**Uterovaginal Junction**
Ab	**Antibody**
h	**Hour**
wk	**Week**
IPVL	**Inner perivitelline layer**
OPVL	**Outer perivitelline layer**
PVL	**Perivitelline layer**
mm	**Micrometer**
SP	**Sperm penetration**
EEM	**Early embryonic mortality**
EM	**Embryonic mortality**
GD	**Germinal disc**
NGD	**Non-germinal disc**
EG&K	**Eyal-Giladi and Kochav**
G&B	**Gupta and Bakst**
C	**Celsius**
RH	**Relative humidity**
BW	**Body weight**
d	**Day(s)**

I. LITERATURE REVIEW*

The ultimate goal in avian reproduction is to produce viable healthy offspring. Hatchability is the number of poults that hatch expressed as a percent of the total number of eggs set in the hatch. Hatchability of both turkey eggs (Van Krey et al., 1967; Nestor et al., 1972; Sexton, 1977) and chicken eggs (Brillard and McDaniel, 1986; de Reviers and Brillard, 1986) deteriorates as the hen ages. Two primary components of hatchability are fertility and embryonic mortality. Fecundity is defined as being able to produce young or reproduce. Fertility refers to the proportion of eggs that are fertilized and calculated as the number of fertilized eggs expressed as a percentage of the total number of eggs produced. The number of eggs used to calculate fertility can be based on a single hen, a group of hens or an entire flock over any given period of time. Fertility declines with the age of the hen in chickens (Crittenden and Bohren, 1962; Smith and Bohren, 1975; Kirk et al., 1980; Fasenko et al., 1992b) and turkeys (Van Krey and Leighton, 1970; Van Krey et al., 1967; Sexton, 1977). While the effects on fertility have been well documented in the literature, embryonic mortality, the second component of hatchability, has not been as extensively reported specifically as related to hen age. This review provides a brief description of the events that contribute to fertilization of the ovum and examines research that has been reported on avian embryonic mortality with an emphasis on turkey embryonic mortality.

* Nomenclature follows the Handbook of Avian Anatomy: Nomina Anatomica Avium (1993). The writing style and form follow the Poultry Science Journal style and form.

AVIAN FERTILIZATION

Oviducal Sperm Transport and Selection

Upon insemination, sperm encounter a series of selection pressures/barriers within the oviduct that cull weak and dead sperm ensuring that only "fit" sperm go on to the site of fertilization located in the infundibulum. It has been hypothesized that motile sperm bypass the vagina at the exclusion of nonmotile sperm (Bakst et al., 1994). Approximately 80% of inseminated spermatozoa are lost within 30 min of insemination (Howarth, 1971a). Selection pressures on the spermatozoa within the oviduct include the vagina (Allen and Grigg, 1957), the presence of an egg (Mimura, 1939), oviduct secretions (Bakst et al., 1994), and the immune response of the hen (Burke and Yu, 1979; Yu and Burke, 1979b; McCorkle et al., 1983; Steele and Wishart, 1992). The presence of an egg in the magnum or isthmus depressed sperm transport, however sperm transport was not affected with an egg present in the uterus (Mimura, 1939). A mechanism exists in the avian oviduct in which cilia of the vagina beat in an abovarian direction. Cilia activity provides movement of luminal secretions, as well as trapped debris, in the direction of the cloaca. Sperm could be expelled from the oviduct when trapped in the secretions and debris that are pushed out (Bakst et al., 1994). It has been suggested that the sperm must possess appropriate characteristics (probably a combination of viability, motility, sufficient metabolic activity, plasmalemma integrity, and possession of certain surface characteristics) in order to traverse the vagina and enter the sperm storage tubules successfully (Bakst et al., 1994). Motility is a requirement as only motile

sperm can traverse the vagina and reach the primary sperm storage tubules (SST) in the utero-vaginal junction (UVJ) (Allen and Grigg, 1957; Wishart, 1984; Van Krey, 1990; Bakst, 1994; Donoghue et al., 1998).

The hen's immune system may contribute to the sperm selection process in the vagina by incapacitating spermatozoa that are recognized as possible antigens. Serum anti-sperm antibodies (Ab) have been detected in both turkeys (Burke and Yu, 1979; Yu and Burke, 1979b; McCorkle et al., 1983) and chickens (Wentworth and Mellen, 1964). McCorkle et al. (1983) reported increased antisperm Ab titers associated with reduced fertility when the oviduct was traumatized during insemination. However, in a similar experiment Kirk et al. (1989a) found that different modes of insemination had no effect on antisperm Ab or fertility. Plasma cells have been observed in the SST epithelium of infertile turkey breeder hens (Van Krey et al., 1987) and were later shown to stain IgG positive, indicating the possibility of a local immune response against spermatozoa as no serum antisperm Ab was detected (Kirk et al., 1989b). Antibodies are found throughout the oviduct, however it appears that the vagina is the primary location in the oviduct where sperm antigen-specific Ab are produced (Kimijima et al., 1990). These results agree with those of Steele and Wishart (1992) where spermatozoa that were inseminated directly into the UVJ were more likely to enter the sperm storage tubules than sperm that were inseminated intravaginally. Sperm that have been recovered from the vagina 20 min after insemination have been found to have IgA and IgG immunoglobulins bound to the plasmalemma and that this was mostly localized in the tail region (Steele and Wishart, 1992). Mast cells can be found in the vaginal histological sections. These

cells produce Ab, particularly IgG, which have been detected on spermatozoa. The Ab that bind to sperm may decrease fertility as a result of reduced motility and faster sperm degeneration as the sperm membrane is degraded. The results of Kapoor et al. (2000) indicate that very large amounts of anti-perivitelline layer (anti-PVL) Ab may be required to reduce fertility however, smaller amounts of anti-sperm Ab may result in reduced fertility. It is possible that the anti-PVL Ab used by Kapoor et al. (2000) was not against the sperm binding receptor because if an Ab was used that was anti-sperm receptor then fertility may be reduced with smaller amounts of Ab than indicated in their study.

Motile and morphologically normal spermatozoa that bypass the vagina enter the SST located at the UVJ. The exact method of how the sperm occupy the SST is still unknown as well as the physiological mechanisms of sperm storage (see Bakst et al., 1994 for review). It has been suggested that sperm are exposed to factors (including zinc, calcium and glutamic acid) in the SST that suppress sperm motility, sperm metabolism, the enzyme acrosin, sperm immunogenicity and stabilize the plasmalemma and other sperm membranes (Bakst, 1994; Holm et al., 2000). Ogasawara et al. (1966) and Bakst (1989) provided evidence of sperm selection at the level of the SST. Young hens retain more sperm in the SST than older hens (Brillard, 1992). It appears that under normal conditions there is only partial filling of the SST in both turkeys (Verma and Cherms, 1965; McIntyre and Christensen, 1983; Bakst, 1994) and chickens (Compton and Van Krey, 1979). Furthermore, the storage capacity of the SST appears to be compromised with the onset of egg production as fewer SST are filled as compared to hens inseminated prior to egg

production (McIntyre and Christensen, 1983). Events in egg formation may dislodge sperm from the SST or might prevent sperm from entering the SST (Bakst et al., 1994). Compton and Van Krey (1979) suggested that only a finite number of spermatozoa are capable of entering the SST in a given period of time. Approximately 1 to 2% of inseminated sperm in the turkey (Brillard and Bakst, 1990) and 1% in the chicken (Brillard, 1993) were found in the SST 24 h after insemination. Bakst (1988) hypothesized that decreasing the volume of semen might influence the number of sperm that reach the UVJ and influence SST filling. In testing that hypothesis, fertility dropped by wk 6 of production indicating that smaller insemination doses did not improve the efficacy of sperm transport and storage in the SST. A greater duration of fertility was demonstrated in hens that were inseminated with larger doses of semen on 2 consecutive days than hens inseminated with smaller doses on 5 consecutive days even though both groups received the same overall number of sperm.

At least three models have been hypothesized for sperm competition in the SST. These models include passive sperm loss, stratification and displacement. Passive sperm loss is the continuous release of sperm from the SST, stratification is the first in/last out hypothesis, and displacement is the replacement of existing sperm with recently inseminated sperm. Passive sperm loss does occur among avian species (Birkhead et al., 1993; Wishart, 1987). The remaining theories have to be reworded to include the passive sperm loss observation. A study is required with proper planning to be able to distinguish between stratification-passive sperm loss and displacement-passive sperm loss (Birkhead and Biggins, 1998). Van Krey et al.

(1981) has provided evidence in favor of the stratification theory using autoradiograms of radio-labeled sperm in the SST. Other observations that appear to support the stratification theory have been demonstrated in both chickens (Compton et al., 1978; Compton et al., 1979) and turkeys (Christensen, 1981). Bakst et al. (1994) suggested that the dominance of the last male producing a majority of offspring could be due to the inability of these sperm to enter the SST and populating other oviduct regions.

Inseminated sperm spread throughout the oviduct and can reach the site of fertilization within 15 min following insemination. Various investigators have suggested that antiperistaltic activity was the primary oviducal sperm transport method anterior to the UVJ (Mimura, 1937; 1939; Allen and Grigg, 1957). Motility is required to migrate through the vagina and enter the SST in the UVJ (Takeda, 1974). In contrast to mammalian fertilization, avian sperm do not appear to require capacitation within the oviduct (Howarth, 1971b; Johnson, 1986). This is supported by the observation that sperm removed from the ductus deferens will yield fertility levels comparable to ejaculated sperm when inseminated (Bakst and Cecil, 1981). However, it has been implied that the avian form of capacitation is the activation that sperm undergo once released from the SST in which there may be increased motility, metabolism, and destabilization of the plasmalemma (Bakst et al., 1994).

Once released from the SST, sperm receive very little resistance in the isthmus and magnum with the exception of an egg being present (Mirmura, 1939). Once the spermatozoon reaches the infundibulum there are several different fates that it could experience. These include fertilization, temporary storage in secondary

sperm storage sites, trapped in the ovum outer perivitelline layer (OPVL), or lost in the body cavity. The infundibulum is divided into three regions: the fimbriated region, the funnel region, and the chalaziferous (distal) region. The secondary sperm storage sites are located in the distal region of the infundibulum (Allen and Grigg, 1957; Bakst, 1981). Although the infundibulum is considered the secondary sperm storage site, there has been very little attention focused on the sperm storage functions and characteristics of this site. The sperm storage sites in this region appear to promote sperm survival. Sperm are passively released from the infundibular sperm storage sites and encounter the egg in the funnel region (Etches, 1996).

Sperm-Ovum Interaction

One of the critical steps of fertilization is the binding of the sperm to the inner perivitelline layer (IPVL) and the subsequent acrosome reaction. The result is a hydrolyzed hole through which sperm enter the ovum. In mammals, the oocyte is encased by an extracellular glycoprotein layer, the zona pellucida, which is similar to the IPVL in avian species (Bakst and Howarth, 1977; Dunbar et al., 1994; Howarth, 1992; Waclawek et al., 1998). The zona pellucida is composed of three major glycoproteins (ZP1, ZP2 and ZP3) that are linked with distinct events in the process of fertilization (Dunbar et al., 1994). ZP1 serves as a cross-linker to maintain the structural integrity of the zona pellucida. ZP2 prevents polyspermy by inducing the zona reaction (Wasserman, 1988). ZP3 is thought to be the primary sperm receptor in human, mouse, pig and rabbit (Florman and Wasserman, 1985; Skinner et al., 1996). Little is known about the avian IPVL glycoprotein function in fertilization.

However, Howarth (1992) demonstrated carbohydrate involvement in sperm-ovum binding. Carbohydrate involvement in sperm binding has also been demonstrated in the mouse (Florman and Wasserman, 1985). Deglycosylation of the solubilized PVL with trifluormethanesulfonic acid inhibited sperm attachment and digestion of intact PVL by 81%. Recently a ZP3 homologue of the mammalian ZP3 glycoprotein has been identified in the chicken IPVL (Takeuchi, 1999). Mammalian ZP3 has been shown to be involved in the activation of the acrosome reaction (Leyton and Saling, 1989) and may possess similar functions in avian species. Future work with avian ZP3 might provide a better understanding of the sperm-ovum interaction in chickens and turkeys. Although the identity of the avian sperm receptor is questionable, many features are known about it. Howarth (1992) showed a reduction in the number of sperm-IPVL interactions, which was blocked by incubating sperm with IPVL that had the carbohydrate removed. This suggested that the IPVL sperm ligand is a carbohydrate. Robertson et al. (2000) reported a decrease in sperm-ovum interaction when the IPVL was pretreated with N-glycanase whereas O-glycanse had no effect, indicating that n-linked glycans (terminal N-acetyl-D-glucosamine) are an essential component in sperm-ovum interactions. This demonstrates that the avian sperm ligand differs from that found in the mouse (Wassarman and Litscher, 1995).

While these studies provide some information about the IPVL sperm ligand, they do not address the preferential hydrolysis of the IPVL area overlying the germinal disc. This physiological event requires more research to elucidate the factors that attract sperm to this area or prevent the binding to nongerminal disc areas. Several studies have examined the nature of the sperm-ovum interaction and

the number of holes hydrolyzed in the IPVL. Although only one spermatozoon is required for fertilization, reports have indicated that a minimum number of sperm entering the ovum may be required for fertilization resulting in greater probabilities that syngamy will occur (Wishart, 1995; Bramwell et al., 1995).

In avian species, fertilization is a polyspermic event where more than one sperm cell will enter the ovum. Birds do not have the fast reacting mechanism to block polyspermy as found in mammals (Carlson, 1988). Both turkey and chicken ova that have 3 sperm penetration (SP) holes in the IPVL have a 50% chance of being fertile where ova that have 6 or more SP holes have a 100% chance of being fertile (Wishart, 1995; 1997). Bramwell et al. (1995) found that greater than 200 SP holes were found in fertile chicken eggs without excessive early embryonic mortality (EEM) conflicting with earlier reports by Bekhtina (1966) who reported that greater than 200 sperm penetrating site of fertilization would increase EEM. In a later study, Bramwell and Howarth (1997) reported increased EEM with increased SP holes in the IPVL. It was suggested that the contradiction between the later study (Bramwell et al., 1995) and the Bekhtina (1966) study could be explained by the assay method. Previous reports have counted sperm pronuclei where in the IPVL binding assay SP does not guarantee sperm entry into the ovum or the formation of a sperm pronucleus. Using unpublished observations Bramwell and Howarth (1995) stated that different hens had different numbers of SP holes throughout both the germinal disc (GD) and non-germinal disc (NGD) regions even when exposed to the same concentration of sperm. They suggested that the number or accessibility of sperm receptors on the IPVL might vary between hens. Wishart (1997) found 25 times

more SP holes in the GD region when compared to other regions of the egg in both chickens and turkeys. Staines et al. (1998) concluded that the IPVL SP assay could be used as a measure for estimating broiler breeder flock mating efficiency. The results of their study indicated that fertility could be quantitated by counting IPVL SP holes just as it could by counting sperm trapped in the OPVL. The arguments for using the IPVL binding assay include the simplicity of the assay when compared to counting sperm trapped in the OPVL, the relative inexpensive materials needed, and the fact that changes could be detected easier by estimating the number of sperm rather than determining fertility on eggs which would require large sample sizes or large differences to pick up changes in sperm quality.

Several factors have been examined that influence the number of SP holes in the IPVL. Broiler breeder age has been found to influence SP. Bramwell et al. (1996) found that eggs from younger broiler breeder hens had higher SP than eggs from older broiler breeder hens. Eggs fertilized by sperm from young broiler breeder males had lower SP than eggs fertilized by sperm from older broiler breeder males. Semen storage has also been reported to affect SP. Turkey hen fertility declines after weekly inseminations with semen stored for greater than 12 h in vitro (see review, Thurston, 1995). In vitro semen storage for 24 h prior to insemination has been shown to reduce sperm numbers at the site of fertilization (Donoghue et al., 1995; Donoghue 1996).

Using ovulated ova (Koyanagi et al., 1988; Howarth, 1990, 1992) and ovarian follicles (Steele et al., 1994) researchers have shown that the IPVL possesses factors that act as sperm receptors and initiate the acrosome reaction. Bramwell

and Howarth (1992b) reported cross-reactivity among glycoproteins involved in sperm-ovum binding for IPVL of chicken, turkey and duck ova. However, they observed no reduction in fertility or SP holes when White Leghorn hens were immunized with anti-quail GD, anti-turkey GD, anti-quail PVL or anti-turkey PVL (Kapoor et al., 2000). Immunoblot analysis indicated that Ab failed to cross-react with any of the chicken IPVL proteins even though Ab were produced in response to injection with these antigen sources. This may indicate that the binding receptors were so similar between the species that they were recognized as self even when the antigen sources were modified with dinitrophenol.

Fertilization and Syngamy

Fertilization in its broadest definition is the entry of the male germ into the ovum. Fertilization has several components. These include (1) membrane contact between the ovum and sperm, (2) entry of the sperm into the ovum, (3) prevention of polyspermy by the ovum, (4) metabolic activation of the ovum, (5) the completion of meiosis by the ovum, and (6) formation and fusion of male and female pronuclei (Carlson, 1988). The ovulated ovum is guided into the ostium of the infundibulum by the fimbriated region of the infundibulum. Bellairs et al. (1963) suggested that sperm encounter the ovum in the funnel of the infundibulum where fertilization occurs. Since some sperm pass through the infundibulum and are lost in the body cavity, it is possible that sperm encounter the ovum prior to entering the ostium of the infundibulum. One or more sperm preferentially attach to the IPVL overlying the germinal disc region and digest a 10-20 μm diameter hole through which they pass to access the oolema (Bakst and Howarth, 1977; Bramwell and Howarth, 1992a;

Birkhead et al., 1994; Wishart, 1997). Kuroki and Mori (1997) demonstrated preferential binding of sperm to the germinal disc region as early as the F1 follicle stage prior to ovulation. Their work also suggested that a structural change in the perivitelline layer occurs between the follicle stage and the oviposited egg. Whether this change is at ovulation or later remains to be determined. At the time of ovulation the female pronucleus is in the metaphase stage of its 2nd meiotic division (Romanoff, 1960; Burley and Vadehra, 1989). The vitelline membrane is composed of two layers separated by a middle continuous layer (Lamina continua), the inner perivitelline layer (Lamina perivitellina) which is deposited during follicular development and the outer perivitelline layer (Lamina extravitellina) which is added in the distal end of the infundibulum after ovulation (Bellairs et al., 1963; Etches, 1996). Spermatozoa penetrate the IPVL within 15 min of ovulation (Fofanova, 1965; Bakst and Howarth, 1977). The spermatozoal outer acrosomal membrane and the overlying plasma membrane expand and fuse together and disintegrate. The apical region of the acrosome opens exposing the acrosome enzymes (acrosin and other trypsin like enzymes) that hydrolyze a hole in the inner perivitelline layer (Howarth and Digby, 1973; Ho and Meizel, 1976; Okamura and Nishiyama, 1978; Koyanagi et al., 1988; Etches, 1996). At this point, the inner acrosomal membrane of the sperm fuses with the oolema creating an opening that the sperm head (minus the plasmalemma), midsection and tail will pass through into the ooplasm. As the ovum moves into the caudal portion of the infundibulum, the middle continuous and the OPVL are secreted on the surface of the IPVL to complete the formation of the perivitelline complex (Bain and Hall, 1969). The OPVL is believed to block additional

sperm from entering the ovum thus reducing the probability of pathological polyspermic fertilization (Howarth, 1971a, Howarth and Digby, 1973, Bakst and Howarth, 1977). The OPVL mechanism of blocking sperm penetration has been questioned in various studies. Steele et al. (1994) suggested that the addition of the OPVL alters the sperm binding function of the IPVL. However, Robertson et al. (1997) indicated that when IPVL was separated from the perivitelline complex after oviposition that it still retained the ability to bind sperm similar to that of ova collected just after ovulation. These results suggested that the block to sperm penetration by the OPVL was more mechanical than chemical and was in agreement with the previous results of Howarth and Digby (1973). During the 15 min period post ovulation, many sperm can enter the ovum through the inner perivitelline membrane as determined by the observation of hundreds of IPVL SP holes (Bramwell et al., 1995). However, a maximum of 60 male pronuclei have been reported, suggesting that every SP hole does not indicate sperm entry into the ooplasm (Fofanova, 1965; Bekhtina, 1966). The sperm move through the hydrolyzed holes to the perivitelline space (Okamura and Nishiyama, 1978).

During the next hour, the male pronuclei transform into an ellipsoid nucleus then into a pear-shape with a long tail which gradually disappears, and finally into a spheroid pronuclei (Perry, 1987). Further expansion to form a spherical nucleus is accompanied by granulation and dispersion of the chromatin. At the same time the female pronucleus completes the second meiotic division and expels the second polar body. During the next two hours the nuclei undergo enlargement. The actual number of supernumerary spermatozoa varies in the literature and may be attributed

to strain, individual hen differences, insemination methods and technology used to quantify. The number of supernumerary spermatozoa ranges from 4 to 24 (Patterson, 1910), 3 to 4 (Olsen, 1942), 1 to 60 (Fofanova, 1965), 20 to 60 (Bekhtina, 1966), 5 to 15 (Perry, 1987), and 5.9 to 26 (Waddington et al., 1998). In their in vitro fertilization studies, Nakanishi et al. (1990) reported 1 to 13 supernumerary spermatozoa. Harper (1904), Bekhtina (1966), Perry (1987), and Waddington, et al. (1998) described the elimination of the supernumerary spermatozoa from the area where the female and male pronuclei were located. Similar observations where made as ova were inseminated in vitro (Nakanishi et al., 1990). Syngamy, which is the union of male and female pronuclei to form a zygote, occurs after the observation of female and male pronuclei juxtaposition (Perry, 1987; Waddington et al., 1998). The next observation reported by Perry (1987) shows the zygote in the mitotic division of anaphase.

The exact timing of pronuclei formation is questionable. Olsen (1942) reported pronuclei formation 15 min after ovulation, Fofanova (1965) reported it at 2 to 5 h post ovulation and Perry (1987) reported it at 3.5 h after ovulation. Nakanishi et al. (1990) observed male and female pronucleus formation 4 h after insemination in an in vitro study. Currently, in the avian species examined, syngamy is believed to occur around 4.5 to 5 h after fertilization and the first mitotic division begins in the zygote. During this time, the remaining supernumerary male pronuclei migrate to the periphery of the germinal disc region, undergo a mitotic division and degrade (Perry, 1987; Etches, 1996).

FERTILITY

The American Heritage Dictionary defines fertility as being able to produce young or reproduce. As stated earlier, fertility in cleodic species can be expressed as the proportion of eggs that are fertilized expressed as a percentage of eggs laid. Fertility is the main component contributing to hatchability; if the ovum is not fertilized then the egg will not hatch (the exception being parthenogenesis). Fertility in birds can be reported in two ways because the hen has the capacity to store semen and lay multiple fertilized eggs with one insemination. First, fertility in hens can include the duration of the fertile period. Second, the percent fertile eggs of all eggs laid can be calculated. The term fertile period was introduced by Lake (1975) who defined it as the period after artificial insemination, or after removal of the male following natural mating, during which the hen would continue to lay fertile eggs.

Factors that affect Fertility

A brief review of the factors that can affect fertility or have been associated with it is useful. This is because of a common problem of determining whether the cause of an egg not hatching is differentiating between infertility and EEM. Factors that have been positively correlated with fertility include sperm concentration of insemination dose (Maeza and Buss, 1976; Donoghue, 1996), ATP levels in avian semen (Wishart, 1982; Wishart and Palmer, 1986), sperm trapped in the OPVL (Wishart, 1997), IPVL SP holes (Bramwell et al., 1995; Robertson et al., 1998; Wishart and Staines, 1999; Hazary and Wishart, 1999), sperm motility (McDaniel and Craig, 1962; Soller et al., 1965; Boone, 1968; Wilson et al., 1979; Wishart and

Palmer, 1986), and sperm mobility (Froman and McLean, 1996; Froman et al., 1997; Donoghue et al., 1998). Sperm concentration and quality play a significant role in fertility. Donoghue (1996) observed higher fertility and hatchability in hens inseminated with larger doses of semen (100×10^6) when compared to hens inseminated with a low dose of semen (10×10^6). Sperm quality deteriorates with in vitro storage beyond 4 h after collection. This deterioration in sperm quality due to in vitro storage prior to insemination has been associated with decreased fertility and increased embryonic mortality (Huyghebaert et al., 1984). McDaniel et al. (1996) demonstrated decreases in broiler breeder fertility when males were exposed to excess ambient heat. Declines were concluded to be due to fewer sperm stored in the UVJ SST. Frozen sperm has been shown to possess reduced motility and fertility in both chickens and turkeys (Bakst and Sexton, 1979). Insemination of irradiated spermatozoa resulted in poor fertility (Kosin, 1944; Wishart and Dick, 1985) even though sperm morphology, ATP content and motility were not effected (Wishart and Dick, 1985).

High protein concentration in seminal plasma has been related to reduced fertility in turkeys (Thurston et al., 1992). Spermiophages, immunoresponsive cells with macrophage characteristics, have been found to actively engulf spermatozoa and bacteria (Thurston et al., 1975; Hess et al., 1986). Previous studies have suggested that increased spermiophages result in reduced fertility and hatchability (Phadke, 1975; Thurston et al., 1975; Hess et al., 1986). However, Barnes et al. (1996) increased spermiophage numbers in fresh and 6 h stored semen by adding

isolated spermiophages, but observed no differences in fertility, hatchability or embryonic mortality.

A fertility decline in turkeys during a 20-25 wk breeding season has been observed which has been called infertility syndrome and was characterized by a dramatic loss of fertility of 10% or greater per wk (Harper and Arscott, 1969; Yu and Burke, 1979a,b). The fertility could be restored by intramagnal insemination and molting, but the restored fertility effect was short term (Yu and Burke, 1979a). Sera from sterile hens have been shown to possess factors that may be involved with infertility. It has been suggested that it may be related to antisperm Ab but no Ab levels were checked (Yu and Burke, 1979b). Late season declines in fertility have a common occurrence and antisperm Ab have been implicated as a possible cause. Yu and Burke (1979a) hypothesized that as the hen aged throughout the production period, antisperm Ab increased to the point that fertility was compromised. Declines in fertility were observed when semen was incubated with the sera from sterile hens as compared with sera from fertile hens. McCorkle et al. (1983) observed declines in fertility of hens with high antisperm Ab titers due to intravenous and intraperitoneal injection of sperm or oviduct trauma. The report was one of the first to indicate that oviduct injury could result in increase antisperm Ab. Late season declines in fertility are observed regularly and have been shown to be associated with a reduction of sperm retention in the SST (Van Krey et al., 1967; Christensen 1981; Pierson et al., 1988) and may have a genetic component (Dunnington et al., 1990). Zheng and Yohsimura (1999) observed an increase in macrophages in the chicken oviduct stroma as the hen aged. The stroma of the magnum was the only section where

older hens had significantly more macrophages as compared to young laying hens. The macrophage numbers did not differ significantly between the two ages in the other oviduct sections. It is possible that sperm lost to engulfing might contribute to late season declines in fertility but more work is needed to determine macrophage phagocytosis of sperm in the oviduct.

McIntyre et al. (1982) reported an initial increased fertility rate when turkey breeder hens were inseminated prior to the start of egg production rather than after. Bakst (1988) demonstrated increased duration of fertility when hens were initially inseminated with larger doses of semen on two consecutive days when compared to hens that were inseminated with smaller doses on five consecutive days. The results of these studies indicate the positive correlation between fertility and the number of sperm stored in the SST. In both studies, increased fertility was observed when the SST received treatments that were associated with maximal filling. Fecund sperm have been shown to be associated with characteristics such as mobility and result in more sperm entering the SST and higher fertility than sperm that are less fecund and have lower mobility (Donoghue et al., 1998; Donoghue, 1999).

Decreased fertility has been observed over the period of egg production when hens were inseminated with semen stored for 24 h (for review, see Thurston, 1995). Donoghue et al. (1995) reported that fewer sperm trapped in the OPVL, which indicated fewer sperm stored in the oviduct SST as a result of in vitro storage. Additional work indicated a reduced number of IPVL SP holes in eggs from hens inseminated with stored sperm but fertility did not differed between the storage treatments in that study (Donoghue, 1996).

One of the strategies to improving fertility has been the identification and culling of subfertile toms by developing ways to categorize toms by semen quality. Some of the factors measured to determine tom differences in fertilizing potential include ATP concentrations (Wishart, 1982; Wishart and Palmer, 1986), motility (Wishart and Palmer, 1986); mobility (Froman et al., 1997; Donoghue et al., 1998) and morphological integrity (Wishart and Palmer, 1986). Another strategy is to improve the fertility of subfertile toms through management or other means such as diliuent additives. Gill et al. (2000) and Donoghue et al. (1997) have demonstrated fertility increases in toms when semen is exposed to a synthetic peptide thought to contain epitopes of universal primary sperm-ovum binding protein. Another strategy that can be used to monitor fertility without having to incubate eggs is by examining the sperm-ovum interaction. Brillard and Bakst (1990) have demonstrated a positive correlation between the number of sperm contained in the oviducal SST and the number of sperm trapped in the OPVL. The number of sperm trapped in the OPVL and the number of SP holes in the IPVL have been compared and found to yield similar results. The method of examining OPVL sperm is difficult and requires equipment to work with fluorescent dyes making the method of counting IPVL SP holes more practical to monitor sperm in the oviducal SST and the subsequent effect on fertility (Wishart, 1997; Staines et al., 1998).

EARLY EMBRYO DEVELOPMENT

The development of the avian embryo is a complex multifaceted process. Discussion of this process in its entirety is beyond the scope of the current review and the reader is referred to Bellairs, 1993; Eyal-Giladi and Kochav, 1976; Kochav et

al., 1980; Eyal-Giladi, 1991; Eyal-Giladi, 1993; Romanoff, 1960; Hamburger and Hamilton, 1951 and Gilbert, 2000 for more detail on this topic. Avian fertilization and development begins prior to oviposition. While the egg descends through the oviduct, two of the important and basic events in embryogenesis occur. The first is cleavage, the process where cells divide and the cytoplasm of the original zygote becomes divided into a cell population called blastomeres. The second process involves the establishment of embryo polarity. Dorso-ventral polarity is established followed by the antero-posterior axis as the eggshell membranes are deposited and calcification occurs (Bellairs, 1993). During this time, the uterine period can be divided into two developmental phases. The first is the transformation from a germinal disc to a blastodisc and the second is the activation of the metabolic machinery (Eyal-Giladi, 1991).

Embryo development can be altered by any change in the incubator environment so it was recognized early that an objective system was required to determine progress of embryo development when eggs were opened prior to hatching such as to determine when embryo death occurred. Phillips and Williams (1944) described the morphology of the turkey from 24 h of incubation through 26 d of incubation. Hamburger and Hamilton (1951) gave detailed morphological descriptions of 46 stages to describe the development of the chick embryo through the entire incubation period. Since turkey and chicken developmental stages are similar but chronologically different, Abbott (1967) correlated the turkey stages of development to those of the chicken as described by Hamburger and Hamilton (1951). Eyal-Giladi and Kochav (1976) described a more detailed staging system of

the chick embryo from cleavage through the formation of the primitive streak. Their staging system consisted of 14 stages (I through XIV EG&K). Gupta and Bakst (1993) described a staging system for the turkey from cleavage through hypoblast formation. Their staging system consisted of 11 stages (I-XI G&B) and lagged behind that of the chicken. Bakst et al. (1997) reported the chronological differences in development between chicken embryos and turkey embryos through hypoblast formation. They reported that at oviposition the chicken embryo is at a Stage X EG&K, where the turkey embryo was at a Stage VII EG&K. This agrees with the results of Gupta and Bakst (1993), Fasenko (1996) and Fasenko et al. (2001) who observed embryos at stage VII at the time of oviposition in turkeys. Completed formation of the turkey area pellucida occurs after incubation (Gupta and Bakst, 1993), where in chickens the completed area pellucida can be seen in the Stage X embryo prior to incubation (Eyal-Giladi and Kochav, 1976; Bakst et al., 1997).

For the purpose of this review, it should be reiterated that there are many processes involving tissue induction during the first week of incubation. If any process is not completed correctly, embryo malformations and death may result. These processes include, but are not limited to, the formation of the neural and circulatory systems. Metabolic and respiratory adjustments are made as nutrient availability changes with development (Romanoff, 1960, 1967; Christensen et al, 1996). For example, the avian embryo is largely dependent on glycogen reserves and glucose during the first few days of development. However, carbohydrate is a limited resource and biochemical and physiological lipolysis and β-oxidation must be functional before the glycogen and glucose are utilized (Freeman, 1965). Improper

formation of the circulatory or extraembryonic components involved in respiration will lead to improper or retarded development and could result in embryonic death.

EMBRYONIC MORTALITY

Periods of Embryonic Mortality

Christensen and Bagley (1989) describe embryonic mortality (EM) as a non-random event with the chance of an embryonic death occurring not being equal on all days of incubation. Turkey EM can be divided into three main periods of mortality. These are pre-oviposital mortality, egg storage prior to incubation mortality and incubation mortality. The current review will focus on the incubation period. Readers are referred to Christensen (2000) for information concerning each period. The periods of turkey EM during incubation are very similar to those reported for the turkey over 65 years ago in both time of occurrence and magnitude, indicating very little improvement in embryonic mortality (Insko and Martin, 1935; MacLaury and Insko, 1953; Christensen, 1978). There are two periods in avian incubation that are associated with high EM. These periods occur during the first or third periods of incubation and can be found in several different avian species including chicken (Payne, 1919; Insko and Martin; 1935; Romanoff, 1972); turkey (Insko and Martin, 1935; MacLaury and Insko, 1953; Christensen, 1978) and doves and pigeons (Riddle, 1930). Romanoff (1949; 1972) listed a third period occurring around 12-14 d of incubation in the chicken. However, this third period of EM is rarely seen with the exception of various nutritional disorders. Jassim et al. (1996) developed a mathematical model for multiphasic analysis to assess infertility and characterize EM distribution in chickens. Using data from broilers, it was demonstrated that the

model worked and gave estimates that were consistent with literature values. It was suggested that this type of model could evaluate management and genetic aspects of hatchability, infertility and EM.

One of the earliest studies of EM in turkeys was conducted utilizing Broad-Breasted Bronze turkeys (Insko and Martin, 1935). Prior to this study the EM of turkey eggs had not been reported in the literature. In that study, the EM of turkey eggs was compared to that of chicken eggs where similar patterns were reported. The EM peaks occurred at comparable periods of incubation for both chicken and turkey eggs (Insko and Martin, 1935). In examining the EM curves obtained from three breeds of turkeys (the Broad Breasted Bronze, Small Type Whites, and Standard Bronze) it was found that all breeds exhibited a peak in EM during the first week of incubation. The day of incubation where this peak occurred tended to vary with breed (MacLaury and Insko, 1953).

In 1993, a study of Large White commercial turkey breeder hens demonstrated that the EEM varied from 6 to 13% of all fertile eggs and that late mortality was 1.5 to 2% (Krueger, 1993). It was stated that the values for late EM were lower than usual and were attributed to the above average hatchability that was observed during the study. Research has focused on late EM mainly due to the ease with which the advanced embryo can be isolated, manipulated and examined.

Working with the embryo at the end of the incubation period has lead to many theories that could influence the amount of late EM. The prediction of the plateau period of oxygen consumption has lead to the concept of matching embryonic metabolism to the ability of the eggshell and eggshell membranes to exchange vital

gases (Rahn et al.,1981; Christensen et al., 1996; Christensen et al., 2000). Early embryonic research was not performed extensively due to the lack of proper techniques, difficulty in obtaining samples and the lack of information on the developmental processes that are occurring during the first week of incubation. The current volume of information concerning this period of development has been expanded and can be used to study why EM occurs during this period and how it could be reduced.

Causes of Embryonic Mortality

If factors that affect EM only affected the mortality that occurs during a specific stage of embryo development, causes of EM would be easier to ascertain and correct or prevent. However, a factor thought to be increasing EM can result in death of the embryo at any of the different stages of development. In instances where large percentages of EM occur at a certain stage, the cause may be indicative of a specific factor. For this reason, the environmental conditions must be recorded in a fashion to help troubleshoot hatchery or breeder problems that result in abnormally high EM. Factors which can have an adverse effect on avian embryogenesis include, but are not limited to, prolonged duration of egg storage (Arora and Kosin, 1966; Coleman and Siegel, 1966; Sittmann et al., 1971; Mather and Laughlin, 1977; Fasenko et al., 1991; Fasenko, 1996; Brake et al., 1997); suboptimal conditions during egg storage (Meijerhof, 1992); season of the year (Kosin and Mun, 1965); nutrition of the hen (see review, Wilson, 1997); nutrient availability to the embryo (Byerly et al., 1932); egg size (Landauer, 1967); and age of the breeders (Christensen, 1978). Intramagnal inseminations have resulted in

increased EEM (Van Krey et al., 1966; Lorenz and Ogasawara, 1968). Death associated with this method of insemination could be due to either "poor" or "unfit" spermatozoa or pathological polyspermic fertilization. By performing intramagnal insemination, the vagina, one of the sperm selection barriers, is bypassed allowing both "fit" and "unfit" sperm to reach the infundibulum. This would probably be a transient effect as "unfit" sperm are unlikely to enter the secondary sperm storage sites located in the infundibulum and would be depleted more rapidly than "fit" sperm. Holleman and Biellier (1976) inseminated hens by placing the insemination straw at a depth 3.0 or 7.5 cm into the oviduct. The treatment with 7.5 cm placed sperm past the UVJ, thus bypassing the sperm selection barriers. A higher embryonic mortality would have been expected as the poor quality sperm would have an opportunity to fertilize the ovum or larger numbers of sperm would have reached the infundibulum creating a situation where pathological polyspermic fertilization could occur. The deep insemination of 7.5 cm resulted in better fertility and lower EM than hens that received a shallow insemination of 3.0 cm. This observation does not agree with results of other studies where intramagnal inseminations resulted in increased EM (Van Krey et al., 1966; Lorenz and Ogasawara, 1968).

As early as 1925, embryonic death has been associated with malpositions throughout the incubation period (Sanctuary, 1925; Hutt and Pilkey, 1930; Hutt and Pilkey, 1934). There are six prominent malpositions exhibited in the last wk of incubation: head between the thighs; head in small end of egg; head to left instead of under right wing; head normal but embryo in a position that puts the beak buried

away from the air cell; feet over head; and beak above right wing rather than below it. These six malpositions can have slight variations (Hutt and Pilkey, 1934). The results of Byerly (1930) and Hutt (1930) suggest that structural abnormalities and teratism account for a small percentage of embryonic deaths. The time of day during when oviposition occurs has been associated with EM. Eggs laid in the afternoon have been reported to have slightly higher EM than eggs laid in the morning (Hutt and Pilkey, 1930) where Funk (1934a) reported better hatchability in afternoon oviposition eggs than in morning oviposition eggs. Moore (1959) observed increased EM in hens that had intervals below or above 26 h. Bernier et al. (1951) reported better hatchability in eggs laid between noon and 2 o'clock compared to eggs laid before or after this time period. The blastoderm size from eggs laid in the first or last clutch positions were more advanced (as measured by blastoderm area) than eggs laid intermediate to these positions. Significant differences were found between individuals in embryo size, but individual differences in oviposition and ovulation, body temperature and genetic factors may have contributed to this difference in embryonic size at oviposition (Bernier et al., 1951). Another factor influencing the blastoderm size at oviposition is nest holding times prior to incubation (Fasenko et al., 1991). Advancement of one developmental stage was observed in turkey eggs held up to 14 d (Bakst and Gupta, 1997). EEM, defined as embryonic mortality during the first 7 days of development, is influenced by egg storage length and temperature when examined in broiler breeder chickens (Scott and Mackenzie, 1993). Riddle (1930) suggested that EEM might be the result of a failure in respiratory adjustment. Sperm stored under sub-optimal conditions prior to

insemination can increase EEM (Huyghebaert et al. 1984, 1985; Sexton, 1988). Increased embryonic death has been associated with embryos fertilized by aged sperm in the chicken (Lodge et al., 1971).

Increased hatchability due to a decrease in EEM has been observed in eggs stored for 4 d at 18 C and 75% RH when compared to eggs stored 1 d under identical conditions (El Jack and Kaltofen, 1969; Kirk et al., 1980). Conversely, EEM has been reported to increase when eggs were stored for longer than 7 d under similar conditions (Waite, 1919; Scott, 1933; Funk, 1934b). The excellent albumen quality found in freshly laid eggs provides greater resistance to gaseous diffusion than the eggshell during early incubation (Meuer and Baumann, 1988). Brake et al. (1993) suggested that EEM in fresh eggs was due to interference of gas flow by the good albumen quality found in those types of eggs. Walsh et al. (1995) reported greater EEM and stored weight loss as the storage temperature and length increased. This theory is supported by the work of Meuer and Baumann (1988). They examined the oxygen pressure in vitelline and intraembryonic blood vessels of chicken eggs and suggested that albumen between the blastoderm and shell was the primary barrier to gas exchange during the first 4 days of incubation, not the shell.

There is a potential error when trying to determine whether infertility or EEM is the cause of poor hatchability. The identification of infertility and EEM can be difficult especially when working with eggs that were not candled out at 14 d of development but were incubated for a full 28 d. Kosin (1951) concluded that in the case of Broad Breasted Bronze turkeys the cause of decreased hatchability was due to excessive

EEM and not infertility. This study was only performed in Broad Breasted Bronze turkeys and should not be applied to today's current genetic lines. Further work in this area is needed to determine EEM and infertility roles in hatch percentages. Egg size has been associated with EM (Hagger et al., 1986; Sewalem and Wilhelmson, 1999). As egg size increases, shell quality decreases, which may contribute to increased embryonic development (Nestor et al., 1972). Selection for increased residual feed intake (feed consumption adjusted to equal BW and BW gain from individual results of a 28-d feeding trial conducted at each generation on all candidates to selection in males. In females feed consumption was also adjusted to equal eggmass) in domestic fowl resulted in decreased hatchability due to an increase of both unfertilized eggs and embryo mortality when compared to the low residual feed intake line. Morisson et al. (1997) suggested that selection for increased residual feed intake may have been associated with an alteration in sperm cell function, as the data indicated that the increased residual feed intake line had fewer sperm cell mitochondria present.

Hen Age Effects on Embryonic Mortality

Early embryonic mortality has generally been found to be greater in hens earlier in lay where late deaths were more prevalent with older hens (Sunde and Bird, 1959; Christensen, 1978; Cherms, 1981; Rahn et al., 1981). Deeming and Van Middlekoop (1999) examined eggs from two different broiler breeder strains at two different ages. They saw decreases in fertility as the flock aged. For the most part, no differences in EEM were found between strain or hen age, however the older birds had significantly higher EEM on d 3 of incubation as compared to younger

flocks. No information on turkey hen age and EM has been reported in the last 20 years but should be examined for possible changes associated with genetic selection to meet market demands. EEM in turkey hens has been reported to decrease as hens are molted and they reach their second and third years of age (Leighton et al., 1971; Woodard et al., 1976; Krueger, 1993). Brake et al. (1993) suggested that EEM in young hens could be due to superior albumen quality that may decrease embryonic gas exchange. EEM has been shown to decrease in broiler breeders with increased hen age during the first 8 weeks of production (O'Sullivan et al., 1991). The results of their study indicated that chicken embryos from older broiler breeder hens weighed more at 18 d of incubation than embryos from younger hens even when egg weight variation was taken into consideration. A possible cause for the decreased embryo weight at 18 d of incubation could be related to the decreased lipid content of the embryo and yolk sac in eggs from younger hens as compared to that from older hens. The percent of lipid in the embryo and yolk sac increased with hen age but there was no similar relationship with liver lipids. The turkey embryo utilizes the majority of the yolk during 22 through 28 d of incubation (Ding et al., 1995). Older hens deposit more yolk in the egg at the expense of albumen. This increased yolk deposition is reflected in the hatchling because poults from older hens have heavier body weights and more residual yolk than poults from younger hens (Applegate and Lilburn, 1996). Noble et al. (1986) observed increased EM in 25 week old chicken hens when compared to hens that were 41 weeks of age. A decreased transfer of lipid or failure in yolk lipid mobilization and uptake by the embryo was observed and suggested as a possible

cause for the increased EM observed in the younger chickens. Yafei and Noble (1990) examined these observations further and not only found similar results, but found that reduced yolk lipid concentration in embryos from younger hens was associated with reduced levels of plasma low-density lipoproteins and altered proportions of the major lipid fractions that include cholesteryl esters, triacylglycerides, free cholesterol and phosphoglycerides. This is a good example of how maternal influences due to hen age might have undesirable effects on embryonic viability.

Lillpers and Wilhelmson (1993) reported that as the hen aged, oviposition intervals within sequence increased, the frequency of missing eggs within a sequence increased, and more than one pause day occurred between sequences. Bernier et al. (1951) reported increased hatchability for eggs laid intermediate to the first and last sequence eggs, which agrees with the results of Bacon and Nestor (1979). This disagrees with the results of Fasenko et al. (1992b) who suggested that the increased EM in older hens might be related to the increase incidence of first-of-sequence eggs. The results of their study indicated that EM and preincubation embryonic development was related more to egg sequence position than hen age. Bacon and Nestor (1979) observed lower hatchability of both all eggs set and fertilized eggs set in first of sequence eggs when compared to other eggs within the clutch. This decrease in hatchability was associated with an increase in 7 d EM. However, Coleman et al. (1964) reported a low correlation between embryonic development after 48 h of incubation and egg sequence position. Increasing hen age has been associated with increased rate of embryonic development in chickens

(Mather and Laughlin, 1979; Shanawany, 1984). Mather and Laughlin (1979) also suggested that the increased embryonic development associated with increasing hen age could be attributed to the greater number of first-of-sequence eggs, which spend longer time on the ovary than subsequent eggs in the sequence (Robinson et al., 1991). Fasenko (1992) observed a more advanced stage of development in first of sequence eggs.

One theory that has been associated with embryonic survival is the developmental stage at oviposition (Hays and Nicolaides, 1934). Embryos from poor hatching eggs were found at pre-gastrula and early gastrula stage at oviposition where eggs that hatched well were at an advanced gastrula stage of development. It is possible that the embryo requires a certain level of maturity at oviposition to survive the rigors of storage and incubation. There are multiple factors that could affect the developmental stage at oviposition that include ambient temperature in the nest prior to egg collection, time the egg is in the nest before collection, whether or not (and if so how long) the hen incubated the egg while in the nest and the preincubation egg storage conditions. Weisbroth and Kosin (1966) observed both increased and decreased embryo diameter after egg storage with the largest percentage increasing in diameter. Advanced embryo development at oviposition and lower EM was reported in hens selected for low nine wk BW when compared to hens selected for high BW (Coleman and Siegel, 1966). Other reports have associated low BW selection with advanced stage of development at oviposition in both chickens (McNary et al., 1960) and turkeys (Kosin and Mun, 1965; Arora and Kosin, 1964, 1966). Young hens may lay eggs that are at an earlier stage of

development (EG&K stage IX) than those laid by older hens (EG&K stage X) (Eyal-Giladi, 1993). Mather and Laughlin (1977) examined fresh, unincubated eggs and reported increased blastoderm area as hens aged. The first and last eggs of clutch sequence have been reported to have more advanced embryos at oviposition and after short periods of incubation than intermediate eggs (Bernier et al., 1951). This is in agreement with Fasenko (1992) who reported advanced stage of development in first of sequence eggs. Kosin (1956) reported improved hatchability from chicken eggs exposed to prestorage warming treatments prior to egg storage. Fasenko et al. (2001) used preincubation treatments to advance embryonic stage of development prior to egg storage but did not observe any differences in hatchability.

Genetic Contributions to Embryonic Mortality

Genetic abnormalities have been associated with EEM (Bloom, 1974). Blastoderm degeneration, an early embryonic failure in dwarf single comb chickens has been identified as a condition that results in embryonic death between Hamburger-Hamilton stages 8 and 9 of development (Savage et al., 1992). Three heritable early embryonic failures during the initial 96 h of incubation have been reported in turkeys. Olsen (1975) first described one embryonic failure, termed parthenogenesis, that is a condition in which eggs from virgin hens develop when placed in normal incubator environments. A second embryonic disorder named ring lethal, is an autosomal trait that is expressed at approximately 48 h of incubation. This embryonic failure is characterized by the presence of a ring of amorphous embryonic tissue (Savage and Harper, 1985). A third embryonic disorder that has been reported is referred to as blood ring, which is an autosomal recessive trait

observed in turkeys after 10 d of incubation. The blood ring lethal is characterized by numerous aggregates of uncoalesced blood islands, an embryo without an extra-embryonic circulatory system and a diffuse *sinus terminalis* situated at the boundary of the yolk and adjacent to the inner shell membrane (Savage and Mirosh, 1989; 1992). Although these three conditions are inheritable, other studies have suggested that EM is not heritable. Shook et al. (1971) saw very low and in some cases negative heritabilities when EM was selected as a trait in broad breasted bronze turkeys. McCartney (1962) found that selection for reduced EM produced little improvement whereas Arora and Kosin (1966) reported improved fertility, and lower pre-oviposital and early post-oviposital EM mortality when selecting for high and low hatchability. Brah et al. (1991) examined Leghorns and reported that EM at various stages of incubation was inherited, as well as total incubation mortality. Byerly et al. (1934) observed improved hatchability in chickens when pure breeds were crossed. The improvement in hatchability was a result of reduced EM during the third week of incubation in chickens. The cross did not reduce EEM. Chromosome abnormalities have also been shown to be responsible for some of the EEM in layer and broiler chickens (Thorne et al., 1991). Inbreeding of the hen or embryo has been reported to have little or no detrimental effect on EM (Hagger et al., 1986; Sewalem and Wilhelmson, 1999).

Embryonic Mortality and Incubation

The four principles of incubation include temperature, relative humidity, physical agitation (turning) and gas exchange (oxygen, carbon dioxide, nitrogen, etc.). These factors are monitored and controlled in modern incubators to maintain

environmental conditions that are conducive to optimal embryo survival. However, if any of these factors fall outside of normal ranges, the incidence of EM can be altered and usually increases. French (1997) stated, “the increased temperature experienced by the developing embryo is dependent on the incubator temperature, the metabolic heat production of the embryo, and the thermal conductance of the egg and surrounding air”. The effect of high temperature on embryonic mortality is dependent on temperature increases, exposure time and the age of the embryo (Deuchar, 1952; Landauer, 1967; Lundy, 1969; Ande and Wilson, 1981). For most poultry species, the optimum temperature is between 37 and 38 C with time dependent deviations from this resulting in reduced hatchability due to increased embryonic death (French, 1997; Wilson, 1991). Increased incubation temperature has resulted in increased embryonic deaths in both the first and last weeks of incubation (Romanoff, 1972). Alsop (1919) observed neural tube and neural fold defects when chick embryos were exposed to low incubation temperatures and neural tube defects and extra somite formation when chick embryos were exposed to high temperatures. Nilsen (1984) reported both extraembryonic and intraembryonic vascular abnormalities when chick embryos were exposed to hyperthermia during the incubation period. Excessive EM has been observed at stages corresponding to 6 to 7 and 19 to 21 d of incubation when chicken eggs were incubated at 36.1 C (Byerly, 1938). Excessive EM was observed on 13 through 15 and 18 through 21 d of incubation when chicken eggs were incubated at a high temperature of 39.2 C (Byerly, 1938).

Relative humidity influences egg hydration during incubation (Ar, 1990). Eggs that are exposed to low RH early in incubation had increased EM as compared with eggs that were exposed to low RH during late incubation (Snyder and Birchard, 1982). However, Meir and Ar (1986) demonstrated that eggs exposed to low RH early could recover and hatch normally. Reduced RH during incubation has resulted in decreased EEM. This was associated with increased water vapor loss during the first 3 days of incubation and was thought to result in an effect where the blastoderm was brought closer to the shell to allow better gas exchange as the albumen thinned (Vick et al., 1993). This may be related to the blocking of gas exchange by albumen as discussed in the previous section. When chicken eggs were incubated in increased RH, late EM was increased (Bruzual et al., 2000)

Eggshell conductance is another factor that is crucial to gas exchange across the eggshell. As the hen ages, eggshell conductance decreases and it has been suggested that the eggs from late cycle hens should be incubated at 7% lower humidity that eggs from early cycle hens (Rahn et al., 1981). When egg weight losses of fertile hatching eggs and fertile nonhatching eggs were compared it was found that embryos dying late in incubation have decreased conductance rates (Christensen and McCorkle, 1982). From this study it was concluded that the eggshells of some turkeys might be inadequate to meet the embryo metabolic requirements especially in the larger eggs of older hens. It was suggested that for each strain of turkey and for each egg weight there might be a unique eggshell conductance that would result in optimum embryo survival.

Mechanical agitation or turning of the egg has also been related to EEM. Insko and Martin (1933) noted a decrease in EEM when the number of times the eggs were turned was increased. In the absence of egg turning, the embryo or the extra-embryonic membranes or both adhere to the shell membranes, which can result in retarded and abnormal development (New, 1957; Freeman and Vince, 1974). Furthermore, eggs that are not turned do not undergo proper formation of the chorioallantois and have more residual albumen which together may be the cause of a reduced rate of embryonic development leading to later hatching times (Tazawa, 1980; Tullett and Deeming, 1987; Deeming, 1989).

From this review it can be ascertained that hen age can influence many variables within avian reproduction. The literature is much more comprehensive for the chicken than the turkey and based on previous reports not everything is similar between these two avian species. There is still a need for research on the effects of hen age in relation to fertility and EM, and in particular lower EEM that could improve hatchability of turkey eggs and possibly increase poult quality at hatching.

DISSERTATION OBJECTIVES

The research completed thus far on turkey embryonic mortality needs to be reevaluated when one takes into consideration the progresses made in genetic selection for increased meat yields. Furthermore, it is assumed in many instances that the physiological and metabolic processes will be similar between chicken and turkey embryos under various conditions. The overall objective for this dissertation was to examine the relationship of embryonic mortality, hen age and fertility in Large White turkeys.

The individual objectives of the five dissertation experiments were:

1. Examine embryonic mortality and fertility between young and old turkey breeder hens.

2. Determine the effects of hen age and sperm dose on in vivo sperm penetration of the inner perivitelline layer.

3. Examine in vitro sperm penetration between two commercial strains of turkeys at two different ages.

4. Determine if sperm penetration of the inner perivitelline layer differs between two turkey lines known to differ in the percentage of early embryonic mortality.

5. Examine genetic, sire, and dam effects on sperm penetration of the inner perivitelline layer and correlate these measurements to embryonic mortality levels.

REFERENCES

Abott, U. K., 1967. Avian developmental genetics. Pages 13-52 *in*: Methods in Developmental Biology. F. H. Wilt and N. K. Wessells ed., Thomas Y. Crowell, Co., New York, NY.

Allen, T. E. and G. W. Grigg, 1957. Sperm transport in the fowl. Aust. J. Agri. Res. 8:788-789.

Alsop, F. M., 1919. The effect of abnormal temperatures upon the developing nervous system in the chick embryos. Anatomical Record 15:307-331.

Ande, T. B., and H. R. Wilson, 1981. Hatchability of chicken embryos exposed to acute high temperature stress at various ages. Poultry Sci. 60:1561-1566.

Applegate, T. J., and M. S. Lilburn, 1996. Independent effects of hen age and egg size on incubation and poult characteristics in commercial turkeys. Poultry Sci 75:1210-1216.

Ar, A., 1990. Egg water movements during incubation. Pages 157-173 *in*: Avian Incubation. S. G. Tullett ed. Butterworth-Heinemann, London.

Arora, K. L., and I. L. Kosin, 1964. Selection for traits in the mature domestic turkey yields correlative responses in embryos. Genetics 50:232-233.

Arora, K. L. and I. L. Kosin, 1966. Developmental responses of early turkey and chicken embryos to preincubation holding of eggs: inter- and intra-species differences. Poultry Sci. 45:958-970.

Bacon, W. L. and K. E. Nestor, 1979. Reproductive traits of first eggs of clutches vs. other clutch positions in turkeys. Poultry Sci. 58:257-258.

Bain, J. M and J. M. Hall, 1969. Observation on the development and structure of the vitelline membrane of the hen's egg: An electron microscopical study. Aust. J. Biol. Sci. 22:653-665.

Bakst, M. R., 1981. Sperm recovery from oviducts of turkey at known intervals after insemination and oviposition. J. Reprod. Fertil. 62:159-164.

Bakst, M. R., 1988. Turkey hen fertility and egg production after artificial insemination and multiple oviduct eversion during the pre-laying period. J. Reprod. Fertil. 83:873-877.

Bakst, M. R., 1989. Oviductal storage of spermatozoa in the turkey: its relevance to artificial insemination technology, Br. Poult. Sci. 30:441-447.

Bakst, M. R., 1994. Fate of fluorescent stained sperm following insemination: New light on oviducal sperm transport and storage in the turkey. Biol. Reprod. 50:987-1992.

Bakst, M. R. and H. C. Cecil, 1981. Changes in the characteristics of turkey ejaculated semen and ductus deferens semen with repeated ejaculations. Reprod. Nur. Develop. 21:1095-1103.

Bakst, M. R. and S. K. Gupta, 1997. Preincubation storage of turkey eggs:impact on rate of early embryonic development. Br. Poult. Sci.. 38:374-377.

Bakst, M. R., S. K. Gupta and V. Akuffo, 1997. Comparative development of the turkey and chicken embryo from cleavage through hypoblast formation. Poultry Sci. 76:83-90.

Bakst, M. R. and T. J. Sexton, 1979. Fertilizing capacity and ultrastructure of fowl and turkey spermatozoa before and after freezing. J. Reprod. Fertil. 55:1-7.

Bakst, M. R., G. Wishart, J. P. Brillard, 1994. Oviducal sperm selection, transport, and storage in poultry. Poultry Science Rev. 5:117-143.

Bakst, M. R. and B. Howarth, 1977. Hydrolysis of the hen's perivitelline membrane by cock sperm *in vitro*. Biol. Reprod. 17:370-379.

Barnes, D. A., R. J. Thurston, T. R. Scott, and N. Korn, 1996. Effect of added spermiophages in pooled turkey semen on fertility, embryonic mortality, and hatchability. Poultry Sci. 75:943-948.

Baumel, J. J., A. S. King, J. E. Breazile, H. E. Evans, and J. C. Vanden Berge, ed., 1993. Handbook of Avian Anatomy: Nomina Anatomica Avium, 2nd ed. Nuttal Ornithological Club, Cambridge, MA.

Bekhtina, V. G., 1968. Morphological features of polyspermy fecundation in hens. In: Summaries from Pushkin Research Laboratory of Livestock Breeding. Leningrad Region, U.S.S.R. Cited by World's Poultry Sci. J. 24:148.

Bellairs, R., 1993. Fertilization and early embryonic development in poultry. Poultry Sci. 72:874-881.

Bellairs, R., M. Harkness, and R. D. Harkness, 1963. The vitelline membrane of the hen's egg: A chemical and electron microscopical study. J. Ultrastruc. Res. 8:339-359.

Bernier, P. E., L. W. Taylor, and C. A. Gunns, 1951. The relative effects of inbreeding and outbreeding on reproduction in the domestic fowl. Hilgardia 20:529-628.

Birkhead, T. R., B. C. Sheldon, and F. Fletcher, 1994. A comparative study of sperm-egg interactions in birds. J. Reprod. Fertil. 101:353-361.

Birkhead, T. R. and J. D. Biggins, 1998. Sperm competition mechanisms in birds: models and data. Behav. Ecol. 9:253-260.

Birkhead, T. R., E. J. Pellatt, and F. Fletcher, 1993. Selection and utilisation of spermatozoa in the reproductive tract of the female zebra finch Taeniopygia guttata. J. Reprod. Fertil. 99:593-600.

Bloom, S. E., 1974. The origins and phenotypic effects of chromosome abnormalities in avian embryos. 15th World Poultry Congress, pp. 316-321 New Orleans, World's Poultry Science Association.

Boone, M. A., 1968. Family differences in semen quality in one strain of White Plymouth Rocks. Poultry Sci. 47:1049-1051.

Brah, G. S., J. S. Sandhu, and M. L. Chaudhary, 1991. Heritability estimates of components of incubation mortality in white leghorns. Br. Poult. Sci. 32:871-874.

Brake, J., T. J. Walsh, C. E. Benton, Jr., J. N. Petitte, R. Meijerhof, and G. Penalva, 1997. Egg handling and storage. Poultry Sci. 76:144-151.

Brake, J., T. J. Walsh, and S. V. Vick, 1993. Relationship of egg storage time, storage conditions, flock age, eggshell and albumen characteristics, incubation conditions, and machine capacity to broiler hatchability—Review and model synthesis. Zootech. Int. 16:30-41.

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

Bramwell, R. K., and B. Howarth, 1992b. Cross-reactivity of sperm binding proteins from chicken, turkey and duck oocytes. Poultry Sci. 71:1927-1932.

Bramwell, R. K. and B. Howarth, 1997. Effect of low or high sperm penetration values at the germinal disc on early embryonic mortality in chicken eggs. Poultry Sci. 76(Suppl. 1):97 (Abstr).

Bramwell, R. K., H. L. Marks, and B. Howarth, 1995. Quantitative determination of spermatozoa penetration of the perivitelline layer of the hen's ovum as assessed on oviposited eggs. Poultry Sci. 74:1875-1883.

Bramwell, R.K., C. D. McDaniel, J. L. Wilson, and B. Howarth, 1996. The effect of male and female broiler breeders on sperm penetration of the perivitelline layer overlying the germinal disc. Poultry Sci 75:755-762.

Brillard, J. P., 1992. Factors affecting oviductal sperm storage in domestic fowl following artificial insemination. Anim. Reprod. Sci. 27:247-256.

Brillard, J. P., 1993. Sperm storage and transport following natural mating and artificial insemination. Poultry Sci. 72:923-928.

Brillard, J. P., and G. R. McDaniel, 1986. The influence of semen dose and frequency of insemination on fertility in dwarf broiler breeder hens. Poultry Sci. 65:2330-2334.

Brillard, J. P. and M. R. Bakst, 1990. Quantification of spermatozoa in the sperm storage tubules of turkey hens and the relation to sperm numbers in the perivitelline layer of eggs. Biol. Reprod. 43:271-275.

Bruzual, J. J., S. D. Peak, J. Brake, and E. D. Peebles, 2000. Effects of relative humidity during incubation on hatchability and body weight of broiler chicks from young breeder flocks. Poultry Sci. 79:827-830.

Burke, W. H. and W. C. Y. Yu, 1979. Infertility in the turkey I. Effects of anti-sperm immune globulins on fertilizing ability of turkey spermatozoa. Poultry Sci. 58:1367-1371.

Burley, R. W. and D. V. Vadehra, 1989. The Avian Egg. John Wiley & Sons, Inc. New York, NY.

Byerly, T. C., 1930. Time of occurrence and probable cause of mortality in chick embryos. Proc. World Poultry Congr. 4:178-186.

Byerly, T. C., 1938. Effect of different incubation temperatures on mortality of chick embryos. Poult. Sci. 17:200-205.

Byerly, T. C., C. W. Knox, and M. A. Jull, 1934. Some genetic aspects of hatchability. Poultry Sci. 13:230-238.

Byerly, T. C., W. G. Helsel, and J. P. Quinn, 1932. Growth of the chick embryo in relation to its food supply. J. Exp. Biol. 9:15-44.

Carlson, B. M., 1988. Patten's Foundations of Embryology. 5th ed. McGraw-Hill, Inc. New York, NY.

Cherms, F. L., 1981. Incidence of embryonic malpositions and terata in turkeys. Poultry Sci. 60:1638 (Abstr).

Christensen, V. L., 1978. Physiological parameters limiting hatchability in domestic fowl (Gallus domesticus) and domestic turkey (Meleagris gallopavo). Ph.D. dissertation. University of Missouri, Columbia, MO.

Christensen, V. L., 1981. Effect of insemination intervals on oviducal sperm storage in turkeys. Poultry Sci. 60:2150-2156.

Christensen, V. L., 2000. Factors Associated With Early Embryonic Mortality. Proc. World Poult. Sci. Cong. Montreal, Canada.

Christensen, V. L., D. T. Ort, S. Suvarna, B. D. Fairchild, and W. J. Croom, 2000. The relationship of egg conductance constants to neonatal poult growth and quality. Poultry Sci. 79(Suppl. 1):79 (Abstr).

Christensen, V. L., and F. M. McCorkle, 1982. Turkey egg weight losses and embryonic mortality during incubation. Poultry Sci. 61:1209-1213.

Christensen, V. L. and L. G. Bagley, 1989. Embryology of the turkey. Pages 69-90 *in*: Recent Advances in Turkey Science. C. Nixey and T. C. Grey ed. Butterworth & Co., London.

Christensen, V. L., W. E. Donaldson, and J. P. McMurtry, 1996. Physiological differences in late embryos from turkey breeders at different ages. Poultry Sci. 75:172-178.

Coleman, J. W., H. S. Siegel, and P. B. Siegel, 1964. Embryonic development of two lines of White Rocks. Poultry Sci. 43:453-458.

Coleman, J. W. and P. B. Siegel, 1966. Selection for body weight at eight weeks of age. 5. Embryonic stage at oviposition and its relationship to hatchability. Poultry Sci. 45:1008-1011.

Compton, M. M. and H. P. Van Krey, 1979. A histological examination of the uterovaginal sperm storage glands in the domestic hen following insemination. Poultry Sci. 58:478-480.

Compton, M. M., H. P. Van Krey and P. B. Siegel, 1978. The filling and emptying of the uterovaginal sperm-host glands in the domestic hen. Poultry Sci 57:1696-1700.

Crittenden, L. B., and B. B. Bohren, 1962. The effects of current egg production, time in production age of pullet and inbreeding on hatchability and hatching time. Poultry Sci. 41:426-433.

Deeming, D. C., 1989. Characteristics of unturned eggs: Critical period, retarded embryonic growth and poor albumen utilisation. Br. Poult Sci. 30:239-249.

Deeming, D. C. and J. H. Van Middlekoop, 1999. Effect of strain and flock age on fertility and early embryonic mortality of broiler breeder eggs. Br. Poult. Sci. 40:S22-S26.

deReviers, M., and J. P. Brillard, 1986. Variations in the sperm production, the sperm output and in the number of sperms to be inseminated in aging broiler breeders. World Poultry Sci. J. 42:98 (Abstr.).

Deuchar, E. M., 1952. The effect of a high temperature shock on early morphogenesis in the chick embryo. J. Anat. 86:443-458.

Ding, S. T., K. E. Nestor, and M. S. Lilburn, 1995. The concentration of different lipid classes during late embryonic development in a randombred turkey population and a subline selected for increased body weight at sixteen weeks of age. Poultry Sci. 74:374-382.

Donoghue, A. M., 1996. The effect of twenty-four hour in vitro storage on sperm hydrolysis through the perivitelline layer of ovipositioned turkey eggs. Poultry Sci. 75:1035-1038.

Donoghue, A. M., 1999. Prospective approaches to avoid flock fertility problems: predictive assessment of sperm function traits in poultry. Poultry Sci. 78:437-443.

Donoghue, A. M., D. R. Holsberger, D. P. Evenson, and D. P. Froman, 1998. Semen donor selection by in vitro sperm mobility increases fertility and semen storage in the turkey hen. J. Androl. 19:295-301.

Donoghue, A. M., M. R. Bakst, D. R. Holsberger, and D. J. Donoghue, 1995. Effect of semen storage on sperm numbers in the perivitelline layer of laid turkey eggs. J. Reprod. Fertil. 105:221-225.

Donoghue, A. M., S. P. S. Gill, and R. P. Amann, 1997. Influencing fertility in turkey: Tom selection by sperm-binding assessment and improved binding with synthetic protein in fresh and store semen. Poultry Sci. 76(Suppl. 1):23. (Abstr).

Dunbar, B. S., S. Avery, V. Lee, S. Prasad, D. Schwan, E. Schwoebel, S. Skinner, and B. Wilkins, 1994. The mammalian zona pellucida: its biochemistry, immunochemistry, molecular biology, and developmental expression. Reprod. Fertil. Dev. 6:331-347.

Dunnington, H. P. Van Krey, R. M. Hulet, and D. M. Denbow, 1990. Genetic influences on seasonal decline in the fertility of female turkeys. Poultry Sci. 69:365-368.

El Jack, M. H., and R. S. Kaltofen, 1969. The effect of high holding and housing temperature on hatchability of chicken eggs. Poultry Sci. 48:1013-1018.

Etches, R. J., 1996. Reproduction in poultry. CAB International, Oxon, UK.

Eyal-Giladi, H., 1991. The early embryonic development of the chick as an epigenetic process. Crit. Rev. Poultry Biol. 3:143-166.

Eyal-Giladi, H., 1993. Early determination and morphogenetic processes in birds. Pages 29-37 in: Manipulation of the Avian Genome, R. J. Etches and A. M. Virrinder Gibbins ed. CRC Press, Inc., Boca Raton, FL.

Eyal-Giladi, H. and S. Kochav, 1976. From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. Dev. Biol. 49:321-337.

Fasenko, G. M., 1992. Factors influencing fertility, preincubation embryo development, and embryo viability in domestic fowl. MS thesis, University of Alberta, Edmonton, AB, Canada.

Fasenko, G. M., 1996. Factors influencing embryo and poult viability and growth during long term storage of turkey eggs. Ph.D. dissertation. North Carolina State University, Raleigh, NC.

Fasenko, G. M. F. E. Robinson, J. G. Armstrong, J. S. Church, R. T. Hardin and J. N. Petitte, 1991. Variability in preincubation embryo development in domestic fowl. 1. Effects of nest holding time and method of egg storage. Poultry Science 70:1876-1881.

Fasenko, G. M., F. E. Robinson, and R. T. Hardin, 1992a. Research Note: Variability in preincubation embryonic development in domestic fowl. 2. Effects of duration of egg storage period. Poultry Sci. 71:2129-2132.

Fasenko, G. M., R. T. Hardin, and F. E. Robinson, 1992b. Relationship of hen age and egg sequence position with fertility, hatchability, viability, and preincubation embryonic development in broiler breeders. Poultry Sci. 71:1374-1383.

Fasenko, G. M., V. L. Christensen, M. J. Wineland, and J. N. Petitte, 2001. Examining the effects of prestorage incubation of turkey breeder eggs on embryonic development and hatchability of eggs stored for four or fourteen days. Poultry Sci. 80:132-138.

Florman, H. M. and P. M. Wasserman, 1985. O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. Cell 41:313-324.

Fofanova, K. A., 1965. Morphological data on polyspermy in chickens. Fed. Proc. (Translation Suppl.) 24:T239-T247.

Freeman, B. M., 1965. The importance of lgycongen at the termination of the embryonic existence of Gallus domesticus. Comp. Biochem. Physiol. 14:217-222.

Freeman, B. M. and M. A. Vince, 1974. Development of the Avian Embryo. Chapman & Hall, London, England.

French, N. A., 1997. Modeling incubation temperature: the effects of incubator design, embryonic development and egg size. Poultry Sci. 76:124-133.

Froman, D. P., A. J. Feltmann, and D. J. McLean, 1997. Increased fecundity resulting from semen donor selection based upon *in vitro* sperm motility. Poultry Sci. 76:73-77.

Froman, D. P., and D. J. McLean, 1996. Objective measurement of sperm motility based upon sperm penetration of Accudenz®. Poultry Sci. 75:776-784.

Funk, E. M., 1934a. Relation of time of laying to hatchability. Poultry Sci. 13:184-187.

Funk, E. M., 1934b. Factors influencing hatchability in the domestic fowl. Missouri Agriculture Experiment Station Bulletin 341, Columbia, MO.

Gill, S. P. S., A. M. Donoghue, and R. P. Amann, 2000. Exposure of turkey sperm to a synthetic peptide before insemination increases fertility. Poultry Sci. 79:426-429.

Gilbert, S. F., 2000. Chapter 11 The early development of vertebrates: fish, birds, and mammals. Pages 339-376 *in*: Developmental Biology 6th ed. Sinauer Associates, Inc., Sunderland, MA.

Gupta, S., and M. K. Bakst, 1993. Turkey embryo development from cleavage through hypoblast formation. J. Morphol. 217:313-325.

Hagger, C., D Steiger-Stafl, and C. Marguerat, 1986. Embryonic mortality in chicken eggs as influenced by egg weight and inbreeding. Poultry Sci. 65:812-814.

Hamburger, V. and H. H. Hamilton, 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88:49-92.

Harper, E. H., 1904. The fertilisation and early development of the pigeon's egg. Am. J. Anat. 3:349-386.

Harper, J. A. and G. H. Arscott, 1969. Seasonal decline in fertility of turkey eggs. Poultry Sci. 48:2109-2113.

Hays, F. A. and C. Nicolaides, 1934. Variability in development of fresh-laid hen eggs. Poultry Sci. 13:74-90.

Hazary, R. C. and G. Wishart, 1999. Assessing the effect of mating ratio in broiler breeder flocks by quantifying sperm-egg interaction. Br. Poult. Sci.. 40:S46.

Hess, R. A., B. L. Hughes, and R. J. Thurston, 1986. Frequency and structure of macrophages and abnormal sperm cells in guinea fowl semen. Reprod. Nutr. Dev. 26:39-51.

Ho, J. J. L. and S. Meizel, 1976. Hydrolysis of the hen egg vitelline membrane by cock sperm acrosin and other enzymes. J. Exp. Zool. 194:429-438

Holleman, K. A. and H. V. Biellier, 1976. Fertility and embryonic livability as influenced by depth of insemination of turkey hens. Poultry Sci. 55:1154-1156.

Holm, L., H. Ekwall, G. J. Wishart, and Y. Ridderstrale, 2000. Localization of calcium and zinc in the sperm storage tubules of chicken, quail and turkeys using X-ray microanalysis. J. Reprod. Fert. 118:331-336.

Howarth, B., 1971a. Transport of spermatozoa in the reproductive tract of turkey hens. Poultry Sci. 50:84-89

Howarth, B, Jr, 1971b. An examination for sperm capacitation in the fowl. Biol Reprod. 3:338-341.

Howarth, B., 1990. Avian sperm-egg interaction: perivitelline layer possesses receptor activity for spermatozoa. Poultry Sci. 69:1012-1015

Howarth, B., 1992. Carbohydrate involvement in sperm-egg interaction in the chicken. J. Recep. Res. 12:255-265.

Howarth, B. and S. T. Digby, 1973. Evidence for the penetration of the vitelline membrane of the hen’s ovum by a trypsin-like acrosomal enzyme. J. Reprod. Fert. 33:123-125.

Hutt, F. B., 1930. On the origin, common types and economic significance of teratological monsters in embryos of the domestic fowl. Proc. World's Poultry Congr. 4:195-202.

Hutt, F. B. and A. M. Pilkey, 1930. Studies in embryonic mortality in the fowl IV: Comparative mortality rates in eggs laid at different periods of the day and their bearing on theories of the origin of monsters. Poultry Sci. 9:194-203.

Hutt, F. B. and A. M. Pilkey, 1934. Studies in embryonic mortality in the fowl, V. Relationships between positions of the egg and frequency of malpositions. Poultry Sci. 13:3-13.

Huyghebaert, G., F. Van Wambeke, and G. De Groote, 1984. The effect of pH of diluent, number of spermatozoa and storage method on fertility and hatchability obtained with turkey semen stored for 6 and 24 hours. Arch. Geflugelk. 48:142-150.

Huyghebaert, G., F. Van Wambeke, and G. De Groote, 1985. The effect of different storage methods of turkey semen on fertility and hatchability. Arch. Geflugelk. 49:205-211.

Insko, W. M., Jr. and J. H. Martin, 1935. Effect of frequent turning on hatchability and distribution of embryo mortality. Poultry Sci. 12:282-286.

Insko, W. M., Jr. and J. H. Martin, 1935. Mortality of the turkey embryo. Poultry Sci. 14:361-364.

Jassim, E. W., M. Grossman, W. J. Koops, and R. A. J. Luykx, 1996. Multiphasic analysis of embryonic mortality in chickens. Poultry Sci. 75:464-471.

Johnson, A. L., 1986. Reproduction in the male. Pages 432-451 *in*: Avian Physiology 4th ed. Ed. P. D. Sturkie. Springer-Verlag New York, NY.

Kapoor, P., M. M. Cooper, and B. Howarth, 2000. Immunization of chickens with quail and turkey perivitelline membrane proteins: Production of antibodies and their effects on fertility. Poultry Sci. 79:245-256.

Kimijima, T., Y. Hashimoto, H. Kitagawa, Y. Kon, and M. Sigimura, 1990. Localization of immunoglobulins in the chicken oviduct. Japanese J. Vet. Sci. 52:299- 305.

Kirk, S., G. C. Emmans, R. McDonald, and D. Arnot, 1980. Factors affecting the hatchability of eggs from broiler breeders. Br. Poultry Sci. 21:37-53.

Kirk, T. A., H. P. Van Krey, R. M. Hulet, E. A. Dunnington and D. M. Denbow, 1989a. Effects of oviductal trauma on humoral anti-sperm antibody production and fertility in turkey breeder hens. Theriogenology 32:315-322.

Kirk, T. A., H. P. Van Krey, R. M. Hulet, E. A. Dunnington and D. M. Denbow, 1989b. The relationship of infertility to antibody production in the uterovaginal sperm storage tubules of turkey breeder hens. Theriogenology 31:955-961.

Kochav, S., M. Ginsburg, and H. Eyal-Giladi, 1980. From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick II. Microscopic anatomy and cell population dynamics. Develop. Biol. 79:296-308.

Kosin, I. L., 1944. Some aspects of the biological action of x-rays on cock spermatozoa. Physiol. Zool. 17:289-319.

Kosin, I. L., 1951. The prevalence of early embryonic mortality in the broad breasted bronze turkeys. Poultry Sci 30:805-814.

Kosin, I. L., 1956. Studies on pre-incubation warming of chicken and turkey eggs. Poultry Sci. 35:1384-1392.

Kosin, I. L. and A. M. Mun, 1965. Some factors affecting the biological quality of turkey hatching eggs. Poultry Sci. 44:31-39.

Kosin, I. L., E. St. Pierre and R. McLaughlin, 1951. The prevalence of early embryonic mortality in the broad breasted bronze turkey. Poultry Sci. 30:805-814.

Krueger, K. K., 1993. Embryo mortality patterns in commercial crossbred parent stock turkeys. Pages 70-74 *in*: Proceedings of the Third International Symposium on Turkey Reproduction. North Carolina State University, Raleigh, NC.

Koyanagi, F., S. Masuda, and H. Nishiyama, 1988. Acrosome reaction of cock spermatozoa incubated with the perivitelline layer of the hen's ovum. Poultry Sci. 67:1770-1774.

Kuroki, M. and M. Mori, 1997. Binding of spermatozoa to the perivitelline layer in the presence of a protease inhibitor. Poultry Sci. 76:748-752.

Lake, P. E., 1975. Gamete production and the fertile period with particular reference to domesticated birds. Symp. Zool. Soc. London 35:225-244.

Landauer, W., 1967. The hatchability of chicken's eggs as influenced by environment and heredity. Storrs Agricultural experiment station Monograph 1. Revised, Storrs, CT.

Leighton, A. T., H. P. Van Krey D. D. Moyer, and L. M. Potter, 1971. Reproductive performance of force-molted turkey breeder hens. Poultry Sci. 50:119-126.

Leyton, L. and P. Saling, 1989. Evidence that aggregation of mouse sperm receptors by ZP3 triggers the acrosome reaction. J. Cell. Biol. 108:2163-2168.

Lillpers, K. and M. Wilhelmson, 1993. Age-dependent changes in oviposition pattern and egg production traits in the domestic hen. Poutlry Sci. 72:2005-2011.

Lodge, J. R., N. S. Fechheimer, and R. G. Jaap, 1971. The relationship of in vivo sperm storage interval to fertility and embryonic survival in the chicken. Biol. Reprod. 5:252-257.

Lorenz, F. W. and F. X. Ogasawara, 1968. Distribution of spermatozoa in the oviduct and fertility in domestic birds. VI. The relations of fertility and embryo normality with site of experimental inseminations. J. Reprod. Fertil. 16:445-455.

Lundy, H., 1969. A review of the effects of temperature, humidity, turning and gaseous environment of the incubator on the hatchability of the hen's egg. pages 143-176 *in:* The Fertility and Hatchability of the Hen's Egg, T. C. Carter and B. M. Freeman, ed., Oliver and Boyd, Edinburgh.

Maeza, A. S. and E. G. Buss, 1976. Sperm concentration and sperm numbers as related to fertility in chickens. Poultry Sci. 55:2059. (Abstr).

MacLaury, D. W., and W. M. Insko, Jr., 1953. A study of turkey hatchability by means of embryo mortality curves. Kentucky Agr. Exp. Sta. Bulletin. 603. Pages 3-32.

Mather, C. M. and Laughlin, K. F., 1977. Storage of hatching eggs: the effect on total incubation period. Br. Poultry Sci. 17:471-479.

Mather, C. M. and Laughlin, K. F., 1979. Storage of hatching egg: the interaction between parental age and early embryonic development. Br. Poultry Sci. 20:595-604.

McCartney, M. G., 1962. Heritabilities and correlations for reproductive traits in randombred population of turkeys. Poultry Sci. 41:168-174.

McCorkle, F. M., V. L. Christensen, and J. P. Thaxton, 1983. Anti-sperm antibodies and fertility of turkey hens. J. Reprod. Immunol. 5:363-370.

McDaniel, C. D., R. K. Bramwell, and B. Howarth, Jr., 1996. The male contribution to broiler breeder heat-induced infertility as determined by sperm-egg penetration and sperm storage within the hen's oviduct. Poultry Sci. 75:1546-1554.

McDaniel, G. R. and J. V. Craig, 1962. Predicting male fertilizing capacity in high and low fertility strains of chickens. Poult. Sci. 41:866-869.

McIntyre, D. R., C. L. Quarles, D. J. Fagerberg, K. K. Krueger, 1982. Fertility of the turkey hen as affected by initial insemination. Poultry Sci. 61:1734-1737.

McIntyre, D. R. and V. L. Christensen, 1983. Filling rates of the uterovaginal sperm storage glands in the turkey. Poultry Sci. 62:1652-1656.

McIntyre, D. R., V. L. Christensen and L. G. Bagley, 1986. Effect of sperm numbers per insemination following early or late initial inseminations in turkeys. Poultry Sci. 65:1400-1404.

McNary, H. W., A. E. Bell, and C. H. Moore, 1960. The growth of inbred and hybrid chicken embryos. Poultry Sci. 39:378-384.

Meir, M., and A. Ar, 1986. Improving turkey poult quality by correcting incubator humidity to match eggshell conductance. Br. Poult. Sci. 28:337-342.

Meijerhof, R., 1992. Pre-incubation holding of hatching eggs. World Poultry Sci. J. 48:57-68.

Meuer, H. J., and R. Baumann, 1988. Oxygen pressure in intra- and extra-embryonic blood vessels of the early chick embryos. Respir. Physiol. 71:331-342.

Mimura, H., 1937. Studies on the ciliary movement of the oviduct of domestic fowl. Okajima's Folia Anat. Japan 15:287-295.

Mimura, H., 1939. On the mechanism of travel of spermatozoa through the oviduct in the domestic fowl, with special reference to the artificial insemination. Okajimas Folia Ant. Japan 17:459-476.

Moore, C. H., 1959. Time interval between successive eggs and its effect on hatchability. Poultry Sci. 38:1230-1231.

Morisson, M., A. Borda, J. M. Petit, C. Jayat-Vignoles, R. Jelien, and F. Minvielle, 1997. Associated effects of divergent selection for residual feed consumption

on reproduction, sperm characteristics and mitochondria of spermatozoa. Poultry Sci. 76:425-431.

Nakanishi, A., K. Utsumi, and A. Iritani, 1990. Early nuclear events of in vitro fertilization in the domestic fowl (Gallus domesticus). Mol. Reprod. Dev. 26:217-221.

Nestor, K. E., K. I. Brown, and S. P. Touchburn, 1972. Egg quality and poult production in turkeys. 1. Variation during a seven month period. Poultry Sci. 51:104-110.

Nestor, K. E., K. I. Brown, and C. R. Weaver, 1972. Egg quality and poult production in turkeys. 2. Inheritance and relationship among traits. Poultry Sci. 51:147-158.

New, D. A. T., 1957. A critical period for the turning of hens' eggs. J. Embryol. Exp. Morph. 5:293-299.

Nilsen, N.O, 1984. Vascular abnormalities due to hyperthermia in chick embryos. Teratology 30:237-251.

Noble, R. C., F. Lonsdale, K. Connor, and D. Brown, 1986. Changes in the lipid metabolism of the chick embryo with parental age. Poultry Sci. 65:406-416.

Ogasawara, F. X., F. L. Lorenz, L. W. Bobr, 1966. Distribution of spermatozoa in the oviduct and fertility in domestic birds. III. Intrauterine insemination of semen from low fecundity cocks. J. Reprod. Fertil. 11:33-41.

Okamura, F. and H. Nishiyama, 1978. The passage of spermatozoa through the vitelline membrane in the domestic fowl, *Gallus gallus*. Cell. Tiss. Res. 188:497-508.

Olsen, M. W., (1942). Maturation, fertilization, and early cleavage in the hen's eggs. J. Morphol. 70:513-533.

Olsen, M. W., 1975. Avian Parthenogenesis. USDA ARS-NE-65 Monograph, USDA Washington, DC.

O'Sullivan, N. P., E. A. Dunnington, and P. B. Siegel, 1991. Relationships among age of dam, egg components, embryo lipid transfer, and hatchability of broiler breeder eggs. Poultry Sci. 70:2180-2185.

Patterson, J. T., 1910. Early development of the hen's egg. J. Morphol. 21:101-134.

Payne, L. F., 1919. Distribution of mortality during the period of incubation. Amer. Assoc. Instr. Invest. Poultry Husb. Jour. 6:9-12.

Perry, M. M., 1987. Nuclear events from fertilization to the early cleavage stages in the domestic fowl (Gallus domesticus). J Anat. 150:99-109.

Phadke, A. M., 1975. Spermiophage cells in man. Fertil. Steril. 26:760-774.

Phadke, A. M., and G. M. Phadke, 1961. Occurrence of macrophage cells in the semen and in the epididymis in cases of male infertility. J. Reprod. Fertil. 2:400-403.

Phillips, R. W. and C. S. Williams, 1944. External morphology of the turkey during the incubation period. Poultry Sci. 23:270-277.

Pierson, E. E., L. M. Krista, and G. R. McDaniel, 1988. Effect of age and physiological status on sperm storage 24 hours after artificial insemination in broiler breeder hens. Br. Poult. Sci. 29:193-197.

Rahn, H., V. L. Christensen, F. W. Edens, 1981. Changes in shell conductance, pores and physical dimensions of egg and shell during the first breeding cycle of turkey hens. Poultry Sci. 60:2536-2541.

Riddle, A., 1930. Studies on the physiology of reproduction in birds. Am. J. Physiol. 94:535-548.

Robinson, F. E., R. T. Hardin, N. A. Robinson, B. J. Williams, 1991. The influence of egg sequence position on fertility, embryo viability and embryo weight in broiler breeders. Poultry Sci. 70:760-765.

Robertson, L., G. J. Wishart, and A. J. Horrocks, 2000. Identification of perivitelline N-linked glycans as mediators of sperm-egg interaction in chickens. J. Reprod. Fertil. 120:397-403.

Robertson, L., H. L. Brown, H. J. Staines and G. J. Wishart, 1997. Characterization and application of an avian in vitro spermatozoa-egg interaction assay using the inner perivitelline layer from laid chicken eggs. J. Reprod. Fertil. 110:205-211.

Robertson, L., Y. I. Wilson, C. Lindsay, and G. J. Wishart, 1998. Evaluation of semen from individual male domestic fowl by assessment of sperm: perivitelline interaction vitro and in vivo. Br. Poult. Sci. 39:278-281.

Romanoff, A. L., 1949. Critical periods and causes of death in avian embryonic development. Auk 66:264-270.

Romanoff, A. L., 1960. The avian embryo: structural and functional development. Macmillan, New York, NY.

Romanoff, A. L., 1967. Biochemistry of the Avian Embryo: A quantitative Analysis of Prenatal Development. John Wiley & Sons, Inc., New York, NY.

Romanoff, A. L., 1972. Pathogenesis of the avian embryo: An analysis of causes of malformation and prenatal death. John Wiley & Sons, Inc., New York, NY.

Sanctuary, W. C., 1925. One cause of dead chicks in the shell. Poultry Sci. 4:141-143.

Savage, T. F. and J. A. Harper, 1985. Ring lethal: an early embryonic failure in medium white turkeys. J. Hered. 76:474-476.

Savage, T. F., M. P. Defrank, and S. E. Brean, 1988. Blood ring – an early embryonic lethal condition in chickens. J. Hered. 79:124-128

Savage, T. F. and L. W. Mirosh, 1989. Blood ring, and early embryonic lethal condition in a second avian species, the turkey. Poultry Sci. 68: (Suppl. 1):130. (Abstr).

Savage, T. F. and L. W. Mirosh, 1992. Inheritance of blood ring, an early embryonic failure in the turkey. Poultry Sci. 71:585-589.

Savage, T. F., L. W. Mirosh, J. L. Jones, and E. T. Schneiderman, 1992. Blastoderm degeneration, an early embryonic failure in dwarf single comb white leghorn chickens. J. Hered. 83:249-254.

Scott, H. M., 1933. The effect of age and holding temperature on hatchability of turkey and chicken eggs. Poultry Sci. 12:49-54.

Scott, T. A. and C. J. Mackenzie, 1993. Incidence and classification of early embryonic mortality in broiler breeder chickens. Br. Poult. Sci. 34:459-470.

Sexton, T. J., 1977. Relationship between number of sperm inseminated and fertility of turkey hens at various stages of production. Poultry Sci. 56:1054-1056.

Sexton, T. J., 1988. Research note: Influence of damaged spermatozoa on the fertility of turkey semn stored 24 h at 5 C. Poultry Sci. 67:1483-1485.

Sewalem, A. and M. Wilhelmson, 1999. Genetic study of embryonic mortality in white leghorn lines selected for egg production traits. Br. Poult. Sci.. 40:467-471.

Shanawany, M. M., 1984. Inter-relationship between egg weight parental age and embryonic development. Br. Poultry. Sci. 25:449-455.

Shook, J. G., A. B. Stephenson, and H. V. Biellier, 1971. Heritability estimates of differences in arbitrary embryonic mortality traits in turkeys. Poultry Sci. 50:1255-1260.

Sittmann, K., H. Abplanalp, and C. F. Meyerdick, 1971. Extended storage of quail, chicken and turkey eggs 1. Hatchability and embryonic mortality. Poultry Sci 50:681-688.

Skinner, S. M., S. V. Prasad, T. M. Ndolo, and B. S. Dunbar, 1996. Zona pellucida antigens: Targets for contraceptive vaccines. Am. J. Reprod. Immunol. 35:163-174.

Smith, K. P., and B. B. Bohren, 1975. Age of pullet effects on hatching time, egg weight and hatchability. Poultry Sci. 54:959-963.

Snyder, G. K., and G. F. Birchard, 1982. Water loss and survival in embryos of the domestic chicken. J. Exp. Zool 192:1-8.

Soller, M., H. Schindler, and S. Bornstein, 1965. Semen characteristics, failure of insemination and fertility, in Cornish and White Rock males. Poultry Sci. 44:424-434.

Staines, H. J., R. C. Middleton, K. F. Laughlin, and G. J. Wishart, 1998. Quantification of a sperm-egg interaction for estimating the mating efficiency of broiler breeder flocks. Br. Poultry. Sci. 39:273-277.

Steele, M. G. and G. J. Wishart, 1992. Evidence for a species-specific barrier to sperm transport within the vagina of the domestic hen. Theriogenology 38:1107-1114.

Steele, M. G., W. Meldrum, J. P. Brillard, and G. J. Wishart, 1994. The interaction of avian spermatozoa with the perivitelline layer in vitro and in vivo. J. Reprod. Fertil. 101:599-603.

Sunde, M. L. and H. R. Bird, 1959. The effect of pullet maturity on fertility and hatchability of eggs. Poultry Sci. 38:272-279.

Takeda, 1974. The transport of spermatozoa in the vagina of the hen. Jap. Poultry Sci. 11:45-54.

Takeuchi, Y., K. Nishimura, N. Aoki, T. Adachi, C. Sato, K. Kitajima, T. Matsuda, 1999. A 42-kDa glycoprotein from chicken egg-envelope, an avian homolog of the ZPC family glycoproteins in mammalian zona pellucida - its first identification, cDNA cloning and granulosa cell-specific expression. Eur. J Biochem. 260:736-742.

Tazawa, H., 1980. Adverse effec of failure to turn the avian egg on embryo oxygen exchange. Resp. Physiol. 41:137-142.

Thorne, M. H., R. K. Collins, and B. L. Sheldon, 1991. Chromosome analysis of early embryonic mortality in layer and broiler chickens. Br. Poultry Sci 32:711-722.

Thurston, R. J. (1995). Storage of poultry semen above freezing for 24-48 hours. Pages 107-122 in: Proceedings of the First International Symposium on Artificial Insemination in Poultry. M. R. Bakst and H. Cecil. ed. Poultry Science Association, Savoy, IL.

Thurston, R. J., R. A. Hess, H. V. Biellier, H. K. Adldinger, R. F. Solorzano, 1975. Ultrastructural studies of semen abnormalities and herpesvirus associated with cultured testicular cells from domestic turkeys. J. Reprod. Fertil. 45:235-241.

Thurston, R. J., R. A. Hess, and N. Korn, 1992. Seminal plasma protein concentration as a predictor of fertility and hatchability in Large White domestic turkeys. J. Appl. Poult. Res. 1:335-338.

Tullett, S. G. and D. C. Deeming, 1987. Failure to turn eggs during incubaiton: Effects on embryo weight, development of the chorioallantois and absorption of albumen. Br. Poult. Sci. 28:239-243.

Van Krey, H. P, 1990. Reproductive biology in relation to breeding and genetics. In Poultry Breeding and Genetics. Ed. R. D. Crawford Elsevier Science Publishing Co. Inc., New York, NY pp 61-90.

Van Krey, H. P., A. T. Leighton, and L. M. Potter, 1967. Sperm gland populations and late seasonal declines in fertility. Poultry Sci. 46:1332. (Abstr.)

Van Krey, H. P., and A. T. Leighton, 1970. Sperm gland populations, oviduct homogenates and late season declines in fertility. Poultry Sci. 49:1477.

Van Krey, H. P., F. X. Ogasawara, and F. W. Lorenz, 1966. Distribution of spermatozoa in the oviduct and fertility in domestic birds. IV. Fertility of spermatozoa from infundibular and uterovaginal glands. J. Reprod. Fertil. 11:257-262.

Van Krey, H. P., G. T. Schuppin, D. M. Denbow, and R. M. Hulet, 1987. Turkey breeder hen infertility associated with plasma cells in the uterovaginal sperm storage glands. Theriogenology 27:913-921.

Van Krey, H. P., R. J. Balander and M. M. Compton, 1981. Storage and evacuation of spermatozoa from the uterovaginal sperm-host glands in domestic fowl. Poultry Sci. 60:871.

Verma, O. P. and F. L. Cherms, 1965. The appearance of sperm and their persistency in storage tubules of turkey hens after a single insemination. Poultry Sci. 44:609-613.

Vick, S. V., J. Brake, and T. J. Walsh, 1993. Relationship of incubation humidity and flock age to hatchability of broiler hatchling eggs. Poultry Sci. 72:251:258.

Waclawek, M., R. Foisner, J. Nimpf, and W. J. Schneider, 1998. The chicken homologue of zona pellucida protein-3 is synthesized by granulosa cells. Biol. Reprod. 59:1230-1239.

Waddington, D., C. Gribbin, R. J. Sterling, H. M. Sang, and M. M. Perry, 1998. Chronology of events in the first cell cycle of the polyspermic egg of the domestic fowl (Gallus domesticus). Int. J. Dev. Biol. 42:625-628.

Waite, R. H., 1919. The effect of age of egg on their hatching quality. Maryland Agricultural Experiment Station Bulletin 233, College Park, MD.

Walsh, T. J., R. E. Rizk, and J. Brake, 1995. Effects of temperature and carbon dioxide on albumen characteristics, weight loss, and early embryonic mortality of long stored hatching eggs. Poultry Sci. 74:1403-1410.

Wasserman, P. M., 1988. Zona pellucida glycoproteins. Annu. Rev. Biochem. 57:415-442.

Wasserman, P. M. and E. S. Litscher, 1995. Sperm-egg recognition mechanisms in mammals. Pages 1-19 *In:* Current Topics in Developmental Biology. R. A. Pedersen and G. P. Schatten ed. Academic Press, Inc., San Diego, CA.

Weisbroth, S. H. and I. L. Kosin, 1966. Gross spatial changes in the turkey blastoderm following extended pre-incubation storage. P. Soc. Exp. Biol. Med. 121:795-800.

Wentworth, B. C. and W. J. Mellen, 1964. Effects of spermatozoal antibodies and method of insemination on the fecundity of domestic hens. Br. Poult. Sci. 5:59-65.

Wilson, H. R., 1991. Physiological requirements of the developing embryo: Temperature and turning. Chapter 9. Pages 145-156 *in*: Avian Incubation. S. G. Tullett, ed. Butterworth-Heinemann, London, Uk.

Wilson, H. R., 1997. Effects of maternal nutrition on hatchability. Poultry Sci. 76:134-143.

Wilson, H. R., N. P. Piesco, E. R. Miller, and W. G. Nesbeth, 1979. Prediction of the fertility potential of broiler breeder males. World Poultry Sci. J. 35:95-118.

Wishart, G. J., 1982. Maintenance of ATP concentrations in and of fertilizing ability of fowl and turkey spermatozoa in vitro. J. Reprod. Fertil. 66:457-462.

Wishart, G. J., 1984. Effect of lipid peroxide formation on sperm motility, ATP content, and fertilizing ability. J. Reprod. Fertil. 71:113-118.

Wishart, G. J., 1987. Regulation of the length of the fertile period in the domestic fowl by numbers of oviductal spermatozoa as reflected by those trapped in laid eggs. J. Reprod. Fertil. 80:493-498.

Wishart, G. J., 1995. New approaches to evaluating male and female fertility. Pages 207-223 in: Proceedings of the First International Symposium on Artificial Insemination in Poultry. M. R. Bakst and G. J. Wishart ed. Poultry Science Association, Savoy, IL.

Wishart, G. J., 1997. Quantitative aspects of sperm;egg interaction in chickens and turkeys. Anim. Reprod. Sci. 48:81-92.

Wishart, G. J. and F. H. Palmer, 1986. Correlation of the fertilising ability of semen from individual male fowls with sperm motility and ATP content. Br. Poult. Sci. 27:97-102.

Wishart, G. J. and H. J. Staines, 1999. Measuring sperm:egg interaction to assess breeding efficiency in chickens and turkeys. Poultry Science 78:428-436.

Wishart, G. J. and L. A. Dick, 1985. Effect of y-radiation on fowl sperm function in vitro and in vivo. J. Reprod. Fertil. 75:617-622.

Woodard, A. E., F. X. Ogasawara, H. Abplanalp, and B. Marquez, 1976. Effect of semen dose, frequency of insemination, age and productivity of the male on duration and levels of fertility and hatchability in the turkey. Poultry Sci. 55:1367-1372.

Yafei, N. and R. C. Noble, 1990. Further observations on the association between lipid metabolism and low embryo hatchability in eggs from young broiler birds. J. Exp. Zool. 253:325-329.

Yu, W. C. Y. and W. H. Burke, 1979a. Infertility in the turkey. II. A description of a spontaneous infertility condition and its alleviation by intramagnal insemination and sexual rest. Poultry Sci. 58:1372-1377.

Yu, W. C. Y. and W. H. Burke, 1979b. Infertility in the turkey. III. effects of sera from sterile and fertile females on the fertilizing ability of spermatozoa. Poultry Sci. 58:1378-1381.

Zheng, W. M., Y. Yoshimura, 1999. Localization of macrophages in the chicken oviduct: Effects of age and gonadal steroids. Poultry Sci. 78: (7) 1014-1018.

II. Hen Age Relationship with Embryonic Mortality and Fertility in Commercial Turkeys[1]

ABSTRACT

Fertility changes due to hen age have been well documented, but the relationship of embryonic mortality (EM) to hen age has not been reported recently with a current commercial strain. Earlier studies may not accurately report early embryonic mortality (EEM) since it may be confused with unfertilized eggs. The purpose of the current study was to examine weekly EM in turkey eggs between two hen ages and to report values that minimize the error of wrongly distinguishing between EEM with unfertilized eggs. Three hatch residue breakouts were performed at two commercial turkey hatcheries for a total of six hatches. Large White turkey eggs from two hen age groups (32-35 weeks of age and 44-50 weeks of age) were set in the same incubator that operated under the incubation profile of each hatchery.

The EEM was significantly greater (P=0.001) in younger hens as compared with older hens. This was true for mortality prior to blood formation and mortality following blood formation. Hatchability, percent internal pips and live pips were not different between the two hen ages. Prepip mortality and the percent dead pips were significantly greater (P=0.0001) in older flocks. Fertility and EEM occurring before blood formation were negatively correlated in younger hens, whereas fertility and pip mortality were positively correlated. Eggs from young hens tended to experience embryonic mortality earlier in incubation than eggs from older hens. The results suggest that hen

[1] Abbreviation Key = BB - Before Blood; AB - After Blood; EM - Embryonic Mortality; EEM - Early Embryonic Mortality

age influences embryonic mortality in modern commercial turkeys, and could be used as an experimental model for further study of embryonic mortality.

INTRODUCTION

The factors that contribute to hatchability are fertility and embryonic mortality. As embryonic mortality increases, hatchability and thus net profit decrease. Factors that have been associated with embryonic mortality include, but are not limited to, prolonged duration of egg storage (Arora and Kosin, 1966; Coleman and Siegel, 1966; Sittmann et al., 1971; Mather and Laughlin, 1977; Fasenko et al., 1992; Fasenko, 1996; Brake et al., 1997); abnormal conditions during egg storage (Meijerhof, 1992); season of the year (Kosin and Mun, 1965); nutrition (for review, see Wilson, 1997); nutrient availability (Byerly et al., 1932); maternal nutrition and egg size (Landauer, 1967); and age of the breeders (Christensen, 1978). Intramagnal inseminations have been associated with increased embryonic mortality (Van Krey et al., 1966; Lorenz, 1968). Embryonic deaths have been reported as a result of malpositions during the last week of incubation as early as 1925 (Sanctuary, 1925; Hutt and Pilkey, 1930; Hutt and Pilkey, 1934).

Much of the work with turkey embryonic mortality is dated and should be reexamined periodically with modern commercial strains to determine changes in mortality patterns. Therefore, the purpose of the current study was to build on the work of Christensen (1978) who used bronze turkeys by testing the hypothesis that embryonic mortality in modern commercial turkeys varies with hen age. Embryonic mortality of eggs from a commercial line of turkey hens from two different ages was examined to determine whether hen age could be used as a model for further analysis of EEM.

MATERIAL AND METHODS

Experimental Protocol

Two commercial turkey hatcheries served as sources of hatch data by providing Nicholas Large White turkey eggs and incubator space. Two flocks of the same strain were identified at each location. One flock was in its first or second week of production (ages varied from 32 to 35 wks of age), whereas the second flock had been in production for at least 12 weeks (ages varied from 44 to 50 wks of age). The eggs were treated by each hatchery's individual protocol for preincubation storage and sanitation. Eggs from each hen age were set in the same incubator with 120 eggs being placed on each tray. Approximately 39 trays in each buggy were utilized providing approximately 4,680 eggs per hen age per trial. The incubators and hatcher were operated utilizing the normal incubation profile of each hatchery.

On the day of hatch, unhatched egg residue was examined by macroscopic examination. Machine cracked, contaminated eggs and the number of cull poults were recorded. Embryonic mortality (EM) was recorded by week of incubation. Early embryonic mortality (EEM), defined as mortality occurring during wk 1 of incubation, was divided in two periods: before blood formation (BB), 0 to 3 d of incubation, and after blood formation (AB), 4 to 7 d of incubation. Mortality during wk 4 was divided into prepipping (21 to 25 d of incubation), internal pipping (IP, 26 days of incubation) and live and dead pips (LP and DP, 27 d of incubation).

Statistical Analysis

All variables were expressed as the percentage of total eggs set on the tray. Tray was used as the experimental unit. All percentage data was subjected to Arc Sine transformation prior to a one way analysis of variance using the General Linear Models

procedure (SAS® Institute Inc., 1989). The correlation of fertility and embryonic mortality was determined using the PROC CORR procedure of SAS® (SAS® Institute Inc., 1989). Significant main effect means were separated using the Least Squared Means procedure. Unless stated otherwise, significance was set at $P \leq 0.05$.

RESULTS

There were no significant differences in the number of cracked eggs, contaminated eggs or cull poults between the two hen ages (Data not shown). Fertility, hatchability of all eggs set and hatchability of fertilized eggs did not differ between the two hen ages (Table 1). The EEM before and after blood formation was significantly greater in younger hens when compared to older hens (Table 2). Due to the infrequent occurrence of mortality during weeks 2 and 3 of incubation the data did not have a normal distribution and were not subjected to statistical analysis. A different embryonic mortality pattern was observed in the last week of incubation (Table 2). A greater number of PP and DP embryonic deaths were observed in older hens when compared to younger hens, whereas no significant differences were noted for IP nor LP.

There was a negative correlation between fertility and EEM occurring before blood formation as well as total EEM (AB + BB) mortality (Table 3). When the flock ages were separated, a negative correlation between fertility and EEM was found in young hens for embryonic mortality occurring before blood formation and total wk 1 mortality. No correlation was found between fertility and EEM in older flocks for embryonic mortality occurring before blood formation, after blood formation or total wk 1 mortality. A positive correlation was observed between fertility and percent live pip

mortality and fertility and percent wk 4 mortality in younger hens. These correlations were not observed in older hens (Table 4).

DISCUSSION

The two peaks in embryonic mortality (Figure 1) that occurred during the first and last wk of incubation agree with earlier studies (Christensen, 1978; Cherms, 1981; Kreuger, 1993). Insko and Martin (1935) demonstrated a similarity between embryonic mortality curves of White Leghorn chickens and Standard Bronze turkeys. Chicken EM peaked at 2 and 19 d of incubation where turkey EM peaked at 4 and 25 d of incubation, therefore EM peaks were observed in the first and last wks of incubation for both chickens and turkeys. Fertility and hatchability of eggs vary with hen age in broilers (Kirk et al., 1980; Fasenko et al., 1992) and turkeys (Van Krey and Leighton, 1970; Van Krey et al., 1967; Sexton, 1977). In addition to changes in fertility, these results indicate that the differences in hatchability as the hen ages may also be attributed to the differences seen in EM between the first and last periods of incubation.

The negative correlation between fertility and EEM suggests that factors that may improve fertility may also lower EEM. When improving fertility, sperm concentration and quality should be considered, in that both have been associated with fertility and embryo mortality. High numbers of inseminated sperm concentrations have been associated with increased sperm penetration of the germinal disc and embryonic death in chickens (Bramwell and Howarth, 1997). Deterioration in sperm quality due to in vitro storage prior to insemination has been associated with decreased fertility and increased EM (Huyghebaert et al., 1984; 1987). This relationship between fertility and EEM may be indirectly related to sperm storage capacity in the sperm storage tubules (SST) of the

hen. Declines in fertility during late season production have been previously related to a reduction of sperm retention in the SST (Van Krey et al., 1967; Christensen, 1981; Pierson et al., 1988). Changes in oviducal SST as hens age may alter the number of sperm cells that can be stored. This may result in fewer sperm at the site of fertilization. This would lead to a condition where less than optimal sperm numbers or quality could contribute to the negative correlation between fertility and EM. The data from this study suggest that as fertility increases, EEM decreases. In terms of sperm concentration, this would mean that larger numbers of sperm cells, which would benefit fertility, result in lower EM. However, there may be a level where sperm concentration could become lethal, at which the large numbers of sperm could result in embryonic death, possibly related to pathological polyspermic fertilization (Bakst and Howarth, 1977). At this time the data are insufficient to ascertain what semen dose is optimal for both high fertility and low EEM. Additional work is needed to further define the relationship between EEM and fertility.

The difference in mortality patterns noted between the two hen ages suggests that the reproductive system of the hen may change with age, which in turn influences embryonic mortality. Microscopic studies have observed no differences in fertility and EEM within the first 36 h of incubation between turkey hens in the first 6 wk of production when compared to the hens in 21 to 24 wk of production (Bakst et al., 1997). Based on these results, Bakst et al. (1997) suggested that increased EEM must occur later than 36 h of incubation. The current study observed that higher EEM occurred both before and after blood formation in young hens when compared to older hens. The increased number of observations in this large commercial study allowed for a more

accurate estimate of the mean, which could explain why increased EEM was observed in younger hens before blood formation. The increased EEM after blood formation complements the earlier microscopic studies performed by Bakst et al. (1997), and provides evidence that the increased mortality is occurring between d 4 and 7 of incubation. These results differ from data collected using broiler breeder eggs from hens of different ages where no significant differences were noted except for d 3 of incubation, in which increased EEM was observed in older hens as compared with younger hens (Deeming and Middlekoop, 1999). This suggests that hen age may be of greater influence on EM in turkeys. It could be argued that some of the infertility was mistaken for EEM and therefore altered the results. However, by classifying the wk 1 deaths into BB and AB, the error of mistaking infertility was minimized in the AB group in the current study.

The increased late embryonic mortality observed in older hens may be attributed to inadequate metabolism of the embryo. Oxygen, which is influenced by eggshell conductance, is one of the limiting factors affecting embryos and hatchability late in the incubation period. Differences in eggshell conductance have been reported due to strain and egg weight during the hen's first breeding cycle (Rahn et al., 1981). Asphyxiation and retarded development due to insufficient water loss has been suggested as causes of late embryonic mortality, which could be related to the conductance constant principle (Christensen and McCorkle, 1982; Rahn and Ar, 1980). Christensen et al. (1994) reported changes in eggshell conductance and the conductance constant as hens aged. Christensen et al. (1999) suggested that the amount of time the turkey embryo spends in the plateau stage of oxygen consumption

and thus the amount of energy that can be utilized in anaerobic conditions could contribute to late embryonic survival. These are some of the factors that have been associated with late EM in turkeys, and may be combined with other factors such as temperature and humidity to influence late embryonic mortality.

There is very little work that attempts to explain differences in EEM between young and old turkey hens. Egg weight has been associated with hatchability in chickens (Landauer, 1967). However, egg weight association with late EM has been variable (Hagger et al., 1968; Sewalem et al., 1998). Selection for increasing egg weight has been shown to be associated with EM but did not indicate whether it was early or late EM (Sewalem and Wilhelmson, 1999). Egg weight is a part of the equation that defines the conductance constant (Ar and Rahn, 1978; Rahn, 1981), and the influence that egg weight may have on EM may be due to its effect on the conductance constant. Christensen and McCorkle (1982) reported lower eggshell conductance for eggs that experienced late EM where eggs that experienced EEM and hatched had higher eggshell conductance. The interaction with the other components of the equation could explain the inconsistent reports concerning egg weight and EM. Hen age differences have been examined in respect to various egg characteristics, which may contribute to EM differences noted in this study. Hen age influences shell, albumen and yolk proportions to embryo mass in chickens (Akbar et al., 1983) and turkeys (French and Shaw, 1989). Percent wet and dry yolk increases at the expense of percent wet and dry albumen as the hen ages (French and Tullet, 1991; Applegate and Lilburn, 1996). Increased serum cholesterol, HDL, LDL, and lower glucose concentrations were reported in chicks from young hens when compared with chicks

from hens at either 36 or 48 wk of age. It was concluded that serum concentrations of lipids and glucose, and relative yolk sac weight in 18 d embryos and newly hatched chicks were influenced by hen age (Latour et al., 1996). A decreased transfer of lipid or failure in yolk lipid mobilization and uptake by the embryo was observed and has been suggested as a possible cause for increased EM observed in eggs from young broiler breeders when compared to older broiler breeders (Noble et al., 1986). The results of these studies indicate that less than optimal nutritional and metabolic substrates may be available to the embryo which could result in embryonic death. Albumen has been shown to contribute resistance to gaseous diffusion in freshly laid eggs (Meuer, and Baumann, 1988). Albumen quality may also be a factor contributing to EEM and has been shown to decrease with hen age (Lapao et al., 1999; Walsh et al., 1995). Although there are several effects of hen age on egg characteristics, reasons as to why EEM is greater in young hens as compared with older hens may not be simply due to a change in egg characteristics that creates less than optimal conditions during incubation. The relationship between fertility and EEM suggests that problems associated with embryo viability in younger hens may relate to fertilization and developmental processes that occur before incubation or even before oviposition.

Conclusion

The results of the current study indicate that EM differs between hen ages. Younger hens had increased EEM whereas older hens experienced increased EM during the fourth week of incubation. Furthermore, a negative correlation between fertility and EEM suggests a potential contribution of sperm cell or egg differences to EEM that requires additional research. These differences in EM patterns between

young and old hens provide an experimental model that could be used in future studies to understand the factors that contribute to EM in turkeys.

REFERENCES

Akbar, M. K., J. S. Gavora, G. W. Friars, and R. S. Gowe, 1983. Composition of eggs by commercial size categories: Effects of genetic group, age and diet. Poultry Sci. 62:925-933.

Applegate, T. J., and M. S. Lilburn, 1996. Independent effects of hen age and egg size on incubation and poult characteristics in commercial turkeys. Poultry Sci. 75:1210-1216.

Ar., A. and H. Rahn, 1978. Interdependence of gas conductance, incubation length and weight of the avian egg. Pages 227-236 *in*: Respiratory Function in Birds, Adult and Embryonic. Springer-Verlag, Berlin, Germany.

Arora, K. L. and I. L. Kosin, 1966. Developmental responses of early turkey and chicken embryos to preincubation holding of eggs: Inter- and intra-species differences. Poultry Sci. 45:958-970.

Bakst, M. R. and B. Howarth, 1977. Hydrolysis of the hen's perivitelline layer by cock sperm in vitro. Biol. Reprod. 17:370-379.

Bakst, M. R., V. Akuffo, D. Harry, and P. Marini, 1997. True fertility and early embryonic mortality in turkeys at onset and after 24 wk of egg production. Poultry Sci. 76(Suppl. 1):116 (Abstr.).

Brake, J., T. J. Walsh, C. E. Benton, Jr., J. N. Petitte, R. Meijerhof, and G. Penalva, 1997. Egg handling and storage. Poultry Sci. 76:144-151.

Bramwell, R. K., and B. Howarth, 1997. Effect of low or high sperm penetration values at the germinal disc on early embryonic mortality in chicken eggs. Poultry Sci. 76(Suppl. 1):97 (Abstr.).

Byerly, T. C., W. G. Helsel, and J. P. Quinn, 1932. Growth of the chick embryo in relation to its food supply. J. Exp. Biol. 9:15-44.

Christensen, V. L., 1978. Physiological parameters limiting hatchability in domestic fowl (Gallus domesticus) and domestic turkey (Meleagris gallopavo). Ph.D. dissertation. University of Missouri, Columbia, MO.

Christensen, V. L., 1981. Effect of insemination intervals on oviducal sperm storage in turkeys. Poultry Sci. 60:2150-2156.

Christensen, V. L., 1999. Length of the plateau and pipping stages of incubation affects the physiology and survival of turkeys. Brit. Poultry Sci. 40:297-303.

Christensen, V. L. and F. M. McCorkle, 1982. Turkey egg weight losses and embryonic mortality during incubation. Poultry Sci. 61:1209-1213.

Christensen, V. L. and K. E. Nestor, 1994. Changes in functional qualities of turkey eggshells in strains selected for increased egg production or growth. Poultry Sci. 73:1458-1464.

Cherms, F. L., 1981. Incidence of embryonic malpositions and terata in turkeys. Poultry Sci. 60(Suppl. 1):1638 (Abstr.).

Coleman, J. W. and P. B. Siegel, 1966. Selection for body weight at eight weeks of age. 5. Embryonic stage at oviposition and its relationship to hatchability. Poultry Sci. 45:1008-1011.

Deeming, D. C. and J. H. Middlekoop, 1999. Effect of strain and flock age on fertility and early embryonic mortality of broiler breeder eggs. Brit. Poult. Sci. 40:S22-S26.

Fasenko, G. M., 1996. Factors influencing embryo and poult viability and growth during long term storage of turkey eggs. Ph.D. dissertation. North Carolina State University, Raleigh, NC.

Fasenko, G. M., R. T. Hardin, and F. E. Robinson, 1992. Relationship of hen age and egg sequence position with fertility, hatchability, viability, and preincubation embryonic development in broiler breeders. Poultry Sci. 71:1374-1383.

French, N. A., and D. J. Shaw, 1989. Changes in egg composition and eggshell characteristics during the first laying cycle of turkey hens. In: Recent Advances in Turkey Science. (Nixey, C. and T. C. Grey, Eds.), London, Butterworths. Pp. 359.

French, N. A., and S. G. Tullett, 1991. Variation in the eggs of poultry species. In. Avian Incubation, Poultry Science Symposium 22 (Tullett, S. G., Ed.) Butterworth-Heinemann, Toronto, pp. 59-77.

Hagger, C., D., Steiger-Stafl, and C. Marguerat, 1986. Embryonic mortality in chicken eggs as influenced by egg weight and inbreeding. Poultry Sci. 65:812-814.

Hutt, F. B. and A. M. Pilkey, 1934. Studies in embryonic mortality in the fowl, V. Relationships between positions of the egg and frequency of malpositions. Poultry Sci. 13:3-13.

Huyghebaert, G., F. van Wambeke, and G. De Groote, 1984. The effect of pH of diluent, number of spermatozoa and storage method on fertility and hatchabilty obtained with turkey semen stored for 6 and 24 hours. Arch. Geflugelkd. 48:142-150.

Huyghebaert, G., F. van Wambeke, E. Ketels, and G. De Groote, 1987. The effect of storage time, frequency of insemination and number of spermatozoa inseminated on reproductive performances of turkeys. Arch. Geflugelkd. 51:161-168.

Insko, W. M., Jr. and J. H. Martin, 1935. Mortality of the turkey embryo. Poultry Sci. 14:361-364.

Kirk, S., G. C. Emmans, R. McDonald, and D. Arnot, 1980. Factors affecting the hatchability of eggs from broiler breeders. Brit. Poultry. Sci. 21:37-53.

Kosin, I. L. and A. M. Mun, 1965. Some factors affecting the biological quality of turkey hatching eggs. Poultry Sci. 44:31-39.

Krueger, K. K., 1993. Embryo mortality patterns in commercial crossbred parent stock turkeys. Pages 70-74 in: Proceedings of the Third International Symposium on Turkey Reproduction. North Carolina State University, Raleigh, NC.

Landauer, W., 1967. The hatchability of chicken's eggs as influenced by environment and heredity. Storrs Agricultural experiment station Monograph 1:68-137.

Lapao, C., L. T. Gama, M. C. Soares, 1999. Effects of broiler breeder age and length of egg storage on albumen characteristics and hatchability. Poultry Sci. 78:640-645.

Latour, M. A., E. D. Peebles, C. R. Boyle, S. M. Doyle, T. Pansky, and J. D. Brake, 1996. Effects of breeder hen age and dietary fat on embryonic and neonatal broiler serum lipids and glucose. Poultry Sci. 75:695-701.

Lorenz, F. W. and F. X. Ogasawara, 1968. Distribution of spermatozoa in the oviduct and fertility in domestic birds. VI. The relations of fertility and embryo normality with site of experimental inseminations. J. Reprod. Fertil. 16:445-455.

Mather, C. M. and Laughlin, K. F., 1977. Storage of hatching eggs: the effect on total incubation period. Brit. Poult. Sci. 17:471-479.

Meijerhof, R., 1992. Pre-incubation holding of hatching eggs. World Poultry Sci. J. 48:58-67.

Meuer, H. J., and R. Baumann, 1988. Oxygen pressure in intra- and extra-embryonic blood vessels of the early chick embryos. Respir. Physiol. 71:331-342.

Noble, R. C., F. Lonsdale, K. Connor, and D. Brown, 1986. Changes in the lipid metabolism of the chick embryo with parental age. Poultry Sci. 65:406-416.

Pierson, E. E., L. M. Krista, and G. R. McDaniel, 1988. Effect of age and physiological status on sperm storage 24 hours after artificial insemination in broiler breeder hens. Brit. Poultry Sci. 29:193-197.

Rahn, H., 1981. Gas exchange of avian eggs with special reference to turkey eggs. Poultry Sci. 60:1971-1980.

Rahn, H., and A. Ar, 1980. Gas exchange of the avian egg: time, structure, and function. Am. Zool. 20:477-484.

Rahn, H., V. L. Christensen, F. W. Edens, 1981. Changes in shell conductance, pores and physical dimensions of egg and shell during the first breeding cycle of turkey hens. Poultry Sci. 60:2536-2541.

Sanctuary, W. C., 1925. One cause of dead chicks in the shell. Poultry Sci. 4:141-143.

SAS Institute, 1989. SAS/STAT® Guide for Personal Computers. 8th ed. SAS Institute Inc., Cary, NC.

Sexton, T. J., 1977. Relationship between number of sperm inseminated and fertility of turkey hens at various stages of production. Poultry Sci. 56:1054-1056.

Sittmann, K., H. Abplanalp, and C. F. Meyerdick, 1971. Extended storage of quail, chicken and turkey eggs 1. Hatchability and embryonic mortality. Poultry Sci. 50:681-688.

Sewalem, A., K. Johansson, A. B. Carigren, M. Wilhelmson, and K. Lillpers, 1998. Are reproductive traits impaired by selection for egg production traits in laying hens? J Anim. Breeding and Genetics 115:281-297.

Sewalem, A. and M. Wilhelmson, 1999. Genetic study of embryonic mortality in white leghorn lines selected for egg production traits. Brit. Poultry Sci. 40:467-471.

Van Krey, H. P., A. T. Leighton, and L. M. Potter, 1967. Sperm gland populations and late seasonal declines in fertility. Poultry Sci. 46:1332. (Abstr.)

Van Krey, H. P., and A. T. Leighton, 1970. Sperm gland populations, oviduct homogenates and late season declines in fertility. Poultry Sci. 49:1477.

Van Krey, H. P., F. X. Ogasawara, and F. W. Lorenz, 1966. Distribution of spermatozoa in the oviduct and fertility in domestic birds. IV. Fertility of spermatozoa from infundibular and uterovaginal glands. J. Reprod. Fertil. 11:257-262.

Walsh, T. J., R. E. Rizk, and J. Brake, 1995. Effects of temperature and carbon dioxide on albumen characteristics, weight loss, and early embryonic mortality of long stored hatching eggs. Poultry Sci. 74:1403-1410.

Wilson, H. R., 1997. Effects of maternal nutrition on hatchability. Poultry Sci. 76:134-143.

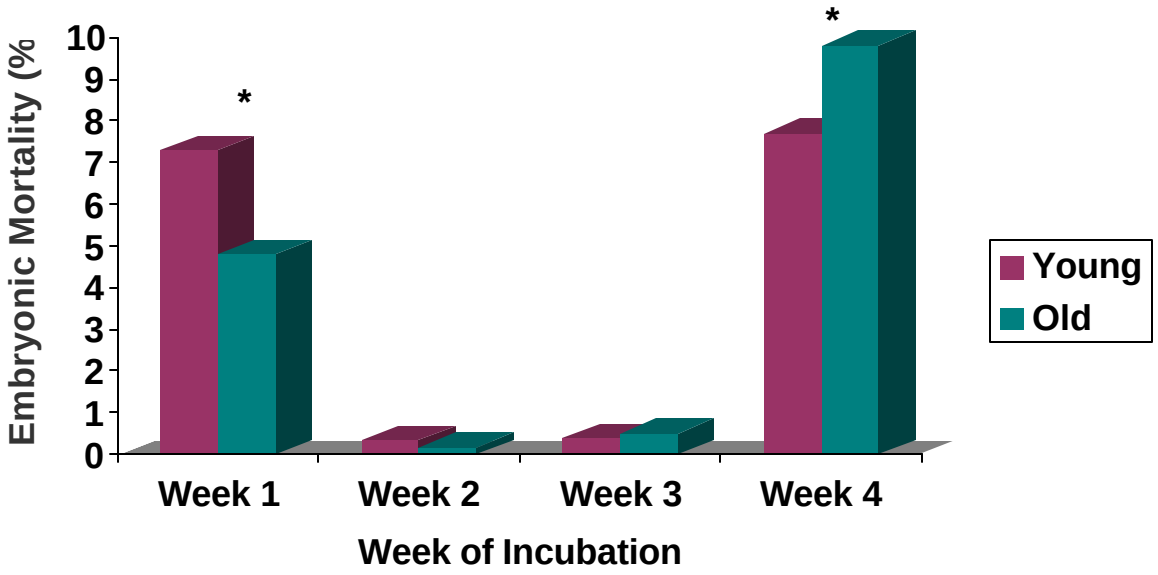

FIGURE 2.1. Weekly percent embryonic mortality during the incubation period from eggs of young (32-35 wks of age) and old (44-50 wks of age) turkey hens (n=51,764). * indicates $P \leq 0.01$.

TABLE 2.1. Fertility and hatchability (hatch) of all eggs set and for fertilized eggs for young (32-35 weeks of age) and old (44-50 weeks of age) commercial turkey hens.

	Fertility	Hatch	Hatch of Fertile Eggs
Young	94.1	79.9	85.3
Old	93.6	80.0	85.0
$\bar{x} \pm$ SEM	93.9 ± 3.6	79.9 ± 6.0	85.2 ± 5.3
P	NS	NS	NS

TABLE 2.2. Embryonic mortality of fertilized eggs from young (32-35 weeks of age) and old (44-50 weeks of age) commercial turkey hens with the first week categorized as before blood (BB) or after blood (AB) formation and the fourth week classified as prepip, internal, live, dead and total pips

	Week 1		Week 2	Week 3	Week 4				
	BB	AB			PrePip	Internal Pip	Live Pip	Dead Pip	Total Pip
Young	4.2^{A}	3.3^{A}	0.3	0.4	2.7^{B}	1.1	3.1	0.8^{B}	3.9
Old	3.0^{B}	1.9^{B}	0.2	0.5	5.0^{A}	1.0	2.5	1.3^{A}	3.8
$\bar{x} \pm$ SEM	3.6 ± 0.1	2.6 ± 0.1	0.3 ± 0.03	0.4 ± 0.03	3.8 ± 0.1	1.1 ± 0.1	2.8 ± 0.1	1.0 ± 0.04	3.9 ± 0.1
P	0.0001	0.0001	-----	-----	0.0001	NS	NS	0.0001	NS

$^{A, B}$ Means within a column with uncommon superscripts differ significantly ($P < 0.01$).

TABLE 2.3. Correlation of fertility and EEM for young (32-35 weeks of age) and old (44-50 weeks of age) commercial turkey hens

	Before Blood (%)	After Blood (%)	Total Week 1 Mortality (%)
	Young and Old Hens Combined		
Correlation Coeff.	-0.244	-0.043	-0.196
P	.0001	NS	.0001
	Young Hens		
Correlation Coeff.	-0.361	-0.105	-0.324
P	.0001	NS	.0001
	Old Hens		
Correlation Coeff.	-0.130	-0.033	-0.121
P	NS	NS	NS

TABLE 2.4. Correlation of fertility and embryonic mortality during the last week of incubation for young (32-35 weeks of age) and old (44-50 weeks of age) commercial turkey hens

	Week 4 (%)	Internal Pip (%)	Live Pip (%)	Dead Pip (%)	Total Pip (%)
	Young and Old Hens Combined				
Corr. Coeff.	0.029	-0.096	0.174	0.028	0.169
P	NS	0.0542	0.0005	NS	0.0007
	Young Hens				
Corr. Coeff.	0.142	-0.084	0.238	0.022	0.229
P	0.0431	NS	0.0006	NS	0.0010
	Old Hens				
Corr. Coeff.	0.010	-0.127	0.014	0.077	0.049
P	NS	NS	NS	NS	NS

III. Insemination Dosage and Hen Age Influence the Number of Holes Hydrolyzed in the Inner Perivitelline Layer of Turkey Eggs

ABSTRACT

Previous work demonstrated a significant increase in early embryonic mortality (EEM) in eggs from young turkey breeder hens as compared to hens in mid production. Preliminary data has indicated that increased insemination concentration decreased the incidence of EEM in eggs from young hens. Possible explanations for decreased EEM following insemination of more sperm include altered sperm cell binding and hydrolyzing of the inner perivitelline layer (IPVL) of eggs from hens of different ages. The current study examines differences in the number of sperm penetration (SP) holes hydrolyzed in the IPVL when hens at two different ages are inseminated with 25, 50, 100, 200, 400 or 800 million viable sperm cells. The hens were inseminated two times on 14 and 21d after onset of photostimulation and were inseminated two more times at 12 and 13 wk of production. The SP holes hydrolyzed in the IPVL were counted in all eggs produced in the 3 wks following each insemination period. There was no interaction between hen age and sperm insemination dose. The number of SP holes hydrolyzed in the IPVL was significantly greater in younger hens than older hens. Furthermore, the number of SP holes was significantly greater with the 400 and 800 million insemination doses as compared with the other four insemination doses. In conclusion, the absence of an interaction between hen age and insemination dose suggests that factors other than sperm binding influence EEM. These factors may be a combination of oviduct influences and IPVL properties that differ among hen age.

INTRODUCTION

Fertility and embryonic viability are required for successful hatching of avian eggs. A number of sperm characteristics have been examined for association with

fertility including semen concentration, semen quality, motility or mobility, and sperm penetration of the inner perivitelline layer (IPVL) (see review, Wishart, 1995). Embryonic viability has been shown to vary with hen age. Young hens were found to have increased early embryonic mortality (EEM) compared to older hens eggs that experienced increased late embryonic mortality (Section II). Preliminary data indicated that EEM decreased when younger hens were inseminated with larger semen doses (L. G. Bagley, 1998, Nicholas Turkey Breeding Farms, P.O. Box Y, 19449 Riverside Dr., Sonoma, CA, 95476-1209, personal communication). This is supported by the observation of a negative correlation between fertility and EEM in young turkey hens (Section II). This indicates that methods used to improve fertility may also affect EEM in young turkeys. Larger insemination doses have been associated with improved fertility (Maeza and Buss, 1976; Bakst, 1988; Donoghue, 1996) and may explain why the preliminary data demonstrated a decrease in EEM as the insemination dose increased. These data suggest that sperm penetration of the IPVL may differ with hen age and might be related to EEM. The hypothesis of the current study was that sperm binding and hydrolyzing of the ovum IPVL differs between hen ages. The objectives were to examine the effects of hen age and insemination dose on sperm penetration (SP) holes of the IPVL. Differences in sperm binding and hydrolyzing of the IPVL may explain differences in EEM noted in the previous study.

MATERIALS AND METHODS

The effects of hen age and insemination dose on sperm binding were examined by obtaining one group of birds and inseminating each bird once at the onset of production (YNG) and again at 12 wks of production (OLD). Nicholas turkey breeder hens were obtained at 30 wks of age from a commercial operation and were placed on

a lighting schedule of 15L:9D. Hens were randomly placed in pens that were assigned to one of six insemination treatments: 25, 50, 100, 200, 400, or 800 million viable sperm cells per insemination. Sperm viability was determined using the ethidium bromide exclusion test (Bakst et al., 1991). Because of the large insemination doses of 400 and 800 million sperm cells, four inseminations were performed on every hen, while allowing the oviduct to revert in between successive inseminations, to ensure that each bird received the desired dose. The volume of each insemination dose was held constant but the sperm concentration differed between treatment groups. The insemination method in Table 1 depicts the insemination scheme. The hens were inseminated twice within 10 d prior to the onset of egg production to ensure maximal filling of the sperm storage tubules (McIntyre and Christensen, 1983). Oviposited eggs were collected to determine sperm binding differences via the number of sperm penetration holes. Midway through production (12 wks of egg production) the hens were inseminated two more times within a 10 d period and eggs were collected again for SP analysis. Eggs laid during the 3 wks following each insemination period were used in the SP analysis.

Sperm Hydrolysis Assay

SP holes hydrolyzed in the IPVL were determined by the method described by Bramwell et al. (1995) and Donoghue (1996). The egg was opened and the albumen was separated from the yolk. The yolk was placed in a pan with the blastoderm positioned on top. Excess albumen was removed by blotting with a kimwipe and 2 mL of 2% NaCl solution was added. The perivitelline layer (PVL) over the blastoderm was removed and immediately rinsed in PBS to remove excess yolk material. The PVL was then placed on a slide and 3 to 4 drops of 3% formalin was added and immediately decanted. The PVL was finally stained with Schiff's Reagent. The IPVL holes were

counted using a light microscope at a magnification of 40x. The germinal disc (blastoderm area) was located on each slide and centered within the field of vision. All holes in this field were counted.

Statistical Analysis

The IPVL SP hole data were arranged in a 2x6 factorial with sources of variation being two hen ages (YNG, OLD) and 6 insemination doses (25, 50, 100, 200, 400, 800) and were then subjected to an analysis of variance using the General Linear Models procedure (SAS® institute, 1989). Because of the large differences from egg to egg within a treatment, all sperm penetration data were transformed by taking the log of holes+1. Means were separated using Least Squared Means. Unless stated otherwise, all statements of significance were assessed using $P \leq 0.05$.

RESULTS

The number of SP holes hydrolyzed in the IPVL was significantly greater when the hens were young during the early production period as compared to when the hens were older in the mid-production period (Table 2). There was a threshold response to insemination dose with doses of $400x10^6$ and $800x10^6$ sperm cells having significantly more hydrolyzed holes than doses of $200x10^6$ or fewer sperm cells (Figure 1). The numbers of SP holes from inseminated doses of $400x10^6$ and $800x10^6$ sperm did not significantly differ from one another. Similarly, SP holes produced by the lower doses of $25x10^6$ through $200x10^6$ sperm cells did not differ from one another. There was no significant interaction between hen age and insemination dose, which indicated that hens from both age groups performed similarly at each dose level.

DISCUSSION

Hen age influences the number of sperm penetration holes in turkey eggs. This is similar to results Bramwell et al. (1996) reported, in which increased SP holes had been observed in young broiler breeder hens as compared to older broiler breeder hens. That study also examined differences in SP holes due to broiler breeder male age. Eggs fertilized by younger broiler breeder males had fewer SP holes than eggs fertilized by older broiler breeder males. The current study did not separately determine male and female effects on SP holes in turkeys. Bramwell et al. (1995) stated that different hens had different numbers of SP holes throughout both the germinal disc (GD) and non-germinal disc (NGD) regions even when exposed to the same concentration of sperm. They suggested that the number or accessibility of sperm receptors on the IPVL might vary between hens. This work has been repeated by Wishart (1997) who reported a 25-fold difference in the number of SP holes of the GD region compared to NGD regions. Another factor not accounted for in the current study or Bramwell et al. (1996) is the difference in SST capacity that might exist between individual hens. There are several factors that could influence the number of sperm cells at the site of fertilization, including egg location within the oviduct (Mimura, 1939) and sperm characteristics such as mobility (Froman et al., 1997; Donoghue et al., 1998). The differences due to hen age noted in the current study and Bramwell et al. (1996) may be attributed to differences in SST storage capacity and sperm retention rather than differences in the number or availability of sperm receptors.

The results of the current study demonstrated a large increase in sperm penetration holes between insemination doses 200 to $400x10^6$ viable sperm cells. The larger insemination dose may be resulting in better filling of the SST allowing more

sperm to be present in the infundibulum for fertilization. There was no significant increase in SP holes from insemination doses of 400 to 800x10^6 sperm cells. This indicates that inseminating with doses larger than 400x10^6 spermatozoa may provide very little improvement in terms of sperm penetration or fertility. Thus, using doses larger than 400x10^6 spermatozoa may not result in optimal use of labor and toms.

Sperm concentrations have been associated with both fertility levels and increased embryonic mortality (Van Krey et al., 1966; Lorenz and Ogasawara, 1968; Bramwell and Howarth, 1997). Intramagnal inseminations, which bypass sperm selection barriers in the vagina and uterovaginal junction, resulted in larger sperm cell concentrations in the infundibulum, which associated with increased embryonic mortality (Van Krey et al., 1966; Lorenz and Ogasawara, 1968). However, deep inseminations of 7.5 cm, that bypassed the uterovaginal junction, exhibited better hatchability than more shallow inseminations of 3.0 cm (Holleman and Biellier, 1976). The deep insemination of 7.5 cm would have been expected to be associated with high numbers of sperm in the infundibulum. High sperm cell concentrations have been associated with increased sperm penetration of the germinal disc and embryonic death (Bekhtina, 1966; Bramwell and Howarth, 1997). These results indicate that some cases of EEM may be due to pathological polyspermic fertilization. Van Krey et al. (1966) and Lorenz and Ogasawara (1968), suggest that excessive EEM might have resulted from excessive pathological polyspermic fertilization. It also could have been due to poor semen quality (Huyghebaert et al., 1984; 1985), as the vagina, a sperm selection barrier (Steele and Wishart, 1992), was bypassed through intramagnal insemination, thereby allowing large numbers of both "unfit" and "fit" sperm to reach the infundibulum.

The results of the current study indicate that EEM in young hens may be a result of pathological polyspermic fertilization. The hens in the early production period had significantly more SP holes hydrolyzed in the IPVL than when they were at least 12 wk into egg production. This does not agree with the preliminary data that indicated increased insemination doses resulted in decreased EEM. If pathological polyspermy were a cause, increasing insemination doses would increase the chances of such an event occurring. However, the SP results could be confounded by oviduct differences between the two periods of production. It is possible that the differences may be due to sperm retention in the sperm storage tubules. In chickens, younger hens have a slower release rate of sperm from the SST, which results in the storage of more sperm for longer periods of time than older hens (Brillard, 1993). Furthermore, the current study indicates that the hen could influence the number of sperm cells that bind and hydrolyze SP holes in the IPVL. This may implicate unknown hen influences on EEM that may result in too many sperm cells penetrating the IPVL.

REFERENCES

Bakst, M. R., 1988. Turkey hen fertility and egg production after artificial insemination and multiple oviduct eversion during the pre-laying period. J. Reprod. Fertil. 83:873-877.

Bakst, M. R., H. C. Cecil, and T. J. Sexton, 1991. Modification of the ethidium bromide exclusion procedure for evaluation of turkey semen. Poultry Sci. 70:366-370.

Bekhtina, V., 1966. Morphological features of polyspermy fecudation in hens. In. Summaries from Pushkin Research Laboratory of Livestock Breeding (Leningrad Region, USSR). Cited by World's Poultry Sci J. 24:148-149, 1968.

Bramwell, R. K., and B. Howarth, 1997. Effect of low or high sperm penetration values at the germinal disc on early embryonic mortality in chicken eggs. Poultry Sci. 76(Suppl. 1):97.

Bramwell, R. K., C. D. McDaniel, J. L. Wilson, and B. Howarth, 1996. The effect of male and female broiler breeders on sperm penetration of the perivitelline layer overlying the germinal disc. Poultry Sci 75:755-762.

Bramwell, R. K., H. L. Marks, and B. Howarth, 1995. Quantitative determination of spermatozoa penetration of the perivitelline layer of the hen's ovum as assessed on oviposited eggs. Poultry Sci. 74:1875-1883.

Brillard, J. P., 1993. Sperm storage and transport following natural mating and artificial insemination. Poultry Sci. 72:923-928.

Donoghue, A. M., 1996. The effect of twenty-four hour in vitro storage on sperm hydrolysis through the perivitelline layer of ovipositioned turkey eggs. Poultry Sci. 75:1035-1038.

Donoghue, A. M., D. R. Holsberger, D. P. Evenson, and D. P. Froman, 1998. Semen donor selection by in vitro sperm mobility increases fertility and semen storage in the turkey hen. J. Androl. 19:295-301.

Froman, D. P., A. J. Feltmann, and D. J. McLean, 1997. Increased fecundity resulting from semen donor selection based upon *in vitro* sperm motility. Poultry Sci. 76:73-77.

Holleman, K. A. and H. V. Biellier, 1976. Fertility and embryonic livability as influenced by depth of insemination of turkey hens. Poultry Sci. 55:1154-1156.

Huyghebaert, G., F. Van Wambeke, and G. De Groote, 1984. The effect of pH of diluent, number of spermatozoa and storage method on fertility and hatchability obtained with turkey semen stored for 6 and 24 hours. Arch. Geflugelk. 48:142-150.

Huyghebaert, G., F. Van Wambeke, and G. De Groote, 1985. The effect of different storage methods of turkey semen on fertility and hatchability. Arch. Geflugelk. 49:205-211.

Lorenz, F. W. and F. X. Ogasawara, 1968. Distribution of spermatozoa in the oviduct and fertility in domestic birds. VI. The relations of fertility and embryo normality with site of experimental inseminations. J. Reprod. Fertil. 16:445-455.

Maeza, A. S. and E. G. Buss, 1976. Sperm concentration and sperm numbers as related to fertility in chickens. Poultry Sci. 55:2059. (Abstr).

McIntyre, D. R. and V. L. Christensen, 1983. Filling rates of the uterovaginal sperm storage glands in the turkey. Poultry Sci. 62:1652-1656.

Mimura, H., 1939. On the mechanism of travel of spermatozoa through the oviduct in the domestic fowl, with special reference to the artificial insemination. Okajimas Folia Ant. Japan 17:459-476.

SAS Institute, 1989. SAS/STAT® Guide for Personal Computers. 8th ed. SAS Institute Inc., Cary, NC.

Steele, M. G. and G. J. Wishart, 1992. Evidence for a species-specific barrier to sperm transport within the vagina of the domestic hen. Theriogenology 38:1107-1114.

Van Krey, H. P., F. X. Ogasawara, and F. W. Lorenz, 1966. Distribution of spermatozoa in the oviduct and fertility in domestic birds. IV. Fertility of spermatozoa from infundibular and uterovaginal glands. J. Rerod. Fertil. 11:257-262.

Wishart, G. J., 1995. New approaches to evaluating male and female fertility. Pages 207-223 *in:* Proceedings First International Symposium on the Artificial Insemination of Poultry. M. R. Bakst and G. J. Wishart ed. Poultry Science Association, Savoy, IL.

Wishart, G. J., 1997. Quantitative aspects of sperm;egg interaction in chickens and turkeys. Anim. Reprod. Sci. 48:81-92.

TABLE 3.1. Number of semen and diluent straws used to achieve each of six increasing sperm concentration insemination doses*

Treatment	Number of straws with semen (sperm cells per straw)	Number of straws with diluent	Total Straws Used
25	1 ($25x10^6$)	3	4
50	1 ($50x10^6$)	3	4
100	1 ($100x10^6$)	3	4
200	1 ($200x10^6$)	3	4
400	2 ($200x10^6$)	2	4
800	4 ($200x10^6$)	0	4

*Total number of straws used in each treatment dose = 4 straws.

TABLE 3.2. The mean and log transformed mean sperm penetration (SP) holes hydrolyzed in the inner perivitelline layer of old and young hens

Age	Mean SP Holes	Log of SP holes + 1
Young	10	1.40^{A}
Old	9	0.77^{B}
Log $\bar{x} \pm$ SEM	1.13 ± 0.03	

$^{A, B}$Means within a column with different superscripts differ significantly ($P \leq 0.01$)

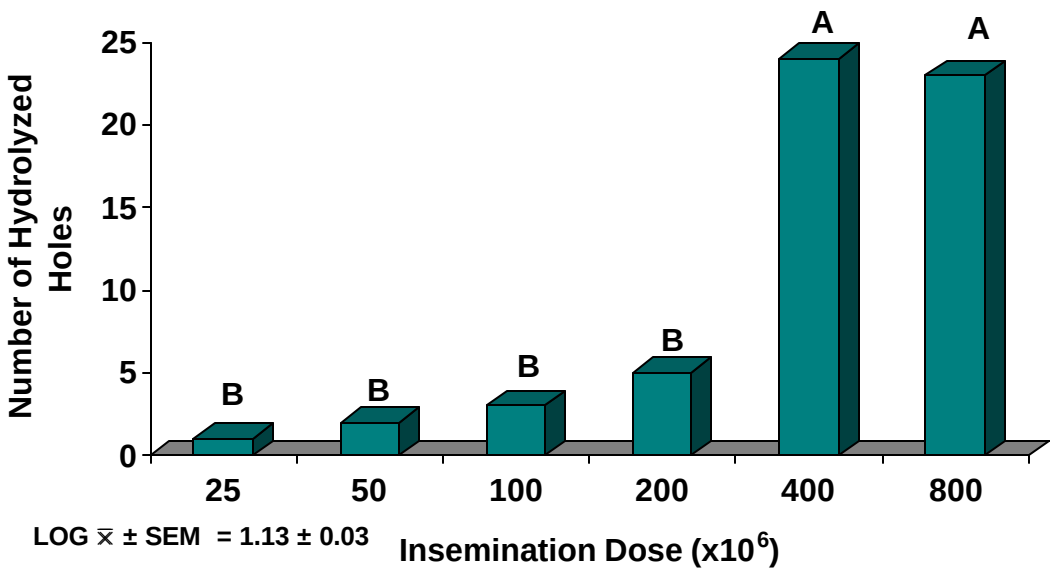

FIGURE 3.1. The number of hydrolyzed holes in the inner perivitelline layer of hens inseminated with six different doses of semen. [A, B]Treatment doses with different letters differ significantly ($P \leq 0.01$)

IV. Influence of Genetic Selection for Egg Production on Sperm Binding and Hydrolyzing of the Perivitelline Complex

ABSTRACT

Early embryonic mortality (EEM), defined as embryonic mortality during the first week of incubation, accounts for 5 % of the mortality that occurs during the incubation period, but can be as high as 15 % in extreme cases. The objectives of the current study were to examine the sperm-ovum relationship between a line of turkeys selected for 180 d egg production (EGG) and its random bred control (RBC1). It was of interest to see if genetic selection influenced the sperm-ovum relationship and to see if the negative relationship between fertility and EEM observed in previous work existed in this experimental model as well. The incubation records from these two lines were examined over a period of four years for fertility, hatchability and EEM differences. The current study examined the sperm-ovum relationship in vivo by ascertaining the number of sperm penetration holes (SP) present in the inner perivitelline layer (IPVL). In vitro experiments were conducted with the intact perivitelline layer (PVL).

The number of SP holes in the IPVL was examined after insemination with $150x10^6$ sperm per weekly artificial insemination. The PVL used in the in vitro studies were obtained from oviposited eggs from infertile hens and incubated with $25x10^6$ sperm to ascertain sperm hydrolysis of the PVL in the absence of oviduct factors.

Examination of incubation records over a four year period revealed that the negative relationship between fertility and EEM existed between a line of turkeys selected for 180 d egg production and their random bred controls. This suggested that the relationship could be valid among several lines of turkeys. To date, only one line of commercial turkeys has been examined and further work is required to determine

whether a similar relationship is true for other turkey lines. Sperm hydrolysis of the IPVL did not differ significantly between the two lines in vivo, whereas in vitro sperm hydrolysis was greater in EGG than RBC1. These results suggest that selection barriers in the oviduct of EGG hens may reduce the numbers of sperm present in the infundibulum, the site of fertilization. When sperm were incubated with PVL from the opposite line, no interaction was observed between males and females of different lines. These data suggest that the increased EEM observed in RBC1 eggs may be due to an excessive number of sperm penetrating the IPVL during fertilization.

INTRODUCTION

Fertility and embryonic mortality have a large impact on hatchability. Previous work has indicated that the number of sperm penetration (SP) holes hydrolyzed in the perivitelline layer was positively correlated with fertility (Bramwell et al., 1995; Wishart, 1997; Wishart and Staines, 1999). Bramwell and Howarth (1997) reported that an increase in SP holes was associated with increased embryonic mortality. A negative relationship between fertility and EEM has been reported from hatch records of commercial hens (Fairchild et al., 1999). Further study of this relationship of increased fertility and decreased EEM may provide more information that would elucidate possible causes of embryonic mortality in turkeys.

This study consisted of two experiments that tested the hypothesis that selection for a trait such as egg production could alter sperm penetration of the IPVL. The objectives of the first experiment were to examine the effect of genetic selection for 180 d egg production compared to its randombred control on fertility, EEM and sperm

binding. The objective of the second study was to examine the effects of reciprocal matings of select breeders to their randombred controls on the same three variables.

MATERIALS AND METHODS

Experiment 1

Using hatch records from EGG and RBC1 flocks from 1996 through 1999 used in studies at the North Carolina State University research unit, fertility, mortality during the first week of incubation (EEM), and hatchability of fertile eggs were analyzed. Flocks were reared under similar conditions each year as described by Christensen et al. (1993). At 31 wk of age, 18 hens from each strain were photostimulated. At 33 wk of age the hens were inseminated with 150 x 10^6 viable sperm prior to onset of egg production followed by weekly inseminations for the remainder of the production period. Approximately 10 hens from each strain were also photostimulated but not inseminated, and used as a source of infertile eggs for in vitro assays. The number of SP holes hydrolyzed in the inner perivitelline layer (IPVL) was determined for 48 eggs collected at 5, 10 and 15 wk of age. All other eggs were incubated to determine fertility, embryonic mortality and hatchability. The number of SP holes in the PVL was determined by the sperm penetration assay of Bramwell et al. (1995) as described in Section III.

In vitro Sperm Penetration Assay

The perivitelline layer obtained from unfertilized oviposited eggs was cut around the equator of the egg and the non-germinal disc region was removed and rinsed repeatedly until the excess yolk material was removed. The perivitelline layer was then placed in PBS and cut into 1.0 cm^2 sections and transferred to beakers containing 1 ml of Dulbecco's Modified Eagle's Medium. Sperm were collected from toms using the

abdominal massage technique (Burrows and Quinn, 1935) and diluted with Minnesota Avian Semen extender. A total of $25x10^6$ sperm in a volume of 20 ul was added to each beaker containing the PVL and incubated for 10 min at 40 C while shaking. After incubation, the PVL was rinsed vigorously in PBS, placed on a glass slide where 2-3 drops of fixative (3% formaldehyde) were added and decanted, then stained with Schiff's reagent. The number of SP holes was counted in 5 fields at a magnification of 400x. The SP holes quantified in the five fields were then averaged and the mean was used as the final observation for that PVL sample. An n of 30 PVL representing five replicates from six eggs was used in this study for each treatment.

Experiment 2

The turkeys used in this study were from the next generation that was selected for 180 d egg production following the generation used for Experiment 1. This experiment was conducted the year following Experiment 1. The breeders were reared using the same protocol as described in Experiment 1. Inseminations were performed as described in Experiment 1 except 200 x 10^6 viable spermatozoa were inseminated each week. Hens from each strain were randomly placed in pens and each treatment had three pens. Each pen received semen from one line, either EGG toms or RBC1 toms. Sperm binding and penetration of the IPVL was determined at early and mid production using 12 eggs from each treatment utilizing the assay described in Section III. The remainder of the eggs produced were placed in incubators on a biweekly basis and incubated to determine fertility, hatchability, and embryonic mortality for each treatment.

Statistical Analysis

Experiment 1

The data from hatch records and in vivo studies were subjected to a one way analysis of variance. Genetic line (EGG and RBC1) was the main factor in this study. Fertility, hatchability, and embryonic mortality means were subjected to arc sine transformation prior to analysis. The data from the in vitro study were arranged in a 2 x 2 factorial with two hen lines (EGG and RBC1) and two tom lines (EGG and RBC1).

Experiment 2

All data were arranged in a 2 x 2 factorial with two hen levels (EGG and RBC1) and two tom levels (EGG and RBC1). Arc sine transformation was performed on all percentage data prior to analysis. The sperm penetration holes were subjected to a log transformation prior to analysis by taking the log of holes + 1. The analysis was conducted using the General Linear Models procedure of SAS® (SAS Institute, 1989). Probability of a significant difference was based on $P \leq 0.05$ unless stated otherwise. Means determined to differ significantly were separated using the Least Squared Means procedure (SAS Institute, 1989).

RESULTS

Experiment 1

The incubation data differed between the two lines (Table 1). EGG fertility was greater as compared to eggs from the RBC1 line. EEM was significantly higher in RBC1 when compared to the EGG line. There was no clear pattern present in the hatchability results, which suggest that changes in the other three weeks of incubation were also contributing to hatchability differences during those four years.

The number of SP in vivo did not differ significantly when RBC1 was compared to EGG (Table 2). However in vitro assays resulted in more SP holes in EGG than RBC1 (Table 3). When sperm from EGG and RBC1 males were incubated on PVL from differing lines, no interaction between the male and females of the two lines was detected (Figure 1). No interaction between tom and hen indicated that for the most part the hen and tom contributions to hydrolyzed holes were similar for both strains in vitro.

Experiment 2

After conducting line to line and reciprocal inseminations, both hen and tom had significant effects on IPVL SP holes in vivo (Table 4). EGG hens and toms had more SP holes than RBC1 hens and toms. EGG breeders exhibited more than double the SP holes as compared to RBC1 breeders. No significant interaction between hen and tom was detected when EGG hens were inseminated with RBC1 semen or when RBC1 hens were inseminated with EGG semen.

The incubation data differed among the insemination treatments (Table 5). Increased fertility was observed in EGG males (84.3%) and females (82.1%) as compared to RBC1 males (73.9%) and females (76.2%). Hatchability exhibited a hen by tom interaction. The reciprocal cross of RBC1 hens inseminated with EGG semen had higher hatchability as compared to EGG hens inseminated with EGG semen or RBC1 hens inseminated with RBC1 semen. The two reciprocal crosses did not differ from one another. Mortality during the first week of incubation (EEM) exhibited both hen and tom differences, but no interaction between hen and tom. Eggs from RBC1 hens (10.9%) and toms (12.2%) experienced more EEM than eggs from EGG hens (8.6%) and toms (7.3%). The distribution of sperm holes in eggs from EGG was more diverse

than from eggs obtained from RBC1 hens (Figure 2). All eggs from RBC1 were within the range of 0-60 SP holes where the EGG strain had 61% of its eggs within this range.

DISCUSSION

The incubation records from previous years indicated that the negative relationship between fertility and EEM exists in this experimental model using genetic selection for 180 d egg production just as it did previously in young hens (Fairchild et al., 1999). This suggests that selection for 180 d egg production has provided this line of turkeys with factors that result in improved fertility and lower EEM, thus resulting in increased hatchability. However, hatchability records did not indicate any differences suggesting that factors other than fertility and EEM were affecting the results. Embryonic mortality during the other three weeks of incubation is affecting hatchability between these two lines. Data from Experiment 1 did not indicate a difference in EEM between EGG and RBC1 (Data not shown). A possible explanation for this discrepancy is the small number of eggs used in this study compared to previous years. Fewer observations were available for hatchability and embryonic data due to the use of some eggs for sperm binding analysis, and the use of fewer hens as compared to previous years. The data from Experiment 2 indicated variability in hatchability due to insemination crosses between the two lines. It appears that neither the line with the highest SP holes (EGG) or the line with the least amount of SP holes (RBC1) were required for greatest hatchability since the reciprocal crosses yielded better hatchability even though they demonstrated an intermediate number of SP holes. This suggests that there may be an optimum number of SP holes for embryo survival that is not

associated with having the most or the fewest SP holes. Although the number of SP holes was associated with differing hatchability percentages between the different lines and their reciprocal crosses, the SP holes did not appear to affect fertility or EEM in the current study. Based on the observations of Bramwell et al. (1995) and Section II of this dissertation, a pattern between fertility, SP holes, and EEM was expected but not observed. These results suggest that factors other than the amount of sperm cell penetrating the IPVL affect hatchability, possibly through deaths at other times during the incubation period.

Donoghue (1996) demonstrated fewer SP holes in the IPVL after storage of sperm in vitro for 24 h prior to insemination. Neither fertility nor hatchability was affected by the reduced hydrolysis of the IPVL. Furthermore, the results of that study suggested that eggs inseminated with stored semen were as capable of embryonic development as eggs fertilized with unstored semen. Those results conflict with unpublished results that indicated insemination with stored sperm resulted in increased embryonic mortality and reduced hatchability (V. L. Christensen, 1992, North Carolina State University, Box 7608, Raleigh, NC 27504, personal communication). The effect of breeder age on sperm penetration of the IPVL has been shown in both broiler breeders (Bramwell et al., 1996) and turkeys (Section III). Bramwell et al. (1996) reported that both males and females could influence SP. The current study demonstrates significant male and female influences on sperm penetration in turkeys.

The results of this study demonstrated that genetic selection could affect sperm penetration of the IPVL both in vivo and in vitro. However, the results varied depending on the system and insemination dose. When $150x10^6$ sperm were inseminated, the

RBC1 SP holes did not differ statistically when compared to EGG line. However, when $200x10^6$ sperm were inseminated EGG had more SP holes than RBC1. This is inconsistent with data found in commercial line hens where hens having greater number of sperm penetration holes in the germinal disc region experienced increased EEM (Bramwell and Howarth, 1997). Ogasawara et al. (1966) and Van Krey et al. (1966) observed large numbers of sperm in the infundibulum after intramagnal and intraperitoneal inseminations that resulted in longer fertility duration and increased EEM. The data from the current demonstrate that an optimal number of sperm may result in the best hatchability with too many or to few sperm resulting in increased embryonic mortality.

When oviduct influences were removed by in vitro examination, RBC1 PVL had lower binding when compared to EGG PVL. Irrespective of hen and tom influences, the in vitro results suggest that EGG have a greater numbers of SP holes than RBC1. When the in vitro data are combined with the in vivo data, the results suggest that the EGG oviduct has developed in such a way as to limit the number of sperm present in the infundibulum for sperm binding and penetration of the IPVL. The differing oviduct factors between these two lines are currently unknown. However, the differing oviduct factors may include differences previously identified in other lines, such as motility to reach sperm storage tubules located in the uterovaginal junction or differences in the storage capacity and sperm release rate. One reason that SP hole differences were detected in vivo in Experiment 2 and not Experiment 1 may be due to insemination dose. Perhaps too few sperm were inseminated in Experiment 1 to detect differences in IPVL SP holes. A larger insemination dose was used in Experiment 2 and significant

differences were detected. The results from Section III indicated a threshold dose effect related to the number of sperm inseminated. This threshold may differ between different turkey lines making insemination dose a factor that could mask possible differences if too many or too few sperm are inseminated.

In summary, a negative relationship between fertility and EEM was observed in EGG and RBC1 line turkeys. RBC1 had lower fertility and increased EEM where EGG had high fertility and lower EEM. In vitro data suggested that EGG actually had higher SP irrespective of male and female influences and that through selection oviduct factors have developed that may limit the number of spermatozoa reaching the infundibulum. In conclusion, there appear to be factors due to selection for 180 d egg production and gender that can influence sperm binding and penetration of the IPVL that may in some part influence EEM.

REFERENCES

Bramwell, R. K., H. L. Marks, and B. Howarth, 1995. Quantitative determination of spermatozoa penetration of the perivitelline layer of the hen's ovum as assessed on oviposited eggs. Poultry Sci. 74:1875-1883.

Bramwell, R. K., and B. Howarth, 1997. Effect of low or high sperm penetration values at the germinal disc on early embryonic mortality in chicken eggs. Poultry Sci. 76(Suppl. 1):97 (Abstr).

Burrows, W. H., and J. P. Quinn, 1935. A method of obtaining spermatozoa from the domestic fowl. Poultry Sci. 14:251-254.

Christensen, V. L., W. E. Donaldson, and K. E. Nestor, 1993. Embryonic viability and metabolism in turkey lines selected for egg production or growth. Poultry Sci. 72:829-838.

Donoghue, A. M., 1996. The effect of twenty-four hour in vitro storage on sperm hydrolysis through the perivitelline layer of ovipositioned turkey eggs. Poultry Sci. 75:1035-1038.

Fairchild, B. D., V. L. Christensen, J. L. Grimes, M. J. Wineland, and L. G. Bagley, 1999. Embryonic mortality differs between hen ages. Poultry Sci. 78 (Suppl. 1):20 (Abstr).

Fairchild, B. D., V. L. Christensen, and L. G. Bagley, 2000. The relationship of insemination sperm concentration and hen age on the number of holes hydrolyzed in the perivitelline membrane. Poultry Sci 79 (Suppl. 1):51 (Abstr).

Ogasawara, F. X., F. W. Lorenz, and L. W. Bobr, 1966. Distribution of spermatozoa in the oviduct and fertility in the domestic birds. III. Intra-uterine insemination of semen from low-fecundity cocks. J. Reprod. Fert. 11:33-41.

SAS Institute, 1989. SAS/STAT Guide for Personal Computers. Version 6 Edition. SAS Institute Inc., Cary, NC.

Van Krey, H. P., F. X. Ogasawara, and F. W. Lorenz, 1966. Distribution of spermatozoa in the oviduct and fertility in the domestic birds. IV. Fertility of spermatozoa from infundibular and uterovaginal glands. J. Reprod. Fert. 11:257-262.

Wishart, G. J., 1997. Quantitative aspects of sperm:egg interaction in chickens and turkeys. Anim. Reprod. Sci. 48:81-92.

Wishart, G. J. and H. J. Staines, 1999. Measuring sperm:egg interaction to assess breeding efficiency in chickens and turkeys. Poultry Science 78:428-436.

TABLE 4.1. Fertility (%), early embryonic mortality (EEM) (%) and hatch (%) of eggs from two lines of turkeys from 1996 through 2000

Line[1]	Fertility	EEM	Hatchability
EGG	93.3^{A}	7.2^{B}	70.6
RBC1	82.7^{B}	12.5^{B}	68.7
$\bar{x} \pm$ SEM	88.6 ± 0.3	9.3 ± 0.3	70.5 ± 0.6
	$P \leq 0.01$	$P \leq 0.01$	NS

^{A,B}Means within a column that have no common superscripts differ significantly ($P \leq 0.01$).

[1]EGG= Egg line breeders selected for 180 d egg production; RBC1= Random bred controls

TABLE 4.2. In vivo data for sperm penetration of the inner perivitelline layer for the EGG and RBC1 lines when inseminated with 150 x 10^6 viable sperm

Line[1]	Hydrolyzed Holes
EGG	32
RBC1	49
Log (holes+1) $\bar{x} \pm$ SEM	3.2 ± 0.2
P	NS

[1]EGG= Egg line breeders selected for 180 d egg production; RBC1= Random bred controls

TABLE 4.3. In vitro data for sperm penetration holes in the perivitelline layer for the EGG and RBC1 when incubated with 25 x 10^6 viable sperm

Strain[1]	Sperm Penetration Holes Due to Hen Effects	Sperm Penetration Holes Due to Tom Effects
EGG	13[a]	14[a]
RBC1	9[b]	8[b]
P	.05	.004
$\bar{x} \pm$ SEM	10 ± 0.79	

[a.b]Means within a column that have no common superscripts differ significantly ($P \leq 0.05$.

[1]EGG= Egg line breeders selected for 180 d egg production; RBC1= Random bred controls

TABLE 4.4. Sperm penetration hole interaction means for hen by tom for two lines of turkeys and their reciprocal crosses when inseminated with 200 x 10^6 viable sperm

Hen[1]	Tom		Hen $\bar{x}$
	EGG	RBC1	
EGG	79	35	57^A
RBC1	27	10	19^B
Tom $\bar{x}$	53^A	22^B	
Log $\bar{x} \pm$ SEM	2.57 ± 0.39		

^{A,B}Means within a column that have no common superscripts differ significantly ($P \leq 0.01$).

[1]EGG= Egg line breeders selected for 180 d egg production; RBC1= Random bred controls

TABLE 4.5. Incubation results (percentage) of eggs from two lines of turkeys, EGG and RBC1, and their reciprocal crosses

Hen[1]	Tom	Incubation Results		
		Fertility	Hatchability	Week 1 Mortality
EGG	EGG	86.8	67.6[bc]	6.9
EGG	RBC1	77.4	72.6[ab]	10.3
RBC1	EGG	81.9	76.2[a]	7.6
RBC1	RBC1	70.4	64.6[c]	14.2
$\bar{x} \pm$ SEM		79.1 ± 1.3	70.3 ± 1.3	9.8 ± 0.6

[a,b]Means within a column that have no common superscripts differ significantly ($P \leq 0.05$).

[1]EGG= Egg line breeders selected for 180 d egg production; RBC1= Random bred controls

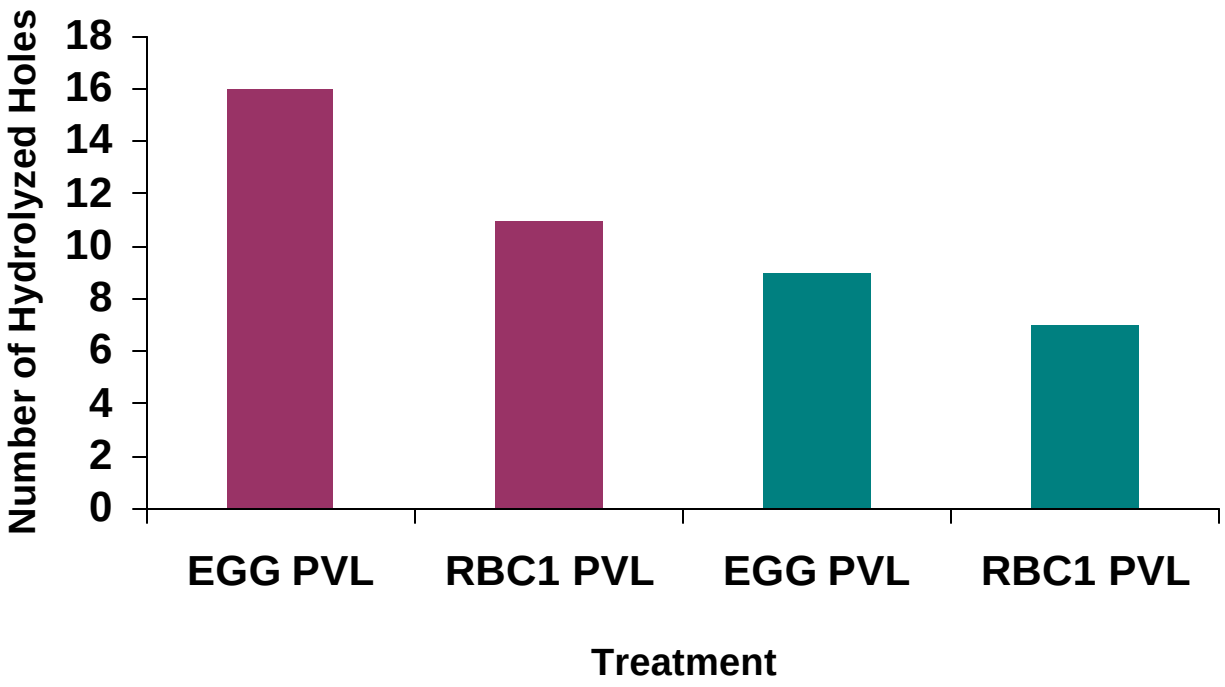

Figure 4.1. Reciprocal incubation of PVL and sperm from a line of turkeys selected for 180 d egg production (EGG) and their random bred control (RBC1). The number of hydrolyzed holes after PVL from both lines were incubated with EGG sperm (Burgundy bars) or with RBC1 sperm (Green bars).

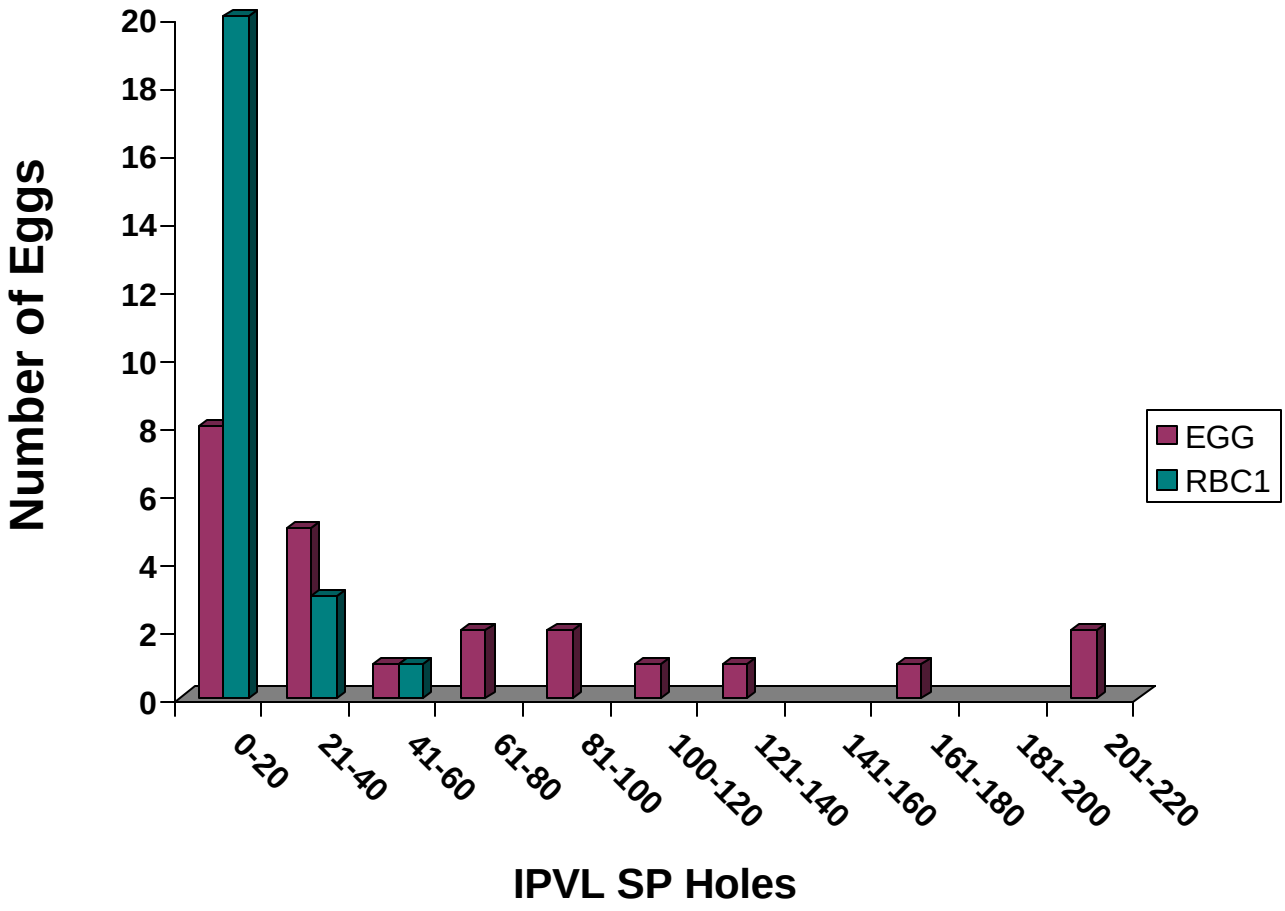

Figure 4.2. Distribution of sperm holes among eggs obtained from a strain of hens selected for 180 d egg production (EGG) and their random bred control (RBC1).

V. The Effects of Hen Age, Strain, Sire and Individual Hen on Sperm Penetration of the Inner Perivitelline Layer and the Relationship to Embryonic Mortality in Turkeys

ABSTRACT

The previous sections have demonstrated the effects of hen age and genetics on embryonic mortality, specifically mortality occurring during wk 1 of incubation, and sperm penetration (SP) of the inner perivitelline layer (IPVL). The purpose of the current study was to examine individual hen effects on IPVL SP holes when inseminated with the semen from an individual tom. Hens from two lines were assigned to toms from a third line for single sire inseminations on a weekly basis. Eggs from each hen were examined at an early egg production period (1 to 4 wks of production) and a mid production period (12-16 wks of production). The data were arranged in a nested set design with hen within tom within strain being the nested factors. The SP hole distribution indicated that 85% of hens had between 21 and 100 holes. There were no significant differences due to the main effects of strain or sire. Depending on the sire, the data exhibited differences due to hen and hen by period interactions. These results indicated that eggs from hens inseminated with semen from the same sire had significantly different numbers of IPVL SP holes. This suggests that the hen can influence sperm binding and hydrolysis of the IPVL. This could provide breeder personnel with another way of increasing fertility and lowering EEM: increasing hatchability by using hens that have an increased ability for IPVL hydrolysis. Furthermore, the hen by period interaction demonstrated that not all hens experienced a decrease in SP holes as they aged. Due to low numbers of eggs, hens in the upper (HI) and lower (LO) third of the flock population were used to analyze sperm hole effects on embryo viability. No differences in fertility or wk 1 mortality were detected between HI and

LO hens. HI hens had higher hatchability and lower wk 4 embryonic mortality as compared to LO hens. It was concluded that hens could have an influence on the relationship among IPVL SP holes and hatchability. Additional studies with larger samples would be required to determine the exact relationship of SP holes and embryonic mortality.

INTRODUCTION

Recent studies have indicated a relationship in young turkey hens in which early embryonic mortality (EEM) decreases as fertility increases. These same investigations suggest that this may be linked to sperm penetration through the inner perivitelline layer (IPVL) (Sections II, III, IV). Furthermore, this previous work has implied that hen age, genetic, male and female factors can interact to affect sperm penetration of the IPVL. However, prior experimental designs have not included all of these factors. Bramwell et al. (1996) reported that sperm penetration holes are affected by the age of both the broiler breeder dam and sire. Other studies have indicated that EEM was associated with excessive male pronuclei (Bekhtina, 1968) and with excessive sperm penetration of the germinal disc area in broilers (Bramwell and Howarth, 1997). Studies have indicated that turkey IPVL sperm penetration differs numerically from that of chickens (Wishart and Staines, 1999) but these studies have not reported the variation that could be observed within a given flock.

The purpose of this current study was to determine the hen variation in sperm penetration within a flock using single sire inseminations and to relate it to embryonic mortality. Sperm penetration of the IPVL, fertility, hatchability and embryonic mortality were compared between different hen ages, dams, sires and genetic lines.

MATERIALS AND METHODS

The breeders used in the current study were housed at Nicholas Turkey Breeder Farms facilities[1]. The breeders were raised according to the Nicholas Turkey Breeder guidelines. At 29 weeks of age, the hens were photostimulated using an 18L:6D photocycle. Two hen lines (A and B) were single sire inseminated using 10 toms from a third line C. Each tom was used to inseminate 10 hens (for a total of 100 hens, 50 per line) weekly throughout the production period. Only hens producing eggs during both production periods were used in the analysis. Semen was collected using the abdominal massage technique (Burrows and Quinn, 1935), and hens were inseminated via the deep vaginal insemination method. The semen volume administered to each tom's hens was $1/10^{th}$ of the collected volume. All eggs were stored in the same room and packed into common cases. The eggs were shipped to North Carolina State University on a weekly basis. Eggs from weeks of production 1-4 (early egg production period) (E) and 13-16 (mid egg production period) (M) were used in this study. Eggs from each hen were used either to determine sperm binding and hydrolysis of the IPVL or to observe fertility, hatchability and embryonic mortality.

The sperm binding and hydrolysis of the IPVL was determined using a modification of the assay used by Bramwell et al. (1995) and Donoghue (1996) (Section III). Eggs were incubated at 99.5 F and 50 % RH and candled on d 14 of incubation. Eggs that appeared clear during candling and unhatched eggs at the end of the incubation period were opened and examined macroscopically for fertility and day of embryonic death.

[1] Nicholas Turkey Breeder Farms, 19449 Riverside Dr., P.O. Box Y, Sonoma, CA, 95476-1111

Statistical Analysis

Factors in the current study were period (E and M), hen line (A and B), sire (n=10 line C toms) and hen (10 hens per sire). A nested set design was used in this experiment with hen within sire within line being the nested factors (Figure 1). The SP hole data were submitted to log transformation prior to analysis by taking the log of holes+1. Due to the infrequent occurrence of embryonic mortality, the number of eggs obtained from each hen was not enough to get an accurate estimate of the mean. Therefore, embryonic mortality means from birds that had SP holes in the highest third or lowest third of the samples were pooled into groups with high SP holes (HI) or low SP holes (LO) and analyzed statistically. The fertility, hatchability, wk 1 and wk 4 embryonic mortality are provided. Arc Sine transformation was performed on all percentage data prior to analysis. Data were analyzed using the General Linear Models procedure of SAS© (SAS Institute, 1989). Significant means were separated using the Least Squared Means procedure. Significance was based on $P \leq 0.05$ unless otherwise stated.

RESULTS

The data demonstrated a period by hen within sire interaction ($P \leq 0.01$). After the interaction was analyzed by sire, sires 2, 3 and 6 were found to have significant hen by period interactions (Table 1). Sires 4, 5, 7, 8 and 9 exhibited hen differences (Table 1). The hen by period interaction means indicated that some hens had an increase in SP holes from the early to mid period of production, others had a decrease in SP holes and some hens did not change. The frequency distribution of hens within a specific range of SP holes is presented in Figure 2. The flock data indicated that 85% of the hens had SP

holes ranging from 21 to 100. Frequency of SP holes outside of this range was three hens or fewer per group.

Fertility did not differ among sires, hen within sire or period. Data from hens having the upper and lower one-third sperm penetration holes exhibited significant differences in hatchability and wk 4 embryonic mortality (Table 2). No significant differences were observed for fertility or wk 1 embryonic mortality.

DISCUSSION

The results of the current study demonstrate that sperm binding and penetration of the IPVL in turkey eggs can differ among hens mated to the same sire. Eggs from 85% of the hens had between 21 and 100 sperm penetration holes. The number of SP holes from early in the production period to midway through the production period was inconsistent and varied with hen. This could be expected in a situation where hens were inseminated with pooled semen, but the results of the current study indicate it was true for hens inseminated with semen from an individual tom. Hens that were inseminated with semen from sires 2, 3, and 6 demonstrated a hen by period interaction. Some hens exhibited a decrease in SP holes from early to mid periods of production, some hens exhibited an increase and some hens did not change. Results from previous sections have indicated that SP holes can be affected by other variables such as insemination dose, genetics, sire and hen age (Sections III and IV). SP holes are also influenced by sperm storage (Donoghue, 1996), sperm mobility (Donoghue et al., 1998), and possibly time of initial insemination. Previous research has demonstrated that optimal filling of the SST occurred when hens were inseminated prior to the onset of egg production (McIntyre and Christensen, 1983). SP holes are positively correlated with numbers of sperm in the SST

(Brillard and Antoine, 1990; Brillard and Bakst, 1990; Wishart, 1995). Therefore it is possible that timing of AI as well as the number of times a hen is inseminated prior to egg production may influence the number of IPVL SP holes. The results in Sections III and IV indicated that young hens had increased sperm penetration both in vivo and in vitro. The results of the current study provided additional information to the results in Section III that showed a significant decrease in the number of IPVL SP holes with hen age. The insemination protocols may explain the variation observed between that study and the current study. The hens used in the study described in Section III were inseminated with specific semen concentrations at a constant volume twice within a 10 d period where the hens in the currently study were inseminated weekly with variable volumes of neat semen. This may have resulted in larger numbers of sperm available for sperm binding and penetration during any given wk of insemination. Differing results between hens at different periods of production could be due to a sperm number difference. Previous studies have indicated that toms that provided increased fertility values and prolonged sperm storage could be selected based on a single trait such as mobility (Donoghue et al., 1998). Several studies have examined semen traits in order to identify a trait that could be used to recognize sires with high fertilizing ability. While various tests that measure semen traits have been correlated with fertility, they have not been very successful in predicting fertility (Wishart, 1995). Recent work has indicated that in pooled semen only one or two toms are producing the progeny (Donoghue et al., 1999). Many of these studies have focused on the tom and have not considered hen influences. The results of the current study indicate that although hen fertility did not differ significantly, the number of IPVL SP

holes, a factor that has been correlated with fertility, differed (Bramwell et al., 1995; Wishart, 1995; Wishart and Staines, 1999).

The current study demonstrates that the hen can influence the IPVL sperm penetration. These observations could be due to either IPVL binding properties or due to different capacities of sperm storage or even a combination of both. Research working with IPVL components on a molecular level has identified glycoproteins that resemble mammalian zona pellucida proteins that may function in the sperm-egg interaction during fertilization (Takeuchi et al., 1999; Takeuchi et al., 2001). As the identification and function of these glycoproteins are revealed, the exact mechanism of why hens differ in the number of IPVL sperm penetration holes may be ascertained.

EEM occurs at a greater frequency in eggs from young hens as compared to eggs from older hens in both turkeys (Section II) and broiler breeders (Deeming and Van Middlekoop, 1999). It has been shown that excessive sperm penetration of the IPVL can result in increased EEM (Bekhtina, 1968; Bramwell and Howarth, 1997) and that younger hens have more sperm penetration than older hens in both chickens (Bramwell et al., 1996) and turkeys (Section III). This suggests that EEM and increased SP of the IPVL may be related and could explain increased EEM in young hens. However, no differences in EEM were detected between high and low SP groups in the current study. The reason no differences were detected in the current study is probably due to sample size. EEM occurs so infrequently that large numbers of eggs are required to obtain accurate estimates of the mean and to show differences between groups that may only differ by 0.5 to 1% in a variable that can range from 2 to 13 % of eggs set (Krueger, 1993; Section I). Hatchability was normally distributed and was higher in the high SP hens as compared to

the low SP hens and wk 4 mortality was lower in the high SP hens as compared to the low SP hens. The increased SP of the IPVL was associated with decreased wk 4 mortality, which suggested there is a benefit from having more sperm penetrate the IPVL than too few. This is in agreement with the results presented in Section IV where treatments having intermediate SP holes compared to treatments with the highest and lowest SP holes resulted in the better hatchability. This may suggest that there is an optimal number of sperm that must penetrate the IPVL that improves embryo survival. The studies of Perry (1987), Waddington et al. (1998) and Nakanishi et al. (1990) have suggested that supernumerary male pronuclei do not participate in the fertilization process, but migrate to the outer periphery undergo one mitotic division and degrade. In spite of these observations it does not rule out the possibility that supernumerary male pronuclei may contribute something to fertilization, whether it be an enzyme, or cell organelle such as mitochondria. In mammalian fertilization, the sperm penetration of the zona pellucida initiates a calcium wave that is associated with egg activation (Schultz and Kopf, 1995). Egg activation in the sea urchin has been associated with both an increase in nitric oxide production and a calcium increase (Shen, 1995; Kuo et al., 1998). Perhaps having a select number of pronuclei may provide the appropriate amount of a substance required for optimum embryonic survival even though fertilization only requires one sperm cell.

In summary, the results of the current study demonstrate the effects that the hen can have on sperm penetration, although it is unknown whether it is due to IPVL properties or due to oviduct sperm storage properties or a combination of the two. In this study, 85% of the hens had between 21 and 100 SP holes. Sperm penetration varied even when hens were inseminated with semen from the same sire, further depicting the hen effect on

sperm binding and penetration of the IPVL. Eggs from hens with high numbers of IPVL SP holes had increased hatchability and decreased wk 4 mortality. No significant EEM or fertility differences were detected between hens with high sperm penetration and hens with low sperm penetration. In conclusion, the results of the current study suggest that although factors such as semen quality, insemination technique and semen concentration could affect sperm penetration of the IPVL, hen influences should also be considered as a factor in the determination of IPVL SP holes.

REFERENCES

Bekhtina, V. G., 1968. Morphological features of polyspermy fecundation in hens. In: Summaries from Pushkin Research Laboratory of Livestock Breeding. Leningrad Region, U.S.S.R. Cited by World's Poultry Sci. J. 24:148.

Bramwell, R. K. and B. Howarth, 1997. Effect of low or high sperm penetration values at the germinal disc on early embryonic mortality in chicken eggs. Poultry Sci. 76(Suppl. 1):97 (Abstr).

Bramwell, R. K., H. L. Marks, and B. Howarth, 1995. Quantitative determination of spermatozoa penetration of the perivitelline layer of the hen's ovum as assessed on oviposited eggs. Poultry Sci. 74:1875-1883.

Bramwell, R.K., C. D. McDaniel, J. L. Wilson, and B. Howarth, 1996. The effect of male and female broiler breeders on sperm penetration of the perivitelline layer overlying the germinal disc. Poultry Sci 75:755-762.

Brillard, J. P. and H. Antoine, 1990. Storage of spermatozoa in the uterovaginal junction and its incidence on the numbers of spermatozoa present in the perivitelline layer of hen's eggs. Br. Poultry Sci. 31:635-644.

Brillard, J. P. and M. R. Bakst, 1990. Quantification of spermatozoa in the sperm storage tubules of turkey hens and the relation to sperm numbers in the perivitelline layer of eggs. Biol. Reprod. 43:271-275.

Burrows, W. H., and J. P. Quinn, 1935. A method of obtaining spermatozoa from the domestic fowl. Poultry Sci. 14:251-254.

Deeming, D. C. and J. H. Van Middlekoop, 1999. Effect of strain and flock age on fertility and early embryonic mortality of broiler breeder eggs. Br. Poult. Sci. 40:S22-S26.

Donoghue, A. M., 1996. The effect of twenty-four hour in vitro storage on sperm hydrolysis through the perivitelline layer of ovipositioned turkey eggs. Poultry Sci. 75:1035-1038.

Donoghue, A. M., M. R. Bakst, P. Drummond, S. Haqque, E. J. Smith, and D. J. Donoghue, 1999. Paternity efficiency in turkeys differs extensively after heterospermic insemination. J. Appl. Poultry Res. 8:214-221.

Donoghue, A. M., D. R. Holsberger, D. P. Evenson, and D. P. Froman, 1998. Semen donor selection by in vitro sperm mobility increases fertility and semen storage in the turkey hen. J. Androl. 19:295-301.

Kuo, R. C., G. T. Baxter, and D. Epel., 1998. Acrosome reaction elicits nitric oxide production. Mol. Biol. Cell 9(Suppl. S):2545 (Abstr).

Krueger, K. K., 1993. Embryo mortality patterns in commercial crossbred parent stock turkeys. Pages 70-74 *in*: Proceedings of the Third International Symposium on Turkey Reproduction. North Carolina State University, Raleigh, NC.

McIntyre, D. R. and V. L. Christensen, 1983. Filling rates of the uterovaginal sperm storage glands in the turkey. Poultry Sci. 62:1652-1656.

Nakanishi, A., K. Utsumi, and A. Iritani, 1990. Early nuclear events of in vitro fertilization in the domestic fowl (Gallus domesticus). Mol. Reprod. Dev. 26:217-221

Perry, M. M., 1987. Nuclear events from fertilization to the early cleavage stages in the domestic fowl (Gallus domesticus). J Anat. 150:99-109.

SAS Institute, 1989. SAS/STAT Guide for Personal Computers. Version 6 Edition. SAS Institute Inc., Cary, NC.

Schultz, R. M. and G. S. Kopf,1995. Molecular basis of mammalian egg activation. Pages 21-62 *in*: Current Topics in Developmental Biology. R. A. Pedersen and G. P. Schatten ed. Academic Press Inc., San Diego, CA.

Shen, S. S., 1995. Mechanisms of calcium regulation in sea urchin eggs and their activities during fertilization. Pages 63-101 *in*: Current Topics in Developmental Biology. R. A. Pedersen and G. P. Schatten ed. Academic Press Inc., San Diego, CA.

Takeuchi, Y., K. Nishimura, N. Aoki, T. Adachi, C. Sato, K. Kitajima, T. Matsuda, 1999. A 42-kDa glycoprotein from chicken egg-envelope, an avian homolog of the ZPC family glycoproteins in mammalian zona pellucida - its first identification, cDNA cloning and granulosa cell-specific expression. Eur. J Biochem. 260:736-742.

Takeuchi, Y., R. Cho, Y. Iwata, K. Nishimura, T. Kato, N. Aoki, K. Kitajima, and T. Matsuda, 2001. Morphological and biochemical changes of isolated chicken egg-envelope during sperm penetration: degradation of the 97-kilodalton glycoprotein is involved in sperm-driven hole formation on the egg-envelope. Biol. Reprod. 64:822-830.

Waddington, D., C. Gribbin, R. J. Sterling, H. M. Sang, and M. M. Perry, 1998. Chronology of events in the first cell cycle of the polyspermic egg of the domestic fowl (Gallus domesticus). Int. J. Dev. Biol. 42:625-628.

Wishart, G. J., 1995. New approaches to evaluating male and female fertility. Pages 207-223 *in*: Proceedings First International Symposium on the Artificial Insemination of Poultry. M. R. Bakst and G. J. Wishart, ed. Poultry Science Association, Savoy, IL.

Wishart, G. J. and H. J. Staines, 1999. Measuring sperm:egg interaction to assess breeding efficiency in chickens and turkeys. Poultry Science 78:428-436.

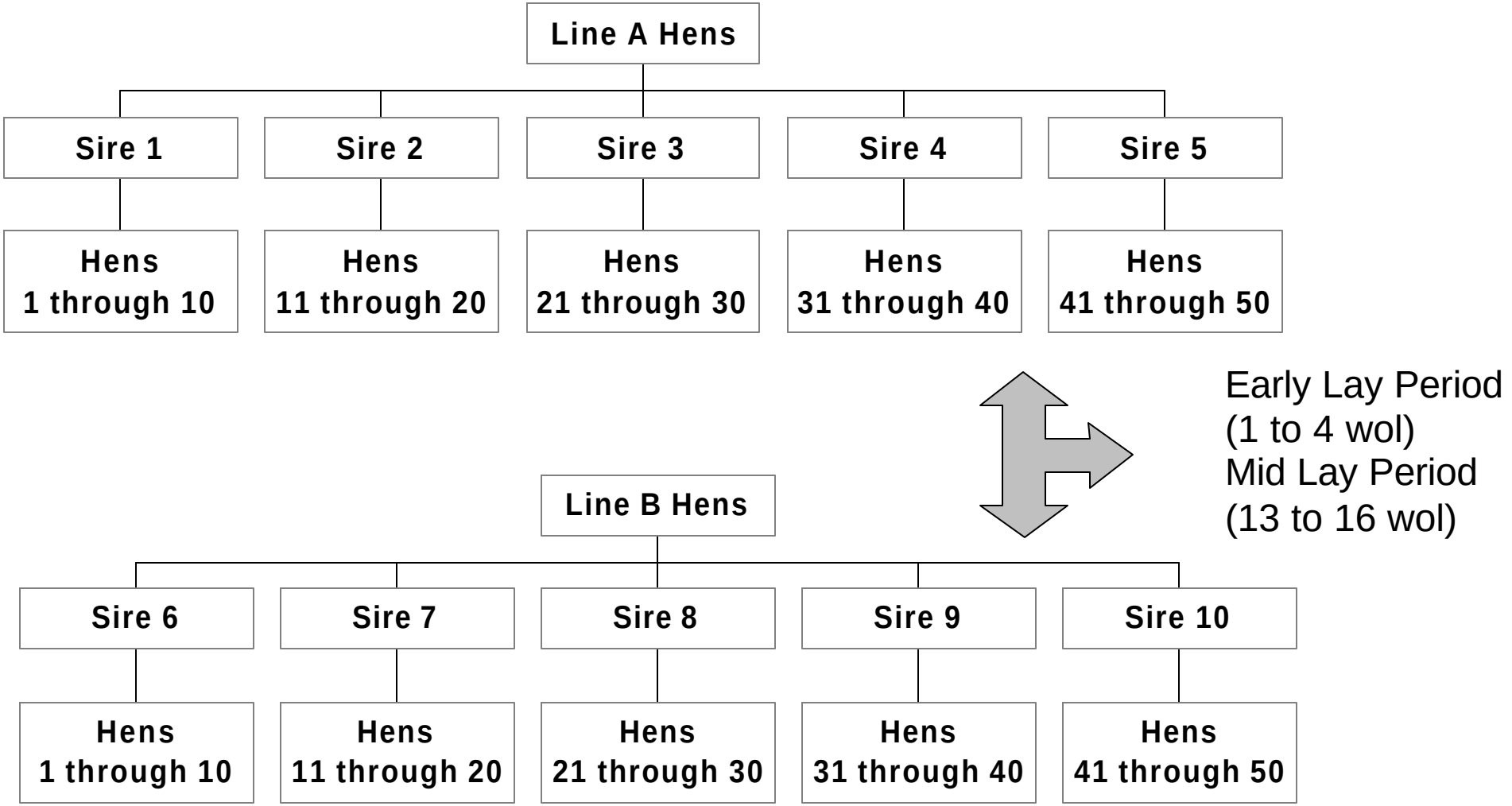

FIGURE 5.1. Schematic of the experimental design used in this study. The study employed a nested set design with hen within sire within line. There were 10 sires and 100 hens with 10 hens from lines A and B being single sire inseminated using sires from line C. Data was collected from eggs laid at two periods within the egg production period (early and mid lay).

TABLE 5.1. Mean sperm penetration holes from eggs produced during the early and mid egg production periods in each hen of Lines A and B that were mated to a single sire from Line C

				Line A												
Hen	Sire 1			Sire 2			Sire 3			Sire 4			Sire 5			
	Early	Mid	$\bar{x}$	Early	Mid	$\bar{x}$	Early	Mid	$\bar{x}$	Early	Mid	$\bar{x}$	Early	Mid	$\bar{x}$	
1	58	111	85	112^{abc}	204^{a}	158	48^{cde}	44^{abcde}	46	122	68	95^{b}	44	95	69^{ab}	
2	85	110	98	35^{de}	93^{bcd}	64	60^{abc}	38^{cde}	49	45	30	38^{c}	56	15	35^{cd}	
3	137	113	126	190^{ab}	112^{abc}	151	90^{ab}	50^{abcd}	70	62	45	53^{bc}	66	47	56^{bc}	
4	67	39	53	96^{abc}	92^{cd}	94	28^{de}	5^{f}	16	112	48	79^{bc}	97	108	103^{a}	
5				38^{ef}	18^{f}	28	29^{cde}	16^{e}	23	48	43	46^{bc}	22	49	36^{cd}	
6				104^{cd}	124^{abc}	114	120^{ab}	140^{a}	130	185	264	225^{a}	45	22	33^{cd}	
7				89^{bc}	46^{cde}	67	49^{cde}	46^{bcde}	47				58	43	51^{bc}	
8													49	56	53^{abc}	
9													33	42	38^{d}	
LOG $\bar{x}$ ±			4.25 ±			4.05 ±			3.43 ±			3.91 ±			3.50 ±	
SEM			0.01			0.08			0.09			0.09			0.08	
P																
Hen			NS			0.0001			0.0001			0.0001			0.0002	
Period			NS			NS			0.03			NS			NS	
Hen by Period			NS			0.05			0.04			NS			NS	

				Line B												
Hen	Sire 6			Sire 7			Sire 8			Sire 9			Sire 10			
	Early	Mid	$\bar{x}$	Early	Mid	$\bar{x}$	Early	Mid	$\bar{x}$	Early	Mid	$\bar{x}$	Early	Mid	$\bar{x}$	
1	106^{a}	62^{ab}	84	83	79	81^{b}	85	121	103^{a}	67	43	55^{b}	56	68	62	
2	75^{ab}	108^{a}	92	92	34	63^{b}	100	76	88^{a}	89	73	81^{ab}	71	43	57	
3	50^{ab}	59^{ab}	55	38	73	56^{b}	73	44	59^{b}	78	145	111^{a}	86	88	87	
4	39^{b}	3^{c}	21	69	37	53^{b}				121	107	114^{a}	53	48	51	
5	93^{ab}	39^{ab}	66	163	106	134^{a}				51	52	51^{b}	82	41	61	
6	48^{ab}	48^{b}	48	108	63	86^{ab}				53	93	73^{ab}	81	85	83	
7										71	111	91^{a}	55	26	41	
8													6	66	36	
LOG $\bar{x}$ ±			3.57 ±			4.12 ±			4.08 ±			4.04 ±			3.74 ±	
SEM			0.10			0.08			0.13			0.10			0.10	
P																
Hen			0.0001			0.0028			0.0071			0.0655			NS	
Period			0.04			NS			NS			NS			NS	
Hen by Period			0.003			NS			NS			NS			NS	

[a-e]Means within a sire with no common superscript differ significantly ($P \leq 0.05$).

TABLE 5.2. Fertility, hatchability and embryonic mortality between hens with high numbers of sperm penetration holes compared to hens with low numbers of sperm penetration holes in the IPVL

			Embryonic Mortality	
	Fertility (%)	Hatchability	Week 1	Week 4
HIGH	97.6	89.9[a]	2.8	3.6[b]
LOW	96.7	85.3[b]	3.0	7.0[a]
P	NS	0.05	NS	0.05
$\bar{x} \pm$ SEM	97.2 ± 0.5	87.6 ± 1.2	2.9 ± 0.5	5.3 ± 0.9

[a,b]Means within a column with no common superscript differ significantly ($P \leq 0.05$)

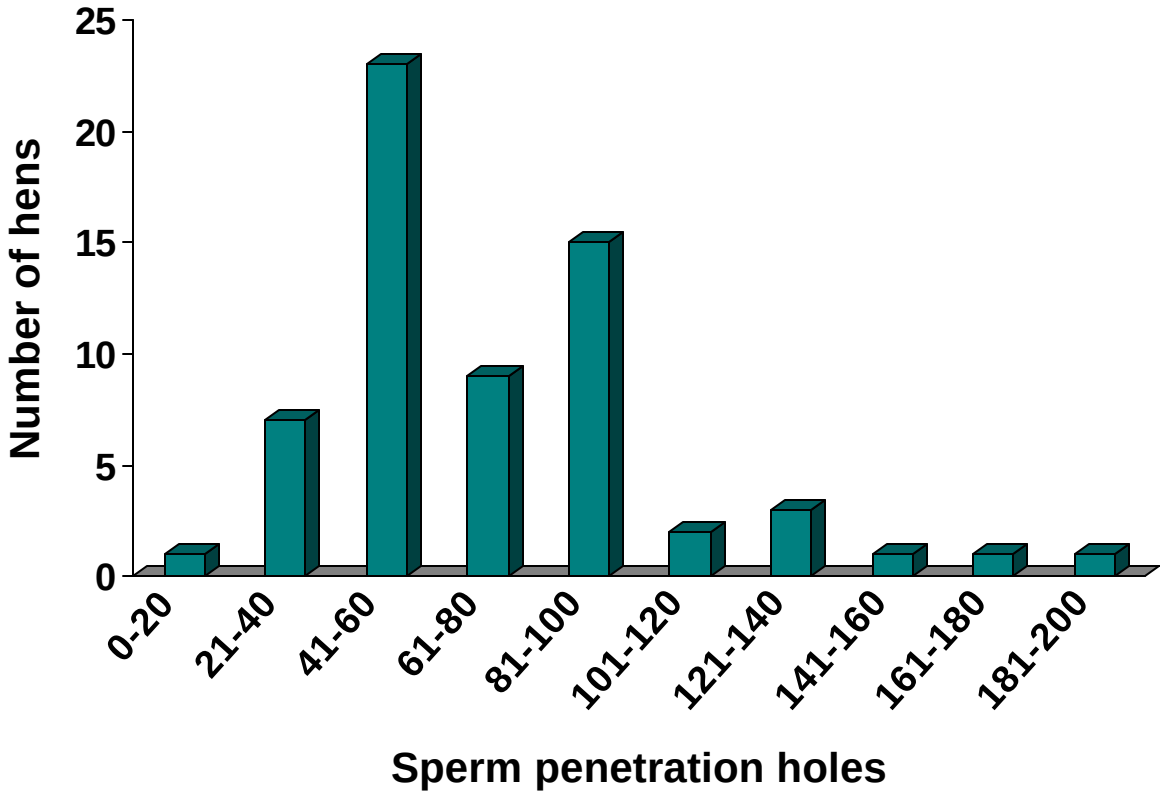

FIGURE 5.2. Frequency distribution showing the average number of sperm penetration holes per hen (n=63).

VI. GENERAL DISCUSSION

A limited amount of knowledge exits on early embryonic mortality (EEM) between different hen ages in commercial turkey lines used today, a problem that accounts for a loss of 3 to 15 % of incubated eggs. A negative relationship was observed between fertility and EEM whereas a positive correlation was observed between fertility and the number of live pips that did not hatch. The focus of these studies was on the negative relationship between fertility and EEM that might be attributed to differences in the hen, tom, or selection for a specific trait such as egg production. EEM is greater in younger hens where late embryonic mortality is greater in older hens. The results reported examined differences in embryonic mortality due to hen age and characterized some factors that may influence the sperm:ovum interaction in turkeys.

The data from the current studies demonstrate a significant hen effect on sperm penetration (SP) of the IPVL. The data presented in Section II and Appendix B indicated a decrease in SP as hens aged. However, when single sire inseminations were used on a group of hens in Section V, the SP varied with hen. Some hens exhibited a decrease in SP and others increased, whereas some did not change between the different ages examined. Data obtained from industry records indicated a negative correlation between the number of SP holes and EEM. No data were available to determine the relationship between SP holes and late embryonic mortality. However, Sections IV and V indicated that high numbers of SP holes were associated with less late embryonic mortality. The extra sperm that do not under syngamy may contribute to both early and late embryo survival in a way that is not

currently understood. Further research on the role of these sperm in avian fertilization and early embryonic development is needed to clarify this hypothesis.

Currently the industry has concentrated on the male influence on sperm penetration. This was done because semen is pooled in industry practices and the semen of one tom may be used to inseminate 10 or more hens depending on the volume and dilution ratio. By focusing more on the tom influence, improvement might be observed across many hens. The current data suggest that hen influences should be considered as well. By identifying both males and females that have superior sperm penetration, improved fertility may be gained while minimizing the occurrence of EEM.

The exact mechanisms for sperm penetration have not been identified. The current data suggest that it may be a combination of IPVL property differences and oviducal influences on inseminated sperm populations. Sperm competition may also influence IPVL sperm penetration. Donoghue et al. (1999) reported that semen from one or two sires fertilize more hens' eggs than other sire semen when pooled and inseminated. A single sire may be responsible for more IPVL sperm penetration when semen from multiple sires is pooled. The current data show, however, that hens respond differently when inseminated with the same source of semen even when the semen source is a single sire. In the wild, turkeys normally mate 40 or more times prior to initial oviposition. A hen will normally lay 12 eggs and incubate them in one season. The production period for wild hens would probably correspond with the early period examined in the current studies. This suggests that all 12 eggs are produced at a time when the sperm penetration is the greatest. If the

relationship between fertility and EEM were relevant to wild turkeys then this would result in the best fertility and possibly the lowest EEM. It would be of interest to examine wild turkeys eggs that were laid over a 12 to 15 week period to observe the effects on sperm penetration of the IPVL. It would be interesting to see if selection for economically important traits in domestic turkeys has resulted in declining sperm penetration as the hen ages or if they are relevant for optimal fertility and embryonic survival in seasonal breeders. Data from the current study demonstrated an increase in sperm penetration due to selection for egg production. The selected line has a smaller body weight than its random bred control. This may suggest a negative relationship between growth and sperm penetration.

Further research is needed to clarify some of the results reported here. The relationship between sperm penetration and EEM is not resolved. At the start of these studies it was thought that either more sperm penetration resulted in lower EEM or sperm penetration was so high that it was resulting in pathological polyspermic fertilization. Polyspermic fertilization is normal in avian species (Fofanova, 1965; Perry, 1987; Waddington, 1998), but there is evidence that if too many spermatozoa penetrate the IPVL and enter the ovum then it could become pathological and result in embryonic death (Bekhtina, 1968; Bramwell and Howarth, 1997). However, these results suggest that neither high or low sperm penetration will lower EEM but a value in between may be the best. This may suggest that both theories are correct. Due to the infrequent occurrence of EEM, a study using a large number of eggs is needed to further examine the relationship between sperm penetration and EEM. A study is needed to determine if IPVL properties actually

differ between hen ages and genetic strains. The recent identification of a mammalian zona pellucida protein homolog (ZPC) in chickens (Takeuchi et al., 1999) provides an opportunity to examine if differences in IPVL exist. If they do not exist then many of the differences noted in these experiments may actually be due to sperm storage differences in the oviduct. If IPVL differences do exist then the differences reported in these experiments may be due to a combination of both IPVL differences and oviduct differences that may be distinguished between in later studies.

In conclusion, these studies have presented several findings on both EEM and SP. Embryonic mortality differs between the different hen ages. The hen can have significant effect on SP of the IPVL. Another observation is the association of sperm penetration and embryonic mortality. The SP differs among hen ages. The SP varied among commercial lines and was greater in a line of turkeys selected for EGG production compared to its random bred control. This may indicate that increasing growth and body weight may be detrimental to IPVL SP. Furthermore, increased numbers of SP holes were not only associated with lowered EEM but also lower wk 4 mortality. The data from these studies indicate there may be a level of sperm penetration that is associated with optimal embryo survival. However, it appears that neither high nor low SP holes are associated with this but a level that is intermediate. Future studies are needed to determine the mechanism of how increased SP of the IPVL improves embryo survival.

REFERENCES

Bramwell, R. K. and B. Howarth, 1997. Effect of low or high sperm penetration values at the germinal disc on early embryonic mortality in chicken eggs. Poultry Sci. 76(Suppl. 1):97 (Abstr.).

Bekhtina, V. G., 1968. Morphological features of polyspermy fecundation in hens. In: Summaries from Pushkin Research Laboratory of Livestock Breeding. Leningrad Region, U.S.S.R. Cited by World's Poultry Sci. J. 24:148.

Donoghue, A. M., M. R. Bakst, P. Drummond, S. Haqque, E. J. Smith, D. J. Donoghue, 1999. Paternity efficiency in turkeys differs extensively after heterospermic insemination. J. Appl. Poultry Res. 8:214-221.

Fofanova, K. A., 1965. Morphological data on polyspermy in chickens. Fed. Proc. (Translation Suppl.) 24:T239-T247.

Kuo, R. C., G. T. Baxter, and D. Epel., 1998. Acrosome reaction elicits nitric oxide production. Mol. Biol. Cell 9(Suppl. S):2545 (Abstr).

Perry, M. M., 1987. Nuclear events from fertilization to the early cleavage stages in the domestic fowl (Gallus domesticus). J Anat. 150:99-109.

Schultz, R. M. and G. S. Kopf,1995. Molecular basis of mammalian egg activation. Pages 21-62 *in*: Current Topics in Developmental Biology. R. A. Pedersen and G. P. Schatten ed. Academic Press Inc., San Diego, CA.

Shen, S. S., 1995. Mechanisms of calcium regulation in sea urchin eggs and their activities during fertilization. Pages 63-101 *in*: Current Topics in Developmental Biology. R. A. Pedersen and G. P. Schatten ed. Academic Press Inc., San Diego, CA.

Takeuchi, Y., K. Nishimura, N. Aoki, T. Adachi, C. Sato, K. Kitajima, T. Matsuda, 1999. A 42-kDa glycoprotein from chicken egg-envelope, an avian homolog of the ZPC family glycoproteins in mammalian zona pellucida - its first identification, cDNA cloning and granulosa cell-specific expression. Eur. J Biochem. 260:736-742.

Waddington, D., C. Gribbin, R. J. Sterling, H. M. Sang, and M. M. Perry, 1998. Chronology of events in the first cell cycle of the polyspermic egg of the domestic fowl (Gallus domesticus). Int. J. Dev. Biol. 42:625-628.

Appendix A. Validation of in vitro Sperm Binding/Hydrolyzing Assay Using the Perivitelline Layer of Oviposited Eggs

INTRODUCTION

The sperm binding receptor in avian eggs is located on the inner perivitelline layer (IPVL) of the ovum. When the continuous and outer perivitelline layers (OPVL) are deposited in the infundibulum, sperm binding is inhibited or reduced (Howarth and Digby, 1973; Bakst and Howarth, 1977). Several factors can affect sperm binding including number of sperm inseminated and the number of sperm reaching the site of fertilization. Once a sperm binds to the IPVL, the acrosome reaction is initiated which results in the release of acrosin and other trypsin-like enzymes that hydrolyze a hole in the IPVL. Therefore, it can be assumed that for every sperm penetration hole present, at least one sperm was bound in that region. However, this is probably an underestimate of the number of sperm binding as more than one sperm cell could bind to the area where a hole was hydrolyzed. Alternatively, the sperm penetration hole does not necessarily indicate that a sperm actually entered into the ooplasm of the ovum. A sperm could bind and hydrolyze a hole in the IPVL but not pass through the oolema into the ovum. An assay has been validated to evaluate sperm holes in oviposited eggs and has been described by Bramwell et al. (1995) and Wishart and Staines (1999). Because the oviduct can exert selection pressures upon sperm after insemination, an in vitro assay was needed to examine sperm-ovum interactions in the absence of oviduct influences. Studies have utilized in vitro methods to examine sperm-ovum relationships by obtaining freshly ovulated ovum from the infundibulum prior to the application of the continuous layer and OPVL (Bakst and Howarth, 1977; Bramwell and Howarth, 1992). This method

involved euthanasia, therefore removing hens from the study. Other studies have utilized chicken IPVL that was isolated from oviposited eggs (Robertson et al., 1997; Robertson and Wishart, 1997) and involved separating the IPVL and OPVL as described by Kido and Doi (1988). This assay allowed the study of sperm-ovum interactions in the absence of oviduct influences. Disadvantages of the methods are the time involvement and relatively low numbers of observations obtained. Thus, a method is needed where the oviposited eggs could be utilized to examine the sperm binding relationship in large numbers of hens and eggs with a reasonable amount of labor. When the technique of Robertson and Wishart (1997) was applied to IPVL from turkeys, the IPVL and OPVL did not separate completely or cleanly. This resulted in the loss of several eggs due to improper layer separation. When incubated with sperm, IPVL were so fragile the sections could not be manipulated onto a slide.

MATERIALS AND METHODS

Isolation of the Perivitelline layer

The current assay is a modification of the Robertson and Wishart (1997) and Phillips et al. (1996) test. Eggs less than 24 h old were opened and the yolk and albumen were separated. The yolk was placed in a vessel with the germinal disc facing down and the excess albumin removed using a kimwipe[1]. The yolk was then rinsed in 2% NaCl and the perivitelline layer (PVL=IPVL+OPVL) was cut around the equator of the yolk. This ensured that non-germinal disc PVL was isolated and used for the assay. Preliminary trials resulted in PVL disintegration when the GD region was used even when incubated with sperm cell numbers as low as $5x10^6$. The

excised PVL was rinsed repeatedly in PBS until all excess yolk was removed and placed in a petridish containing PBS. The PVL was then sectioned into 1.0 cm^2 pieces. Each section was placed in a 5 mL disposable beaker containing 1 mL of Dulbecco's Modified Eagles Medium.

Semen collection and dilution

Semen was collected separately from toms of two lines using the abdominal massage technique (Burrows and Quinn, 1935) and diluted 1:1 with Minnesota Turkey Semen Extender[2]. Concentration was determined via packed cell volume and viability was determined using the ethidium bromide exclusion test (Biligili and Renden, 1984; Bakst et al., 1991). Using concentration, viability, and volume measurements, semen was diluted further to 50 x 10^6 spermatozoa in 20 μL. Using this as the stock solution, all other semen concentrations were made by adding semen from the stock and diluting with semen extender.

PVL Hydrolyzed Hole Assay

Prior to incubation, 20 μL of diluted semen was placed into each beaker. The PVL and semen were then incubated at 37-40 C for 10 min while shaking gently. After incubation, the PVL was removed, rinsed vigorously in PBS, placed on a microscope slide, fixed with 3% formalin and stained with Schiff's reagent. After drying, the hydrolyzed holes in five fields from each PVL section were counted at a magnification of 400x and averaged to give a value for each PVL section.

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[1] Kimwipes® EX-L, Kimberly-Clark Corporation, Roswell, GA, 30076-2199
[2] Minnesota Turkey Growers Association, 2830 Wycliff St., St. Paul, MN, 55114

RESULTS AND DISCUSSION

The assay demonstrated linearity with the linear curve fitting with a $r^2 = 0.95$ (Figure 1). The sensitivity of the assay was determined by performing multiple dilutions until reaching a dose where results became inconsistent. Using the decreased doses the sensitivity was estimated to be between concentrations of 3 and 6 x 10^6 spermatozoa. To provide a better estimate of sensitivity and to determine intra-assay precision, 10 samples were incubated with 24 x 10^6 spermatozoa. Sensitivity was estimated by calculating three times the standard deviation. The sensitivity was found to be 24 hydrolyzed holes. Intra-assay precision was found to have coefficient of variation of 54.1%. Inter-assay precision for the current assay had a coefficient of variation of 43.6%. The number of hydrolyzed holes did not differ between fresh and stored PVL sections. Accuracy may be determined by spiking samples with known amounts of sample, but in this case that was not possible. The accuracy of the assay was determined by incubating 24 x 10^6 spermatozoa with PVL sections isolated from eggs of two lines that have different sperm penetration (EGG & RBC1). When plotted, parallel lines were exhibited demonstrating the accuracy of the assay (Figure 2).

The exact mechanism behind the sperm binding in this assay is not known. As stated earlier, the sperm receptors are located on the IPVL and the OPVL serves as a block to sperm attachment after it is applied to the egg. Three possible explanations exist describing sperm attachment and hydrolysis of the intact PVL. One explanation is that the sperm receptor is a transmembrane type receptor and

the sperm cell is binding to the portion on the yolk side of the PVL. Alternatively, incubating the IPVL in Dulbecco's Modified Eagle's Medium and rinsing it in PBS are creating mechanical separations between the IPVL and OPVL allowing enough space for sperm to enter and bind to the IPVL. Lastly, it is possible that some protein similar to the sperm receptor (maybe the sperm receptor itself) is also deposited in the OPVL. This assay met the requirements of an assay, which included linearity, sensitivity, accuracy and precision. Further work may result in answers to why sperm are binding and hydrolyzing the intact PVL.

REFERENCES

Bakst, M. R. and B. Howarth, 1977. Hydrolysis of the hen's perivitelline membrane by cock sperm *in vitro*. Biol. Reprod. 17:370-379.

Bakst, M. R., H. C. Cecil, and T. J. Sexton, 1991. Modification of the ethidium bromide exclusion procedure for evaluation of turkey semen. Poultry Sci. 70:366-370.

Biligili, S. F. and J. A. Renden, 1984. Fluorometric determination of avian spermatozoa viability and concentration. Poultry Sci. 63:2275-2277.

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

Bramwell, R. K., H. L. Marks, and B. Howarth, 1995. Quantitative determination of spermatozoa penetration of the perivitelline layer of the hen's ovum as assessed on oviposited eggs. Poultry Sci. 74:1875-1883.

Burrows, W. H., and J. P. Quinn, 1935. A method of obtaining spermatozoa from the domestic fowl. Poultry Sci. 14:251-254.

Howarth, B. and S. T. Digby, 1973. Evidence for the penetration of the vitelline membrane of the hen's ovum by a trypsin-like acrosomal enzyme. J. Reprod. Fert. 33:123-125.

Kido, S and Y. Doi, 1988. Separation and properties of the inner and outer layers of the vitelline membrane of hens eggs. Poultry Sci. 67:476-486.

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

Robertson, L. and G. W. Wishart, 1997. 4. Sperm motility and metabolism VII. *In vitro* sperm-egg interaction assay utilizing inner perivitelline layer from chicken eggs. Pages 64-67 In: Techniques for semen evaluation, semen storage, and fertility determination. Ed. M.R. Bakst and H.C. Cecil, Poultry Science Association, Savoy IL 61874.

Robertson, L., H. L. Brown, H. J. Staines and G. J. Wishart, 1997. Characterization and application of an avian in vitro spermatozoa-egg interaction assay using the inner perivitelline layer from laid chicken eggs. J. Reprod. Fertil. 110:205-211.

Wishart, G. J. and H. J. Staines, 1999. Measuring sperm: egg interaction to assess breeding efficiency in chickens and turkeys. Poultry Sci. 78:428-436.

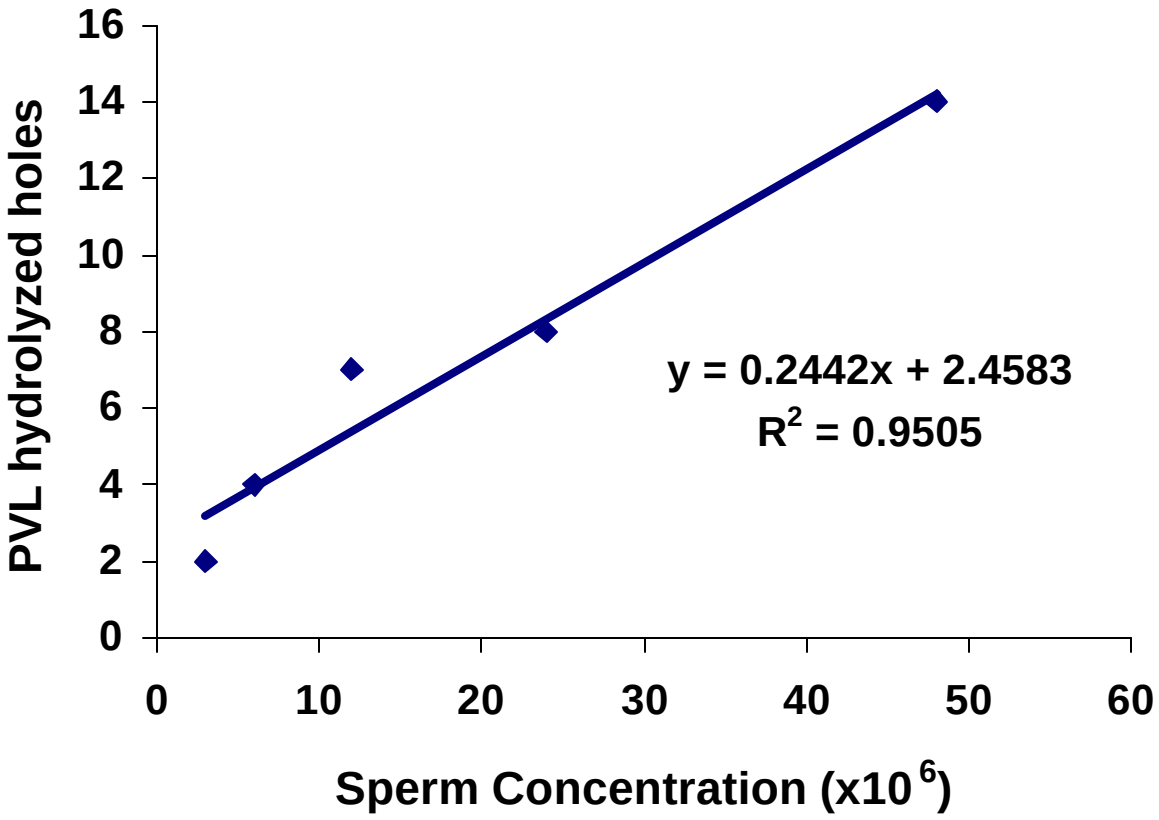

Figure A.1. Perivetilline (PVL) hydrolyzed hole assay linearity after PVL was incubated with varying concentrations of sperm for 10 min at 37 C.

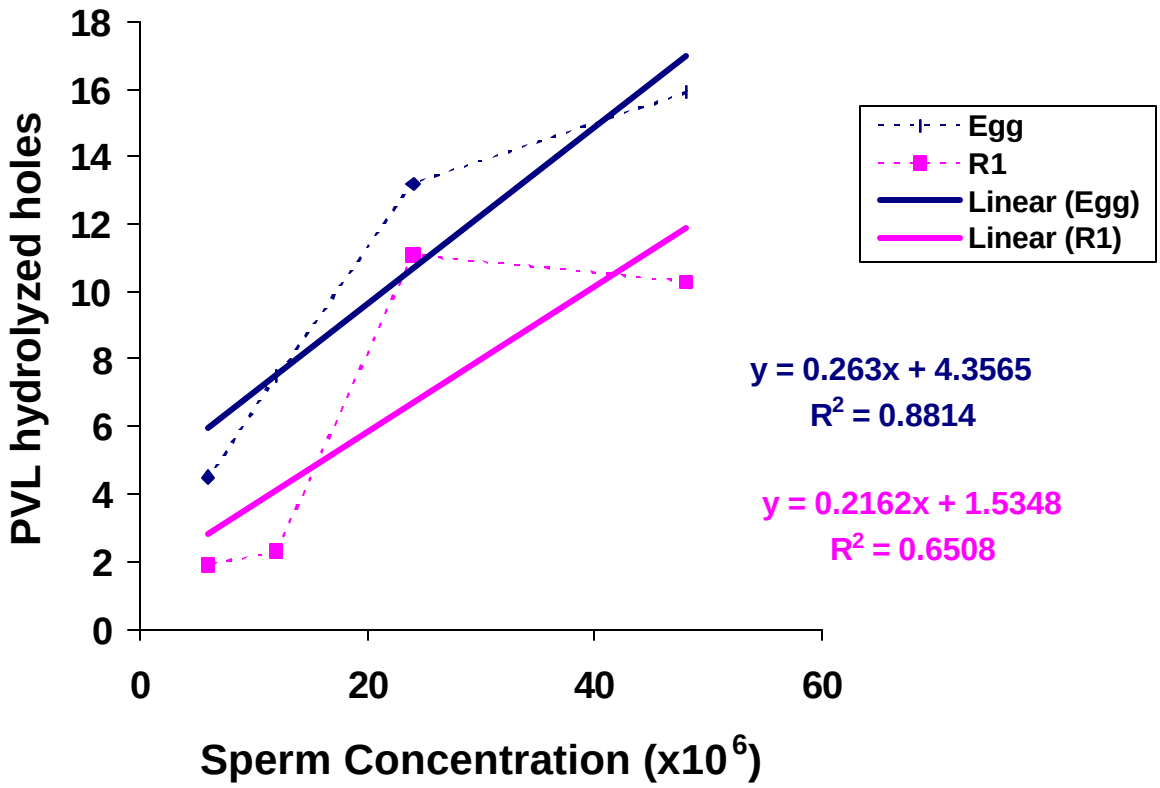

Figure A.2. Perivetilline (PVL) hydrolyzed hole assay accuracy when varying concentration of sperm were incubated with PVL sections isolated from eggs of two different turkey strains known to have differing in vivo sperm penetration (EGG & RBC1). Each PVL was incubated with sperm obtained from the same source.

Appendix B. Differences in in vitro Sperm Hydrolysis of the Perivitelline Layer between two Commercial Lines of Turkeys

ABSTRACT

Sperm penetration of the ovum inner perivitelline layer (IPVL) is positively correlated with fertility. Greater numbers of sperm penetration holes in the IPVL is indicative of successful insemination and can be positively associated with optimal filling of the sperm storage tubules in the uterovaginal region of the oviduct. The hypothesis tested was that in vitro sperm hydrolysis of the IPVL in the absence of oviductal factors would differ between two commercial turkey lines at two different periods in production. The objectives were to determine strain, hen age, tom and hen influence on sperm hydrolysis of the IPVL in turkeys. Two experiments were conducted. In Experiment 1, strain and hen age were examined. Two strains (N & H) and two hen ages within the laying period (early period=1-4 weeks of lay and mid period=12-16 weeks of lay) were used as factors in a 2 x 2 factorial arrangement. In Experiment 2, Tom (HM and NM) and Hen (HF and NF) were arranged factorially to examine male and female influence on sperm hydrolysis. Eggs were obtained at oviposition from non-fertilized hens. The perivitelline layer (PVL) was isolated from non-germinal disc regions of the egg, then incubated with $25x10^6$ viable sperm cells in vitro. In Experiment 1, young hens had significantly more hydrolyzed holes than older hens and H hens had more hydrolyzed holes than N hens. No interaction between age and commercial line was detected indicating that both lines performed similarly at both production periods examined. In Experiment 2, HM and HF had significantly more sperm hydrolyzed holes than NM and NF. The results of these

two experiments suggest that strain, hen age, sire and hen have significant effects on sperm hydrolysis of the PVL. Reciprocal incubation of sperm and PVL from toms and hens of different lines provided no significant differences in the number of sperm hydrolyzed holes formed in vitro.

INTRODUCTION

Mammalian fertilization can be divided into six steps that include: (1) membrane contact between the egg and sperm cells, (2) entry of the sperm cell into the egg, (3) prevention of polyspermy by the egg, (4) metabolic activation of the egg, (5) the completion of meiosis by the egg, and (6) formation and fusion of male and female pronuclei (Carlson, 1988). Avian fertilization involves many of the same processes with different terminology and structures which include: (1) contact between sperm and the ovum inner perivitelline layer (IPVL), (2) sperm entry into the ovum, (3) prevention of pathological polyspermy, (4) ovum activation, (5) completion of meiosis by the female germ cell, (6) formation and fusion of the male and female pronuclei. Sperm binding and hydrolysis of the inner perivitelline layer (IPVL) are processes in avian fertilization. The sperm binding receptor in avian eggs is located on the IPVL of the ovum, and when the continuous and outer perivitelline layers are applied in the infundibulum, sperm binding is inhibited or reduced. This appears to be the main mechanism to block pathological polyspermic fertilization in avian species (Bakst and Howarth, 1977). Previous studies with chickens have suggested an increased incidence of early embryonic mortality (EEM) when excessive sperm penetration of the IPVL was observed (Bramwell and Howarth, 1997). In turkeys, EEM has been shown to be negatively correlated with fertility in young hens (Section

II) and that the number of IPVL sperm penetration (SP) holes differed with hen age. Bramwell et al. (1996) found differences in SP holes due to age of both the sire and the dam. The results of the study by Bramwell et al. (1996) and the study in Section II could be due to differences in sperm-ovum interaction or it could be differences in oviduct properties that have an effect on spermatozoa populations in the infundibulum. The work in turkeys concerning sperm binding and hydrolyzing of the IPVL is sparse and with artificial insemination being performed in all turkey breeder operations it would be useful to better understand the factors that influence these fertilization processes that would in turn affect fertility and EEM.

The current study consisted of two experiments. The objective of Experiment 1 was to examine IPVL SP differences utilizing intact perivitelline sections of two commercial turkey strains at two ages in vitro to remove any oviduct effects that may influence SP. The objective of Experiment 2 was to examine male and female influences in vitro on the number of SP holes hydrolyzed in the IPVL utilizing intact perivitelline sections.

MATERIALS AND METHODS

Experiment 1

Eggs were obtained from turkey hens of two different commercial strains of hens (N[1] and H[2]) that had not been inseminated. Hens (n=5) were obtained at 30 weeks of age and placed on a photo cycle of 15.5L:8.5D. Hens were not inseminated at any time during the study and therefore provided a source of unfertilized eggs for in vitro assays. Feed and water were provided ad libitum.

[1] Nicholas Turkey Breeder Farms, 19449 Riverside Dr., P.O. Box Y, Sonoma, CA, 95476-1111
[2] Hybrid Turkeys Inc.,9 Centennial Drive, Kitchener, Ontario, N2B 3E9

Toms from each strain were obtained and were maintained on a 12L:12D photocycle. Eggs from hens were examined at two periods during egg production, which were 1 to 4 (32 to 36 weeks of age) (YNG) and 12 to 16 weeks of egg production (44 to 48 weeks of age) (OLD) using the in vitro sperm hydrolysis assay.

Experiment 2

Unfertilized eggs were obtained from hens of two different commercial strains (N and H) that had not been inseminated. Hens were maintained in facilities as described in Experiment 1. The semen was obtained from N and H toms. Reciprocal incubations of hen PVL of each strain were made with the semen of each strain to determine the effect of strain, tom and hen effects on sperm binding and hydrolysis of the PVL.

In Vitro PVL Hydrolyzed Hole Assay

Semen Collection And Dilution. Semen was collected from one tom of each strain using the abdominal massage technique (Burrows and Quinn, 1935) and diluted 1:1 with Minnesota turkey semen extender[3]. Sperm cell concentration was determined via packed cell volume and viability was determined by the ethidium bromide exclusion test (Bakst et al., 1991). Using the packed cell volume, viability and semen volume, the semen samples were further diluted to 25 x 10^6 spermatozoa per 20 μL.

Perivitelline Layer Preparation. The current assay is a modification of the assay used by Phillips et al. (1996) and Robertson and Wishart (1997). The assay validation was discussed in Appendix A. Only eggs oviposited within 24 h of

[3] Minnesota Turkey Growers Association, 2830 Wycliff St., St. Paul, MN, 55114

performing the assay were used. Eggs were opened and the yolk and albumen were separated. The yolk was placed in a pan with the germinal disc facing down. Excess albumen was gently removed and the yolk was rinsed in 2% NaCl. The perivitelline layer, the term used for both the inner, continuous and outer perivitelline layers, (PVL) was cut along the equator of the egg and the non-germinal disc area was removed. The excess yolk was removed by rinsing the PVL in PBS then placed in a petri dish containing PBS and sectioned into 1 cm^2 pieces. The sections were then placed in disposable beakers containing 1 mL Dulbecco's Modified Eagles Medium.

Assay Incubation and Hole Quantification. Diluted semen (25x 10^6 in 20 μL) was added to each beaker and incubated at 37-40 C for 10 min while shaking gently. After incubation, the PVL was removed and rinsed vigorously in PBS, placed on a microscope slide, fixed with 3% formalin and stained with Schiff's reagent. After drying, the number of hydrolyzed holes in five fields was determined at a magnification of 400x using a light microscope.

Statistical Analysis

Data from both experiments were arranged into a 2 x 2 factorial. In Experiment 1, the factors were hen age (YNG & OLD) and strains (N & H). In Experiment 2, the factors were sire (N & H) and dam (N & H). The data were compared using the General Linear Models procedure of SAS (SAS Institute, 1989). Significance was based on $P \leq 0.05$ unless otherwise stated.

RESULTS

In Experiment 1, the number of holes hydrolyzed in the PVL was significantly greater in the H strain as compared to the N strain (Table 1). Independent of strain, there were significantly more PVL hydrolyzed holes when spermatozoa were incubated with PVL of young hens compared to older hens (Table 2). There was no interaction of strain and hen age indicating that both strains performed similarly at both periods of production.

In Experiment 2 (Table 3), significant differences between toms and hens of each strain were observed, but there was no interaction between the tom and hen, indicating that incubation with semen from either strain performed similarly on PVL from hens of each strain. The HM and HF had significantly more hydrolyzed holes as compared to NM and NF.

DISCUSSION

Previous studies have indicated increased SP holes in the IPVL of young hens as compared to older hens (Section III). This suggested that this might contribute to pathological polyspermic fertilization resulting in increased EEM observed in younger hens (Section II). However, the results of Section III were unclear as to whether the cause of increased IPVL SP holes was due to properties of the IPVL or to oviductal influences as well. The current study removed the oviductal influences by incubating sperm with PVL sections in vitro. This allowed the comparison of IPVL properties that may affect sperm binding and hydrolysis. The results indicate that both females and males make contributions that affect SP significantly. The IPVL of eggs from young hens had greater hydrolyzed holes than

the IPVL of eggs from older hens. This was observed independent of strain effects. This suggests that the IPVL of eggs from young hens may have increased sperm binding potential whereas sperm binding occurs prior to the acrosome reaction. It may also suggest that acrosmal enzymes digest the IPVL of younger hens more easily than IPVL of older hens. Regardless of the mechanism, this suggests that IPVL properties alter with hen age and may not only influence fertility but also have an effect on embryonic viability.

Genetics can also influence fertility and EEM (Section IV). The results of the current study demonstrate differences between commercial strains on the number of sperm hydrolyzed holes. H consistently had more hydrolyzed holes than N at both production periods examined. Both male and female factors influence this significantly. This is in agreement with the results of section (IV) and demonstrates that genetic influences between commercial strains can also influence sperm hydrolyzed penetration holes in the ovum IPVL. More work is required to determine whether this would impact fertility and EEM.

The results of the current study indicate that sperm hydrolyzed holes in the ovum IPVL can be influenced by female, male, genetic and age factors. It is possible that a combination of those factors may increase sperm penetration to the point that pathological polyspermic fertilization may occur. Further work is needed to determine whether or not increased numbers of sperm penetration holes can be associated with increased male pronuclei. It is possible that sperm could bind and hydrolyze a hole in the IPVL without entering the ovum and forming a male pronucleus. Nevertheless, sperm penetration has been associated with increased

EEM and may explain a portion of the EEM that occurs among different strains and hen ages.

REFERENCES

Bakst, M. R. and B. Howarth, 1977. Hydrolysis of the hen's perivitelline membrane by cock sperm *in vitro*. Biol. Reprod. 17:370-379.

Bakst, M. R., H. C. Cecil, and T. J. Sexton, 1991. Modification of the ethidium bromide exclusion procedure for evaluation of turkey semen. Poultry Sci. 70:366-370.

Bramwell, R. K. and B. Howarth, 1997. Effect of low or high sperm penetration values at the germinal disc on early embryonic mortality in chicken eggs. Poultry Sci. 76(Suppl. 1):97 (Abstr).

Bramwell, R.K., C. D. McDaniel, J. L. Wilson, and B. Howarth, 1996. The effect of male and female broiler breeders on sperm penetration of the perivitelline layer overlying the germinal disc. Poultry Sci 75:755-762.

Burrows, W. H., and J. P. Quinn, 1935. A method of obtaining spermatozoa from the domestic fowl. Poultry Sci. 14:251-254.

Carlson, B. M., 1988. *Patten's Foundations of Embryology*. 5th ed. McGraw-Hill, Inc. New York, NY.

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

Robertson, L., and G. J. Wishart, 1997. Sperm motility and metabolism VII. In vitro sperm-egg interaction assay utilizing inner perivitelline layer from laid chicken eggs. Pages 64-67 *in.* Techniques for Semen Evaluation, Semen Storage, and Fertility Determination. M. R. Bakst and H. C. Cecil ed. Poultry Science Association, Inc., Savoy, IL.

SAS Institute, 1989. SAS/STAT Guide for Personal Computers. Version 6 Edition. SAS Institute Inc., Cary, NC.

TABLE B.1. The mean sperm hydrolyzed holes in the perivitelline layer of two commercial turkey lines when incubated in vitro

Turkey Line[1]	Hydrolyzed Holes
H	16[a]
N	12[b]
$\bar{x} \pm$ SEM	14 ± 0.71
P	0.0134

[a, b]Means within a column that do not have common superscripts differ significantly ($P \leq 0.05$)

[1] N = Nicholas Turkey Breeder Farms, 19449 Riverside Dr., P.O. Box Y, Sonoma, CA, 95476-1111

H = Hybrid Turkeys Inc.,9 Centennial Drive, Kitchener, Ontario, N2B 3E9

TABLE B.2. The mean sperm hydrolyzed holes in the perivitelline layer of two commercial turkey lines at two different ages (young = 1 to 4 weeks of lay and old = 12 to 16 weeks of lay) when incubated in vitro

Age	N	H	Combined
Old	11^{b}	13^{b}	12^{b}
Young	14^{a}	20^{a}	17^{a}
$\bar{x} \pm$ SEM	12 ± 0.91	16 ± 1.11	14 ± 0.71

[a, b]Means within a column that do not have common superscripts differ significantly ($P \leq 0.05$)

[1] N = Nicholas Turkey Breeder Farms, 19449 Riverside Dr., P.O. Box Y, Sonoma, CA, 95476-1111
H = Hybrid Turkeys Inc.,9 Centennial Drive, Kitchener, Ontario, N2B 3E9

TABLE B.3. Sperm hydrolyzed holes in the perivitelline layer of two commercial turkey lines when reciprocal incubations were made with semen of both strains in vitro. Hens from each strain differed significantly P=0.0431 and toms differed P=0.0001 but there was no significant interaction between tom and hen

Turkey Line	HM	NM	Combined F$\bar{x}$
HF	21	18	20[a]
NF	20	14	16[b]
Combined M$\bar{x}$	20 [a]	17 [b]	Log 2.9 $\pm$ 0.021

[a, b]Means within a column that do not have common superscripts differ significantly ($P \leq 0.05$)

[1] N = Nicholas Turkey Breeder Farms, 19449 Riverside Dr., P.O. Box Y, Sonoma, CA, 95476-1111
H = Hybrid Turkeys Inc., 9 Centennial Drive, Kitchener, Ontario, N2B 3E9

Appendix C. Inner Perivitelline Layer Sperm Penetration Assay Protocol

Materials:

2% NaCl Solution	Petri Dish x 2	Small trash bags
PBS 1x	Slides*	Rubber Nitrile Gloves*
Small Aluminum Pans*	Schiff's Reagent*	Sharpie marker
Small Scissors	Pasteur Pipets	Kim Wipes*
250 ml Beaker	500 ml Beaker	Forceps (blunt)
5-6 20 ml Beakers	Paper Towels	3% Formaldehyde*
NaCl*	TES*	Forceps (Watch maker)
Na_2HPO_4*	KH_2PO_4*	KCL*

* Items have company and catalog numbers listed in next table

Item	Company	Catalog #	Size
Small Aluminum Pans	Fisher	08-732	
Frosted Slides	Fisher	12-550-34	
Schiff's Reagent	Sigma	S5133	500 ml
Rubber Nitrile Gloves	Fisher	6005PFL -11-388-32	Large
		6005PFM - 11-388-31	Medium
		6005PFS – 11-388-30	Small
Formaldehyde 37%	Fisher	F79-500	500 ml
NaCl	Fisher	S271-1	1 kg
TES	Fisher	BP309-100	100 g
KCl	Fisher	BP366-500	500 g
Na_2HPO_4	Fisher	S-374-500	500 g
KH_2PO_4	Fisher	P-285-400	500 g
Kim Wipes	Fisher	06-666A	

PBS 1x

NaCl	8.0g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	1.24g

Add the above ingredients to 800 ml of dH_2O
pH solution to 7.4 using HCl
Bring up solution up to 1 L with dH_2O

Procedure:

1. Break open egg and collect yolk while removing excess albumin
2. Place egg in pan with germinal disc or blastoderm facing up
3. Blot dry with a kim wipe

4. Pour 2% NaCl solution over yolk making sure the entire yolk is wet

   ***Care should be taken when pouring so that the perivitelline layer does not break

5. Use scissors to cut a square around the germinal disc
6. Use forceps to remove the membrane with as little yolk as possible
7. Repeatedly rinse the membrane in PBS until yolk free
8. Place membrane in petri dish containing PBS
9. Float membrane onto the slide and wick dry excess fluid
10. Place 3-4 drops of 3% formaldehyde on membrane
    ****(just enough to cover the membrane)
11. Immediately remove excess formaldehyde from the slide by pouring into a waste container
12. As quickly as possible place 2-3 drops of undiluted Schiff's reagent on the membrane
13. As soon as the membrane turns magenta remove excess Schiff's reagent by pouring into a waste container
14. Lean slide against something so that it can dry while allowing excess fluid to drain off
15. Allow slide to dry then count the number of holes at 40x

To determine the field of view area (FOV):

$$\text{FOV diameter (mm)} = \frac{\text{Field of view\# on eyepiece}}{\text{Magnification of objective x optivar}}$$

This will give you the diameter, divide by 2 to get the radius then input insert radius into the area equation:

$$\text{Area} = \pi r^2$$

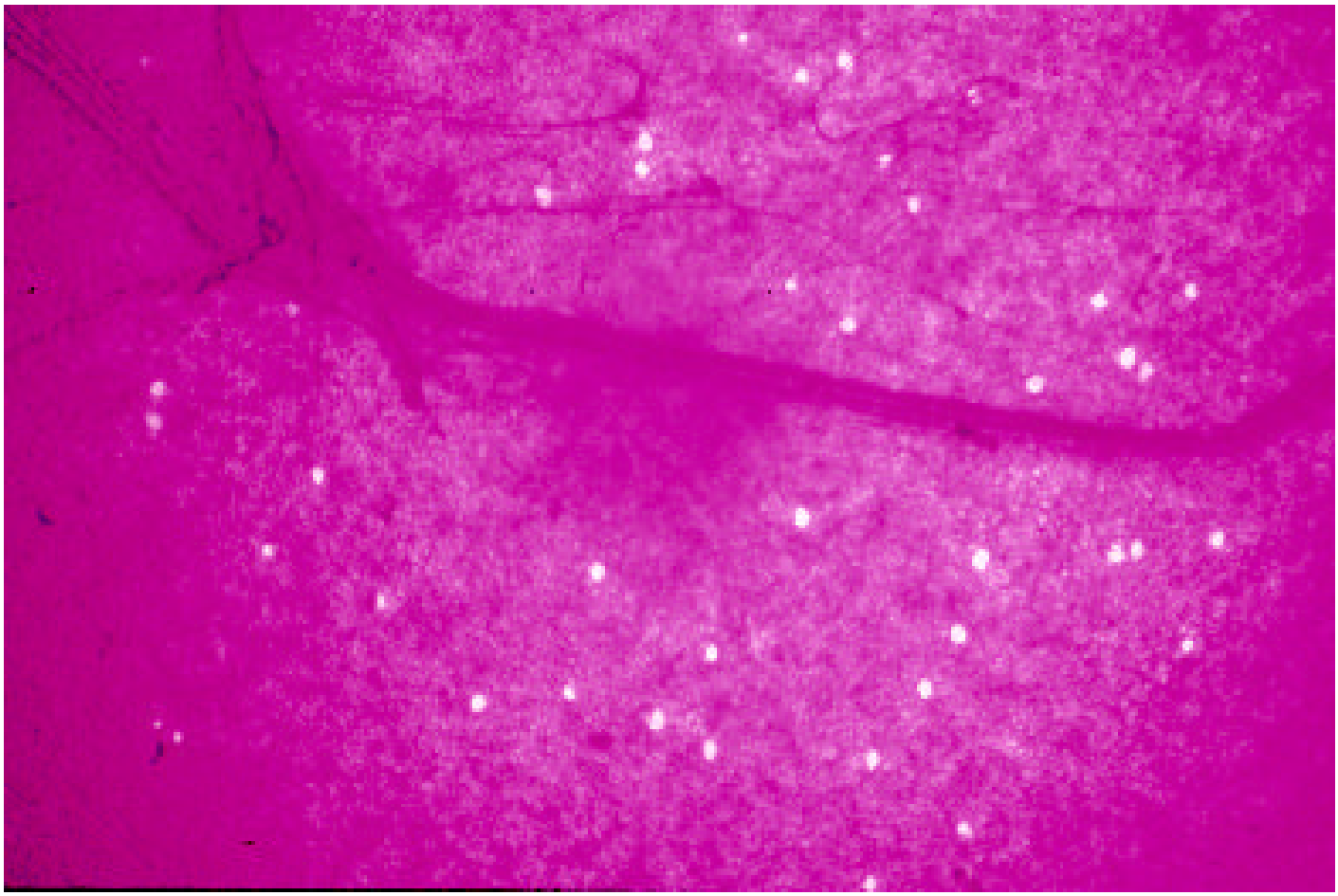

Figure C.1. Slide displaying sperm penetration holes in the germinal disc region of the inner perivitelline layer.

Appendix D. *In Vitro* Sperm Hydrolysis of the Inner Perivitelline layer Assay Protocol

Materials:

2% NaCl Solution	Petri Dish x 2	Small trash bags
PBS 1x	Slides*	Rubber Nitrile Gloves*
Small Aluminum Pans*	Schiff's Reagent*	Sharpie marker
Small Scissors x 2	Pasteur Pipets	Kim Wipes*
250 ml Beaker	500 ml Beaker	Forceps (sharp point)
5-6 20 ml Beakers	Paper Towels	3% Formaldehyde*
Dulbecco's MEM*	5 ml disposable beakers*	Shaker
Incubator oven	Timer	Watch Maker Forceps
Disposable Pipet (10 ml)	Pipet bulb	Pipet (20ul) and tips
NaCl*	Bullet Tube (0.5-2 ml)	Slide Box
Na_2HPO_4*	KH_2PO_4*	
TES*	KCL*	

* Items have company and catalog numbers listed in next table

Item	Company	Catalog #	Size
Small Aluminum Pans	Fisher	08-732	
Dulbecco's MEM	Sigma	D6171	100 ml
Frosted Slides	Fisher	12-550-34	
Schiff's Reagent	Sigma	S5133	500 ml
Disposable Beakers	Fisher	02-544-30	5 ml
Rubber Nitrile Gloves	Fisher	6005PFL -11-388-32 6005PFM - 11-388-31 6005PFS – 11-388-30	Large Medium Small
Formaldehyde 37%	Fisher	F79-500	500 ml
NaCl	Fisher	S271-1	1 kg
TES	Fisher	BP309-100	100 g
KCl	Fisher	BP366-500	500 g
Na_2HPO_4	Fisher	S-374-500	500 g
KH_2PO_4	Fisher	P-285-400	500 g
Kim Wipes	Fisher	06-666A	

PBS 1x

NaCl	8.0g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	1.24g

Add the above ingredients to 800 ml of dH_2O
pH solution to 7.4 using HCl
Bring up solution up to 1 L with dH_2O

Procedure:

This experiment consists of two parts.

1. First the perivitelline membrane sections to be incubated in the presence of sperm must be collected.

2. The second part includes semen collection and dilution as well as the incubation of sperm cells with the PVL followed by the subsequent rinsing and staining of the PVL.

Collection of the perivitelline layers

1. Break open egg and collect the yolk while removing excess albumin
2. Place yolk in pan with germinal disc or blastoderm facing down
3. Blot dry with a kim wipe
4. Pour 2% NaCl solution over yolk making sure the entire yolk is wet

   ***Care should be taken when pouring so that the Perivitelline layer does not break
5. Use one pair of scissors to cut the perivitelline layer around the equator of the yolk.
6. Use blunt forceps to remove the membrane with as little yolk as possible
7. Repeatedly rinse the membrane in PBS until yolk free
8. Place membrane in petri dish containing PBS
9. Use the second pair of scissors to cut the PVL into small square sections. This step is subjective in that different size squares may be used, but larger squares appear to be easier to mount on the slide for staining and do not fold during incubation with sperm cells
10. The PVL sections can be stored overnight in PBS or NaCl + TES, or the assay may be performed immediately after collection.

Incubation of PVL with sperm

1. Setup one disposable beaker with 1 ml of Dulbecco's MEM for each PVL section.
2. Place one PVL section in each beaker.
3. Collect semen.
4. Determine semen viability (Et Br. Procedure) and concentration (Spermatocrit).
5. Dilute semen to 25 x 10^6 / ml *** This is falls in the middle of the validated Standard curve.
6. Pipet 20 µl into beaker and incubate at 37-40 C for 10 min while gently shaking.
7. Remove beaker from oven and rinse the PVL in PBS repeatedly until pink medium residue is absent.

Slide mounting and staining of the PVL

1. Using PBS, float membrane onto the slide

***Note with this assay the PVL sections are small enough for multiple sections to be place on each slide

2. Wick dry excess fluid

3. Place 3-4 drops of 3% formaldehyde on membrane (just enough to cover the membrane)

4. Immediately remove excess formaldehyde from the slide by pouring into a waste container

5. As quickly as possible place 2-3 drops of undiluted Schiff's reagent on the membrane

6. As soon as the membrane turns magenta remove excess Schiff's reagent by pouring into a waste container

7. Lean slide against something so that it can dry while allowing excess fluid to drain off

8. Allow slide to dry then count the number of holes at 400x

To determine the field of view area (FOV):

$$\text{FOV diameter (mm)} = \frac{\text{Field of view\# on eyepiece}}{\text{Magnification of objective x optivar}}$$

This will give you the diameter, divide by 2 to get the radius then input insert radius into the area equation:

$$\text{Area} = \pi r^2$$

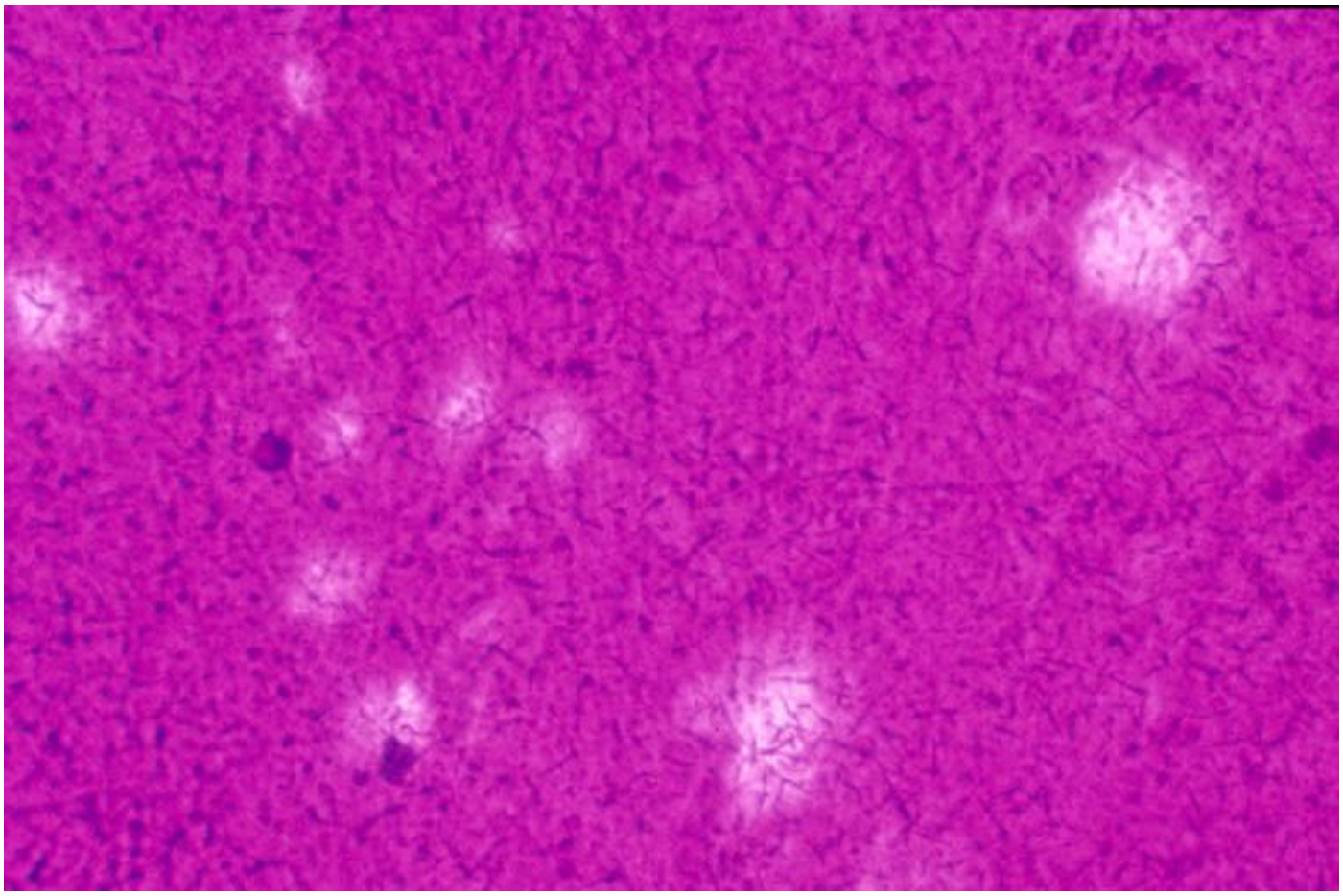

FIGURE D.1. Slide displaying sperm penetration holes at 400x in the perivitelline layer obtained from ova of oviposited eggs.