THE RELATIONSHIP BETWEEN HUMORAL AND LOCAL ANTI-SPERM ANTIBODY PRODUCTION AND INFERTILITY IN TURKEY BREEDER HENS

by

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(ABSTRACT)

Two experiments were conducted to determine the role of the turkey hen's immune system in the so-called seasonal decline in fertility. Results from Experiment I demonstrated that trauma to the oviduct induced either at the time of the initial artificial insemination, or during subsequent inseminations had no effect upon hen fertility, or serum anti-sperm antibody titers. No differences in fertility were found between hens showing a positive or negative anti-sperm antibody response.

Experiment II demonstrated that only relatively infertile turkey hens had antibody positive cells within the uterovaginal sperm storage gland epithelium (UVSG), while fertile turkey hens had no such cells. Antibody positive amorphous material was found within the lumen of the UVSG of relatively infertile, but not fertile turkey hens. Blood serum from relatively infertile hens showed minimal anti-sperm antibody titers. It was concluded that the seasonal decline in fertility in turkey hens is not due to a humoral immune response against spermatozoa. However, a local immune response against spermatozoa appears to exist in the
uterovaginal sperm storage glands of the turkey hen, which has a detrimental effect upon fertility.
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INTRODUCTION

A factor affecting the overall success of turkey egg producers is seasonal decline in fertility of turkey breeder hens. Seasonal decline in fertility is defined as the gradual decrease in turkey breeder hen fertility as the egg production season progresses. In commercial turkey breeder flocks experiencing this phenomenon, it is not unusual to observe a 20 to 25 percent decrease in flock fertility, although egg production remains unaffected. The production of infertile eggs is costly to the turkey producer. Therefore, it is economically desirable to maintain high fertility levels throughout the egg production cycle.

It has been established that a seasonal decline in fertility is a female-related problem and results from an increase in the number of infertile hens in a flock (Almquist, 1964). Because the affected hens appear healthy and show no overt signs, a producer is not able to remove infertile hens from the flock. To maintain individual fertility records is not feasible, and, therefore, infertile hens cannot be identified. Thus, a producer has no alternative but to maintain all egg-producing hens in the flock.

The underlying cause of seasonal declines in fertility remains controversial. It has been shown that infertile hens have a decreased ability to store spermatozoa within the uterovaginal sperm storage glands (UVSG) (Van Krey et al., 1967a; Van Krey and Leighton, 1970), but the reason was not identified. It has been suggested that a hostile environment within the hen’s oviduct may be responsible for the lack of sperm storage in infertile turkey hens (Van Krey and Leighton, 1970; Christensen, 1981).
It has been demonstrated that infertility could be induced in the turkey hen by immunization against spermatozoa (Burke et al., 1971) and an immune response against spermatozoa by the turkey hen was suggested to have a role in seasonal declines in fertility (McCorkle et al., 1983). Furthermore, a relationship between induced oviductal trauma and the production of serum anti-sperm antibodies was also demonstrated in turkey breeder hens (McCorkle et al., 1983). To date there has been no study to determine if, during artificial insemination, sufficient trauma is applied to the oviduct of the turkey breeder hen to evoke an anti-sperm immune response.

Recent studies suggest the occurrence of a local immune response against spermatozoa at the level of the UVSG (Schuppin et al., 1985; Van Krey et al., 1987) in infertile turkey breeder hens. It has been shown in human females that local immunity to sperm can occur without the presence of serum anti-sperm antibodies (Behrman and Menge, 1980). Bakst (1987a) observed IgG positive cells within the UVSG of fertile turkey breeder hens, but he did not investigate IgG production in infertile hens. Therefore, the significance of serum anti-sperm antibodies in the turkey hen remains questionable. Whether serum anti-sperm antibodies or locally secreted antibodies are the cause of seasonal declines in fertility has yet to be determined.
LITERATURE REVIEW

Avian Sperm Storage

Prolonged fertility in domestic fowl following natural mating or artificial insemination has long been known to exist. This prolonged fertility is attributed to microscopic sperm storage tubules located in two sites within the hen oviduct. Infundibular sperm storage nests were identified by Van Drimmelen (1946), and (UVSG) were described by Bobr et al. (1962; 1964), Fujii (1963), and Fujii and Tamura (1963). Bobr et al. (1962; 1964) suggested that the UVSG were the primary oviductal sperm storage sites in chickens. Verma and Cherms (1965) and Ogasawara and Fuqua (1972) concluded that the UVSG, and not the infundibular sperm glands were the primary sperm storage sites in the turkey.

The UVSG are found within the mucosal folds of the vaginal epithelium just distal to the uterovaginal sphincter (Fujii, 1963; Gilbert et al., 1968a; Tingari and Lake, 1973). Mero and Ogasawara (1970) measured the dimensions of the UVSG in the chicken. They observed that glands ranged from 24.8 to 52 microns in diameter, with the average length being 500 microns, and suggested that tubular enlargement may play a role in sperm release from the gland.

The sperm storage gland epithelium differs from that of the surface vaginal epithelium. At the proximal, lumenal end of a gland, the epithelium resembles that of the oviduct surface epithelium and consists exclusively of ciliated columnar cells (Van Krey et al., 1967a; Tingari and Lake, 1973). Deeper in the neck region of a gland, the epithelium consists of alternating ciliated columnar cells with apical nuclei, and
non-ciliated columnar cells having basal nuclei (Tingari and Lake, 1973). Friess and co-workers (1978) described the proximal portion of the UVSG epithelium in quail as being lined by pseudostratified epithelium, consisting of high ciliated columnar cells, and small, irregular basal cells. The epithelium deeper into a UVSG consists of non-ciliated, simple, columnar cells with elongate apical microvilli (Van Krey et al., 1967a; Tingari and Lake, 1973; Friess et al., 1978). The cells also contain numerous cytoplasmic granules.

In domestic fowl, cytoplasmic vacuoles containing lipid have been observed in the true glandular epithelium of the UVSG (Fujii, 1963; Gilbert et al., 1968a; Friess et al., 1978). Histochemical analysis detected cholesterol, but no phospholipids in the UVSG of domestic fowl (Fujii, 1963). Schuppin et al. (1984), Bradley et al. (1986), and Bakst (1987b) observed supernuclear vacuoles in the UVSG epithelium of turkeys, but not in the epithelium of the uterovaginal surface. They reasoned that these vacuoles contained lipid material. Renden and co-workers (1981) reported that the presence of lipids in the UVSG of Japanese quail was not altered by the presence or absence of spermatozoa, but was reduced when an egg was present in the uterus.

Ultrastructural studies by Tingari and Lake (1973) demonstrated glycogen within the UVSG cells of chickens. Renden et al. (1981) reported that in Japanese quail, stained sperm glands indicated the lack of complex carbohydrates, which agrees with the earlier studies of Fujii (1963) and Gilbert et al. (1968a). Schuppin et al. (1984) found limited glycogen in turkey UVSG cells. Bakst (1987b) found the UVSG epithelial cells stained positive for alpha-L-fucose, which was also present in the cells.
of the uterovaginal junction epithelium. Thus, distinct species differences exist with respect to cell glycogen content.

The epithelium of the UVSG is bounded by a basal lamina in chickens (Tingari and Lake, 1973) and turkeys (Schuppin et al., 1985). The glands are surrounded by connective tissue (Van Krey et al., 1967a). No evidence of contractile elements such as myoepithelial cells have been found surrounding the UVSG (Schindler et al., 1967; Van Krey et al., 1967a; Burke et al., 1972; Tingari and Lake, 1973; Friess et al., 1978). However, Tingari and Lake (1973) observed that the non-ciliated cells within the glands contain abundant of cytoplasmic filaments, which resemble tonofibrils. They suggested that these filaments may affect sperm gland contraction by responding to chemical stimuli. Ultrastructural observations did not detect neural innervation surrounding the UVSG (Van Krey et al., 1967a; Tingari and Lake, 1973), nor did histochemical tests (Gilbert et al., 1968a). There is an extensive capillary network surrounding the UVSG, which is associated with the venous portal system that supplies the oviductal epithelium (Gilbert et al., 1968b).

The mechanism by which spermatozoa enter the UVSG has not yet been determined. Schindler et al. (1967) observed that the accumulation of spermatozoa within a UVSG was greatest one day following insemination. Compton and Van Krey (1979a) suggested only a finite number of spermatozoa are capable of entering a UVSG during a given time period. Compton et al. (1978) found that once spermatozoa enter a UVSG, they produce a permanent stratification and do not mix within the gland.
Spermatozoa stored within a UVSG lumen have been found to lie parallel to the long axis of the gland, with their heads pointing towards the distal end of the gland (Van Krey et al., 1967a; Schindler et al., 1967; Tingari and Lake, 1973; Friess et al., 1978; Van Krey et al., 1981). Van Krey and co-workers (1967a) found spermatozoa deeply embedded among the distal cell microvilli. Schindler et al. (1967) observed an occasional association of spermatozoa with the epithelial lining of the gland, but observed most spermatozoa to lie free in the gland lumen. Friess et al. (1978) agreed with this finding, but also reported that spermatozoa were arranged in bundles in quail sperm glands. Tingari and Lake (1973) observed that spermatozoa stored within a UVSG were not intimately associated with the gland's epithelium, but adhered to other spermatozoa within the gland. Van Krey et al. (1981) observed in vitro that spermatozoa exhibited "head to head" agglutination and proposed that loss of the ability to agglutinate may be a factor in release of spermatozoa from a gland.

The basic mechanism by which spermatozoa are released from a UVSG has been investigated, but conflicting results have been reported. Grigg (1957) suggested that mechanical pressure from a passing ovum had an effect on the release of spermatozoa from the infundibulum. Burke and Ogasawara (1969) found spermatozoa present in the oviduct of the hen in the absence of both oviposition and ovulation, and suggested that spermatozoa are released on a continuous basis. These results were confirmed by Compton and Van Krey (1979b).

Compton and Van Krey (1979b) observed that in hens treated with pregnant mare serum gonadotropin (PMSG), the rate of sperm-gland emptying
was not significantly different from that found in untreated laying hens, suggesting that ovulation and oviposition do not have an effect on sperm release. In a subsequent study, Bushman et al. (1985) observed that sperm glands emptied over time in normal cyclic hens, but not in anovulatory hens which were treated with PMSG. Therefore, the effect of oviposition and/or ovulation on the release of sperm from the UVSG has not yet been established. The fact that the first egg laid by a hen can be fertile suggests that oviposition does not play a role in sperm release (Benoff and Renden, 1978). Bakst (1981) supported this observation in the turkey hen, reporting that spermatozoa are present in the oviduct during various stages of the hen's reproductive cycle. He also suggested that that spermatozoa could be released from the UVSG at specific times and may travel up the oviduct at different speeds. Finally, Van Krey and co-workers (1981) suggested the continuous release of spermatozoa from the UVSG, and may be due to the occasional disassociation of agglutinated spermatozoa within the glands. Sperm release from the oviductal sites was reviewed recently by Zavaleta and Ogasawara (1987).

Artificial Insemination and Fertility in Turkey Hens

Prolonged fertility in the domestic hen depends primarily upon the retention of adequate numbers of spermatozoa within the UVSG. Lorenz (1950) reported that the average fertility of turkey hens following artificial insemination was three weeks, and also noted that occasionally fertile eggs could be observed as long as 10 weeks after insemination. In order to achieve maximum fertility levels from turkey breeder hens, it is important that proper management of artificial insemination be
practiced. Certain variables during artificial insemination, including semen volume, insemination frequency, and insemination technique, can affect the number of spermatozoa reaching the sperm glands, and consequently alter fertility levels.

Research examining the optimal semen dose for turkey breeder hens has been inconsistent. In early studies, McCartney (1954) did not find any differences in fertility between turkey hens inseminated with 0.025 and 0.050 ml of semen at either two or three week intervals. He recommended the insemination of 0.025 ml of semen at three week intervals for commercial turkey breeders hens. van Tienhoven and Steele (1957) reported that the insemination of 11.9 to 15.2 million spermatozoa produced fertility levels which were equivalent to the insemination of 157 to 228 million spermatozoa. Twenty years later, Van Krey and co-workers (1976) cited poor insemination practices as the cause of chronic low fertility in breeder flocks, and suggested that the insemination of larger volumes of semen produced more consistent fertility levels. Although 0.025 ml of semen was shown to be adequate for optimum fertility, they stated that a greater semen volume could compensate for inconsistent insemination techniques. Wall and Jones (1977) found no differences in turkey hens which were inseminated with either 32.4, 59.6, or 180 million spermatozoa. Sexton (1977a) reported that the insemination of 50 million spermatozoa into turkey hens was adequate for producing optimal fertility in the early stages of egg production. Recently, Lake and Ravie (1987) obtained high levels of fertility in chickens fowl with only 5.5 million spermatozoa per
insemination. However, insemination with lesser quantities of semen can lead to an increased number of misinseminations (Lake, 1983).

Overall, weekly inseminations have been demonstrated to result in the highest fertility levels (Meyer et al., 1980; Ansah et al., 1984). Meyer et al. (1980) reported that weekly insemination of 0.025 ml semen resulted in the highest average level of fertility, but weekly inseminations also adversely affected egg production when compared to biweekly inseminations. Ansah et al. (1984) reported that a weekly dose of 100 million spermatozoa resulted in maximum fertility levels.

After the insemination of an adequate number of spermatozoa, subsequent fertility levels depend upon the filling of the uterovaginal sperm storage glands for later release into the hen oviduct. Any factor that reduces the number of spermatozoa entering these glands will have an adverse effect on fertility. If oviposition occurs shortly after artificial insemination, many spermatozoa may be swept out of the vagina before they gain access to the UVSG (Johnston and Parker, 1970; Geison et al., 1980). Also, if insemination occurs near the time of oviposition, when oviductal motility is maximal, sperm transport to the storage sites is diminished and fertility is decreased (Brillard et al., 1987). It is for these reasons that the time of day of artificial insemination has been reported to affect fertility levels in chicken hens (Moore and Byerly, 1942; Gracewki and Scott, 1943; Parker, 1945; Johnston and Parker, 1970; Parker and Arscott, 1965, 1971; Geison et al., 1980) and in turkey hens (Wyne et al., 1959; Christensen and Johnston, 1975, 1977).

Higher fertility levels may result from initial inseminations being performed prior to the start of egg production. McIntyre et al. (1982)
reported that hens inseminated prior to the onset of lay had higher fertility levels than hens inseminated after egg production began. McIntyre and Christensen (1983) examined histological sections of the UVSG of such hens and noted that the glands of early-inseminated hens were nearly filled to capacity, while the late-inseminated hens were not. McIntyre and Christensen (1985) proposed that the higher fertility levels in these hens results from an enhanced receptiveness to spermatozoa in the oviduct before egg production commences. Recent observations by Wentworth and Lapp (1986) failed to support this concept, for they found no differences in fertility levels of hens initially inseminated before or after the start of egg production. Ultrastructural observations by Schuppin et al. (1984) also showed that there are no differences in the UVSG of turkey hens at different stages of the egg production cycle.

Poor insemination techniques, such as improper depth of insemination, and rough handling of the hens are known to have negative effects upon turkey hen fertility. Biellier et al. (1961) obtained higher fertility when turkey hens were inseminated relatively deep in the vagina (8 cm) as compared to shallow inseinations (2 and 5 cm). They suggested that deeper inseminations deposited semen closer to the UVSG. Paton et al. (1971) and Wentworth et al., (1975) reported that shallow inseminations (2 cm) resulted in higher overall fertility and longer duration of fertility following a single insemination than deep inseminations (7 cm). However, Paton et al. (1971) and Wentworth et al. (1975) were depositing semen directly into the uterus when utilizing deep inseminations. Ogasawara and Fuqua (1972) demonstrated that intrauterine inseminations led to relatively poor fertility in turkeys. Later,
Holleman and Biellier (1976a) reported that when turkey hens were inseminated at two and three week intervals, higher fertility resulted when hens were inseminated relatively deep into the vagina.

Improper handling of hens during artificial insemination has been shown to affect fertility levels. MacPherson et al. (1977) observed lower fertility in hens which were thrown onto litter following insemination, and suggested that this may be due to an excess of semen being forced out of the vagina. Holleman and Biellier (1976b) suggested that the practice of oviduct relaxation immediately prior to semen deposition prevents the semen from following the insemination tube when it is withdrawn. Howarth (1971) observed that under normal conditions up to 87% of live spermatozoa inseminated into turkey hens was found in vaginal excretions within sixty minutes.

Seasonal Declines in Fertility

A phenomenon known as seasonal decline in fertility has been observed in commercial turkey breeder flocks. It is characterized by an approximate 25% drop in flock fertility levels over the course of the breeding season, while egg production remains unaffected. Knox and Marsden (1954) first reported a decline in flock fertility after four months of egg production. Harper and Arscott (1969) observed 94% fertility during the first six weeks of egg production, which decreased to 68% after twenty weeks.

One of the characteristics of seasonal decline in fertility is that the problem is female related. It is due to an increase in the number of infertile hens in the flock (Almquist, 1964). Attempts to restore
fertility to the high initial levels by inseminating with semen of proven quality did not prevent the decline in fertility (Green et al., 1963; Ogasawara and Rooney, 1966; Lorenz, 1970; Sexton, 1977a). Almquist (1964) utilized semen from younger toms late in the egg laying season, and did not restore high fertility levels. Burrows and Marsden (1938), Almquist (1964), Harper and Arscott (1969) and Sexton (1977a) increased the frequency of insemination but could not increase fertility in the latter stages of egg production. Sexton (1977a) did not observe an increase in fertility by increasing the number of spermatozoa per insemination.

Attempts to restore initial fertility levels in a second egg production cycle following force-molting yielded temporary success. Moyer et al. (1966) observed an initial increase in fertility after previously infertile hens were force-molted, but fertility levels declined again after only eight weeks of lay. Leighton and co-workers (1971) reported that force-molting did not prevent the recurrence of seasonal declines in fertility. Hens that became infertile during the initial egg production cycle showed the same inclination during the second egg production cycle. Ogasawara and Rooney (1966) cited faulty insemination techniques as the cause for low fertility, and improved fertility in a flock by paying careful attention to insemination technique. Previous work by Burrows and Marsden (1938), however, showed that some hens remained infertile, even when they were repeatedly and carefully inseminated.

Infectious agents have been implicated in infertility of turkey hens, but conclusive evidence has yet to be demonstrated. Green et al. (1963) were unable to isolate pathogenic organisms from a commercial flock
where fertility had dropped below 30%. Singh et al. (1964) observed that the infertility condition could be transferred from one hen to another by use of the same inseminating syringe. They inoculated hens with various infectious agents, but did not depress fertility. Nestor and Brown (1968) used a common inseminating straw and did not find any fertility difference when compared to hens that were inseminated with individual straws. Ball et al. (1969) were unable to isolate bacterial or viral agents from a flock of low fertility hens. Metz et al. (1985) reported that reproductive performance was not depressed in four flocks of breeder hens suffering from venereal pox. Almquist (1964) and Ogasawara and Rooney (1966) treated hens from a low fertility flock with antibiotics and were not able to increase fertility. More recently, Opengart (1985) was successful in increasing flock fertility levels by treating hens with the antibiotic oxytetracycline. At present, the role of pathogenic agents in seasonal decline in fertility remains questionable.

Infertile hens may be unable to store spermatozoa within the uterovaginal sperm storage glands. Van Krey and co-workers (1967b) suggested that late in the breeding season the hen's oviduct is less capable of providing a favorable environment for the normal survival of spermatozoa. Christensen (1981) observed that the percentage of empty sperm storage glands in infertile hens increased over the course of egg production, and suggested that seasonal decline in fertility may result from a change to a hostile environment in the hen's oviduct. However, ultrastructural analyses by Schuppin et al. (1984) demonstrated that, although the sperm storage glands of infertile hens contained few, if any
spermatozoa, they were morphologically indistinct from the glands of fertile turkey hens.

**Immunity Against Spermatozoa in Chicken and Turkey Hens**

A relationship between the production of anti-sperm antibodies and poor fertility has been investigated. As early as 1923, McCartney injected fresh rooster testicular material into laying hens and observed that hens experienced a temporary infertile period. Lamoreux (1940), however, attempted to duplicate McCartney's study by injecting homogenates of fresh rooster testes into hens. He observed that hens immunized against this material developed high titers of anti-sperm antibodies without a reduction in fertility.

More recently, Wentworth and Mellen (1964) demonstrated that anti-sperm antibodies were produced in domestic hens following natural mating or artificial insemination, and that these antibodies increased with successive inseminations. Anti-sperm antibodies were highest in hens which were immunized by intraperitoneal or intrauterine injections of spermatozoa. They reported a negative correlation between antibody titer and fertility.

In Japanese laying flocks, Saeki et al. (1965) reported that fertility declined in chicken hens 3-5 months following the start of artificial insemination. They observed no differences in anti-sperm agglutination titers between hens having fertility above 90 percent and hens having fertility below 49 percent. They did however, observe higher anti-sperm antibody titers in low fertility hens after inseminating spermatozoa from different males. Itagaki and coworkers (1966) reported
that a decline in fertility occurred in domestic hens, especially after heteroplasmic matings, but could not conclude that sperm agglutinins caused the production of infertile eggs. Abe et al. (1965) observed that hens immunized against spermatozoa had reduced fertility and that there was a negative relationship, between anti-sperm agglutination titers and fertility. They also noted that immunized hens had a shorter duration of fertility. However, Abe and co-workers (1966) did not observe lower fertility in hens passively immunized with heteroimmune sperm-agglutinating antibodies. They could not conclude that low fertility in hens following successive inseminations was due to the production of anti-sperm antibodies.

Burke et al. (1971) reported that when turkey breeder hens were immunized against spermatozoa, fertility was reduced, but they were not able to detect anti-sperm antibodies using a series of standard assays. They noted that differences in fertility between immunized and non-immunized hens were greater when hens were mated to males of another line. Burke and Rieser (1972) incubated spermatozoa in sera obtained from turkey hens immunized against spermatozoa. When these spermatozoa were inseminated into recipient hens, lower fertility was observed. The lowest fertility was observed when spermatozoa were incubated in sera from non-immunized hens with a history of low fertility, suggesting a possible relationship between anti-sperm antibody production and infertility conditions.

Evidence for an antibody-fertility relationship was supported by Yu and Burke (1979b), who showed that sera from turkey hens exhibiting infertility syndrome carried a substance that reduced the fertilizing
ability of spermatozoa. Burke and Yu (1979) demonstrated that anti-sperm antibodies impaired sperm function, but they did not define a role of anti-sperm antibody production and infertility conditions in the turkey hen. McCorkle et al. (1983) measured anti-sperm antibody titers in commercial turkey hens from three different flocks using an adaptation of the microagglutination technique of Frieberg (1974), and observed high antibody titers in the flock having the lowest fertility. Anti-sperm antibodies increased over time, which may explain the common gradual decrease in flock fertility as the egg production season progresses. A relationship between anti-sperm antibody production and a single injury to the hen's oviduct was also reported by McCorkle et al. (1983). They proposed that anti-sperm antibody titers in hens may be induced through trauma to the hen's oviduct at the time of artificial insemination.

Histological observations suggest that the anti-sperm immune response in the hen may occur at the level of the oviduct. Ball et al. (1969) examined the oviduct of hens experiencing an infertility condition, and found a heavy infiltration of plasma cells in the lamina propria of the infundibulum and isthmus, despite an overall normal appearance in oviduct morphology. Yu and Burke (1979a) also observed a lymphocytic accumulation in the isthmus of low fertility hens. Schuppin et al. (1985) and Van Krey et al. (1987) examined the sperm storage glands from fertile and infertile turkey breeder hens, and found that plasma cells infiltrated the baso-lateral clefts between contiguous cells of the glands in infertile hens only. It was postulated that these plasma cells could be responsible for local anti-sperm antibody production, which could prevent spermatozoa from entering the sperm storage glands. Morris
and Howarth (1985) showed a decrease in the number of spermatozoa present in the sperm storage glands of hens inseminated with spermatozoa incubated in IgG fragments obtained from rabbits immunized against rooster Wolfian duct fluid. These results suggest that the presence of anti-sperm antibodies in the hen's reproductive tract may prevent spermatozoa from entering the sperm storage glands, thereby reducing the fertility of the hen. Bakst (1987a,b) observed IgG positive cells within the sperm storage glands of relatively fertile turkey breeder hens, but could not explain their significance.

Detection of Anti-sperm Antibodies

**Agglutination Tests.** The development of *in vitro* methods to detect and measure antisperm antibodies has, for the most part, been directed towards understanding infertility problems in humans. Agglutination tests were the first tests which were adapted for detecting anti-sperm antibodies. Agglutination techniques are based on the principle that at the appropriate antigen and antibody concentrations, sperm specific antibodies binding to spermatozoa will form a "lattice". If the lattice becomes heavy enough to settle to the bottom of the tube, the reaction can then be observed macroscopically. Kibrick and co-workers (1952) developed a gelatin agglutination test that was able to detect anti-sperm antibodies in serum. Slide agglutination was later used by Rumke and Hellinga (1959) and Franklin and Dukes (1964), though this method did not offer any significant advantages over tube agglutination. Capillary tube agglutination was also adapted for detecting anti-sperm antibodies (Shulman and Hehman, 1971). Though not as sensitive as gelatin
agglutination, this method utilized smaller quantities of reagents, and did not require motile spermatozoa.

The tray agglutination technique (TAT) was developed by Frieberg (1974). Like capillary agglutination, the TAT utilized micro quantities of reagents, but it also appeared to be more sensitive than the gelatin agglutination test. Frieberg suggested, however, that the increased sensitivity observed in the TAT may be due to non-immunologic spermagglutinins. Other variations of the agglutination method have been adapted for the detection of anti-sperm antibodies including exchange agglutination (O'Rand and Metz, 1974), and passive agglutination (Garabedian and Syverton, 1960). The utilization of these assays has been limited.

Complement Dependent Assays. Sperm immobilization assays were developed to employ the mechanism of complement activation by anti-sperm antibodies and its effect upon sperm motility. The major disadvantage of these tests is that they are not useful in detecting antibody subclasses other than IgG and IgM. The first sperm immobilization tests were reported by Fjallbrant (1968) and by Isojima and co-workers (1968). A sperm cytotoxicity test consisting of counting dead sperm cells after incubation in anti-sperm antibody sera was developed by Hamerlynck and Rumke (1968). The capillary tube mucous penetration test was designed by Kremer (1965), to specifically measure anti-sperm antibody activity in semen. This test was later modified by Fjallbrant (1968) to test cervical mucous from infertile women for the presence of anti-sperm antibodies.
Labeled Antibody Techniques. Radioimmunoassays (RIA's) have been adapted to detect and measure anti-sperm antibodies in humans (Haas et al., 1980) and in rabbits (Hill and Hampton, 1980). The RIA was reported to be more sensitive than agglutination tests (Hill and Hampton, 1980). The RIA had the capability to yield quantitative estimates of anti-sperm antibodies, whereas existing assays could only determine relative quantities, or titers of antibody. Previously, the quantitation of anti-sperm antibodies could only be performed by immunoelectrophoresis, after separation of antibodies from the sera or secretions by affinity chromatography (Mettler et al. 1980). The major drawback of the RIA over the existing techniques was the use of radioactive compounds.

The use of an immunobead binding assay to detect anti-sperm antibodies was reported by Bronson and coworkers (1981; 1984). Spermatozoa were first incubated in the test sera, mixed with the colored immunobeads that had been coupled with anti-human IgG or IgA, and then examined microscopically for binding to the immunobeads. The advantage of the immunobead assay is that one could tell against what sites on the sperm cells the antibodies were directed.

The labeling of antibodies with fluorescent dyes became the next class of immunological assays to be modified for the detection of anti-sperm antibodies. Indirect immunofluorescence (Hjort and Hansen, 1971) involved incubating methanol fixed spermatozoa with the test sera on a slide, after which antisera conjugated with a fluorescent dye is added. If antibody in the test sera was bound to the sperm, fluorescence could then be observed.
Enzyme assays have been the most recent immunological test adapted for detecting anti-sperm antibodies. The ELISA, or enzyme-linked immunosorbant assay was first described in the literature in 1971 (Engvall and Perlman). The first report of its use to detect anti-sperm antibodies (Rajgopal and Rao, 1979) was for measuring anti-sperm antibodies in infertile men and women. The advantages of the ELISA over other standard assays were that it could be interpreted objectively, expressed quantitatively, and correlated well with the results of other tests.

Other versions of the anti-sperm ELISA have been reported for use in mice (Allen et al., 1986), and rabbits (Wicher et al., 1987). All versions of the ELISA for anti-sperm antibodies basically start with spermatozoal antigens bound to wells of the microtiter plate, followed by subsequent incubations with the test sera, an enzyme labeled second antibody, and substrate. However, tests vary in the preparation of spermatozoa, reagents, and incubation times. Few tests use whole unfixed spermatozoa as the antigen source (Allen et al., 1986). Most ELISA's used to detect anti-sperm antibodies required coating the plates with whole spermatozoa fixed with either paraformaldehyde (Lynch et al., 1986; Lynch and Howe, 1987), glutaraldehyde (Zanchetta et al., 1982; Alexander and Bearwood, 1984), or Cytofix spray (Ing et al., 1985). Other tests use sperm homogenates (Paul et al., 1983) or sperm antigens obtained from frozen-thawed sperm (Rajigopal and Rao, 1979). Wolf et al. (1982) compared three methods of adhering whole sperm cells to plates. After adding whole spermatozoa to the test wells of the ELISA plates, the plates were either air dried, centrifuged, or centrifuged after pretreatment with glutaraldehyde. Sperm recovery in the test wells was greatest in
the plates which were air dried. These researchers noted that the recovery of the sperm decreased as the number of cells initially added to the plated decreased.
EXPERIMENT I

THE EFFECTS OF OVIDUCTAL TRAUMA ON THE PRODUCTION OF
ANTI-SPERM ANTIBODIES AND FERTILITY IN TURKEY BREEDER HENS
INTRODUCTION

A factor affecting the efficiency of fertile turkey egg production is the gradual decrease in breeder hen fertility as the egg production season progresses. In commercial turkey breeder flocks that experience this phenomenon, it is not unusual to observe a 20% to 25% decrease in flock fertility after several months of egg production, even though egg production remains unaffected. This has been termed seasonal decline in fertility.

Attempts to alleviate seasonal declines in fertility have been unsuccessful. The high levels of fertility found in the earlier stages of egg production could not be restored by increasing insemination frequency (Almquist, 1964; Sexton, 1977a), or by increasing the number of spermatozoa inseminated (Sexton, 1977a).

It has been established that seasonal decline in fertility is a female-related problem, but the cause of the problem has not been determined. Disease, and poor insemination technique have been shown not to be a factor. Van Krey et al. (1967b) found that seasonal decline in fertility was the result of a reduced ability of infertile hens to store spermatozoa within the uterovaginal sperm storage glands. Harper and Arscott (1969) obtained similar results. Based on the observation that the percentage of empty sperm storage glands in infertile hens increase over the course of the egg production season, Christensen (1981) suggested
that seasonal declines in fertility may be the result of a change to a hostile environment within the hen's oviduct.

A relationship between the hen's humoral immune system and fertility has been demonstrated. It has been shown that turkey hens which are actively immunized with spermatozoa exhibited reduced levels of fertility (Burke et al., 1971; Burke and Reiser, 1972). In addition, Wentworth and Mellen (1964) reported a negative correlation between anti-sperm antibody titers and fertility in chickens which were artificially inseminated. However, the mechanism by which anti-sperm antibodies are produced in the avian oviduct following repeated matings or inseminations has yet to be determined. This will be necessary if the role of the immune system in seasonal decline in fertility is to be defined.

McCorkle et al, (1983) reported that a single injury to the oviduct of the turkey early in the egg production season resulted in anti-sperm antibody production and a reduction in fertility. They also reported that turkey breeder hens which were inseminated using standard commercial techniques also produced anti-sperm antibodies. Furthermore, they found that anti-sperm antibody titers increased over the course of the egg production season. These data suggest that trauma to the oviduct during routine insemination may stimulate anti-sperm antibody production by the hen, thereby having a detrimental affect on hen fertility.

The use of different insemination tools may affect the level of trauma to the oviduct during artificial insemination. To date, there has
been no evaluation of artificial insemination equipment and its effect on anti-sperm antibody production in turkey breeder hens.

The objectives of this study were:

1. To develop an enzyme-linked immunosorbant assay (ELISA) to detect and measure humoral anti-sperm antibody titers in turkey breeder hens.

2. To determine the effects of different types of commercial insemination straws and varied degrees of oviductal trauma induced during insemination on turkey breeder hen fertility and humoral anti-sperm antibody production.

3. To determine the effects of a single, traumatic injury to the oviduct at the time of initial insemination on turkey breeder hen fertility and humoral anti-sperm antibody production.

4. To determine whether differences exist between turkey breeder hens in their first or second cycle of egg production with respect to anti-sperm antibody production and fertility levels.
MATERIALS AND METHODS

Elisa Validation: To verify the capability of the ELISA that was developed to detect and measure turkey anti-sperm antibody titers, a preliminary experiment was performed. Five virgin turkey breeder hens were injected intravenously at weekly intervals with one ml of washed, pooled turkey spermatozoa diluted one to four with physiological saline, and five virgin hens received physiological saline. Approximately five ml of blood were obtained from each hen via the brachial vein prior to any injections, and at weekly intervals thereafter. Blood was centrifuged at 1380 g for 25 minutes, and the serum was removed and stored at -20° C for subsequent analysis. The serum was analyzed for anti-sperm antibodies using an enzyme-linked immunosorbant assay (ELISA) adapted from the procedure of Lynch et al. (1986) (Appendix I). ELISA titers were calculated based on absorbance values of the pre-immune serum sample from each hen. Absorbance values which were 150% of the pre-immune value were considered positive for anti-sperm antibody, as described by Allen et al. (1986). Titer was expressed as the reciprocal of the highest dilution of the serum that yielded a positive result.

Trial 1: Large White Nicholas turkey breeder pouls were reared (0-8 weeks) in floor pens and exposed to a photoperiod of 24 hours light per day. At 9 weeks of age, the photoperiod was reduced to 12 hours per day. During the prebreeder blackout period (20 to 30 weeks) hens were exposed to a photoperiod of 6 hours per day at a light intensity of 43 lux. For the initial 8 weeks of the blackout period, the hens were
maintained in floor pens, but were transferred to cages for the final two weeks of the blackout period. At time of transfer (28 weeks), 48 hens were randomly assigned to individual cages in two ventilated, unheated cage rooms. At 30 weeks of age, hens were exposed to a photostimulatory period of 14 hours light per day, at a light intensity of 43 lux, to induce egg production.

Semen used in the experiment was collected from sixteen Large White Nicholas toms maintained in floor pens. The males were exposed to photoperiods similar to that of the females with the exception that they were exposed to these photoperiods two weeks earlier than the females.

Immediately prior to the start of egg production (32 weeks), hens were randomly assigned to one of four treatments. Hens receiving Treatment I were inseminated mid-vaginally with commercial insemination straws having non-beveled, relatively sharp edges. The latter straws are currently being used by some turkey breeders. Treatment II hens were inseminated mid-vaginally with a different type of commercial insemination straw having beveled, dull edges. Treatment III hens were inseminated mid-vaginally in a rough manner with a coarse serrated insemination straw in an attempt to induce relatively severe trauma to the oviduct. Hens receiving Treatment IV were inseminated mid-vaginally with a beveled insemination straw, taking care to deposit semen very gently onto an everted oviduct. All hens were inseminated weekly with .05 ml of pooled, fresh semen, diluted (1:1) with Beltsville Semen Extender (Sexton, 1977b).
Eggs were collected daily, beginning two days after the initial artificial insemination, throughout a 16-week period. Eggs were incubated on a weekly basis and were candled at 25 days of incubation to establish embryonic development. Eggs that appeared infertile were broken out and examined macroscopically to distinguish between early dead embryos and true infertile eggs.

Blood samples were taken from each hen prior to the initial artificial insemination, and at 4-week intervals thereafter for the remainder of the egg production season. Blood serum samples were collected and processed as described previously. The serum was analyzed for anti-sperm antibodies using a modification of the microagglutination method of McCorkle et al. (1983) (Appendix II), and an enzyme linked immunosorbant assay (ELISA) adapted from the procedure of Lynch et al. (1986).

Fertility data for each four-week period of egg production were combined to make up a fertility period. Percent fertility was transformed to arc sin square roots to normalize the data. Anti-sperm antibody titers were transformed to square roots. Fertility and anti-sperm antibody differences due to insemination treatment were analyzed by analysis of variance. Differences in the number of serum anti-sperm antibody positive hens between insemination treatments and by blood collection periods were analyzed using Chi-square. Differences in fertility between hens positive and negative for serum anti-sperm antibodies were analyzed using the analysis of variance procedure. Significance is $P \leq .05$. 
**Trial 2:** Forty-eight Large White Nicholas Turkey breeder hens were raised and subjected to photostimulation as described in Trial 1. In addition, forty-eight Large White Nicholas turkey breeder hens that had recently completed an egg production cycle were molted by the procedure of Hulet (1986). At 12 weeks post-molt, the hens were subjected to photostimulation as described in Trial 1. Equal numbers of hens from both groups were randomly assigned to individual cages in four ventilated, unheated cage rooms. Immediately prior to the start of egg production hens were randomly assigned to one of two insemination treatments in a 2 X 2 randomized block design. At the time of the initial insemination, oviducts of hens from the first treatment were everted, and traumatized by scraping with a probe to induce bleeding (McCorkle et al., 1983). Non-traumatized hens served as a control group, and were artificially inseminated in a conventional manner. Hens in both treatments were inseminated again seven days later, and were inseminated thereafter at 14-day intervals. Hens in both treatments were inseminated mid-vaginally with .05 ml of pooled, fresh semen, diluted 1:1 with Beltsville Semen Extender (Sexton, 1977b). All inseminations were performed with commercial insemination straws having dull, beveled edges. Semen used in the experiment was collected from 30 British United Turkey toms maintained in floor pens and exposed to a photoperiod of 14 hours light per day.

During the egg production period, eggs were collected daily, identified by hen number, and incubated to monitor hen fertility as described in Trial 1. Venous blood was obtained from each hen prior to
the initial insemination, and at 4-week intervals thereafter. Blood serum samples were processed as described previously, and analyzed for anti-sperm antibodies using an ELISA. Statistical analyses were identical to those described in Trial 1, with the exception that differences in the number of serum anti-sperm antibody positive hens between first and second cycle hens were analyzed using Chi-square.
RESULTS AND DISCUSSION

**Elisa Validation**

The results of the preliminary ELISA experiment showed that hens injected with semen had significantly higher ($P < .05$) serum anti-sperm antibody titers than saline-injected hens eight weeks following the initial injections (Table 1). Although the increases in serum antibody titers were significant, the titers never did attain high levels. Because antibody titers did not attain high levels in the turkey breeder hens, an adjunct experiment was set up with chickens to verify the sensitivity of our ELISA. Hens from a line of chickens genetically selected for high antibody response (Siegel and Gross, 1980) were immunized against spermatozoa, as were the turkey breeder hens. After eight weeks, the ELISA detected anti-sperm antibody titers of 5000-10,000, indicating that the ELISA developed was indeed sensitive enough to detect high titers of serum anti-sperm antibodies.

Low levels of anti-sperm antibodies were also detected in the serum of saline-injected hens, but these titers did not increase over the test period. It is likely that antibody titers in the saline-injected hens were due to a non-specific binding of serum antibodies to the spermatozoa as described by Witkin *et al.* (1980).

Microagglutination tests (McCorkle *et al.*, 1983) were also performed on the serum and were negative. The lack of agglutination suggests that the increase in anti-sperm antibody titers detected by the ELISA probably represented poor-agglutinating antibodies.
The source of spermatozoa used in an ELISA test has been suggested to be a factor in the consistency between different anti-sperm ELISAs. Ackerman et al. (1981) found higher reproducibility (89%) in ELISA's which utilized multiple semen donors compared to assays which used a single semen donor (51%). Lynch and Howe (1987) addressed the heterogeneity of different sperm sources, and suggested that spermatozoa between individuals may vary in their antigenic expression, explaining why the results of ELISAs conducted by different laboratories using the same reference sera may be inconsistent. They also suggested that the correlation of agglutination tests to ELISA's would be improved by using the same sperm suspension in each assay. In our experiment, all semen used in artificial insemination and immunological assays consisted of a pooled sample which was obtained from a minimum of 10 toms of the same strain, which should have enhanced our reproducibility.

Trial 1

Artificial insemination treatment did not have a significant effect on hen fertility in Trial 1, nor did hen fertility change significantly over the egg production cycle (Table 2). Overall flock fertility was excellent, and did not decrease during the 16-week trial, indicating that the classic seasonal decline in fertility had not occurred. Although the fertility of several hens decreased during the trial, only one hen approached zero percent fertility during the final four weeks of egg production. It is possible that the duration of the experiment was too short to observe the characteristic decline in fertility, but many of the
hens ceased egg production at the end of the trial, and it was not feasible to extend the trial beyond 16 weeks. Conceivably, potentially infertile hens went out of egg production prior to becoming infertile.

The microagglutination test for serum anti-sperm antibodies was negative for all hens throughout the egg production cycle. Even the hens inseminated with a serrated straw in a rough manner did not show an increase in the production of anti-sperm antibodies, which is contrary to the results of McCorkle et al. (1983). Previous studies by these researchers linked oviductal trauma to increased anti-sperm antibody titers. McCorkle et al. (1983) also reported that anti-sperm antibody titers existed in hens which were inseminated under normal conditions, suggesting that a humoral anti-sperm response in the turkey hen can occur using standard artificial inseminating techniques. Because we obtained negative results with the microagglutination test, and because the microagglutination test is a relatively insensitive test, the more sensitive ELISA was adopted to measure anti-sperm antibodies in these hens.

The results obtained with the ELISA test were similar to those obtained with the microagglutination test. There were no significant differences in serum anti-sperm antibody titers due to artificial insemination treatment, nor did titers change over the egg production cycle (Table 3). The number of serum anti-sperm antibody positive hens was not significantly different among artificial insemination treatments, nor were there any significant differences among blood collection periods.
No relationship appeared to exist between serum anti-sperm antibody titers, as determined by the ELISA, and fertility levels in this experiment (Table 4). The average percent fertility was 94.8% for antibody positive hens and 94.3% for negative hens. The one hen in this trial which did exhibited a sharp decrease in fertility was negative for serum anti-sperm antibodies as determined by ELISA. Thus, it was concluded that no clinically significant levels of anti-sperm antibodies were produced by the hens. The presence of low levels of anti-sperm antibodies in fertile hens is not without precedence. It has been demonstrated that intermediate titers of serum anti-sperm antibodies can exist in women without adversely affecting fertility levels (Bronson et al., 1984).

It is possible that antibodies detected in our experiment were either low affinity antibodies or they were not sperm-specific and were cross-reacting, for it has been suggested that non-specific reactions with irrelevant antigens may affect the reliability of the ELISA. Witkin and co-workers (1980) demonstrated the presence of IgG-Fc receptors (crystalline fragments) on human and bovine spermatozoa. Mettler et al. (1985) evaluated different ELISA methods from eight laboratories and found that ELISA's utilizing whole spermatozoa had a high frequency of positive reactions, and they suggested that non-specific binding of IgG-Fc to spermatozoa, or the detection of low affinity antibodies may be occurring. However, Lynch and co-workers (1986) found that utilizing whole spermatozoa as the antigen source yielded ELISA results comparable to those obtained with standard assays. In our assay, by using the
pre-insemination serum as a reference, antibody titers detected in the post-insemination sera should not be the result of non-specific binding of IgG-Fc to turkey spermatozoa.

The greater sensitivity of the ELISA over agglutination tests may explain why occasionally the anti-sperm antibody titers between the ELISA test and other standard assays may differ. Wolf et al. (1982) reported that the ELISA is as sensitive as the radioimmunoassay (RIA) in detecting anti-sperm antibodies. Paul et al. (1983) observed that the sensitivity of the ELISA was one thousand times that of the tray agglutination test. Ing et al. (1985) found anti-sperm antibody titers determined by ELISA to be 10 to 20 fold greater than titers found in the agglutination test. Thus, it was assumed in our study, that the ELISA detected minute quantities of antibody that were not detected by agglutination. It may also be possible that the ELISA detected several types of immunoglobulins, including IgG which is not a good agglutinating antibody (Barrett, 1983).

**Trial 2.**

Among the control non-traumatized hens in their first egg production cycle, fertility decreased significantly as the egg production season progressed, but fertility did not decrease in the traumatized hens (Table 5). When both treatments were combined for each time interval, fertility again decreased as the egg production season progressed. Fertility among the second-cycle hens did not decrease in either treatment group, nor did it decrease overall. Fertility was not significantly different between traumatized and non-traumatized hens (Table 5). Among
the second-cycle hens, fertility did not decrease over the egg production season in either treatment group, nor were there any significant differences between traumatized and non-traumatized hens. Therefore, contrary to the results of McCorkle et al. (1983), traumatization of the oviduct prior to the initial insemination had no significant effect on subsequent fertility of turkey hens.

Anti-sperm antibody titers for hens in their first egg production cycle were not significantly different between traumatized and non-traumatized hens and titers did not increase over the course of the egg production cycle (Table 6). The number of anti-sperm antibody positive hens was not significantly different between traumatized and non-traumatized hens, nor were there any differences among blood collection periods.

There were no significant differences in anti-sperm antibody titers between traumatized and non-traumatized second-cycle hens. The ELISA detected minimal serum anti-sperm antibody titers in the recycled hens, with only two hens showing a relatively low positive response.

The number of first cycle hens (21) positive for serum anti-sperm antibodies was significantly greater than the number of second-cycle hens (3). The reason why a greater number of hens in their first cycle of egg production produced serum anti-sperm antibodies when compared to those in their second cycle is unknown.

As was true for Trial 1, there was no relationship between serum anti-sperm antibodies as detected by the ELISA and fertility (Table 7). After 10 to 12 weeks of egg production, several of the hens showed a
gradual decrease in fertility, and at the end of the egg production cycle, their fertility ranged from 0 to 50 percent. Nevertheless, these relatively infertile hens were negative for serum anti-sperm antibodies. In addition, hens which were positive for serum anti-sperm antibody activity were found to have moderate to high fertility levels.

Previous studies have demonstrated a relationship between the hen immune system and fertility in chickens (Wentworth and Mellen, 1964), and turkeys (Burke et al., 1971; Burke and Reiser, 1972; McCorkle et al., 1983). However, Lamoreux (1940) demonstrated normal fertility levels in hens immunized against homogenates of rooster testes, which is consistent with our studies. Saeki and co-workers (1965) reported that fertility declined in chickens 3 to 5 months following the start of artificial insemination. However, they observed no differences in anti-sperm titers between hens with relatively high fertility and hens with poor fertility. Abe et al. (1966) did not observe lower fertility in hens passively immunized with heteroimmune sperm-agglutinating antibodies. Furthermore, it has been shown that it is possible to have serum anti-sperm antibody production and normal fertility in women (Bronson et al., 1984; Ohashi et al., 1987) and in men (Jager et al., 1987).

It has also been shown in humans that a local immune response against spermatozoa can occur without the presence of a systemic antibody response (Behrman and Menge, 1980; Moghissi et al., 1981). It is, therefore, possible that an anti-sperm immune response may be occurring within the reproductive tract of the turkey hen. Previous work by Schuppin et al. (1985) and Van Krey et al. (1987) support this concept,
for they found plasma cells within the UVSG of infertile, but not fertile turkey breeder hens.

In summary, the results of this study suggest there is no relationship between oviductal trauma at the time of artificial insemination and the production of serum anti-sperm antibodies. Thus, it was concluded that seasonal declines in fertility are not a result of poor artificial insemination practices. In addition, it is doubtful that the decreased fertility observed in turkey breeder hens over the course of the egg production cycle is the result of a humoral response against spermatozoa. It is possible, however, that a local immune response may be occurring in the turkey hen that is affecting fertility.
Table 1. Serum anti-sperm antibody titers for sperm and saline injected hens as determined by ELISA.

<table>
<thead>
<tr>
<th>Material Injected</th>
<th>Weeks following initial immunization</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>4-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermatozoa</td>
<td>40.0 ± 23.1</td>
<td>40.0 ± 23.1</td>
<td>90.0 ± 41.2</td>
<td>140.0 ± 68.3</td>
<td>160.0 ± 56.6</td>
<td>98.0 ± 21.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(4)$^4$</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(4)</td>
<td>(20)</td>
</tr>
<tr>
<td>Saline</td>
<td>16.0 ± 9.8</td>
<td>48.0 ± 18.6</td>
<td>40.0 ± 17.9</td>
<td>24.0 ± 9.8</td>
<td>40.0 ± 12.6</td>
<td>33.6 ± 5.9</td>
<td></td>
</tr>
<tr>
<td>Combined</td>
<td>26.7 ± 11.5</td>
<td>53.3 ± 17.3</td>
<td>62.2 ± 21.1</td>
<td>75.6 ± 34.9</td>
<td>93.3 ± 32.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(9)</td>
<td>(9)</td>
<td>(9)</td>
<td>(9)</td>
<td>(9)</td>
<td>(9)</td>
<td></td>
</tr>
</tbody>
</table>

$^1$ Mean ± standard error.

$^2$ Means having different superscripts are significantly different (P < .05) between sperm- and saline-injected hens.

$^3$ Enzyme-linked immunosorbant assay.

$^4$ Number of observations.
Table 2. Percent fertility\(^1\)\(^2\) by artificial insemination (A.I.) treatment (Trial 1).

<table>
<thead>
<tr>
<th>A.I. Treatment</th>
<th>1-4</th>
<th>5-8</th>
<th>9-12</th>
<th>13-16</th>
<th>1-16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sharp Straw</td>
<td>91.4</td>
<td>97.7</td>
<td>98.2</td>
<td>99.2</td>
<td>96.3 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>(12)</td>
<td>(12)</td>
<td>(11)</td>
<td>(7)</td>
<td>(42)</td>
</tr>
<tr>
<td>Dull Straw</td>
<td>97.8</td>
<td>94.9</td>
<td>95.7</td>
<td>98.4</td>
<td>96.6 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>(12)</td>
<td>(12)</td>
<td>(10)</td>
<td>(8)</td>
<td>(38)</td>
</tr>
<tr>
<td>Rough A.I.</td>
<td>91.4</td>
<td>92.1</td>
<td>93.1</td>
<td>96.6</td>
<td>92.7 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>(12)</td>
<td>(11)</td>
<td>(10)</td>
<td>(5)</td>
<td>(38)</td>
</tr>
<tr>
<td>Gentle A.I.</td>
<td>93.2</td>
<td>95.2</td>
<td>94.9</td>
<td>82.5</td>
<td>92.8 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>(12)</td>
<td>(12)</td>
<td>(8)</td>
<td>(5)</td>
<td>(37)</td>
</tr>
<tr>
<td>Combined</td>
<td>93.4 ± 1.6</td>
<td>95.0 ± 1.2</td>
<td>95.6 ± 1.3</td>
<td>95.1 ± 3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(48)</td>
<td>(47)</td>
<td>(39)</td>
<td>(25)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Mean ± standard error.

\(^2\) Means are not significantly different (P < .05) among treatments or among 4-week intervals.

\(^3\) Number of observations.
Table 3. Serum anti-sperm antibody titers\(^1\)\(^2\) by artificial insemination (A.I.) treatment and by blood collection period as determined by ELISA\(^3\) (Trial 1).

<table>
<thead>
<tr>
<th>A.I. Treatment</th>
<th>Blood collection period(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Sharp Straw</td>
<td>21.7</td>
</tr>
<tr>
<td>(12)(^5)</td>
<td>(12)</td>
</tr>
<tr>
<td>Dull Straw</td>
<td>53.3</td>
</tr>
<tr>
<td>(12)</td>
<td>(11)</td>
</tr>
<tr>
<td>Rough A.I.</td>
<td>21.8</td>
</tr>
<tr>
<td>(11)</td>
<td>(9)</td>
</tr>
<tr>
<td>Gentle A.I.</td>
<td>0.0</td>
</tr>
<tr>
<td>(11)</td>
<td>(8)</td>
</tr>
<tr>
<td>Combined</td>
<td>24.8 ± 9.2</td>
</tr>
<tr>
<td>(46)</td>
<td>(40)</td>
</tr>
</tbody>
</table>

\(^1\) Mean ± standard error.

\(^2\) Means not are significantly different among treatments (P < .05) or among 4-week intervals.

\(^3\) Enzyme-linked immunosorbant assay.

\(^4\) Blood collection periods are at 4-week intervals.

\(^5\) Number of observations.
Table 4. Percent fertility\(^1,2\) of hens testing positive or negative for serum anti-sperm antibodies (Trial 1).

<table>
<thead>
<tr>
<th>Serum Antibody Response</th>
<th>Percent Fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>94.8 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>(32)(^3)</td>
</tr>
<tr>
<td>Negative</td>
<td>94.3 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>(96)</td>
</tr>
</tbody>
</table>

\(^1\) Mean ± standard error.

\(^2\) Means are not significantly different (\(P < .05\)) between positive and negative hens.

\(^3\) Number of observations.
<table>
<thead>
<tr>
<th>A.I. Treatment</th>
<th>Weeks of egg production</th>
<th>1-4</th>
<th>5-8</th>
<th>9-12</th>
<th>13-16</th>
<th>1-16</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First-cycle hens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traumatized²</td>
<td>88.7</td>
<td>89.1</td>
<td>88.9</td>
<td>81.5</td>
<td>87.3 ± 1.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(23)</td>
<td>(21)</td>
<td>(21)</td>
<td>(18)</td>
<td>(83)</td>
<td></td>
</tr>
<tr>
<td>Non-traumatized</td>
<td>93.9</td>
<td>87.9²</td>
<td>86.3²</td>
<td>80.7³</td>
<td>87.5 ± 1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(22)</td>
<td>(23)</td>
<td>(21)</td>
<td>(18)</td>
<td>(84)</td>
<td></td>
</tr>
<tr>
<td>Combined</td>
<td>91.3 ± 1.9²</td>
<td>88.4 ± 2.1³</td>
<td>87.6 ± 2.2²³</td>
<td>81.1 ± 3.3³</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(45)</td>
<td>(44)</td>
<td>(42)</td>
<td>(36)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Second-cycle hens</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Traumatized</td>
<td>94.9</td>
<td>88.1</td>
<td>83.3</td>
<td>78.2</td>
<td>87.7 ± 2.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(22)</td>
<td>(20)</td>
<td>(14)</td>
<td>(11)</td>
<td>(67)</td>
<td></td>
</tr>
<tr>
<td>Non-traumatized</td>
<td>94.7</td>
<td>89.8</td>
<td>92.4</td>
<td>89.8</td>
<td>91.9 ± 1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(24)</td>
<td>(24)</td>
<td>(21)</td>
<td>(16)</td>
<td>(85)</td>
<td></td>
</tr>
<tr>
<td>Combined</td>
<td>94.9 ± 1.4³</td>
<td>89.0 ± 2.4³</td>
<td>88.7 ± 3.4³</td>
<td>85.1 ± 5.4³</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(46)</td>
<td>(44)</td>
<td>(35)</td>
<td>(27)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Mean ± standard error.
² Means in a row having different superscripts are significantly different (P < .05).
³ Vagina superficially lacerated with a blunt probe at the time of the initial insemination.
⁴ Number of observations.
Table 6. Serum anti-sperm anti-body titers\(^{1,2}\) of first-cycle hens by initial insemination treatment (A.I.) and by blood collection period as determined by ELISA\(^{3}\) (Trial 2).

<table>
<thead>
<tr>
<th>A.I. Treatment</th>
<th>Blood collection period(^{4})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Traumatized(^{5})</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>(23)(^{6})</td>
</tr>
<tr>
<td>Non-traumatized</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>(21)</td>
</tr>
<tr>
<td>Combined</td>
<td>4.5 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>(44)</td>
</tr>
</tbody>
</table>

\(^{1}\) Mean ± standard error.

\(^{2}\) Means are not significantly different (P < .05) between treatments or among 4-week intervals.

\(^{3}\) Enzyme-linked immunosorbant assay.

\(^{4}\) Blood collection periods are at 4-week intervals.

\(^{5}\) Vagina superficially lacerated with a blunt probe at the time of the initial insemination.

\(^{6}\) Number of observations.
Table 7. Percent fertility\textsuperscript{1,2} of first-cycle hens testing positive and negative for serum anti-sperm antibodies (Trial 2).

<table>
<thead>
<tr>
<th>Serum Antibody Response</th>
<th>Percent Fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>85.8 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>(21)\textsuperscript{3}</td>
</tr>
<tr>
<td>Negative</td>
<td>87.6 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>(145)</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Mean ± standard error.

\textsuperscript{2} Means are not significantly different (P < .05) between positive and negative hens.

\textsuperscript{3} Number of observations.
EXPERIMENT II

RELATIONSHIP OF INFERTILITY TO ANTI-SPERM ANTIBODIES IN THE
UTEROVAGINAL SPERM STORAGE GLANDS IN TURKEY BREEDER HENS
INTRODUCTION

Seasonal decline in fertility in the turkey breeder hen is known to be due to the lack of viable spermatozoa in the oviductal sperm storage glands (Van Krey et al., 1967b; Harper and Arscott, 1969). However, the reason spermatozoa are not retained by the oviduct remains equivocal.

The relationship between the avian immune system and infertility has been investigated. Wentworth and Mellen (1964) reported that serum anti-sperm antibodies were produced in chicken hens following natural mating or artificial insemination, and that a negative correlation existed between antibody titer and fertility. Burke et al. (1971) reported that when turkey hens were immunized against spermatozoa, fertility was depressed. Burke and Rieser (1972) observed a reduction in fertility in turkey hens which were inseminated with spermatozoa that had been incubated in sera obtained from turkey hens that had been immunized against spermatozoa.

Additional evidence that humoral antibodies can affect fertility was obtained by Yu and Burke (1979b). They demonstrated that sera from turkey hens experiencing infertility contained a substance that reduced the fertilizing ability of spermatozoa. Burke and Yu (1979) found that serum immune globulins impaired sperm function and diminished fertility in turkey hens, but the mechanism whereby anti-sperm antibodies affected fertility was not determined. McCorkle et al. (1983) measured anti-sperm
antibody titers in turkey hens utilizing an adaptation of the microagglutination technique of Frieberg (1974), and reported a negative correlation between antibody titer and fertility. Nevertheless, when employing a relatively sensitive enzyme-linked immunosorbant assay (ELISA) in Experiment I of this dissertation, no correlation was found between serum anti-sperm antibodies and fertility in turkey breeder hens.

A relationship between infertility and local antibody production has been established in humans. Parish et al. (1967) suggested that a local immune response against spermatozoa existed in the female genital tract after finding higher antibody titers in cervical mucous than in serum. Hutcheson et al. (1974) reported an increase in the number of IgA producing cells in the vaginal/endocervical mucous membranes of women experiencing infertility of an unknown cause.

Recent ultrastructural studies also support the concept of a local anti-sperm immune response in the turkey hen. Van Krey et al. (1987) found plasma cells interspersed between the lateral walls of adjacent sperm gland cells in infertile, but not fertile turkey breeder hens. It was postulated that these plasma cells were producing anti-sperm antibodies, thereby preventing spermatozoa from entering the oviductal sperm storage sites, resulting in decreased fertility.

Bakst (1987a,b) incubated histological sections of turkey UVSG with anti-turkey IgG and reported finding IgG positive cells in the sperm storage gland epithelium. However, these tissues were obtained from highly fertile hens (above 85%), which casts doubt on a relationship between a localized cellular antibody response and fertility. To date
there has been no study to determine if differences exist in local antibody production within the UVSG of fertile and infertile turkey breeder hens. Thus, the objective of this study was to establish a possible immunological basis at the cellular level for infertility in turkey breeder hens.

MATERIALS AND METHODS

Commercial turkey breeder hens, maintained in individual cages, were artificially inseminated with .05 ml of pooled semen diluted 1:1 with Beltsville Semen Extender (Sexton, 1977b) at 14-day intervals for an 18-week period. Eggs were collected daily, identified by hen number, and incubated weekly to monitor hen fertility. Venous blood was obtained from each hen at the onset of egg production and at 4-week intervals thereafter. The blood was centrifuged at 1380 g for 25 minutes, and the serum was decanted and stored at -20 C for subsequent analysis. Antibody titers were determined using an ELISA as described in Experiment I.

After 18 weeks of egg production, hens were classified as being relatively fertile or infertile based on the percentage of fertile eggs produced during the final four weeks of egg production. Hens having greater than 90 percent fertility for this time period were classified as relatively fertile, while hens having less than 50 percent fertility were classified as relatively infertile. Three fertile and eight infertile hens were necropsied, and tissue containing the (UVSG) was dissected free of the oviduct and placed in 10% neutral buffered formalin.
Following overnight fixation, the tissues were processed using standard histological techniques (Appendix III). Tissue sections (3-4 microns) were collected at 100 micron intervals, with a minimum of 10 sections being prepared from each hen. The sections were stained for IgG positive cells using an immunohistochemical staining technique (Appendix IV) which consisted of incubating sections with anti-turkey IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD.), followed by staining with peroxidase anti-peroxidase (Vector Labs, Burlingame, CA). Sections were examined for IgG positive cells within the uterovaginal sperm storage glands, connective tissue surrounding the glands, and the oviductal surface epithelium. Sections were also examined for IgG positive material within the lumen of the sperm glands.

Differences in anti-sperm antibody titers by fertility group and blood collection period, and overall anti-sperm antibody titer by fertility group were analyzed by analysis of variance. Differences in number of hens observed to have IgG positive cells within the UVSG by fertility group, and the number of hens having IgG positive material within the UVSG lumen were compared by Chi-square. Significance is P<.05.
RESULTS AND DISCUSSION

One of the characteristics of seasonal decline in fertility is a progressive increase in the number of infertile hens in a flock. In our flock, the mean fertility during the last four weeks of egg production was 89 percent (Table 8), and ranged from 79-100 percent for those hens classified as fertile. For those hens classified as infertile, the mean fertility was 35 percent (Table 8), and ranged from 13-50 percent. There were no significant differences in anti-sperm antibody titers between hens classified as relatively fertile and infertile during the egg production cycle. While only one hen in this study was totally infertile by the last week of egg production, many appeared to be in the early stages of such a decline. If these birds had been allowed to continue egg production they might have become totally infertile.

ELISA tests showed no differences in serum anti-sperm antibody titers between hens classified as fertile and infertile at the time of necropsy (Table 9). Low levels of serum anti-sperm antibodies were detected in two fertile hens, but no antibodies were found in infertile hens (Table 9). The results indicate that the reduced fertility in the infertile hens was not due to a humoral immune response against spermatozoa. This is consistent with the results obtained in Experiment I where no correlation was found between serum anti-sperm antibodies and fertility in turkey breeder hens. The results were also consistent with earlier reports by Burke et al. (1971) and Burke and Rieser (1972) who were unable to demonstrate anti-sperm antibodies in turkey breeder hens by complement fixation, ouchterlony double diffusion, or sperm
agglutination tests. These results are not, however, in agreement with those of McCorkle et al. (1983) who utilized a microagglutination test to demonstrate increasing anti-sperm antibody titers over the course of the egg production cycle. In Experiment I, anti-sperm antibody titers were not detected with the microagglutination test.

The results of the immunohistochemical test are presented in Table 9. Because the primary antibody used in the immunohistochemical staining procedure was directed against IgG heavy and light chains, all antibody subclasses stained positive. Therefore, positive staining can indicate the presence of any of the antibody classes (personal communication, Kirkegaard and Perry Labs).

Scattered lymphocytes were located baso-laterally between contiguous cells of the UVSG of both fertile and infertile hens. The incidence of lymphocytes was variable. Tissue sections of some glands did not have any lymphocytes, while other glandular sections contained numerous such cells. The lymphocytes generally did not stain positive, except in sections which were overstained, suggesting an artifactual response.

The finding of lymphocytes in the UVSG of both fertile and infertile turkey breeder hens was consistent with ultrastructural observations of Schuppin et al. (1984), and by Van Krey et al. (1987). Although it cannot be concluded from the results of the immunohistochemical stain whether the lymphocytes within the UVSG are of B or T cell origin, it has been suggested that they may be suppressor T cells, that prevent the occurrence of an immune response against spermatozoa at the level of the sperm glands.
Bakst (1987c) reported that the number of lymphocytes found within the UVSG epithelium increased over the egg production cycle, whether or not there were spermatozoa present within the gland lumen. He suggested that these lymphocytes may act to sequester spermatozoal antigens, thus preventing an immune response. He also proposed that a breakdown in this system may result in an anti-sperm immune response.

In most of the sections, the surface epithelium of the vagina was found to be coated with antibody and contained positively staining cells, suggesting that antibodies are secreted at this level. This appears to be a general response, for cells staining positive for antibody production were present near the basal lamina in the vaginal surface epithelium of both fertile and infertile hens. These positive staining cells are most likely the source of the antibody which was found to coat the epithelial surface.

It has been established in humans that the primary immune defense mechanism in the female genital tract is by local secretion of IgA. The IgA, which is produced by plasma cells in the lamina propria, is found in the fallopian tube, the uterus, and especially the uterine cervix (Rebello et al., 1975). Because the primary antibody used in our study is also capable of staining IgA, it is assumed that the antibody lining the surface epithelium of hen oviduct is IgA, which has been secreted by the plasma cells located near the basal lamina.

The loose connective tissue surrounding the UVSG stained positive, indicating that antibody had been secreted into the connective tissue.
Plasma cells were found within the connective tissue, and were most likely the source of the immunoglobulins. This finding is consistent with that of Bakst (1987a), who observed IgG positive cells in the loose connective tissue of fertile turkey hens. Because plasma cells and antibody secretion were found in the connective tissue of both fertile and infertile hens in this study, it can be concluded neither was the result of an immune response against spermatozoa.

Cells staining positive for cytoplasmic antibodies were found within the UVSG (Plates 4, 6) of six infertile hens. Such cells were not present within any of the glands of the fertile hens. There was a significant difference in the number of hens with IgG positive cells by fertility group. The absence of IgG positive cells in the UVSG of fertile hens conflicts with the recent observations of Bakst (1987b), who reported finding IgG positive cells in the UVSG of highly fertile hens. In our study, positive cells were found in the glands of fertile hens only when a section was overstained due to incubation in excess anti-turkey IgG.

When found, positive staining cells were located near the orifice of a sperm storage gland, which is analogous to the site where Van Krey et al. (1987) reported finding plasma cells in the UVSG of infertile turkey breeder hens. On that basis, it was assumed that the positive cells were plasma cells. Glands observed to contain plasma cells were not found to contain spermatozoa; however, limited spermatozoa were found in other nonstaining glands in the same tissue section. The presence of spermatozoa within some glands of infertile hens explains why they were still capable of producing a limited number of fertile eggs. The presence
of occasional spermatozoa and plasma cells may be representative of the early stages of a local immune response against spermatozoa. Bakst (1987a,b) reported IgG positive cells in the sperm storage glands of highly fertile hens. However, the hens had been in egg production for 10-12 weeks, so it is conceivable that some of those hens may also have been in the early stages of an immune response.

Occasionally amorphic positive staining material was found within the lumena of UVSG from infertile hens. The difference in the number of hens having this material was significant between fertile and infertile hens. It could not be concluded, however, if the positive staining material represented anti-sperm antibody bound to spermatozoa, or was merely an accumulation of surface secretion.

Previous observations in humans, where a local immune response in the female reproductive tract can occur without the presence of serum antibodies, support the results of this study. Behrman and Menge (1980) suggested that a local immune response against spermatozoa can occur without the presence of a systemic antibody response. Moghissi et al. (1981) failed to demonstrate serum anti-sperm antibodies in infertile women who were shown to have anti-sperm antibodies present in cervico-vaginal secretions. Vaginal exposure to antigens resulted in local IgA production, but not in serum antibody production (Waldman et al., 1972; Ogra and Ogra, 1973). Ogra and Ogra (1973) were able to demonstrate serum antibodies only in individuals immunized intramuscularly.
In summary, the results of the study do not support the concept that seasonal decline in fertility in turkey breeder hens is the result of a humoral immune response against spermatozoa. The ELISA test for serum anti-sperm antibodies was negative, indicating the absence of a humoral immune response against spermatozoa.

The immunohistochemical test for a local immune response showed antibody production within the oviductal connective tissue surrounding the UVSG and along the vaginal surface epithelium in all hens. However, only infertile hens were observed to have plasma cells and antibody secretion at the level of the sperm glands. This latter observation is consistent with those made by Van Krey et al. (1987). Thus, a localized immune response may be occurring.
Table 8. Summary of fertility data and serum anti-serum antibody titers as determined by ELISA\(^1\) for hens classified as relatively fertile or infertile.

<table>
<thead>
<tr>
<th>Fertility Classification</th>
<th>Fertile eggs produced last 4 wk production</th>
<th>Anti-sperm antibody titer(^2,3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertile</td>
<td>31/35</td>
<td>16.0 ± 10.9</td>
</tr>
<tr>
<td>(3)*</td>
<td>(89%)</td>
<td></td>
</tr>
<tr>
<td>Infertile</td>
<td>38/109</td>
<td>14.0 ± 6.9</td>
</tr>
<tr>
<td>(8)</td>
<td>(35%)</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Enzyme-linked immunosorbant assay.

\(^2\) Mean ± standard error.

\(^3\) Anti-sperm antibody titers are expressed as log\(_2\).

\(^4\) Number of hens.
Table 9. Summary of ELISA¹ and immunohistochemical stain for hens classified as relatively fertile or infertile.

<table>
<thead>
<tr>
<th>Fertility Group</th>
<th>Anti-sperm antibody titer at necropsy²,³</th>
<th>Percent hens with antibody positive cells in the UVSG⁴</th>
<th>Percent hens with antibody positive material in lumena of UVSG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertile (3)⁵</td>
<td>53.0 ± 53.0</td>
<td>0⁶</td>
<td>0⁶</td>
</tr>
<tr>
<td>Infertile (8)</td>
<td>0 ± 0</td>
<td>75⁶</td>
<td>50⁶</td>
</tr>
</tbody>
</table>

¹ Enzyme-linked immunosorbant assay.
² Mean ± standard error.
³ Anti-sperm antibody titers are expressed as log₂.
⁴ Uterovaginal sperm storage glands.
⁵ Number of hens.
SUMMARY

Two experiments were conducted to determine the role of the turkey hen's immune system in the so-called seasonal decline in fertility. Results from Experiment I demonstrated that trauma to the oviduct induced either at the time of the initial artificial insemination, or during subsequent inseminations had no effect upon hen fertility, or serum anti-sperm antibody titers. No differences in fertility were found between hens showing a positive or negative anti-sperm antibody response.

Experiment II demonstrated that only relatively infertile turkey hens had antibody positive cells within the uterovaginal sperm storage gland epithelium (UVSG), while fertile turkey hens had no such cells. Antibody positive amorphous material was found within the lumen of the UVSG of relatively infertile, but not fertile turkey hens. Blood serum from form infertile hens showed minimal anti-sperm antibody titers. It was concluded that the seasonal decline in fertility in turkey hens is not due to a humoral immune response against spermatozoa. However, a local immune response against spermatozoa appears to exist in the uterovaginal sperm storage glands of the turkey hen, which has a detrimental effect upon fertility.
REFERENCES


Wentworth, B.C., and A. Lapp, 1986. Cumulative fertility of turkey hens inseminated before or after the first egg is laid. Poultry Sci. 65(Suppl.1):142. (Abstr.)


PLATES
1 - 6
PLATE 1

ANTIBODY POSITIVE CELLS LOCATED BASALLY IN THE VAGINAL EPITHELIUM OF A RELATIVELY FERTILE TURKEY HEN.
PLATE 2

ANTIBODY SECRETION WITHIN CELLS OF THE VAGINAL EPITHELium OF A RELATIVELY INFERTILE TURKEY HEN.
PLATE 3

AGGLUTINATED SPERMATOZOA SURROUNDED BY ANTIBODY POSITIVE MATERIAL WITHIN THE LUMEN OF THE OVIDUCT OF A RELATIVELY INFERTILE TURKEY HEN.
PLATE 4

ANTIBODY POSITIVE CELLS AND SECRETIONS WITHIN A GROOVE LEADING INTO A UTEROVAGINAL SPERM STORAGE GLAND OF A RELATIVELY INFERTILE TURKEY HEN.
PLATE 5

ANTIBODY POSITIVE SECRETIONS AND SPERMATOZOA WITHIN A UTEROVAGINAL SPERM STORAGE GLAND OF A RELATIVELY INFERTILE TURKEY HEN. THE ABBREVIATED CILIA SUGGESTS THE GLAND WAS SECTIONED NEAR THE IT'S ORIFICE.
PLATE 6

ANTIBODY POSITIVE CELL WITHIN A UTEROVAGINAL SPERM STORAGE GLAND OF A RELATIVELY INFERTILE TURKEY HEN. ANTIBODY POSITIVE MATERIAL IS LOCATED BOTH BASALLY AND APICALLY WITHIN THE SPERM GLAND CELLS.
APPENDIX I

ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA)
ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA) FOR DETECTING ANTISPERM ANTIBODIES IN TURKEY BREEDER HEN SERUM

1. PREPARATION OF SPERM SOLUTION

A. Solutions required:

1. Phosphate buffered saline (PBS)-Ethylene glycol tetra-acetic acid (EGTA) buffer

Prepare PBS (see ELISA solutions). Add 38 mg ethylene glycol tetra-acetic acid per 100 ml PBS rinsing buffer.

2. Stock paraformaldehyde:

8 g paraformaldehyde in 100 ml distilled water

Under a fume hood, heat solution to 70° C, stirring constantly. Add 10 N NaOH dropwise until paraformaldehyde is dissolved. Filter solution, and store at 4° C in a foil-wrapped bottle for up to 2 weeks.

3. Coating buffer:

1.59 g Na₂CO₃
2.93 g NaHCO₃

Dissolve in 1 liter distilled H₂O, adjust pH to 9.6 with 10 N NaOH.

B. Procedure:

1. Dilute a pooled turkey semen sample 1:10 with PBS-EGTA. Mix with a Pasteur pipette and centrifuge 25 min at 3000 g.

2. Discard supernatant, resuspend with warm 37° C PBS-EGTA, mix with a pipette and centrifuge 10 min at 3000 g.

3. Repeat Step #2.

4. Discard supernatant and resuspend sperm cells in 30 ml of PBS-EGTA in a beaker.

5. Add 10 ml stock paraformaldehyde. Mix with a pipette, breaking apart any aggregates. Let mixture sit at room temperature for 10 minutes. Centrifuge 10 min at 3000 g.

7. Pass the sperm suspension through 20 gauge needle, then through a 23 gauge needle to disrupt large aggregates of spermatozoa.

8. Pour the sperm solution to a graduated cylinder, and let settle for 10 minutes. Aspirate off the upper 95% to be used as the stock solution. Remove any large aggregates. Stock solution can be stored at 4°C for up to 2 months.

9. Determine the concentration of the sperm stock solution. Dilute the stock solution with coating buffer to a final concentration of 20,000 spermatozoa/ml.
2. ELISA SOLUTIONS

1. PBS-Tween 20 Rinsing Buffer:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>0.899 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>

Dissolve in 1 liter distilled water, adjust pH to 7.4. (Use 1N NaOH for adjustment) Add 0.5 ml Tween 20. Approximately 1 liter of this solution is used per 12 Elisa plates.

2. PBS-Tween 20-Gelatin Blocking Solution:

1 liter PBS-Tween 20 Rinsing Buffer
2.5 g gelatin

Dissolve gelatin at 80°C. Let cool.

3. Primary antibody - anti-turkey IgG conjugated with peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD.). Prepare a 1:2500 dilution with PBS-Tween-20-Gelatin. Need approximately 10 ml per Elisa plate.

4. O-phenylenediamine dihydrochloride substrate.

Mix together:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>citric acid</td>
<td>5.106 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>7.296 g</td>
</tr>
</tbody>
</table>

1 liter distilled water

Add 40-mg O-phenylenediamine dihydrochloride per 100 ml citric acid/Na$_2$PO$_4$ solution. One or two minutes before using, add 0.15 ml 30% hydrogen peroxide per 100ml citric acid/Na$_2$PO$_4$ solution.

5. Stopping solution - .2M Sulfuric acid solution:
3. ELISA PROCEDURE

1. Add 100 microliters diluted sperm suspension to each well of the ELISA plate using the octapipette. Seal with laminated paper (or stack plates with an empty plate on top) and incubate overnight (16 hrs) at room temperature.

2. Remove laminated paper and remove excess liquid from the well. Gently rinse plate once with PBS-Tween 20 rinsing buffer. Flick out liquid in sink. Blot dry by gently tapping inverted plates on paper towels.

3. Add 100 microliters warm (37° C) PBS/Tween-20/Gelatin (blocking solution) to each well. Incubate uncovered 1 hr at room temperature.


5. Add 100 microliters blocking solution in wells B1 to H12. Put 190 microliters blocking solution in row A. Add 10 microliters serum to each well in row A to make a 1:20 dilution. Run duplicates of each serum sample. Make a series of two-fold dilutions of the sera/blocking solution down the plate through row G. Row H serves as the background control. Incubate for 30 minutes at 37° C.

6. Flick out serum in sink. Rinse 3 times with rinsing buffer. Blot dry.

7. Add 100 microliters diluted enzyme labelled anti-turkey IgG to each well. Incubate at 37° C for 30 minutes.

8. Flick out in sink. Rinse 3 times with rinsing buffer, and blot dry.

9. Add 100 microliters OPD substrate solution to each well. Incubate at room temperature in the dark for 30 minutes.

10. Stop reaction by adding 25ul .2M sulfuric acid to each well. Measure absorbance at 492 nm.

11. Subtract background absorbance (Row H) from each well. Calculate average absorbance between duplicate samples at each serum dilution.

12. A positive titer is expressed as the reciprocal of the highest dilution of the serum which has an absorbance 1.5 times that of the negative control.
APPENDIX II

MICROAGGLUTINATION METHOD
Microagglutination Method for the Detection of Turkey Anti-sperm Antibodies

1. Place 25 microliters of Beltsville Semen Extender (Sexton, 1977b) in each well of a V-bottomed microtitration plate.

2. Put 25 microliters of serum sample into row 1 of the plate.

3. Make a series of 2-fold dilutions of the serum across the plate. Do not add serum to row 12 of the plate, which will serve as the negative control.

4. Obtain a fresh, pooled turkey semen sample.

5. Determine the semen concentration.

6. Prepare the antigen solution by diluting the semen sample to a 1\% sperm solution with Beltsville Semen Extender. Add 10 microliters of green food coloring per 100 ml of solution.

7. Add 25 microliters of sperm solution to each well of the plate.

8. Incubate in a sealed container for 30 minutes at 37° C.


10. Determine the antibody titer by taking the reciprocal of the highest dilution where there is visible agglutination of spermatozoa.
APPENDIX III

PROCESSING SCHEDULE FOR
UTEROVAGINAL SPERM STORAGE GLANDS
TIMING SCHEDULE FOR PARAFFIN PROCESSING OF UTEROVAGINAL SPERM STORAGE GLANDS

1. Fix 1-2 pieces of tissue overnight in 10% neutral buffered formalin.
2. Transfer tissues to 70% ethyl alcohol: 1 hour.
3. Transfer tissues to 80% ethyl alcohol: 1 hour.
4. Transfer tissues to 95% ethyl alcohol: 2 changes of 45 minutes each.
5. Transfer tissues to absolute ethyl alcohol: 2 changes of 45 minutes each.
6. Transfer tissues to toluene No.1: 45 minutes.
7. Transfer tissues to toluene No.2: 1 hour.
8. Transfer to melted paraffin* No.1: 45 minutes.
9. Transfer to melted paraffin* No.2: 1 hour.
10. Embed in melted paraffin*

* Temperature of melted paraffin not to exceed 58° C.
APPENDIX IV

IMMUNOPEROXIDASE STAIN FOR TURKEY IgG
STAINING PROCEDURE FOR ANTI-TURKEY IgG IN UTEROVAGINAL SPERM STORAGE GLANDS IN TURKEY BREEDER HENS

SOLUTIONS REQUIRED

1. Washing Buffer: NaCl 8.0 grams, KH₂PO₄ 0.2 grams, Na₂HPO₄ 0.89 grams, KCl 0.2 grams. Dissolve in 1 liter of distilled water, adjust pH to 7.4 (use 1N NaOH for adjustment).

2. Peroxide substrate solution: Mix together an equal volume of 0.02% hydrogen peroxide (made in distilled water from 30% stock) and 0.1% (1 mg/ml) diaminobenzidine tetrahydrochloride (DAB) made in 0.1 M Tris buffer, pH 7.2. This solution should be prepared immediately before use.

3. Normal rabbit serum, biotinylated anti-goat IgG, ABC reagent provided in Goat Vecta Stain Kit (Vector Laboratories, Burlingame, Ca.).

4. Anti-turkey IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD.).
STAINING PROCEDURE FOR ANTI-TURKEY IgG IN UTEROVAGINAL SPERM STORAGE GLANDS OF TURKEY BREEDER HENS

Controls: Turkey spleen with and without primary antibody. Test section without primary antibody.

1. Place sections in a 57° C oven for 60 minutes or a 37° C oven overnight.

2. Deparaffinize in two changes of xylene, 10 minutes each. Hydrate sections through graded alcohol series.

3. Quench endogenous peroxidase by incubating sections for 30 minutes in 0.3% hydrogen peroxide in methanol. At this time, prepare reagents provided in Vecta Stain kit.

4. Wash in buffer for 20 minutes.

5. Incubate sections for 20 minutes with normal rabbit serum from Vecta Stain kit (Vector Labs).

6. Blot excess serum from sections. Incubate sections for 30 minutes with anti-turkey IgG (Kirkegaard and Perry Labs) diluted 1:1300 in buffer.

7. Wash slides for 10 minutes in buffer.

8. Incubate sections for 30 minutes with diluted biotinylated antibody from Vectastain kit.

9. Wash slides for 10 minutes in buffer.

10. Incubate sections for 30 minutes with Vecta Stain ABC reagent.

11. Wash slides for 10 minutes in buffer.

12. Incubate sections for 4 minutes in peroxidase substrate solution.

13. Wash sections for 5 minutes in tap water.

14. Stain sections in hematoxalin for 1 minute. Wash in tap water, dip sections twice in 1% HCL, wash under running tap water for 3 minutes, dip in dilute ammonium hydroxide for 10 seconds, wash in running tap water 2 min.

15. Dehydrate, clear and mount.
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