AN ABSTRACT OF THE THESIS OF


Title: Demonstration of a Link Between Seminal Plasma Proteins and Male Fertility in the Domestic Fowl (Gallus Domestius).

Abstract Approved: Redacted for Privacy

The objective of this research was to clarify the basis of subfertility in Delaware roosters. It was anticipated that a sensitive method would be needed to compare seminal plasma protein composition between subfertile and fertile roosters. Consequently, the applicability of two-dimensional electrophoresis was tested as a tool for the analysis of chicken seminal plasma proteins. Two-dimensional electrophoresis resolved 95 ± 4.4 derivative polypeptides from seminal plasma proteins of fertile roosters, whereas one-dimensional electrophoresis resolved only 23 ± 0.4. Thus, two-dimensional electrophoresis was found to be a useful tool for seminal plasma protein analysis. Seminal plasma composition was compared between subfertile Delaware and fertile roosters. Seminal plasma from subfertile roosters was characterized by an imbalance of proteins, electrolytes, and amino acids (P<0.05). Neither type of seminal plasma contained proteolytic activity. No difference (P>0.05) was observed in seminal
plasma osmolality. Differences in seminal plasma composition were attributed to a dysfunction of the excurrent ducts of the testis. This realization lead to experiments designed to modulate subfertility. Hemicastration exacerbated (P<0.001) subfertility, whereas supplementation of spermatozoa with seminal plasma proteins from fertile roosters ameliorated (P<0.001) subfertility. Addition of seminal plasma proteins from subfertile roosters to spermatozoa from fertile roosters had no effect (P>0.05) on fertility. Therefore, subfertility was attributed to protein deficiency in seminal plasma rather than the presence of some agent that induces subfertility. The study of subfertile Delaware roosters has helped establish a link between seminal plasma proteins and fertility in the domestic fowl.
Demonstration of a Link Between Seminal Plasma Proteins and Male Fertility in the Domestic Fowl (Gallus domesticus).

by

Abdulwali M. Al-Aghbari

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Redacted for Privacy

Head of Department of Animal Sciences

Redacted for Privacy

Dean of Graduate School

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Al-Aghbari Abdulwali
DEDICATION

This Doctoral Dissertation is dedicated to my parents, my wife and my children, Amal, Pacheer and Belaal. I couldn't have accomplished this without your love, patience and support. Thanks a lot for everything.
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$$y(x) = \frac{98.5}{1+e^{-5261(12.6-x)}}$$
and

$$y(x) = \frac{103.4}{1+e^{-3749(7.4-x)}}.$$

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$$y(x) = \frac{[97.9]}{[1+e^{-5143(8.42-x)}]}$$

and

$$y(x) = \frac{[95.9]}{[1+e^{-6068(10.76-x)}]}.$$
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\[
y(x) = [97.9]/[1+e^{-3.303(9.2-x)}],
\]

\[
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\]

and

\[
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\[ y(x) = \frac{66.7}{1 + e^{-0.1+6.5579(5.01-x)}} \]
and
\[ y(x) = \frac{85.5}{1 + e^{-0.4736(8.35-x)}} \].

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A1.2 Duration of fertility following intravaginal insemination of Single Comb White Leghorn hens with spermatozoa suspended either in Beltsville Poultry Semen Extender (BPSE; ○), or BPSE supplemented with the high molecular weight fraction of seminal plasma ( ● ). Each suspension contained 2 x 10^8 viable spermatozoa per ml. Each hen was inseminated with 10x10^6 spermatozoa. Solid lines represent the functions

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- R/r+ sd+/sd+ ( □)
- R/r+ Sd+/sd+ ( ▽)
- R/R sd+/sd+ ( ○)
- R/R Sd+/sd+ ( △)

roosters. Each hen (n = 60 per treatment) was inseminated with 1x10^8 spermatozoa. Solid lines represent the functions

\[ y(x) = \frac{94.7}{1 + e^{-0.5228(12.1-x)}} \],
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CHAPTER I

INTRODUCTION

A primary goal of research in reproductive physiology of male domestic animals is the efficient use of semen. This includes maximizing the fertilizing ability of spermatozoa and using the smallest number of spermatozoa for maximal fertility. Chicken spermatozoa can survive in the oviduct for 2-3 weeks (Hodges, 1974; Johnson, 1986), and as a result, a hen is expected to lay many fertilized eggs following a single insemination. When a hen is bred naturally or by artificial insemination, spermatozoa are deposited within the initial segment of the oviduct, i.e. the vagina. Following spermatozoal deposition in the lumen of the vagina, spermatozoa enter specialized storage sites known as the uterovaginal glands (Bobr et al., 1964). Thereafter, spermatozoa are released gradually over a period of weeks back into the lumen of the oviduct (Zavaleta and Ogasawara, 1987). Some of these spermatozoa reach the site of fertilization (Olsen and Neher, 1948) in the terminal segment of the oviduct known as the infundibulum.

In general, spermatozoa undergo a number of changes between their release from the seminiferous epithelium of
the testis and the site of fertilization in the oviduct. Unlike mammalian testicular spermatozoa, rooster spermatozoa do not undergo any morphological changes during their passage through the excurrent ducts of the testis (Tingari, 1973). Rather, rooster spermatozoa undergo biochemical changes that constitute sperm maturation. These include the acquisition of protein (Esponda and Bedford, 1985), an ability to become motile at the time of ejaculation (Ashizawa and Sano, 1990), and changes in electrolytes associated with spermatozoa (Ashizawa et al., 1988).

Esponda and Bedford (1985) discovered that protein, apparently secreted from excurrent duct epithelial cells, coats the surface of spermatozoa as they pass through the excurrent ducts of the testis, i.e. these proteins are not found on testicular spermatozoa. These proteins remain attached to the surface of spermatozoa sequestered within the uterovaginal glands (Morris et al., 1987). These researchers postulated that maturation proteins may be essential to spermatozoal storage or survival in the oviduct. They tried to use antibodies as a tool to study the relationship between maturation proteins and fertility. However, neither IgG nor F\textsubscript{ab} were acceptable tools because of adverse effects on spermatozoa.

Subfertile Delaware roosters appeared to provide an alternative approach (Froman, 1990). Spermatozoa from these
roosters die prematurely either within the excurrent ducts of testis (Froman and Bernier, 1987) or the oviduct following insemination (Kirby et al., 1989). Spermatozoal degeneration has been associated with malformed efferent ducts (Kirby et al., 1990). These ducts are characterized by an aberrant surface to volume ratio. In fertile roosters the efferent ducts constitute 60 percent of excurrent duct volume within the epididymis (Aire, 1979), and absorb 86 percent of testicular fluid in quail (Clulow and Jones, 1988).

Therefore, this research was designed to further clarify the basis of subfertility of Delaware roosters. Based upon previous research, it was anticipated that subfertile roosters would be characterized by an abnormal complement of seminal plasma proteins and that this phenomenon would be related to spermatozoal survival.
REFERENCES


CHAPTER II

LITERATURE REVIEW

ANATOMY AND HISTOLOGY OF THE MALE REPRODUCTIVE TRACT

The anatomy and histology of the male reproductive tract in *Gallus domesticus* has been thoroughly described (for reviews see Lake, 1971; Hodges, 1974; Lake, 1981; Johnson, 1986). Therefore, a general overview follows. The testes in the male fowl are paired and intra-abdominal are loosely attached to the dorsal abdominal wall by short peritoneal folds, or mesorchia (Gray, 1937; Parker et al., 1942; Kumaran and Turner, 1949). They are supplied medially, together with their contiguous ducts, with blood from the cranial renal artery, which is a branch of the dorsal aorta. Blood is drained by the testicular vein which discharges into caudal vena cava (Kumaran and Turner, 1949; Lake, 1957; Hodges, 1974; Johnson, 1986). The nerve plexus of the testis is a branch of the dorsal aortic plexus (Tingari and Lake, 1972). The size and weight of the testes vary according to age and breed. For example, in adult White Leghorn roosters, the length and width of the right testis range between 1.5 to 4.5 cm and 0.7 to 1.7 cm, respectively. The weight ranges from 7 to 30 g (Gray, 1937; Parker et al., 1942; Kumaran and Turner, 1949). Oftentimes the left testis is slightly larger than the right
Lake, (1981). In general, testis weight constitutes approximately 1% of total body weight (Parker et al., 1942; Kumaran and Turner, 1949). Testes are white and bean-shaped in sexually mature roosters. Each testis is surrounded by a thin layer of fibro-elastic tissue called the tunica albuginea (Hodges, 1974; Johnson, 1986). Because the fowl testis has no septa, the parenchymal tissue is not divided into lobules (Nonidez, 1925; Gray, 1937; Parker et al., 1942; Kumaran and Turner, 1949). Instead, thin connective tissue extends from the tunica albuginea to separate the seminiferous tubules from each other. This region is referred to as interstitial tissue and contains Leydig cells, which are the primary steroidogenic cells of the testis (Nonidez, 1925; Sluiter and Van Oordt, 1947; Nicholls and Graham, 1972). The seminiferous tubules constitute the parenchyma of the testis. The seminiferous epithelium contains somatic and germinal cells (Gray, 1937; Lake, 1957; Hodges, 1974).

The Sertoli cell, or sustentacular cell, is the only type of somatic cell within the seminiferous epithelium. These cells have large well-developed basal nuclei (Rothwell, 1973; Cooksey, 1973; Hodges, 1974) and are attached to the basal lamina of the seminiferous tubule (Gray, 1937; Cooksey, 1973; Johnson, 1986). The Sertoli cell has numerous functions. According to a recent review
by Bardin et al. (1988), the Sertoli cells help direct the process of spermatogenesis by secreting numerous compounds and by partitioning the seminiferous epithelium into two functional compartments. The barrier between these compartments is known as the blood-testis barrier (Osman et al., 1980; Bergmann and Schindelmeiser, 1987). The opposing membranes of adjacent Sertoli cells are adhesive at multiple levels (Cooksey, 1973; Osman et al., 1980; Bergmann and Schindelmeiser, 1987). Because of the blood-testis barrier, the chemical milieu in the basal compartment of the seminiferous epithelium is distinct from that of the adluminal compartment (Bardin et al., 1988).

Germ cells include spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa. These cells are found in distinct associations within the seminiferous epithelium. Each association is referred to as a stage of spermatogenesis. Spermatogonia exist within the basal compartment of the seminiferous epithelium and are thus adjacent to the basal lamina. As spermatogenesis proceeds, cells derived from spermatogonia are moved towards the lumen of the seminiferous tubule.

Spermatogonia are diploid cells that give rise to stem cells or primary spermatocytes. The latter undergo the first meiotic division to yield secondary spermatocytes. The secondary spermatocytes undergo the second meiotic
division to yield spermatids. The spermatid undergoes several morphological changes as it is transformed into a mature spermatozoon. These changes include the following: chromosomes condense into a cylindrical compact nucleus, the Golgi body is transformed into an acrosome, an axoneme develops, mitochondria form a helix around the initial portion of the axoneme, and cytoplasmic volume is reduced. Spermatogenesis in the domestic fowl requires approximately 18 days (de Reviers, 1975; de Reviers and Williams, 1984). Unlike mammals, spermatogenesis proceeds at deep body temperature (Williams, 1958; Herin et al., 1960). The end result of spermatogenesis is a vermiform spermatozoon that is approximately 0.5 μm wide and 80 to 100 μm long (Bakst and Howarth, 1975; Thurston and Hess, 1987).

Spermiation is defined as the release of mature spermatozoa from the seminiferous epithelium into the lumen of the seminiferous tubule. Unlike mammalian testicular spermatozoa, fowl testicular spermatozoa have no cytoplasmic droplets (Tingari, 1973a). Therefore, the morphology of testicular fowl spermatozoa is identical to that of ejaculated spermatozoa. Consequently, no morphological change occurs during spermatozoal passage through the excurrent ducts of the testis (Tingari, 1973a). Nonetheless, a profound functional change occurs during spermatozoal passage through the excurrent ducts. Unlike
mammalian spermatozoa, this change does not pertain to the acquisition of fertilizing ability; for fowl testicular spermatozoa are capable of fertilizing oocytes if they are surgically deposited within the oviduct above the uterovaginal junction (Howarth, 1983; Kirby et al., 1990). Rather, the functional change is related to spermatozoal storage within the uterovaginal glands. When testicular spermatozoa are placed within the vagina, oocytes are not fertilized (Munro, 1938; Howarth, 1983; Kirby et al., 1990). In contrast, ejaculated spermatozoa enter the uterovaginal glands (Bobr et al., 1964) and fertilize oocytes over a period of weeks following a single insemination (Hodges, 1974; Johnson, 1986).

The excurrent ducts include the rete testis, efferent ducts, connecting ducts, epididymal duct, and deferent duct. As stated above, there are no septa in the fowl's testis. As a result, the seminiferous tubules form a complex anastomotic network (Gray, 1937; Lake, 1957), which opens at the border of the rete testis. Thus, the rete testis connects the seminiferous tubules with the excurrent ducts of the epididymis. Like the testis, the epididymis is enclosed by connective tissue that is continuous with the tunica albuginea. The epididymis contains the following types of ducts: rete testis, efferent ducts, connecting ducts, and epididymal duct. In addition, the epididymis
contains two types of vestigial ducts: the appendix epididymis and paradidymal ducts (Aire, 1979, 1980; Bakst, 1980; Johnson, 1986). According to Budras and Sauer (1975), the vestigial ducts are scattered throughout the epididymis, and they appear to be nonfunctional. An appendix epididymis is short blind-ending duct. It emerges from the epididymal duct, and its blind end is connected dorsally to the edge of the adrenal gland. As implied by the name, paradidymal ducts are found alongside the epididymal duct but are not connected to it (Budras and Sauer, 1975; Lake, 1981).

The rete testis is located at the border of the testis and epididymis. It conveys spermatozoa and testicular fluid from the seminiferous tubules to the efferent ducts. The rete testis is a mass of irregular channels and constitutes 22.6% of the epididymal excurrent duct volume (Aire, 1979). These channels are embedded in fibrous connective tissue and are connected to seminiferous tubules at several points along the edge of the testis (Gray, 1937; Tingari, 1971, 1973a; Budras and Sauer, 1975; Aire, 1980; Bakst, 1980).

The rete testis is lined with a squamous to low cuboidal epithelium. Cuboidal cells are approximately 6 μm in height (Tingari, 1971, 1972; Aire, 1980). These cells possess large nuclei, which occupy most of the cytoplasm (Tingari, 1972). Cuboidal cells appear to phagocytose
spermatozoa (Tingari, 1971, 1972). Recently, Nakai et al. (1989) observed spermiophagy in the rete testis by macrophages. It appears that spermatozoa spend a very short period of time in the rete testis. For example, Japanese quail spermatozoa spend only 25 seconds in this region prior to their passage into the efferent ducts (Clulow and Jones, 1988).

An abrupt change from cuboidal into pseudostratified columnar epithelium is evident at the juncture of the rete testis and efferent ducts. Efferent ducts emerge along the whole length of the rete testis. The efferent ducts have been classified as proximal or distal based on their diameter and location (Tingari, 1971; Budras and Sauer, 1975; Aire, 1979, 1980; Bakst, 1980). The proximal efferent ducts are large ducts, approximately 500 μm in diameter. The epithelium of the proximal efferent ducts is characterized by high mucosal folds that occupy much of the cross-sectional area of any given duct (Tingari, 1971; Kirby et al., 1990). The extensive epithelial folds generate a large surface to volume ratio within the duct. In contrast, distal efferent ducts are narrow, about 100 μm in diameter, and are characterized by lower mucosal folds than that of the proximal efferent ducts (Tingari, 1971; Aire, 1979; Bakst, 1980; Kirby et al., 1990). The pseudostratified columnar epithelium is approximately 26 μm in height in
either the proximal or distal efferent ducts (Tingari, 1971, 1972; Aire, 1980).

Studies of the ultrastructure of proximal efferent duct epithelial cells have demonstrated the presence of three types of cells: non-ciliated type I columnar cells, ciliated cells, and basal cells (Tingari, 1972, 1973a; Aire, 1980). The primary morphological features of the predominant non-ciliated type I columnar cells have been examined in detail by Tingari (1972). These cells are characterized by microvilli, bundles of filaments in the apical cytoplasm, numerous mitochondria, cisternae of well-developed smooth and rough endoplasmic reticula, numerous lysosomes and secretory vesicles as well as abundant electron-dense secretory granules (Tingari, 1972, 1973a; Aire, 1980). Such morphological attributes denote secretory and absorptive activity (Tingari, 1972; Aire, 1980; Bakst, 1980).

The ciliated cells are characterized by the presence of numerous cilia protruding into the lumen from the apical cytoplasm. They have few microvilli, small Golgi complexes, few rough endoplasmic reticula, and lack smooth endoplasmic reticula. The mitochondria are numerous but smaller than those of the non-ciliated type I epithelial cells. Ciliated cells have irregularly shaped nuclei, large electron-dense bodies, and very scanty lysosomes (Tingari, 1972, 1973a;
Aire, 1980). These cells are not secretory but could have absorptive activity (Tingari, 1972).

The basal cells have been considered as the precursors of the other epithelial cells (Tingari, 1972). Basal cells have an irregular cuboidal shape and large nuclei occupying most of cytoplasm. They possess few mitochondria, scanty Golgi complexes, and few rough endoplasmic reticula (Tingari, 1972, 1973a; Aire, 1980).

In contrast, the epithelium of the distal efferent ducts contains non-ciliated type II columnar cells, numerous ciliated cells, and basal cells Tingari (1972; Aire, 1980). The non-ciliated type II cells are characterized by numerous microvilli on their apical surfaces, numerous mitochondria, cisternae of smooth endoplasmic reticula, along with only a few electron-dense secretory granules and lysosomes (Tingari, 1972, 1973a; Aire, 1980). The structural features denote absorptive activity (Tingari, 1972; Aire, 1980). The morphological features of the ciliated and basal cells have been described above.

The efferent ducts are the primary excurrent ducts in the rooster's epididymis. In fact, the efferent ducts constitute approximately 60% of the epididymal excurrent duct volume (Aire, 1979). Studies with Japanese quail have shown that the efferent ducts absorb 86% of testicular fluid that enters them (Clulow and Jones, 1988). Fluid absorption
by rooster efferent ducts has been confirmed by Nakai et al. (1989). Spermatozoa are concentrated and apparently mixed with secretions as they pass through the efferent ducts. Based upon a study with Japanese quail (Clulow and Jones, 1988), spermatozoal transit through the efferent ducts is rapid. Clulow and Jones (1988) estimated that quail spermatozoa pass through the efferent ducts within eight minutes.

Connecting ducts link the distal efferent ducts with the epididymal duct. The connecting ducts constitute only 4% of the epididymal excurrent duct volume (Aire, 1979). Unlike the efferent ducts, the inner lining of the connecting ducts has a smooth contour (Tingari, 1971). Initially, lumens are narrow (60 μm in diameter) but gradually increase in width (200 μm) as the connecting ducts anastomose with epididymal duct (Gray, 1937; Tingari, 1971; Aire, 1980; Bakst, 1980; Lake, 1981). The epithelial cells of the connecting ducts are similar to those of the distal efferent duct except that the pseudostratified columnar cells decrease from 26 to 15 μm in height (Tingari, 1971; Aire, 1980). In Japanese quail, spermatozoa pass through the connecting tubules within 22 minutes (Clulow and Jones, 1988).

The epididymal duct is the last type of excurrent duct within the epididymis. While it would seem likely to be the
primary duct within the epididymis, the epididymal duct actually constitutes only 12.9% of the epididymal excurrent duct volume (Aire, 1979). The tortuous epididymal duct has a smaller luminal diameter than that of the proximal efferent duct. The luminal diameter is 300 μm at the cranial end of the testis and enlarges to 400 μm at the caudal end (Tingari, 1971). The mucosa is characterized by low folds (Bakst, 1980). The pseudostratified columnar epithelium is approximately 30 μm in height (Gray, 1937; Tingari, 1971). The epididymal duct is lined by only two types of epithelial cells: non-ciliated type II columnar cells and basal cells Tingari (1972, 1973a). The morphological features of the non-ciliated type II cells and basal cells are similar to those found in the distal efferent ducts. In the Japanese quail, spermatozoal transit through the epididymis requires 80 minutes. A single epididymal duct projects caudally from the connective tissue of the tunica albuginea to become the deferent duct (Gray, 1937; Tingari, 1971, 1973a).

The deferent duct is very convoluted and is enclosed by a thick layer of fibrous connective tissue (Gray, 1937). It advances caudally over the kidney, runs along the ureter, and opens into the urodeum (Lake, 1957; Tingari, 1971). The deferent duct is poorly vascularized and is characterized by a muscular wall (Gray, 1937; Tingari, 1971). This smooth
muscle layer may help propel semen caudally by peristalsis (Gray, 1937; de Reviers, 1975). Upon dissection, the deferent duct is the most obvious of the excurrent ducts of the testis. Its luminal width increases gradually along its length. The luminal diameter is 400 μm at the cranial end, 550 μm at the middle of the duct, and 900 μm at the terminal end of the duct (Tingari, 1971).

The deferent duct is lined by a pseudostratified columnar epithelium, which contains non-ciliated type II and basal cells Tingari (1972, 1973a). The former are 30 μm in height within the cranial section of the duct. Cell height progressively decreases to 20 μm in the middle and then to 15 μm in the terminal end of the duct (Tingari, 1971). The ultrastructure of the non-ciliated type II cells resembles that described previously for distal efferent ducts. The basal cells become more abundant toward the caudal end of the deferent duct (Gray, 1937; Lake, 1957; Tingari, 1971). Beneath the basal cells is the posterior retractor muscle, which appears important for ejaculation (Gray, 1937; Lake, 1957; Nishiyama and Fujishima, 1961).

An expanded region at the caudal end of the deferent duct is known as the receptaculum (Gray, 1937; Lake, 1957; Johnson, 1986). The receptaculum is the widest section of the deferent duct and is embedded in the fascia and muscle of the urodeum (Bakst, 1980). According to Bakst (1980) the
receptaculum is a sac-like structure and contains primary as well as secondary mucosal folds. It opens into the cloaca through the ejaculatory organ or papilla (Wolfson, 1952; Lake, 1957; Tingari, 1971; Johnson, 1986). The ejaculatory duct is about 1.5 mm in height and 2.0 mm in width (Lake, 1957; Bakst, 1980). The mucosa of the ejaculatory duct is characterized by low longitudinal folds and is lined by a pseudostratified columnar epithelium containing non-ciliated type II and basal cells (Tingari, 1972; Bakst, 1980). The ejaculatory duct is associated with the vascular bodies (Lake, 1957, Nishiyama and Ogawa, 1961), which are responsible for secretion of lymph-like fluid called transparent fluid into the proctodeal folds of the cloaca (Wolfson, 1952; Lake, 1957; Nishiyama and Fujishima, 1961; Nishiyama and Ogawa, 1961). It appears that spermatozoa spend more time in the deferent duct than any other excurrent duct. In Japanese quail, spermatozoa require 22 hours for passage through the deferent duct (Clulow and Jones, 1988).

Unlike mammals, the rooster's reproductive tract lacks accessory sex glands such as the prostate, ampulla, seminal vesicles and bulbourethral glands (Lake, 1957; Hodges, 1974; Budras and Sauer, 1975; Lake, 1966; 1981; Johnson, 1986). Therefore, the composition of the rooster's ejaculate exclusively reflects the function of the seminiferous
tubules and excurrent ducts.

SEMINAL PLASMA

Seminal plasma may be defined as the natural biological fluid that surrounds spermatozoa at the time of ejaculation (Lake and Ravie, 1960; Lake, 1966). A rooster's ejaculate ranges in volume from 0.01 to 0.9 ml (Lake, 1971; Lake, 1984; Johnson, 1986). Approximately 90% of any given ejaculate's volume is seminal plasma. The chemical composition of seminal plasma has been thoroughly described (for reviews see Lake, 1966, 1971, 1981, 1984). However, the biological functions of seminal plasma components and their effects upon spermatozoal fertilizing ability are poorly understood.

Seminal plasma obtained from an ejaculate collected by abdominal massage (Burrows and Quinn, 1937) is a composite, which appears to be derived, in part, from blood plasma components (Stratil, 1970; Esponda and Bedford, 1985; Hinton and Turner, 1988), the secretory and absorptive activities of excurrent duct epithelial cells (Tingari, 1972; Clulow and Jones, 1988; Nakai et al., 1989), and transparent fluid (Lake and Ravie, 1960; Nishiyama and Fujishima, 1961). Transparent fluid deserves special note. During sexual excitation induced by abdominal massage, transparent fluid exudes from the proctodeal folds of the cloaca (Lake and Ravie, 1960; Nishiyama and Fujishima, 1961; Nishiyama and
Ogawa, 1961). Some researchers, such as Nishiyama and Fujishima (1961), have considered transparent fluid to be a natural component of semen. However, others (Lake and Ravie, 1960; Lake, 1966) regard transparent fluid as a contaminant. In either case, seminal plasma, derived from semen collected by abdominal massage, contains substances derived from transparent fluid.

The blood-testis barrier and excurrent duct epithelia control the chemical composition of the fluid that surrounds spermatozoa at any level of the reproductive tract (Lake and Ravie, 1960; Osman et al., 1980, Bergmann and Schindelmeiser, 1987; Hinton and Turner, 1988; Jones et al., 1989; Lake, 1989). Processes include active transport (Hinton and Turner, 1988), endocytosis (Nakai et al., 1989), exocytosis (Tingari, 1972; Budras and Sauer, 1975; Bakst, 1980), and transudation as in the case of transparent fluid (Lake and Ravie, 1960; Nishiyama and Fujishima, 1961). While most seminal plasma components appear to be derived from the bloodstream, some components originate from the excurrent duct epithelial cells (Stratil, 1970; Esponda and Bedford, 1985) or as metabolic products of spermatozoa (Lake, 1966).

In the past, the general significance of seminal plasma to fowl spermatozoa has been viewed according to the following functions: 1) it acts as a buffer (Petitjean and
Servouse, 1981; Austin and Natarajean, 1989), 2) it contains nutrients and metabolic enzymes (Hammond et al., 1965; Lake and Hatton, 1969; McIndoe and Lake, 1974), 3) it contains protective proteins, such as the acrosin inhibitor identified by Lessley and Brown (1978), and 4) it provides a medium that facilitates spermatozoal passage through the excurrent ducts. However, recent research (Esponda and Bedford, 1985; Morris et al., 1987), with spermatozoal maturation proteins has shown that some seminal plasma proteins, may be important for spermatozoal storage or survival in the oviduct.

COMPOSITION OF SEMINAL PLASMA

ENZYMES

Seminal plasma contains the following enzymes: acid and alkaline phosphomonoesterase (Wilcox, 1961; Bell and Lake, 1962a,b; Hammond et al., 1965; McIndoe and Lake, 1974), acidic proteinase (Droba, 1986), aminopeptidase-like, cathepsin-like, (Bernon and Buckland, 1975), arylsulphatase (Droba and Droba, 1981, 1983), B-galactosidase (Droba and Droba, 1987), lactic dehydrogenase, glutamic-oxaloacetic transaminase, leucine-aminopeptidase, glutamic-pyruvic transaminase (GPT) (Hammond et al., 1965), and B-N-acetylglucosaminidase (McIndoe and Lake, 1974).

Acid and alkaline phosphomonoesterase activity of fowl
seminal plasma has been assayed with 4-nitrophenyl phosphate as substrate (Wilcox, 1961; Bell and Lake, 1962a,b; Hammond et al., 1965; McIndoe and Lake, 1974). The activity of either enzyme represents the amount of 4-nitrophenol released when seminal plasma is added. The acid phosphomonoesterase (acid phosphatase) activity is maximum at a pH of 4.9 whereas, the maximal activity of alkaline phosphatase is observed at a pH of 10 (Wilcox, 1961; Bell and Lake, 1962a,b; Hammond et al., 1965). Phosphatases are activated by Mn$^{+2}$, Mg$^{+2}$, Fe$^{+2}$, and Co$^{+2}$, and are inhibited by Ca$^{+2}$, Cu$^{+2}$, and Hg$^{+2}$, (Bell and Lake, 1962a; Mohan et al., 1989). Rooster seminal plasma has an extremely high acid phosphatase activity. In a comparative study including the rooster, turkey, boar, bull, rabbit and man, Bell and Lake (1962b) discovered that rooster seminal plasma contains the highest acid phosphatase activity second to that of man. However, the alkaline phosphatase activity of rooster seminal plasma was the lowest. Petitjean and Servouse (1981) reported that seminal plasma of subfertile RR (Rose Comb) chicken breeds contains significantly lower acid phosphatase than that of fertile roosters. However, no relationship was found between the activity of this enzyme and variables such as spermatozoal fertilizing ability, motility or semen volume (McIndoe and Lake, 1974; Petitjean and Servouse, 1981). It is believed that the major source
of rooster acid phosphatase is the deferent duct (Lake, 1962; Hammond et al., 1965).

Fowl seminal plasma is characterized by an acidic proteolytic activity (Droba, 1986). This activity has been measured by mixing seminal plasma with a hemoglobin solution. Proteolytic activity is inversely related to the concentration of hemoglobin remaining following incubation. Hemoglobin concentration can be determined spectrophotometrically (Droba, 1986). According to Droba (1986) the maximal activity of proteinase is observed at a pH of 3.5. Proteolytic activity is absent at neutral pH. Earlier, Lessley and Brown (1978) had suggested that the source of seminal plasma proteinase activity probably was damaged spermatozoa. However, the proteinase that has been isolated from spermatozoal acrosomes has a maximal activity at pH 8 (Langford and Howarth, 1974). Apparently, the activity of the acidic proteinase is restricted by the neutral pH of seminal plasma, and the activity of the acrosomal proteinase is inhibited by the polypeptide proteinase inhibitor of seminal plasma (Lessley and Brown, 1978).

Aminopeptidase-like and cathepsin-like enzyme activity also has been observed in fowl seminal plasma (Bernon and Buckland, 1975). This activity was assayed using L-leucine-p-nitroanilide and glycyl-L-phenylalaninamide as substrates,
respectively. The origin of these enzymes, their pH optima, and other biological properties are unknown.

Arylsulphatase activity has been detected in fowl seminal plasma (Droba and Droba, 1981). According to these authors, this enzyme catalyzes the transformation of nitrocatechol sulphate to a free sulfate and nitrocatechol. This activity is maximal at pH 5 to 6 and can be inhibited by phosphate and chloride ions. Based on DEAE-cellulose chromatography, two forms of arylsulphatase have been identified in fowl seminal plasma: arylsulphatase A and B. Their molecular weights range between 59,000 and 105,000 Kd (Droba and Droba, 1983). Dudkiewicz (1984) purified arylsulphatase from boar spermatozoal acrosomes. Therefore, degenerate acrosomes as well as the deferent duct epithelial cells may be the most likely source of arylsulfatase in rooster seminal plasma.

A β-galactosidase has been isolated from fowl seminal plasma by Sephacryl S-300 chromatography (Droba and Droba, 1987). It has a molecular weight of 100,000 kd, and a pI of 4. Enzyme activity has been assayed by incubating seminal plasma with p-nitrophenyl-β-D-galactopyranoside as substrate and measuring released p-nitrophenol. Maximal activity was observed between pH 3.6 and 4 (Droba and Droba, 1987). This activity can be induced by Cl⁻. This enzyme hydrolyzes the non-reducing galactose residue of complex glycoproteins
(Droba and Droba, 1987). Two forms of this enzyme have been purified from fowl spermatozoa (Droba, 1988), and these have similar properties to those of seminal plasma β-galactosidase.

Other enzymes found in rooster seminal plasma include the following. Lactic dehydrogenase (LDH; Hammond et al., 1965) activity is considered essential for anaerobic energy production and can be measured in vitro by the production of oxidized Nicotinamide Adenine dinucleotide (NAD) as follows: pyruvic acid + NADH → lactic acid + NAD⁺ (Hammond et al., 1965; Lake, 1966). Leucine-aminopeptidase hydrolyses peptide chains at leucine residues (Hammond et al., 1965). Glutamic-oxaloacetic transaminase (GOT) catalyses the following reaction:

\[
\text{alpha-keto-glutaric acid} + \text{aspartic acid} \rightarrow \text{oxaloacetic acid} + \text{glutamic acid}.
\]

Hammond et al. (1965) suggested that GOT and glutamic-pyruvic transaminase (GPT) probably are responsible for the high level of glutamic acid in seminal plasma. The LDH and GOT might be derived from spermatozoa (Hammond et al., 1965; Lake, 1966; Matsumoto et al., 1985). β-N-acetylglucosaminidase activity has been found in both seminal plasma and rooster spermatozoa (McIndoe and Lake, 1974). The latter have greater activity than that of seminal plasma. According to McIndoe and Lake (1974), the maximal activity of this enzyme is observed between pH 4 and
4.8.

In summary, most seminal plasma enzymes are active at acidic pH, and many of these enzyme activities are comparable to those associated with lysosomes (Pitt, 1975; Holtzman, 1976). Therefore, it appears that many seminal plasma enzymes may originate from the lysosomes of epithelial cells (Tingari, 1972; Aire, 1980) or from the acrosomes of spermatozoa (Lake, 1966, 1971).

LIPIDS

Compared to research performed with mammalian seminal plasma, relatively few analyses have been made of fowl seminal plasma lipid content. Resseguie and Hughes (1984a) identified three types of phospholipids in fowl seminal plasma: phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine. Lake (1966) suggested that phospholipids might be utilized as an alternative source of energy when glucose is deficient. However, phospholipids are not utilized by spermatozoa as a source of energy (Resseguie and Hughes, 1984a,b). In contrast, free fatty acids may serve as source of energy for spermatozoa (Wechsung et al., 1981; Resseguie and Hughes, 1984a,b; Lake, 1984).

Prostaglandins exist in rooster seminal plasma (Wechsung et al., 1981; Lake, 1984). It has been demonstrated that PGE₂, PGF₂α, and PGD₂ are synthesized from
free arachidonic acid, and this takes place in the deferent duct, but neither in the testes nor epididymis (Wechsung et al., 1981). Spermatozoal transport within the deferent duct is believed to depend upon peristalsis of smooth muscle (de Reviers, 1975; Wechsung et al., 1981; Lake, 1981), and prostaglandins may affect smooth muscle contractility.

Several researchers have measured the steroid hormones of poultry seminal plasma. Zeman et al. (1986) reported that testosterone concentration averaged 1.57 and 1.34 ng per ml in chicken seminal plasma and deferent duct fluid, respectively. Zeman et al. (1986) demonstrated that the testosterone concentration in seminal plasma does not undergo a circadian rhythm as does testosterone in blood plasma. The Leydig cell is believed to be the source of this hormone. The possible catabolism of testosterone into androstenedione via 17-β-hydroxysteroid dehydrogenase in the male reproductive tract has been noted (Tingari, 1973b; Lake, 1981; Zeman et al., 1983; Cecil and Bakst, 1988). This may provide an explanation for the gradual decline in the concentration of testosterone along the length of the male reproductive tract (Scott et al., 1963; Zeman et al., 1983; Cecil and Bakst, 1988). No correlation has been found between semen volume or spermatozoal concentration and testosterone within the seminal plasma or blood plasma (Cecil and Bakst, 1988).
AMINO ACIDS

Fowl seminal plasma contains free amino acids in small amounts, similar to that of blood plasma, except for glutamic acid, which is found in a much greater concentration than that of blood (Lake and McIndoe, 1959; Ahluwalia and Graham, 1966). According to Lake and McIndoe (1959), Lake and Hatton (1969), and Lake and Wishart (1984), the most prominent amino acid in seminal plasma is glutamic acid, which represents 70 to 90 percent of the total amino acids. The concentration of this amino acid in seminal plasma is 400 to 500 times greater than that of blood plasma (Ahluwalia and Graham, 1966; Lake and Hatton, 1969; Lake and Wishart, 1984). Therefore, the epithelial cells of the excurrent ducts appear to regulate the concentration of glutamic acid in seminal plasma.

The significance of the high concentration of glutamic acid as well as the remaining free amino acids is unknown (Thurston et al., 1982b). However, a number of researchers have speculated that the seminal plasma free amino acids may play an essential role in maintaining seminal plasma osmolality (Lake, 1966; Lake and Hatton, 1969; Thurston et al., 1982b).

ELECTROLYTES

Spermatozoa, like other cells, are surrounded by a
balance of inorganic elements, and these elements affect cellular function (El Jack and Lake, 1969; Lake and Wishart, 1984). Because reproductive tract luminal fluid contains different concentrations of electrolytes than those of blood plasma (Lake et al., 1958; Ashizawa et al., 1987, 1988; Hinton and Turner, 1988), the electrolyte composition of reproductive tract fluid must be controlled by the epithelia of the male reproductive tract. Ashizawa et al. (1988) demonstrated that Na⁺, K⁺, Cl⁻, and Ca²⁺ concentrations decrease gradually during spermatozoal passage from the seminiferous tubules to the terminus of the male excurrent ducts. In contrast, the concentration of Mg²⁺, Cu²⁺, and Zn²⁺ around spermatozoa increases gradually as spermatozoa approach the receptaculum (Ashizawa et al., 1988).

The most abundant electrolyte in seminal plasma is Na⁺, followed by Cl⁻ (El Jack and Lake, 1969; Lake and Wishart, 1984). The only electrolyte of excurrent duct fluid that has a significantly higher concentration than that of blood plasma is K⁺ (Lake et al., 1958; Lake and Wishart, 1984). It has been demonstrated (Lake et al., 1958) that the concentration of this ion is about two-fold greater than that of blood plasma. According to Clulow and Jones (1988), approximately 86 percent of the testicular fluid in Japanese quail is absorbed in the efferent ducts. The removal of water from the lumen of these ducts seems to depend upon the
active transport of Na\(^+\) and Cl\(^-\) (Hinton and Turner, 1988). The latter authors have noted that about 90 percent of testicular fluid Na\(^+\) is removed in the efferent ducts. Perhaps K\(^+\) is exchanged for Na\(^+\). Another electrolyte species that changes in concentration within the excurrent ducts is HCO\(_3\)^-. There is a decrease in luminal fluid pH between the efferent ducts and the deferent duct, which appears to be due to the removal of HCO\(_3\)^- from the luminal fluid (El Jack and Lake, 1966, 1969; Lake and Wishart, 1984; Hinton and Turner, 1988).

Blesbois and Mauger (1989) recently determined the concentration of zinc in seminal plasma. They demonstrated that the zinc concentration ranges from 1 to 3 \(\mu\)g per ml. This trace element acts as a cofactor for some hydrolytic and proteolytic enzymes. In addition, copper has been found in seminal plasma at a concentration of 0.06 \(\mu\)g per ml (Ashizawa et al., 1987, 1988; Blesbois and Mauger, 1989).

**PROTEINS**

Based on the electrophoretic studies of Stratil (1970) and Thurston et al. (1982a,b), the general classes of seminal plasma proteins are as follows: prealbumin, albumin, alpha, beta, and gamma. Pytasz and Klymiuk (1961) reported that seminal plasma protein concentration ranges between 7 to 9 mg per ml (Pytasz and Klymiuk, 1961). Stratil (1970) as well as Harris and Sweeney (1971) reported
that fowl seminal plasma contains 8 to 12 proteins. Most of these proteins were believed to be derived from blood plasma, but four were found to originate within the male reproductive tract (Stratil, 1970). Recently, Esponda and Bedford (1985) demonstrated that proteins, apparently secreted by epididymal epithelial cells, and coat the surface of spermatozoa during their passage through the excurrent ducts. It has been hypothesized that these proteins play a role in spermatozoal survival or storage in the oviduct (Morris et al., 1987). Using antibodies to these proteins, Morris et al. (1987) failed to identify a relationship between these proteins and spermatozoal fertilizing ability. Because antibodies caused agglutination of spermatozoa and decreased motility (Morris et al., 1987), it was concluded that neither IgG nor Fₐ₄ can be used to elucidate the relationship between proteins secreted by the male reproductive tract and fertility.

A proteinase inhibitor has been purified from fowl seminal plasma by affinity chromatography (Lessley and Brown, 1978). It has a similar amino acid composition to that of mammalian reproductive tract inhibitors, and has a molecular weight of 6100 daltons (Lessley and Brown, 1978). According to Lessley and Brown (1978), the inhibitor is capable of suppressing acrosin, trypsin, and plasmin activity in vitro. As a result, these authors suggested
that the proteinase inhibitor might protect epithelial cells and spermatozoa from acrosin associated with or released from dead or damaged spermatozoa.

OTHER COMPONENTS

A high concentration of creatine has been detected in seminal plasma at ranged from 70 to 100 mg per 100 ml (Lake and McIndoe, 1959). This amount is much greater than that reported for mammalian seminal plasma or fowl blood plasma (Lake and McIndoe, 1959). It has been suggested that the testis of the fowl is a possible source of creatine. However, its physiological role has not been determined.

Acetyl carnitine and carnitine have been found in seminal plasma at a concentration ranging from 10 to 20 times that of blood plasma (Lake, 1984). While these molecules may promote fatty acid transport into spermatozoal mitochondria, this role has not been demonstrated to date. Uric acid is another substance commonly found in fowl seminal plasma. The concentration of uric acid is influenced by urine contamination. Its concentration ranges from 19 mg per 100 ml in uncontaminated seminal plasma to as much as 379 mg per 100 ml in contaminated seminal plasma (Lake, 1966; Petitjean and Servouse, 1981; Austin and Natarajean, 1989). This variation has no significant effect on duration of fertility but it may decrease the pH of
seminal plasma.

The pH of seminal plasma ranges from 7.0 to 7.6 (Lake, 1966). The pH is not only influenced by the level of urine contamination, but also by lactic acid concentration. Lactic acid ranges from 27 to 100 mg per 100 ml, and its concentration is dependent upon anaerobic glycolytic activity (Lake, 1966; Petitjean and Servouse, 1981; Austin and Natarajean, 1989).

A growth factor has been detected recently in Japanese quail testicular fluid (Jones et al., 1989). It has a molecular weight between 14,000 and 20,000 daltons. This growth factor may regulate spermatogenesis or the secretory activity of excurrent duct epithelial cells (Jones et al., 1989).

SUBFERTILE DELAWARE ROOSTERS

Delaware chickens have been maintained at the Oregon Agricultural Experiment Station since 1946. The subfertility of Delaware roosters was recognized first in 1964 by Drs Bernier and Parker (Bernier, personal communication). Through extensive breeding and selection which proceeded until 1977, a heritable basis for subfertility was established. Following Dr. Bernier's retirement, selection for this trait ceased until 1984 when selection resumed.

In 1987, Froman and Bernier identified the cause of
heritable subfertility. Subfertility was attributed to spermatozoal degeneration, which was observed to occur within the deferent duct. Ejaculated semen or semen obtained from the mid- to distal deferent duct was characterized by 40 to 60% degenerated spermatozoa (Froman and Bernier, 1987). Froman and Bernier (1987) demonstrated that the proportion of spermatozoal degeneration, as evidenced by ethidium bromide uptake (Bilgili and Renden, 1984), can be decreased to less than 3% by daily ejaculation. Based upon this observation, Froman and Bernier (1987) concluded that spermatozoal degeneration was not due to aberrant spermatogenesis but a malfunction of the deferent duct.

Because degenerate spermatozoa were characterized by disrupted plasma membranes (Froman and Bernier, 1987) and zinc appears to promote membrane stability (Bettger and O'Dell, 1981), Kirby and Froman (1988) hypothesized that dietary supplementation with zinc might promote spermatozoal viability. Feeding Delaware roosters a ration containing 25 times the daily requirement of zinc did not decrease spermatozoal degeneration. Because their hypothesis had to be rejected, Kirby et al. (1989) tested the hypothesis that spermatozoa degenerate within the oviduct as well as the excurrent ducts of the testis. They proposed that a diminished duration of fertility would be symptomatic of
spermatozoal degeneration within the oviduct. Kirby et al. (1989) obtained viable spermatozoa by frequent ejaculation and then performed comparative and competitive fertility trials. Kirby et al. (1989) observed that spermatozoa from subfertile Delaware roosters are characterized by a shortened life in the oviduct. In other words, spermatozoal degeneration was not limited to the excurrent ducts of the testis. Kirby et al. (1989) hypothesized that spermatozoal degeneration was attributable to a premature activation of spermatozoal metabolism within the deferent duct.

An alternative hypothesis was autoimmunity to spermatozoa. Based upon the study of Classen (1977), the symptoms of autoimmunity to fowl spermatozoa include IgY associated with spermatozoa and increased numbers of lymphocytes within the excurrent ducts. Froman et al. (1990) hypothesized that heritable spermatozoal degeneration might be associated with an autoimmune condition. These authors evaluated the immunoglobulin content of reproductive tracts from subfertile roosters via immunofluorescence microscopy. In this study no such symptoms were observed. Consequently, the autoimmunity hypothesis was rejected as the basis for heritable spermatozoal degeneration.

Based on the study of Kirby et al. (1989), heritable spermatozoal degeneration was attributed to a spermatozoal
defect. It was hypothesized that this defect could be either inherent or acquired. In other words, the effect of an inherent defect could be latent at the time of spermiation or the defect could be acquired during spermatozoal passage through the excurrent ducts. Therefore, the objectives of Kirby et al. (1990) were to determine if spermatozoal passage through the excurrent ducts was associated with spermatozoal degeneration and to study the histology of the excurrent ducts. By using intramagnal insemination of ejaculated and testicular spermatozoa, duration of fertility could be assessed for spermatozoa that had and had not passed through the excurrent ducts. Kirby et al. (1990) discovered that the fertilizing ability of testicular spermatozoa from subfertile roosters did not differ from that of fertile roosters. However, ejaculated spermatozoa from subfertile roosters were characterized by a diminished duration of fertility. Consequently, these authors demonstrated that spermatozoal passage through the excurrent ducts of the testis contributes to premature death of spermatozoa from subfertile Delaware roosters.

Based on a morphometric examination of epididymal excurrent ducts, Kirby et al. (1990) demonstrated that the proximal efferent ducts of subfertile roosters are characterized by a greater luminal cross-sectional area and
a diminished height of mucosal folds. A diminished mucosal surface appears to create an aberrant surface to volume ratio that could contribute to spermatozoal degeneration (Kirby et al., 1990).

As described previously, the efferent ducts of normal, i.e. fertile roosters, are characterized by high mucosal folds (Tingari, 1971; Aire, 1980). The extensive epithelial folds create a large surface to volume ratio, which seemingly would contribute to the secretory and absorptive activities of the efferent ducts (Tingari, 1971, 1972; Clulow and Jones, 1988; Nakai et al., 1989). Because the principal functions of these ducts include testicular fluid absorption (Clulow and Jones, 1988; Nakai et al., 1989), endocytotic activity (Nakai et al., 1989), and apocrine secretion (Tingari, 1971; Bakst, 1980), Kirby et al. (1990) inferred that spermatozoa within the efferent ducts of subfertile roosters may not be adequately mixed with epithelial secretions.

As described above, Kirby et al. (1989) hypothesized that heritable spermatozoal degeneration is caused by a premature activation of spermatozoal metabolism within the deferent duct. Consequently, the study of Kirby and Froman (1991) was to assay spermatozoal metabolic activity. Spermatozoal metabolism was measured by reduction of 2-((p-iodophenyl)-3-((p-nitrophenyl))-5-phenyl tetrazolium chloride
(INT) to red formazan pigment. Cyanide was added to block cytochrome oxidase, which optimizes intermediate electron transfer to INT. The concentration of formazan was determined spectrophotometrically. Kirby and Froman (1991) did not observe a difference in metabolic activity between spermatozoa from subfertile roosters and those of fertile roosters at body temperature. They suggested that heritable spermatozoal degeneration may be most likely due to abnormal maturation during spermatozoal passage through the excurrent ducts.

Based on the study of Esponda and Bedford (1985) and Morris et al. (1987), secretory proteins are involved with spermatozoal maturation and may affect spermatozoal survival in the oviduct. Consequently, an efferent duct dysfunction in Delaware roosters may contribute to abnormal spermatozoal maturation during their passage through the excurrent ducts and a subsequent shortened life span within the deferent duct as well as the oviduct.
REFERENCES


CHAPTER III

TWO-DIMENSIONAL ELECTROPHORESIS OF CHICKEN SEMINAL PLASMA PROTEINS

ABDULWALI M. AL-AGHBARI, HAROLD N. ENGEL, JR.
AND DAVID P. FROMAN

Department of Animal Sciences and
College of Veterinary Medicine
Oregon State University
Corvallis, Oregon 97331
USA

Running Head: Two-Dimensional Electrophoresis
Section: Physiology and Reproduction

1Oregon State University Technical Paper Number _____.
2To whom correspondence should be addressed.
ABSTRACT

Chicken seminal plasma contains an array of proteins. These proteins may be partially resolved by electrophoresis. The present work describes a method for separating chicken seminal plasma proteins by two-dimensional electrophoresis. When used in conjunction with silver staining, this technique afforded the detection of 95 ± 4.4 polypeptides (n = 7 roosters). In comparison, conventional SDS-polyacrylamide gel electrophoresis followed by silver staining revealed only 23 ± 0.4 polypeptide bands (n = 7 roosters). As evidenced by the polypeptide map, the majority of polypeptides resolved by two-dimensional electrophoresis were neutral polypeptides.
INTRODUCTION

Chicken seminal plasma, like blood plasma, contains a mixture of proteins. While blood plasma proteins have been well-characterized (Butler, 1983), seminal plasma proteins have not. According to Lake (1984), little is known about the biological role of chicken seminal plasma proteins. Because proteins specific to reproductive tract fluid and spermatozoa have been identified (Stratil, 1970; Esponda and Bedford, 1985; Morris et al., 1987), it is likely that some seminal plasma proteins have a role in extra-gonadal maturation of spermatozoa. Others, such as the protease inhibitor described by Lessley and Brown (1978) probably play a protective role. The characterization of chicken seminal plasma proteins would be facilitated by the enumeration and identification of their physical properties. Electrophoresis is a highly suitable method for this purpose. To date, the characterization of poultry seminal plasma proteins by electrophoresis has been limited to either native or sodium dodecyl sulfate (SDS) one-dimensional electrophoresis (Harris and Sweeney, 1971; Thurston et al., 1982a; Thurston et al., 1982b; Hess et al., 1984; Esponda and Bedford, 1985; Morris et al., 1987).

In general, the resolving power of two-dimensional electrophoresis is much greater than that of one-dimensional electrophoresis (Dunbar, 1987). This is due to an initial
separation of proteins on the basis of isoelectric point followed by a secondary separation on the basis of molecular weight. Therefore, the objectives of the present work were: 1) to separate chicken seminal plasma proteins by one- and two-dimensional electrophoresis in order to compare the resolving power of the two techniques, and 2) to describe the polypeptide map generated by two-dimensional electrophoresis of chicken seminal plasma proteins.
MATERIALS AND METHODS

SEMINAL PLASMA

An ejaculate was collected from each of seven New Hampshire roosters according to Burrows and Quinn (1937). Each ejaculate was pipetted into a 1.5-mL microcentrifuge tube. Tubes were centrifuged at 15,600 x g for 5 min in an Eppendorf Model 5414 microcentrifuge (Brinkmann Instruments, Inc., Westbury, NY). Following centrifugation, seminal plasma supernatants were transferred to 1.5-mL microcentrifuge tubes. A 100-μl subsample was removed for determination of protein concentration with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was used as the standard. Residual seminal plasma was prepared for electrophoresis by the two methods described below.

ONE-DIMENSIONAL ELECTROPHORESIS

Separation of seminal plasma proteins by one-dimensional electrophoresis was performed according to a modification of the technique of Laemli (1970). A 30% (wt/vol) acrylamide stock solution was prepared with deionized water. This solution contained acrylamide and N'N'-bis-methylene-acrylamide at a ratio of 36 to 1. Thirty milliliters of stock solution were diluted 1:2 with 1.5 M Tris-HCl, pH 8.8, and this mixture was degassed prior to the addition of SDS and polymerization reagents. The final
monomer solution contained 12% (wt/vol) acrylamide, 0.1% (wt/vol) SDS, 0.05% (wt/vol) ammonium persulfate, and 0.05% (vol/vol) N,N,N',N'-tetramethylethylenediamine (TEMED). Immediately after mixing, 32 mL of this solution was poured between 16 x 20 cm and 18.3 x 20 cm glass plates, which were separated by 1.5 mm teflon spacers. The acrylamide solution was overlaid with water-saturated t-amyl alcohol and 1 h was allowed for polymerization. Following polymerization, the top of the gel was rinsed with deionized water. Residual water was removed by absorption into filter paper.

A 6% (wt/vol) acrylamide solution was prepared as above with the exception that the stock solution was diluted with 0.5 M Tris-HCl, pH 6.8. A teflon comb was inserted into 8 mL of the monomer solution after it had been poured on top of the polyacrylamide slab, and 1 h was allowed for polymerization. After removal of the teflon comb, each well was rinsed with deionized water. The gel sandwich was then attached to a Protean™ II slab cell (Bio-Rad Laboratories, Richmond, CA).

Prior to being pipetted into wells within the 6% (wt/vol) polyacrylamide gel, each seminal plasma sample had been treated as follows. Seminal plasma was diluted 1:4 with sample buffer and then heated at 95° C for 4 min in an Eppendorf Model 5320 constant temperature heater (Brinkmann Instruments, Inc., Westbury, NY). The sample buffer
contained 2% (wt/vol) SDS, 5% (vol/vol) β-mercaptoethanol, 0.13% (wt/vol) bromphenol blue, and 10% (vol/vol) glycerol in 0.0625 M Tris-HCl, pH 6.8. After cooling, 120-μL of each dilution was pipetted into a well and then overlaid with 50 μL of electrode buffer. The electrode buffer, pH 8.3, contained 0.1% (wt/vol) SDS and 0.192 M glycine in 0.025 M Tris-HCl.

Electrophoresis was performed at 25 mA per gel until the bromphenol blue had entered the 12% (wt/vol) polyacrylamide gel. Thereafter, current was maintained at 35 mA per gel. During electrophoresis the temperature of the slab gel was maintained at 10° C with a VWR Model 1165 refrigerated constant temperature circulator (VWR Scientific, Seattle, WA). Electrophoresis was terminated when the band of bromphenol blue was 1 cm from the bottom of the gel.

After disassembly of the gel sandwich and dissociation from the 6% (wt/vol) polyacrylamide gel, the 12% (wt/vol) polyacrylamide gel was immersed for 1 h in a 50:40:10 mixture of water, methanol, and acetic acid in order to fix proteins. Thereafter, proteins were stained with the Bio-Rad silver stain kit (Bio-Rad Laboratories, Richmond, CA). The stained gel was digitized with the Macvision System™ (Version 2.0; Koala Technologies, Scotts Valley, CA), as follows. The gel was placed on a glass plate and
illuminated from beneath. The image was recorded with a video camera equipped with a macro lens. A standard RCA jack coupled the camera to the digitizer unit, which in turn was coupled to a Macintosh II computer. The digitized image was enhanced with Image Studio™ (Version 1.52; Letraset Graphic Design, Paramus, NJ).

TWO-DIMENSIONAL ELECTROPHORESIS

Separation of seminal plasma proteins by two-dimensional electrophoresis was performed according to a modification of the technique of O'Farrell (1975). A 100-mL volume of 10% (vol/vol) Triton X-100 was deionized by the addition of 5 g AG 501-X8 ion exchange resin beads (Bio-Rad Laboratories, Richmond, CA). After stirring for 1 h, the detergent solution was decanted and used to prepare an isoelectric focusing (IEF) gel solution, which contained 6% (wt/vol) acrylamide, 2% (vol/vol) Triton X-100, 8 M urea, 4.5% (vol/vol) Bio-Lyte 5/7 (Bio-Rad Laboratories, Richmond, CA), and 0.5% (vol/vol) Bio-Lyte 3/10 (Bio-Rad Laboratories, Richmond, CA). The ratio of acrylamide to N'N'-bis-methylene-acrylamide was 36:1. The IEF gel solution was subdivided and frozen at -75° C.

After thawing, the IEF gel solution was degassed for 15 min. Polymerization was initiated by the addition of ammonium persulfate and TEMED at concentrations of 0.01% (wt/vol) and 0.1% (vol/vol), respectively. These reagents
were added as solutions, but the change in volume of the IEF gel solution was negligible. Glass tubes 180 mm in length with an inner diameter of 2 mm were sealed at one end with Parafilm (Bio-Rad Laboratories, Richmond, CA) and then filled with 480 μL of IEF gel solution. Each tube was filled from the bottom with a 185 mm fine gauge needle, and 1 h was allowed for polymerization.

Following polymerization, the Parafilm was removed from the bottom of the tube and the top of the gel was rinsed with deionized water. Thereafter, the IEF gel was overlaid with 30 μL of 0.2% (vol/vol) Triton X-100 containing 4.5 and 0.5% (vol/vol) Bio-Lyte 5/7 and 3/10, respectively. Then tubes were secured within the Protean™ II tube gel adaptor (Bio-Rad Laboratories, Richmond, CA), and this assembly was positioned with the Protean™ II electrophoresis cell so that the bottoms of the IEF gels were submerged in 0.06% (vol/vol) phosphoric acid.

Prior to casting IEF gels, seminal plasma had been processed as follows. After saturation with urea, seminal plasma was mixed 1:5 with a solution containing 33% (vol/vol) Triton X-100, 30% (vol/vol) Bio-Lyte 5/7, 17% (vol/vol) β-mercaptoethanol, 3.3% (vol/vol) Bio-Lyte 3/10, and 1.7% (wt/vol) SDS. Diluted seminal plasma was centrifuged at 15,660 x g for 15 sec. Following centrifugation, 90 μL of each supernatant was injected
slowly underneath the solution overlaying an IEF gel. Then each tube was filled to the top with degassed 0.1 N NaOH. Prior to electrophoresis, each tube was inspected to insure that the bottom was free of air bubbles. Electrophoresis was performed at 400 V for 14.5 h and then 800 V for 2 h. Throughout electrophoresis, gels were maintained at 10° C as above.

Following electrophoresis, the top and bottom of each tube was rinsed with deionized water. Gels were extruded from tubes by hydrostatic pressure induced by injection of deionized water in between the gel and surrounding tube via a beveled fine gauge needle (Bio-Rad Laboratories, Richmond, CA). Each gel was extruded into a plastic weigh boat containing 4 mL of the sample buffer described for one-dimensional electrophoresis. After submersion for 15 min, each gel was placed in a screw cap culture tube. These tubes were immersed immediately in an ethanol:dry ice bath. Frozen gels were stored at -20° C prior to vertical slab electrophoresis.

The second dimension slab gel was cast as described above with the exception that only 2 mL of the 6% (wt/vol) acrylamide solution was overlaid on the 12% (wt/vol) polyacrylamide slab and the teflon comb was omitted. Therefore, the 6% (wt/vol) polyacrylamide gel assumed the dimensions of a 2 x 20 cm rectangle. Immediately after
thawing, an IEF gel was laid on top of the 6% (wt/vol) polyacrylamide slab. Then the remaining space between the glass plates was filled with electrode buffer. Electrophoresis, silver staining, and digitization of gels were performed as described above for one-dimensional electrophoresis. Protein bands were counted in each replicate lane and gel for one- and two-dimensional electrophoresis, respectively. Band numbers were compared by single classification analysis of variance (Sokal and Rohlf, 1981).
RESULTS AND DISCUSSION

When seminal plasma proteins were reduced by β-mercaptoethanol, denatured by heat, and coated with SDS, one dimensional electrophoresis resolved 23 polypeptide bands (Table III. 1). This number is comparable to that observed by Esponda and Bedford (1985) following one dimensional electrophoresis of chicken deferent duct fluid. However, these authors did not evaluate variation among males. In the present work, the coefficient of variation for polypeptide bands was 5%. Therefore, because the pattern shown in Figure III.1 was consistent among males, both the number of bands and the molecular weights of these polypeptides were comparable among males.

Based upon band size and stain density, nine major bands were observed (Figure III.1). The molecular weight of polypeptides within these bands ranged from 12 to 80 kd. Polypeptides in the largest band, were characterized by a molecular weight of 29 kd. This differed from the results of Esponda and Bedford (1985), who observed a predominant band corresponding with a molecular weight of 60 kd. However, these authors resolved polypeptides in a 10% (wt/vol) polyacrylamide gel and did not show the distribution of their molecular weight markers.

Two-dimensional electrophoresis resolved approximately 4 times more polypeptide bands (P<.001) than were resolved
by one-dimensional electrophoresis (Table III. 1). This is attributed to the high resolution of IEF in the first dimension followed by separation according to polypeptide molecular weight (Dunbar, 1987). As observed for one-dimensional electrophoresis, little variation was observed among males (Table III. 1). A representative polypeptide map is shown in Figure III.2. Based upon staining density, band size, and band position, the majority of the polypeptides resolved by two-dimensional electrophoresis were neutral polypeptides. As also shown in Figure III.2, the majority of polypeptides tended to appear in arrays of comparable molecular weight but of differing pIs. Such arrays may denote isozymes.

The present research demonstrates that the polypeptide composition of chicken seminal plasma is much more complex than that evidenced by one-dimensional electrophoresis. Therefore, two-dimensional electrophoresis may be useful in systematic studies of neutral and acidic seminal plasma proteins. The technique may be particularly useful when used in conjunction with immunological techniques.
TABLE III.1  Polypeptides from fowl seminal plasma resolved by one (1-D) and two-dimensional electrophoresis (2-D).

<table>
<thead>
<tr>
<th>Method</th>
<th>Polypeptides&lt;sup&gt;1&lt;/sup&gt; (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-D</td>
<td>23 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-D</td>
<td>95 ± 4.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Means within a column bearing different superscripts are different (P<.001).
<sup>1</sup>Each value represents a mean ± SEM from 7 replicate males.
FIGURE III.1 Molecular weight markers and polypeptides from chicken seminal plasma separated by one-dimensional electrophoresis. Lane A is a digital image of a lane from a polyacrylamide gel containing molecular weight markers purchased from Bio-Rad Laboratories, Richmond, CA. These proteins included rabbit phosphorylase b (97.4 kd), bovine serum albumin (66.2 kd), chicken ovalbumin (45 kd), bovine carbonic anhydrase (31 kd), soybean trypsin inhibitor (21.5 kd), and chicken albumen lysozyme (14.4 kd). Lane B is a digital image of a representative lane from a polyacrylamide gel containing polypeptides from chicken seminal plasma proteins.
FIGURE III. 2 Digital image of a representative polyacrylamide gel containing polypeptides from chicken seminal plasma resolved by two-dimensional electrophoresis.
REFERENCES


CHAPTER IV

ANALYSIS OF SEMINAL PLASMA FROM SUBFERTILE DELAWARE ROOSTERS

ABDULWALI M. AL-AGHBARI, HAROLD N. ENGEL, JR.
AND DAVID P. FROMAN

Department of Animal Sciences and College of Veterinary Medicine
Oregon State University
Corvallis, Oregon 97331
USA

Running Head: Subfertile Delaware roosters

1Oregon State University Technical Paper Number _____.
2To whom correspondence should be addressed.
The objective of the present study was to compare the composition of seminal plasma from subfertile and fertile roosters. All comparisons were made with seminal plasma procured from semen characterized by equivalent spermatozoal concentration and spermatozoal viability but differing in spermatozoal fertilizing ability. Seminal plasma from subfertile roosters was characterized by an imbalance of proteins, electrolytes, and amino acids (P<0.05). Proteolytic activity was not detected in seminal plasma from either subfertile or fertile roosters. Differences in seminal plasma composition were attributed to excurrent duct dysfunction.
INTRODUCTION

Heritable subfertility in Delaware roosters was first described by Froman and Bernier (1987). Subfertility was attributed originally to spermatozoal degeneration within the deferent ducts. Froman et al. (1990) rejected autoimmunity as the cause of spermatozoal degeneration. Kirby et al. (1990) inferred that spermatozoal degeneration was attributable to a dysfunction of the excurrent ducts of the testis rather than an inherent spermatozoal defect. These authors also discovered malformed efferent ducts in subfertile roosters. Efferent ducts constitute 60% of the epididymal excurrent duct volume in the fowl (Aire, 1979). Because the fowl lacks secondary sex glands, the composition of seminal plasma necessarily is indicative of excurrent duct function. The composition of fowl seminal plasma differs from that of blood plasma with respect to free amino acids and electrolytes (Freeman, 1984) as well as proteins (Stratil, 1970; Esponda and Bedford, 1985). We reasoned that if the composition of seminal plasma from subfertile roosters differed from that of fertile roosters, then excurrent duct dysfunction would be unequivocal. Therefore, the objective of the present study was to compare the composition of seminal plasma of subfertile roosters to that of fertile roosters.
MATERIALS AND METHODS

Following 1 wk of sexual rest, subfertile Delaware roosters (n=6) and fertile New Hampshire roosters (n=6) were ejaculated daily for 25 consecutive days. On each of the first 10 days as well as the day when the last ejaculate was collected, spermatozoal viability was determined by ethidium bromide exclusion (Bilgili and Renden, 1984). In the case of subfertile roosters, mean spermatozoal viability was plotted as a function of time. Because data points conformed to a logistic function, parameters of

\[ y(x) = \frac{\gamma}{1 + e^{\beta(x-r)}} \]

were estimated by iterative least squares (Freund and Littell, 1986). The hypothesis that spermatozoal viability stabilized at 100% was tested with the extra sums of squares F test as described by Kirby and Froman (1990).

After spermatozoal viability stabilized at 100%, the fertility of each rooster was determined and seminal plasma procured as follows. Spermatozoal concentration was determined fluorometrically with a 10-μl subsample from a single ejaculate as described by Bilgili and Renden (1984). Semen was diluted to 2.0 x 10⁹ viable spermatozoa per ml with Beltsville Poultry Semen Extender, pH 7.5 (a gift from Dr. T. Sexton, USDA, Beltsville, MD). Each spermatozoal suspension was used to intravaginally inseminate 15 Single
Comb White Leghorn hens with $1 \times 10^8$ viable spermatozoa per hen. Eggs were incubated, fertility determined, and time of half-maximal fertility estimated as described Kirby and Froman (1990).

Seminal plasma was procured by centrifugation of ejaculates at 12,800 x g for 5 minutes in an Eppendorf Model 5412 microcentrifuge (Brinkmann Instruments, Inc., Westbury NY). Seminal plasma samples were used immediately for determining protein concentration, electrophoresis, and the protease assay. In contrast, samples designated for amino acid and electrolyte analyses were frozen -20° C and thawed immediately before analysis. After thawing, such samples were pooled by rooster in order to have an adequate volume for analysis.

Protein concentration was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo) was used as the standard. Concentrations of Na⁺, K⁺, and Cl⁻ were assayed with a Ciba Corning Model 664 electrolyte analyzer (Gilford System, Oberlin, OH). Concentrations of Ca²⁺ and Mg²⁺ were measured spectrophotometrically with Gilford Systems diagnostic kits. Data were analyzed by single classification analysis of variance (Sokal and Rohlf, 1981). Amino acid analysis was performed by the AAA-Laboratory, Mercer Island, WA. In brief, proteins were precipitated by
the addition of sulfosalicylic acid to a concentration of 4.5% (wt/vol) prior amino acid analysis. Following centrifugation, supernatants were applied to a column of sulfonated polystyrene. Amino acids were eluted by increasing the pH and ionic strength of the eluting buffer. Eluted amino acids were detected colorimetrically following their reaction with ninhydrin reagent. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional electrophoresis were performed as described by Al-Aghbari et al. (1992). Proteolytic activity was evaluated with the Bio-Rad protease assay. Osmolality was determined with a Wescor Model 5500 vapor pressure osmometer (Wescor, Inc., Logan, Utah).
RESULTS

As shown in Figure IV.1, Delaware spermatozoal viability improved following 1 wk of sexual rest when roosters were ejaculated daily. The hypothesis that $\gamma$, an estimate of maximal spermatozoal viability, was 100% was not rejected at ($P<0.05$). Therefore, when seminal plasma was saved for analysis, Delaware spermatozoal viability was equivalent to that of New Hampshire roosters. As shown in Figure IV.2 and Table IV.1, Delaware roosters were subfertile relative to New Hampshire roosters. The difference in $\tau$, which represents time of half-maximal fertility, was 5.3 d (Table IV.1).

As shown in Table IV.2, spermatozoal concentration did not differ ($P>0.05$) between subfertile and fertile roosters. However, both seminal plasma protein concentration (Table IV.3) and electrolyte composition (Table IV.4) differed ($P<0.05$) between subfertile and fertile roosters. Likewise, the concentration of seven species of amino acids differed ($P<0.05$) between subfertile and fertile roosters (Table IV.5).

Representative patterns of polypeptide bands separated by SDS-PAGE are shown in Figure IV.3. When 80 $\mu$g of protein were applied per lane, different patterns were observed for subfertile and fertile roosters. Polypeptides of 40 Kd and 17-22 Kd were in excess in seminal plasma from subfertile
roosters. In contrast, polypeptides of 10-16 Kd were diminished in seminal plasma from subfertile roosters. These differences were confirmed by two-dimensional electrophoresis (Figure IV.4). Differences in polypeptide patterns were not attributed to proteolytic activity in seminal plasma from subfertile roosters (Figure IV.5). As shown in Table IV.6 osmolality (mmol/kg) of seminal plasma did not differ (P>0.05) between subfertile and fertile roosters.
TABLE IV.1 Time of half-maximal fertility, $\tau$, of Single Comb White Leghorn hens following a single intravaginal insemination with $1 \times 10^8$ viable spermatozoa from either Delaware or New Hampshire roosters.

<table>
<thead>
<tr>
<th>Breed of rooster</th>
<th>$\tau^1$ (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delaware</td>
<td>7.2 ± 0.62$^a$</td>
</tr>
<tr>
<td>New Hampshire</td>
<td>12.5 ± 0.34$^b$</td>
</tr>
</tbody>
</table>

$^1$Each value represents a mean ± SEM from 6 replicate roosters. $^a,b$ Means within a column bearing different superscripts are different (P<0.0001).
TABLE IV.2 Comparison of spermatozoal concentration in semen ejaculated from subfertile Delaware and fertile New Hampshire roosters.

<table>
<thead>
<tr>
<th>Breed of rooster</th>
<th>Spermatozoal concentration (x 10^9/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delaware</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td>New Hampshire</td>
<td>5.7 ± 0.2</td>
</tr>
</tbody>
</table>

Each value represents a mean ± SEM from 6 replicate roosters.
### TABLE IV.3
Comparison of protein concentration in seminal plasma from subfertile Delaware and fertile New Hampshire roosters.

<table>
<thead>
<tr>
<th>Breed of rooster</th>
<th>Protein concentration(^1) (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delaware</td>
<td>4.8 ± 0.4(^a)</td>
</tr>
<tr>
<td>New Hampshire</td>
<td>3.2 ± 0.3(^b)</td>
</tr>
</tbody>
</table>

\(^1\)Each value represents a mean ± SEM from 6 replicate roosters. 
\(^a,b\)Means within a column bearing different superscripts are different (P<0.05).
### TABLE IV.4
Comparison of electrolyte concentrations in seminal plasma from subfertile Delaware and fertile New Hampshire roosters.

<table>
<thead>
<tr>
<th>Breed of rooster</th>
<th>Electrolyte concentration ( ^1 ) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{Na}^+ )</td>
</tr>
<tr>
<td>Delaware</td>
<td>139±1.9(^a)</td>
</tr>
<tr>
<td>New Hampshire</td>
<td>134±0.9(^b)</td>
</tr>
</tbody>
</table>

\(^1\)Each value represents a mean ± SEM from 6 replicate roosters.
\(^{a,b}\)Means within a column bearing different superscripts are different (P<0.05).
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration (mM)</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Delaware</td>
<td>New Hampshire</td>
</tr>
<tr>
<td>Alanine</td>
<td>.80±0.1*a</td>
<td>.30±0.02*b</td>
</tr>
<tr>
<td>Arginine</td>
<td>.12±0.02</td>
<td>.08±0.01</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>.96±0.04*a</td>
<td>.78±0.03*b</td>
</tr>
<tr>
<td>Cystine</td>
<td>.02±0.003</td>
<td>.02±0.004</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>49.00±3.2*a</td>
<td>69.00±4.5*b</td>
</tr>
<tr>
<td>Glycine</td>
<td>.70±0.04*a</td>
<td>.50±0.02*b</td>
</tr>
<tr>
<td>Histidine</td>
<td>.11±0.01*a</td>
<td>.08±0.01*b</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>.03±0.004</td>
<td>.04±0.003</td>
</tr>
<tr>
<td>Leucine</td>
<td>.13±0.01</td>
<td>.12±0.01</td>
</tr>
<tr>
<td>Lysine</td>
<td>.23±0.01</td>
<td>.23±0.01</td>
</tr>
<tr>
<td>Methionine</td>
<td>.03±0.003</td>
<td>.03±0.002</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>.06±0.01</td>
<td>.05±0.02</td>
</tr>
<tr>
<td>Proline</td>
<td>.20±0.01</td>
<td>.20±0.01</td>
</tr>
<tr>
<td>Serine</td>
<td>.90±0.1*a</td>
<td>.40±0.04*b</td>
</tr>
<tr>
<td>Threonine</td>
<td>.40±0.04*a</td>
<td>.30±0.01*b</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>.03±0.002</td>
<td>.02±0.01</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>.10±0.003</td>
<td>.10±0.01</td>
</tr>
<tr>
<td>Valine</td>
<td>.10±0.003</td>
<td>.10±0.01</td>
</tr>
</tbody>
</table>

1Each value represents a mean ± SEM from 6 replicate roosters.

*a,bMeans within a column bearing different superscripts are different (P<0.05).
TABLE IV.6  Comparison of osmolality in seminal plasma from subfertile Delaware and fertile New Hampshire roosters.

<table>
<thead>
<tr>
<th>Breed of rooster</th>
<th>Osmolality' (mmol/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delaware</td>
<td>295.5 ± 2.5</td>
</tr>
<tr>
<td>New Hampshire</td>
<td>295.2 ± 2.7</td>
</tr>
</tbody>
</table>

'Each value represents a mean ± SEM from 6 replicate roosters.
FIGURE IV.1 Spermatozoal viability plotted as a function of time. Each point represents a mean from 6 Delaware roosters. The solid line represents the function

\[ y(x) = \frac{101.6}{1 + e^{0.8615277-x}} \]

Roosters were ejaculated daily following 1 wk of sexual rest.
FIGURE IV.2  Duration of fertility following intravaginal insemination of Single Comb White Leghorn hens with spermatozoa from either a New Hampshire (△) or Delaware (●) rooster. Each hen (n=15) was inseminated with 1 x 10^8 viable spermatozoa. Solid lines represent the functions

\[
y(x) = \frac{98.5}{1 + e^{-526(12.6-x)}}
\]

and

\[
y(x) = \frac{103.4}{1 + e^{-3749(7.4-x)}}.
\]

Each curve is representative of the response observed with semen from either New Hampshire or Delaware roosters.
FIGURE IV.3 Standard molecular weight markers and seminal polypeptides following sodium dodecyl sulfate polyacrylamide gel electrophoresis and silver staining. Lane A represents molecular weight markers. Lanes B-E are representative of fertile New Hampshire roosters. Lanes F-I are representative of subfertile Delaware roosters. Seminal plasma was applied at 80 µg protein per lane.
FIGURE IV.4 Digital images of representative polyacrylamide gels containing polypeptides from subfertile Delaware (A) and fertile New Hampshire rooster seminal plasma (B) resolved by two-dimensional electrophoresis.
FIGURE IV.4
FIGURE IV.5  Agar gel containing casein used to assess the proteolytic activity of seminal plasma. Wells A–C and D–F are representative of fertile and subfertile roosters, respectively. Each well received 15 μl of seminal plasma. Well G received 15 μl of 10 mg bovine serum albumin per ml. Well H received 15 μl of 20 μg bovine trypsin per ml. Wells I received 15 μl deionized water. The gel was incubated for 24 h at 24° C before proteins were denatured by overlaying the gel with 3% (vol/vol) acetic acid. As evidenced by a halo around well H, only bovine trypsin tested positive for proteolytic activity at pH 7.4.
DISCUSSION

The objective of the present study was to compare the composition of seminal plasma from subfertile Delaware roosters to that of fertile roosters. This objective was based upon the following observations. First, Froman and Bernier (1987) demonstrated that spermatozoa in sexually rested Delaware roosters undergo degeneration within the deferent duct. These authors also found that spermatozoal viability could be improved by daily ejaculation. Second, Froman et al. (1990) dismissed autoimmunity as a basis for spermatozoal degeneration. Third, Kirby et al. (1990) inferred that spermatozoal degeneration more likely was attributable to abnormal spermatozoal maturation within the excurrent ducts of the testis. Because the rooster lacks secondary sex glands, a difference in seminal plasma composition necessarily would be indicative of excurrent duct dysfunction, providing that the effect of such a dysfunction was not confounded with the effect of spermatozoal degeneration. Therefore, before any analysis could be made in the present study, roosters had to be ejaculated daily to improve spermatozoal viability. As shown in Figure IV.1, daily ejaculation maintained spermatozoal viability at 100% over a period of weeks. This observation corroborated that of Froman and Bernier (1987), Kirby et al. (1989), and Kirby et al. (1990) with respect to
the relationship between spermatozoal viability and ejaculation frequency. All analyses were performed with semen characterized by 100% spermatozoal viability as determined by ethidium bromide exclusion (Bilgili and Renden, 1984).

As reported previously (Kirby et al., 1989; Kirby et al., 1990) Delaware roosters characterized by 100% spermatozoal viability due to frequent ejaculation were nevertheless subfertile. In contrast to the previous studies, which utilized pooled semen, this study evaluated subfertility on an individual basis. Representative curves are shown in Figure IV.2. Subfertility was attributable to a diminished duration of fertility. This variable can be quantified as \( \tau \), time of half-maximal fertility (Kirby and Froman, 1990) and differed (\( P<0.0001 \)) by 5.3 d (Table IV.1). In summary, seminal plasma was procured from semen samples characterized by equivalent spermatozoal viability but different spermatozoal fertilizing ability.

Due to the fowl's blood-testis barrier (Bergmann and Schindelmeiser, 1987), it is likely that the composition of seminiferous tubule fluid differs from that of blood plasma. Likewise, it is likely that the composition of deferent duct fluid differs from that of seminiferous tubule fluid. This difference may be attributed to the processes of water absorption, pinocytosis, and secretion within the excurrent
ducts of the testis. In the Japanese quail (Clulow and Jones, 1988), the efferent ducts absorb approximately 86% of seminiferous tubule fluid. Absorption of water appears to depend upon active transport of Na⁺ and Cl⁻ (Hinton and Turner, 1988). Nakai et al. (1989) observed pinocytotic activity within the efferent ducts of the fowl. Finally, apocrine secretion has been observed in micrographs of fowl efferent ducts (Tingari, 1971; Tingari, 1972; Budras and Sauer 1975; Bakst, 1980). Based upon these studies, and the fact that the fowl's efferent ducts constitute 60% of excurrent duct volume within the epididymis (Aire, 1979), the efferent ducts probably play a critical role in the formation of deferent duct fluid, and hence seminal plasma.

Kirby et al. (1990) discovered that subfertile Delaware have malformed efferent ducts and postulated that spermatozoal degeneration was probably caused by excurrent duct dysfunction. While spermatozoal concentration did not differ (P>0.05) between subfertile and fertile roosters (Table IV.2), seminal plasma composition differed with respect to protein concentration (Table IV.3), electrolytes (Table IV.4), free amino acids (Table IV.5), and protein composition (Figure IV.3; Figure IV.4). Seminal plasma from subfertile roosters contained more protein per ml (P<0.05) than seminal plasma from fertile roosters. Because spermatozoal viability was 100% when samples were collected
(Figure IV.1), this difference cannot be attributed to spermatozoal degeneration. Due to the decreased surface to volume ratio of efferent ducts in subfertile roosters (Kirby et al., 1990), this difference may be attributable to reduced absorption of proteins from seminiferous tubule fluid. This explanation might also account for differences in protein composition. Neither spermatozoal degeneration nor proteolytic activity within seminal plasma (Figure IV.5) can account for the differences in polypeptide patterns following electrophoresis (Figure IV.3; Figure IV.4).

The electrolytes in seminal plasma from fertile roosters (Table IV.4) were comparable in concentration to those reported previously for fowl deferent duct fluid (Freeman, 1984; Lake and Wishart, 1984). Seminal plasma from subfertile roosters was characterized by higher concentration (P<0.05) of Na⁺, K⁺ and Cl⁻ (Table IV.4) than was observed for fertile roosters. As explained above, these differences cannot be attributed to spermatozoal degeneration. Rather, these differences must be attributable to excurrent duct dysfunction. For example, approximately 90% of testicular fluid Na⁺ is absorbed by the efferent ducts (Hinton and Turner, 1988). Therefore, the reduced surface to volume ratio within the efferent ducts of subfertile roosters (Kirby et al., 1990) may contribute to the observed differences in electrolyte concentration.
The amino acids in seminal plasma from fertile roosters were comparable in concentration to previously reported values (Lake and Wishart, 1984). Glutamic acid was the principal amino acid in seminal plasma from both subfertile and fertile roosters (Table IV.5). However, seminal plasma from subfertile roosters had 29% less (P<0.05) glutamic acid than seminal plasma from fertile roosters. In contrast, alanine, aspartic acid, glycine, histidine, serine, and threonine were found at a higher concentration (P<0.05) in seminal plasma from subfertile roosters (Table IV.5). Perhaps these differences may also be attributable to the decreased surface to volume ratio in the efferent ducts of subfertile roosters (Kirby et al., 1990). The concentration of glutamic acid in deferent duct fluid is typically 400-500 times greater than that found in blood plasma (Lake and Mcindoe, 1959; Lake and Hatton, 1968; Lake and Wishart, 1984). Therefore, glutamic acid is secreted into the effluent of the male reproductive tract. In the case of subfertile roosters, the extent of glutamic acid secretion may be reduced. Conversely, a higher concentration of other amino acids could be attributable to decreased absorption, if these amino acids are selectively secreted into testicular fluid and then absorbed within the epididymis.

When osmolality of seminal plasma from fertile and
subfertile was evaluated, no difference (P<0.05) was observed (Table IV.6). Consequently, a difference in seminal plasma osmolality cannot account for spermatozoal degeneration in subfertile roosters.

In conclusion, Kirby et al. (1990) inferred that spermatozoal degeneration was attributable to spermatozoal passage through the efferent ducts of the testis. These authors also discovered the malformation of the proximal efferent ducts within the epididymis of subfertile Delaware roosters. The present study demonstrates that the efferent ducts of subfertile Delaware roosters are dysfunctional. However, the relationship between this dysfunction and spermatozoal degeneration remains to be elucidated. The relationship between spermatozoal degeneration and spermatozoal maturation proteins (Esponda and Bedford; Morris et al., 1987) presents an intriguing possibility.
REFERENCES


CHAPTER V

MODULATION OF FOWL SPERMATOZOAL FERTILIZING ABILITY BY HEMICAstration AND SUPPLEMENTING SPERMATOZoa WITH SEMINAL PLASMA PROTEINS¹

ABDULWALI M. AL-AGHBARI, HAROLD N. ENGEL, JR.¹
DAVID P. FROMAN²

Department of Animal Sciences and College of Veterinary Medicine¹
Oregon State University
Corvallis, Oregon 97331
USA

Running Head: Subfertile Delaware roosters

¹Oregon State University Technical Paper Number _____.
²To whom correspondence should be addressed.
ABSTRACT

The objectives of the present study were to determine the effect of hemicastration and supplemental seminal plasma proteins on the fertilizing ability of spermatozoa from subfertile Delaware roosters. In Experiment 1, the removal of the left testis induced (P<0.0001) compensatory growth of the right testis and exacerbated (P<0.001) subfertility. In Experiment 2, spermatozoa from subfertile roosters were suspended in a protein-free semen extender, extender containing bovine serum albumin (BSA), or extender containing seminal plasma proteins from fertile roosters prior to insemination. Only the seminal plasma proteins ameliorated (P<0.001) subfertility. In Experiment 3, spermatozoa from fertile roosters were suspended in a protein-free extender, extender containing BSA, or extender containing seminal plasma proteins from subfertile roosters prior to insemination. Protein supplementation of spermatozoa from fertile roosters had no effect (P>0.05) on fertility. In summary, we have established a link between seminal plasma proteins and fertility in Gallus domesticus and hypothesize that subfertile Delaware roosters can be used to identify proteins that promote spermatozoal survival.
INTRODUCTION

When subfertile Delaware roosters are ejaculated weekly, their semen contains only 40-60% viable spermatozoa (Froman and Bernier, 1987). While daily ejaculation can increase spermatozoal viability to ≥ 97%, subfertility is still observed following insemination of hens with such spermatozoa (Kirby et al., 1989; Kirby et al., 1990; Al-Aghbari et al., 1992). Subfertile Delaware roosters are characterized by malformed proximal efferent ducts (Kirby et al., 1990). This malformation may contribute to an imbalance in seminal plasma composition (Al-Aghbari et al., 1992). Kirby et al. (1990) demonstrated that spermatozoal degeneration was dependent upon spermatozoal passage through the excurrent ducts of testis. Al-Aghbari et al. (1992) inferred that spermatozoal degeneration is attributable to excurrent duct dysfunction.

In normal roosters, the efferent ducts constitute 60% of the excurrent duct volume within the epididymis (Aire, 1979). The proximal efferent duct of poultry appears to be a duct in which spermatozoa are mixed with epithelial secretions (Tingari, 1971, 1972; Budras and Sauer, 1975; Bakst, 1980) while spermatozoa are concentrated (Clulow and Jones, 1988). The efferent ducts may be the source of the spermatozoal maturation proteins described by Esponda and Bedford (1985). Morris et al. (1987) postulated that these
proteins have a role in spermatozoal storage or survival in the female reproductive tract.

Because of the reduced surface to volume ratio within the proximal efferent ducts of subfertile Delaware roosters (Kirby et al., 1990), we hypothesized subfertility would be exacerbated by increasing daily spermatozoal production. Daily spermatozoal production can be increased by hemicastration (Onura, 1985; 1987). Thus, our first objective was to determine the effect of hemicastration on the fertilizing ability of spermatozoa from subfertile Delaware roosters. Because subfertile roosters are characterized by excurrent duct dysfunction (Al-Aghbari et al., 1992) and because spermatozoal maturation proteins are known to exist in the domestic fowl (Esponda and Bedford, 1985) we hypothesized subfertility would be ameliorated by supplementing spermatozoa with proteins obtained from seminal plasma of fertile roosters. Consequently, our second objective was to determine the effect of supplemental seminal plasma proteins on the fertilizing ability of spermatozoa from subfertile Delaware roosters. Our third objective was to determine the effect of supplemental seminal plasma proteins obtained from subfertile roosters on the fertilizing ability of spermatozoa from fertile roosters.
MATERIALS AND METHODS

EXPERIMENT 1

Delaware rooters (n=25) were hemicastrated at 5 wk of age. Each bird was anesthetized by injecting 0.04 ml xylazine* (20 mg per ml; Haver-Lockahrt, Shawnee, KS) into the right cutaneous ulnar vein. Laparotomy was performed in order to remove the left testis. Following recovery from surgery, hemicastrates were double wing-banded and then reared with intact controls.

Beginning at sexual maturity, 4 ejaculates were collected from each male at weekly intervals. Following the fourth ejaculation, spermatozoal viability was determined by ethidium bromide exclusion (Bilgili and Renden, 1984). The hypothesis that percentages of viable spermatozoa were equivalent between hemicastrate and intact Delaware roosters was tested by the Kruskal-Wallis test (Sokal and Rohlf, 1981a).

The fertilizing ability of viable spermatozoa was evaluated as follows. Roosters were ejaculated daily for 5 days following 1 wk of sexual rest in order to maximize spermatozoal viability (Froman and Bernier, 1987). Ejaculates were pooled according to treatment, i.e. hemicastration or control. Spermatozoal viability and spermatozoal concentration were determined by ethidium bromide exclusion (Bilgili and Renden, 1984). Semen was
diluted to $2.0 \times 10^8$ viable spermatozoa per ml with Beltsville Poultry Semen Extender, pH 7.5 (BPSE; a gift from Dr. Tom Sexton, USDA, Beltsville, MD). Each spermatozoal suspension was used to inseminate 44 Single Comb White Leghorn (SCWL) hens. The insemination dose was $1 \times 10^8$ viable spermatozoa per hen. Data collection and analysis were performed as described by Kirby and Froman (1990).

After the fertility trials were concluded, roosters were euthanized. The right testis of each rooster was dissected from the body cavity and weighed. The hypothesis that testicular weights were equivalent between hemicastrate and intact Delaware roosters was tested by single classification analysis of variance (Sokal and Rohlf, 1981b).

EXPERIMENT 2

Approximately 50 ml of semen was collected from 130 fertile crossbred roosters. semen was pipetted into 1.5-ml microcentrifuge tubes and then immediately centrifuged at 12,800 $\times$ g for 5 min in an Eppendorf Model 5412 microcentrifuge (Brinkmann Instruments, Inc., Westbury, NY). Following centrifugation, supernatants were pooled and filtered through 0.45 $\mu$m acrodisc filters (VWR Scientific).

A 35-ml volume of filtered seminal plasma, was applied to a 1.6 x 18 cm column of Cibacron Blue-Sepharose (Bio-Rad, Richmond, CA) at a flow rate of 20 ml per hour. Albumin was
separated from other seminal plasma proteins by affinity chromatography at 4° C according to Travis et al. (1976). Prior to sample application, the column had been equilibrated with degassed 0.05 M NaCl in 0.05 M Tris-HCl, pH 8.0. Once sample application was complete, the column was washed with 100 ml of the Tris-HCl buffer. The eluate was collected in 8.5 ml fractions using siliconized tubes. When elution of non-bound protein had ceased, the column was washed with approximately 100 ml of the Tris-HCl buffer at a flow rate of 60 ml per hour. Albumin was eluted from the column with degassed 0.2 M NaSCN in 0.05 M Tris-HCl, pH 8.0, at a flow rate of 60 ml per hour.

A 25-μl volume from each albumin-depleted fraction was placed in a microtiter plate well and mixed with 275 μl of Bio-Rad dye reagent previously diluted 1:5 with deionized water. The fractions which contained the highest concentration of protein, as evidenced by an intense blue color, were pooled. Albumin-depleted proteins were desalted by gel filtration with a 2.5 x 20 cm of Sephadex G-25 column. Proteins were eluted from the column in distilled water. The protein solution was then placed in dialysis tubing (MW cut off 6000-8000 daltons) and concentrated to half the original volume at 4° C by overlaying the membrane with polyethylene glycol flakes (MW 15,000-20,000 daltons; VWR Scientific). After 5-6 hours of dehydration, the
protein solution was lyophilized and stored at -20°C. This procedure was repeated and albumin-depleted protein pooled. The efficacy of affinity chromatography was evaluated by native polyacrylamide gel electrophoresis.

Fertility trials were performed by intravaginally inseminating three groups of SCWL hens with control or treated spermatozoa from subfertile Delaware roosters. Viable spermatozoa were procured after ejaculating roosters daily as above. Spermatozoal viability and concentration were determined as described above. Ejaculates were pooled and semen was diluted with BPSE to provide a suspension with $4 \times 10^9$ viable spermatozoa per ml. Diluted semen was partitioned into 3 volumes. The first was further diluted with BPSE to yield a suspension containing $2.0 \times 10^9$ viable spermatozoa per ml. The second was diluted with BPSE containing bovine serum albumin (BSA) so that the suspension containing $2.0 \times 10^9$ viable spermatozoa and 10 mg BSA per ml. The third was diluted with BPSE so that the suspension contained $2.0 \times 10^9$ viable spermatozoa and 10 mg albumin-depleted proteins per ml. Each suspension was used to inseminate 39-42 hens. The insemination dose was $1 \times 10^8$ viable spermatozoa per hen. Data collection and analysis were performed as described by Kirby and Froman (1990).

EXPERIMENT 3

Subfertile Delaware roosters ($n=30$) were ejaculated
daily in order to maximize spermatozoal viability (Froman and Bernier, 1987). Semen was pooled, centrifuged, and filtered as above. Filtrates were stored at -20° C. A 35-ml volume of filtered seminal plasma was thawed, desalted, concentrated, and lyophilized as described above. Lyophilized protein was stored at -20° C.

Fertility trials were performed by inseminating three groups of SCWL hens with spermatozoa from fertile roosters suspended in protein-free BPSE, BPSE containing BSA, or BPSE containing seminal plasma proteins from subfertile Delaware roosters (SPPD). The final spermatozoal suspensions contained 2.0 x 10⁹ viable spermatozoa per ml, and in the protein supplemented treatments, either 10 mg BSA or 10 mg SPPD per ml. Each suspension was used to inseminate 46-50 hens. Insemination, data collection, and analysis were performed as described above.
RESULTS

EXPERIMENT 1

After 1 wk of sexual rest, spermatzoal viability averaged 31 and 41 % for hemicastrates and control (Table V.1). Even though spermatzoal viability appeared to be lower for hemicastrates, no difference (P>0.05) in spermatzoal viability was observed. Duration of fertility of hemicastrates and control roosters is shown in Figure V.1. The time of half-maximal fertility, or $t$, for hemicastrates was 2.34 d less (P<0.001) than that of controls. Fertility also differed (P<0.001) between the two groups of roosters. As shown in Table V.2, the fertility of hemicastrates was 15 percentage units less than that of controls. Table V.3 compares right testis weights between hemicastrates and controls. Hemicastration increased the size of the right testis by 43% (P<0.0001).

EXPERIMENT 2

As shown in Figure V.2, affinity chromatography with Cibacron Blue-Sepharose was effective in removing albumin from seminal plasma proteins. As shown in Figure V.2, when equivalent amounts of protein from the enriched albumin and albumin-depleted fractions were evaluated by native polyacrylamide gel electrophoresis, distinct patterns were observed. When compared to the normal array of seminal plasma proteins (Figure V.2), the former fraction was indeed
enriched with albumin and contained non-albumin contaminants as well. In contrast, the albumin-depleted fraction contained only a trace of albumin and was enriched with non-albumin proteins.

When spermatozoa from subfertile Delaware roosters were suspended in diluent containing albumin-depleted proteins and then inseminated intravaginally (Table V.4), fertility improved (P<0.001). As also shown in Table V.4, BSA was without effect (P>0.05). Duration of fertility is shown in Figure V.3. The time of half-maximal fertility, or $\tau$, was equivalent between non-protein supplemented and BSA-supplemented spermatozoa. In contrast, $\tau$ increased (P<0.001) by 2.7 d when spermatozoa were supplemented with albumin-depleted seminal plasma proteins.

EXPERIMENT 3

As shown in Table V.5, supplementing spermatozoa from fertile roosters with seminal plasma proteins from subfertile roosters had no effect (P>0.05) on fertility.
TABLE V.1  
Comparison of percentages of viable spermatozoa between hemicastrated and control subfertile roosters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spermatozoal viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemicastration</td>
<td>31 ± 3.3</td>
</tr>
<tr>
<td>Control</td>
<td>41 ± 5.3</td>
</tr>
</tbody>
</table>

¹Each value represents a mean ± SEM from 20 replicate males.
TABLE V.2  
Fertility over a 21-day egg collection interval following a single intravaginal insemination\(^1\) of Single Comb White Leghorn hens with semen from either control or hemicastrated subfertile roosters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hens (n)</th>
<th>Eggs (n)</th>
<th>Fertility(^2) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30</td>
<td>600</td>
<td>48 ± 1.9(^a)</td>
</tr>
<tr>
<td>Hemicastration</td>
<td>30</td>
<td>590</td>
<td>38 ± 2.8(^b)</td>
</tr>
</tbody>
</table>

\(^1\) Each hen was inseminated with 1 x 10^8 viable spermatozoa.  
\(^2\) Each value represents a mean ± SEM.  
\(^a,b\) Means bearing different superscripts are different (P<0.01).
TABLE V.3  Comparison of right testis weight between control and hemicastrated subfertile roosters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Testis' weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.1 ± 1.05a</td>
</tr>
<tr>
<td>Hemicastration</td>
<td>17.3 ± 1.13b</td>
</tr>
</tbody>
</table>

*a, b*Means bearing different superscripts are different (P<0.0001).

*'Each value represents a mean ± SEM from 20 replicate males.
TABLE V.4  Fertility over a 21-day egg collection interval following a single intravaginal insemination\(^1\) of Single Comb White Leghorn hens with spermatozoa from subfertile roosters suspended beforehand in Beltsville Poultry Semen Extender (BPSE), BPSE containing bovine serum albumin (BSA)\(^2\), or BPSE containing albumin-depleted seminal plasma proteins\(^3\) obtained from fertile roosters.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hens (n)</th>
<th>Eggs (n)</th>
<th>Fertility(^4) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41</td>
<td>760</td>
<td>41 ± 0.50(^a)</td>
</tr>
<tr>
<td>BSA</td>
<td>42</td>
<td>732</td>
<td>45 ± 0.52(^a)</td>
</tr>
<tr>
<td>Albumin-Depleted</td>
<td>37</td>
<td>669</td>
<td>54 ± 0.53(^b)</td>
</tr>
</tbody>
</table>

\(^1\)Each hen was inseminated with 1 x 10\(^8\) viable spermatozoa.
\(^2,3\)10 mg per ml.
\(^4\)Each value represents a mean ± SEM.
\(^ab\)Means bearing different superscripts are different (P<0.001).
TABLE V.5  
Fertility over a 21-day egg collection interval following a single intravaginal insemination of Single Comb White Leghorn hens with spermatozoa from fertile roosters suspended beforehand in Beltsville Poultry Semen Extender (BPSE), BPSE containing bovine serum albumin (BSA)$^2$, or BPSE containing seminal plasma proteins obtained from subfertile Delaware roosters (SPPD)$^3$.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hens (n)</th>
<th>Eggs (n)</th>
<th>Fertility$^4$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPSE</td>
<td>49</td>
<td>973</td>
<td>47 ± 2.11</td>
</tr>
<tr>
<td>BSA</td>
<td>50</td>
<td>950</td>
<td>53 ± 2.35</td>
</tr>
<tr>
<td>SPPM</td>
<td>46</td>
<td>916</td>
<td>51 ± 2.02</td>
</tr>
</tbody>
</table>

$^1$Each hen was inseminated with 1 x $10^8$ viable spermatozoa.
$^2,3$10 mg per ml.
$^4$Each value represents a mean ± SEM.
FIGURE V.1  Duration of fertility following intravaginal insemination of Single Comb White Leghorn hens with spermatozoa from either hemicastrated ( □ ) or control ( ▲ ) subfertile roosters. Each hen was inseminated with $1 \times 10^8$ viable spermatozoa. Solid lines represent the functions

$$y(x) = \frac{[97.9]}{[1 + e^{-0.6068(10.76-x)}]}$$

and

$$y(x) = \frac{[95.9]}{[1 + e^{-0.6068(10.76-x)}]}.$$
FIGURE V.2  Seminal plasma proteins following native polyacrylamide gel electrophoresis and silver staining. A) Enriched albumin removed from seminal plasma proteins by an affinity chromatography Cibacron Blue-Sepharose column and eluted with 0.2 M NaSCN in Tris-HCl buffer. B) Albumin-depleted proteins eluted from Cibacron Blue-Sepharose column with 0.05 M NaCl in Tris buffer. C) Whole seminal plasma. Lane A is enriched with albumin and contained contaminating non-albumin proteins while lane B contained only a trace amount of albumin.
FIGURE V.3  Duration of fertility following intravaginal insemination of Single Comb White Leghorn hens with spermatozoa from Delaware roosters suspended beforehand in Beltsville Poultry Semen Extender (BPSE; □ ), BPSE containing 10 mg per ml bovine serum albumin (BSA; ○ ), and BPSE containing 10 mg per ml albumin-depleted proteins ( ▲ ) obtained from fertile roosters. Each hen was inseminated with $1 \times 10^8$ viable spermatozoa. Solid lines represent the functions

\[ y(x) = \frac{97.9}{1 + e^{-3.303(9.2-x)}} , \]
\[ y(x) = \frac{103}{1 + e^{-3.718(8.7-x)}} , \]
and
\[ y(x) = \frac{98.6}{1 + e^{-6.333(11.9-x)}} . \]
DISCUSSION

Fowl spermatozoa mature during their passage through the excurrent ducts of the testis (Munro, 1938; Howarth, 1983; Ashizawa and Sano, 1990). Munro (1938) concluded that passage was essential to acquisition of fertilizing ability. However, Howarth (1983) differentiated between fertilizing ability and the ability of spermatozoa to be sequestered in the uterovaginal glands. Howarth demonstrated that while testicular and ejaculated spermatozoa are capable of fertilizing oocytes, only spermatozoa that have passed through the excurrent ducts have the ability to reside within the uterovaginal glands.

This ability has generally been attributed to spermatozoal motility. However, it also may be related to spermatozoal maturation proteins. In 1970, Stratil reported 4-5 proteins that are unique to fowl seminal plasma. In 1985, Esponda and Bedford corroborated Stratil's observation and demonstrated that these proteins are not associated with testicular spermatozoa. Rather, these protein associate with spermatozoa as they enter the epididymis. Morris et al. (1987) demonstrated that spermatozoal maturation proteins remain associated with spermatozoa that are sequestered in the hen's uterovaginal glands. These researchers postulated that spermatozoal maturation proteins have a role in spermatozoal storage or survival within the
oviduct.

Based upon stereological data published by Aire (1979), the efferent duct is the principal duct of the fowl's epididymis. Subfertile Delaware roosters are characterized by malformed efferent ducts (Kirby et al., 1990) and abnormal seminal plasma composition (Al-Aghbari et al., 1992). Subfertility has been attributed to a shortened spermatozoal life span (Froman and Bernier, 1987; Kirby et al., 1989). Froman (1990) proposed that spermatozoal degeneration is attributable to a decreased functional capacity of the efferent duct. Therefore, we suspected that the fertilizing ability of spermatozoa from subfertile Delaware roosters could be manipulated by two experimental approaches: hemicastration and supplementing spermatozoa with seminal plasma protein from fertile roosters.

When sexually immature male fowl are hemicastrated by removal of the left testis, the right testis of sexually mature hemicastrates is 38-56% larger than that of intact roosters of the same age, and daily spermatozoal production is 3.7 times greater in hemicastrates Onura (1985; 1987). We reasoned that heightened daily spermatozoal production would exacerbate subfertility in males with malformed efferent ducts. In Experiment 1, spermatozoal viability, fertility, and right testicular weights were compared between subfertile hemicastrates and subfertile controls.
As shown in Table V.1, no difference (P>0.05) was observed in spermatozoal viability between treatment groups. In contrast, the fertility of hemicastrates was less (P<0.001) than that of controls. Overall fertility differed by 15 percentage units (Table V.2). This difference in fertility was attributed to a difference in duration of fertility (Figure V.1) as the time of half-maximal fertility of hemicastrates was 2.34 d less (P<0.001) than that of the control (Figure V.1). Following the fertility trial, roosters were euthanized and right testes weighed. The right testis weight of hemicastrates was 43% greater (P<0.0001) than that of controls (Table V.3). This difference was comparable to that reported by Onura (1985; 1987).

Froman (1990) proposed that spermatozoal degeneration in subfertile Delawares is attributable to aberrant excurrent duct secretory activity that affects spermatozoal surface properties. This was based, in part, upon the discovery that the efferent ducts of subfertile roosters are characterized by a reduced surface to volume ratio (Kirby et al., 1990). This hypothesis remains tenable in view of the abnormal seminal plasma composition reported for subfertile Delaware roosters by Al-Aghbari et al. (1992) and the effect of hemicastration on fertilizing ability (Table V.2; Figure V.1). In view of the work of Stratil (1970), Esponda and
Bedford (1985) and Morris et al. (1987), a link between spermatozoal survival and secretory proteins seemed likely. This supposition served as the basis for Experiment 2. Experiment 2 was designed to test the effect of seminal plasma proteins obtained from fertile roosters on the fertilizing ability of spermatozoa from subfertile roosters. Based upon the work of Stratil (1970), and Esponda and Bedford (1985), albumin may be discounted as a spermatozoal-maturation protein. Therefore, we chose to use albumin-depleted proteins as a source of protein.

As shown in Figure V.2, albumin was removed from a mixture of proteins by affinity chromatography with Cibacron Blue-Sepharose. As also shown in Figure V.2, most of the albumin was removed from seminal plasma proteins. This increased the effective concentration of other seminal plasma proteins. The fertilizing ability of spermatozoa supplemented with albumin-depleted proteins was compared to that of non-supplemented spermatozoa and spermatozoa supplemented with BSA (Table V.4; Figure 3). No difference (P>0.05) was observed in fertility between the BSA treatment and the non-supplemented control (Table V.4). Albumin-depleted proteins increased (P<0.001) the fertilizing ability of spermatozoa from subfertile roosters (Table V.4). Time of half-maximal fertility was 2.7 d more than that of the control (Figure V.3). Therefore, supplementation of
spermatozoa with albumin-depleted proteins obtained from fertile roosters ameliorated subfertility. These proteins apparently promoted the survival of spermatozoa within the female reproductive tract.

Experiment 3 was conducted to examine the effect of seminal plasma proteins obtained from subfertile Delaware roosters on the fertilizing ability of spermatozoa from fertile roosters. Thus, it was designed to determine if seminal plasma from subfertile roosters contained a protein component that decreased spermatozoal fertilizing ability. As shown in Table V.5 the seminal plasma proteins of subfertile roosters had no influence upon the fertilizing ability of spermatozoa from fertile roosters.

We conclude that seminal plasma from subfertile Delaware roosters is deficient in proteins that promote spermatozoal storage within the oviduct. This conclusion is based upon the following lines of evidence. First, subfertile roosters are characterized by a malformation of the efferent ducts (Kirby et al., 1990) and abnormal excurrent duct function (Al-Aghbari et al., 1992). Second, subfertility has been shown to depend upon spermatozoal passage through the excurrent ducts (Kirby et al., 1990) and this effect is exacerbated by hemicastration. Third, admixture of spermatozoa with seminal plasma proteins obtained from fertile roosters ameliorates subfertility.
Fourth, seminal plasma proteins of subfertile Delaware roosters do not reduce the fertilizing ability of spermatozoa that matured within the excurrent ducts of normal roosters. The present study demonstrates a link between seminal plasma proteins and fertility in *Gallus domesticus*. We hypothesize that the Delaware model can be used to identify proteins that promote spermatozoal survival.
REFERENCES


CHAPTER VI

CONCLUSIONS

A seminal plasma polypeptide map was described in Chapter III. Two-dimensional electrophoresis revealed that the composition of chicken seminal plasma proteins is much more complex than that inferred from one-dimensional electrophoresis. Two-dimensional electrophoresis resolved approximately 4 times more polypeptide bands (P<.001) than were resolved by one-dimensional electrophoresis (23 ± 0.4 bands). This technique was used to help compare the seminal plasma protein composition of fertile and subfertile roosters in Chapter IV.

The experiments in Chapter IV were designed to compare the composition of seminal plasma from fertile and subfertile roosters. Because the fowl's reproductive tract lacks secondary sex glands, seminal plasma composition reflects the function of the reproductive tract itself rather than the function of the tract and accessory organs. Sexually rested Delaware roosters are characterized by a large number of degenerate spermatozoa within the deferent duct. Consequently, these cells release intracellular material that is not found in the seminal plasma of fertile birds. However, this problem can be eliminated by daily ejaculation. Under this condition, the hypothesis that
spermatozoal viability was 100% was not rejected (P<0.05). Therefore, analysis of seminal plasma from subfertile roosters was not confounded by the presence of material from spermatozoa.

Spermatozoal concentration, seminal plasma proteolytic activity, and osmolality (mmol/kg) did not differ (P>0.05) between subfertile and fertile roosters. However, seminal plasma protein concentration as well as amino acid and electrolyte composition differed (P<0.05) between subfertile and fertile roosters. When one-dimensional electrophoresis was used to separate seminal plasma polypeptides, different patterns were observed for subfertile and fertile roosters. These differences were confirmed by two-dimensional electrophoresis. These differences were attributed to dysfunctional excurrent ducts in subfertile Delaware roosters.

In order to investigate the relationship between excurrent duct function and fertility, three experiments were performed in Chapter V. The objectives of these experiments were: 1) to determine the effect of hemicastration on the fertilizing ability of spermatozoa from subfertile Delaware roosters, 2) to determine the effect of supplemental seminal plasma proteins on the fertilizing ability of spermatozoa from subfertile Delaware roosters, and 3) to determine the effect of supplemental
seminal plasma proteins from subfertile roosters on the fertilizing ability of spermatozoa from fertile roosters. When sexually immature Delaware roosters were hemicastrated by removal of the left testis, the right testis of sexually mature hemicastrates was 38-56% larger than that of intact roosters. Time of half-maximal fertility, or \( \tau \), for hemicastrates was 2.34 d less (\( P<0.001 \)) than that of intact Delaware roosters and the percentage of fertilized eggs also differed (\( P<0.001 \)) between groups. Thus, hemicastration exacerbated subfertility. This was attributed to the interaction of increased daily spermatozoal production induced by hemicastration with excurrent duct dysfunction. Albumin-depleted proteins from fertile roosters were obtained by affinity chromatography with Cibacron Blue-Sepharose. Subfertility was ameliorated (\( P<0.001 \)) by admixture of albumin-depleted proteins with spermatozoa from Delaware roosters prior to insemination. Time of half-maximal fertility increased (\( P<0.001 \)) when spermatozoa were supplemented with albumin-depleted seminal plasma proteins. In contrast, supplementing spermatozoa from fertile roosters with seminal plasma proteins from subfertile roosters had no effect (\( P>0.05 \)) on fertility. Therefore, subfertility was attributed to a protein deficiency rather than the presence of a protein that induces subfertility.

In summary, this thesis reports a greater complexity of
seminal plasma proteins than that previously known, a confirmation that the excurrent ducts of subfertile Delaware roosters are dysfunctional, and that the subfertility of Delaware roosters may stem from a deficiency of seminal plasma protein.
BIBLIOGRAPHY


APPENDIX 1

INCLUSION OF HIGH MOLECULAR WEIGHT FRACTION OF FOWL SEMINAL PLASMA IN SEMEN EXTENDER AUGMENTS THE FERTILIZING ABILITY OF EXTENSIVELY DILUTED SEMEN

DAVID P. FROMAN AND ABDULWALI M. AL-AGHBARI

Department of Animal Sciences
Oregon State University
Corvallis, Oregon 97331
USA

Running Head: Seminal plasma and fertility

1Oregon State University Technical Paper Number ______.
2To whom correspondence should be addressed.
ABSTRACT

Experiment 1 was designed to test the efficacy of fowl seminal plasma as an extender. Seminal plasma from fertile roosters was procured by centrifugation. Residual cells and particulate matter were removed by filtration. Semen from fertile roosters was diluted with either Beltsville Poultry Semen Extender (BPSE) or seminal plasma and hens were inseminated with only 10% of a conventional insemination dose in order to optimize the probability of detecting a difference between extenders. The seminal plasma extender yielded greater fertility \((P<0.001)\). In Experiment 2, the high molecular weight fraction of seminal plasma was obtained by gel filtration through Sephadex G-25. This fraction was concentrated and then added back to BPSE so that the final concentration of the additive was equivalent to the concentration of the high molecular weight fraction in seminal plasma. Hens were inseminated as above with semen extended with either supplemented BPSE or BPSE. Supplemented BPSE yielded higher fertility \((P<0.001)\). Therefore, the high molecular weight fraction of seminal plasma contains a factor that promotes spermatozoal sequestration in the hen's uterovaginal glands.
Seminal plasma is the natural medium that surrounds fowl spermatozoa at the time of ejaculation (Lake and Ravie, 1960; Lake, 1966). A number of researchers (Munro, 1938; Weakley and Shaffner, 1952; Fewlass et al., 1975; Terada et al., 1984; Lake and Ravie, 1987; Sexton, 1988) have reported that seminal plasma is efficacious as an extender for fowl spermatozoa. In fact, Lake and Ravie (1987) reported that spermatozoal fertilizing ability could be maintained even when semen was diluted as much as 1:46 with seminal plasma. Thus, use of seminal plasma as an extender can counter the deleterious effect of excessive semen dilution on fertility.

High fertility has also been observed with semen stored in an extender containing the high molecular weight fraction of seminal plasma (Blesbois, 1990). This observation is interesting in view of the work of Al-Aghbari et al. (1992). These authors reported that the fertilizing ability of spermatozoa from subfertile Delaware roosters could be improved by mixing spermatozoa with seminal plasma proteins obtained from fertile roosters prior to insemination. Esponda and Bedford (1985) discovered that the extra-gonadal maturation of fowl spermatozoa entails acquisition of protein within the excurrent ducts of the testis. Morris et al. (1987) observed that such protein remains associated with spermatozoa sequestered within the uterovaginal glands.
These authors postulated that spermatozoal maturation proteins may have a role in spermatozoal storage or survival within the oviduct. When the discovery of spermatozoal maturation proteins is viewed along with the fertility data cited above, it seems likely that the efficacy of seminal plasma as an extender may depend upon specific high molecular weight components found in seminal plasma.

Reproductive efficiency as well as genetic gain could be enhanced by reducing the insemination dose in artificial insemination programs. The most practical approach would be to extensively dilute semen. While Lake and Ravie (1987) demonstrated that this can be accomplished by using seminal plasma as a diluent, these researchers did not identify the component or components in seminal plasma that were responsible for this effect. Therefore, the objectives of the present research were to see if the observation of Lake and Ravie (1987) could be confirmed and if so, to test the efficacy of the high molecular weight fraction of seminal plasma.
MATERIALS AND METHODS

EXPERIMENT 1

Ejaculates from 60 fertile roosters were pooled in order to obtain a 20-ml volume of semen. Seminal quality was assessed by ethidium bromide exclusion (Bilgili and Renden, 1984). Semen was centrifuged for 5 min at 15,600 x g in an Eppendorf microcentrifuge (Brinkman Instruments Co., Westbury, NY). Seminal plasma was filtered through 0.45 μm Acrodiscs (VWR Scientific, Seattle, WA). The filtrate was kept in a siliconized test tube kept in ice for 5 h before use. Beltsville Poultry Semen Extender (BPSE), pH 7.5, was prepared according to Sexton and Fewlass (1978).

Approximately 30 min before insemination, the ejaculates of an additional 10 fertile roosters were pooled. Spermatozoal concentration and viability were determined according to Bilgili and Renden (1984). A subsample of semen was diluted with either filtered seminal plasma or BPSE in order to provide spermatozoal suspensions containing 2 x 10⁶ spermatozoa per ml. Each suspension was used to inseminate 60 Single Comb White Leghorn hens. Each hen received 10 x 10⁶ viable spermatozoa in a volume of 50 μl.

Eggs were collected throughout a 21-day interval, which commenced on the second day following insemination. Eggs were incubated for 4 days and then broken open in order to determine whether the oocyte had been fertilized. Data were
discarded from any given hen that failed to lay a single fertilized egg during the egg collection interval. Fertility data were analyzed according to Kirby and Froman (1990).

EXPERIMENT 2

A 49-ml volume of semen was centrifuged and seminal plasma filtered as above. Thirty-eight ml of filtered seminal plasma were placed in dialysis tubing (molecular weight cutoff = 6,000 to 8,000 daltons). The filled tube was placed prone on polyethylene glycol flakes (20,000 daltons; VWR Scientific) in a glass pan. Then the bag was covered with additional polyethylene glycol flakes and the pan was refrigerated at 5° C for several hours. Volume was measured following dehydration, and the concentrated seminal plasma was stored overnight in a siliconized test tube kept in ice.

On the following afternoon, BPSE was prepared according to Sexton and Fewlass (1978). A PD-10 column (Pharmacia LKB, Alameda, CA) was equilibrated with BPSE. The 2.9 fold concentrate was diluted with BPSE to provide a 1.4 fold concentrate. A 2.5-ml volume of the 1.4 fold concentrate was applied to the PD-10 column. This volume was followed by 3.5 ml of BPSE and 3.5 ml of eluate were collected. This solution was designated as supplemented BPSE.

Ejaculates from 10 fertile roosters were pooled, and
spermatozoal concentration and viability determined as above. Semen was diluted to $2 \times 10^8$ spermatozoa per ml with either BPSE or supplemented BPSE. Each suspension was used to inseminate 60 hens. Inseminations, egg collection, and data analysis were performed as above.
RESULTS AND DISCUSSION

Experiment 1 demonstrated (Table A1.1) that seminal plasma was superior (P<0.001) to BPSE as an extender when semen was diluted to $2 \times 10^8$ spermatozoa per ml. This concentration is approximately 20 times less than that observed in undiluted semen and is 10 times less than that of extended semen typically used to inseminate hens. Duration of fertility is shown in Figure A1.1. Both initial fertility, or $\gamma$, and time of half-maximal fertility, or $\tau$, differed (P<0.001) between extenders. Initial fertility was 66.7 and 85.5% for spermatozoa suspended in BPSE and seminal plasma, respectively. Likewise, time of half-maximal fertility was 5.0 and 8.4 days.

Experiment 2 demonstrated (Table A1.2) that BPSE supplemented with the high molecular weight fraction of seminal plasma was superior (P<0.001) to BPSE when semen was diluted to $2 \times 10^8$ spermatozoa per ml. Duration of fertility is shown in Figure A2.2. In contrast to the difference in initial fertility observed in Experiment 1, initial fertility did not differ (P>0.05) between extenders in Experiment 2. Nonetheless, time of half-maximal fertility did differ (P<0.001), and this appeared to account for the difference in overall fertility observed in Experiment 2.

The difference in the fertilizing ability of
spermatozoa extended in BPSE in Experiments 1 and 2 was an enigma. Based upon previous research with fertile roosters that were comparable genetically to those used in the present study (Froman and Engel, 1989; Kirby et al., 1989; Kirby et al., 1990; Kirby and Froman, 1990), the inter-assay coefficient of variation for $t$ following intravaginal insemination of SCWL hens was 6.1\% ($n = 4$). However, these studies utilized an insemination dose of $1 \times 10^8$ spermatozoa per hen, which is the recommended insemination dose for chickens (Sexton, 1983) and is ten times greater than that used in the present study. Perhaps the effect of experimental errors in either the determination of spermatozoal concentration or semen dilution are magnified when a marginal insemination dose is used.

Even though the present study did not replicate the exact experiments performed by Lake and Ravie (1987), it corroborates their results. Lake and Ravie (1987) diluted fowl semen 1:10 with either an aqueous diluent or seminal plasma and used an insemination dose of $1 \times 10^7$ spermatozoa per hen. In the present study, while semen was diluted 1:20, the insemination dose was $1 \times 10^7$ spermatozoa per hen. Therefore, fertility data may be compared. According to Lake and Ravie (1987), fertility over the first 7 days of egg collection was 84 and 91\% for semen extended with an aqueous diluent and seminal plasma, respectively. In the
present study, Experiment 2 yielded results that were most comparable to those of Lake and Ravie (1987). Fertility over the first 7 days of egg collection in this experiment was 69 and 80% for semen extended with BPSE and supplemented BPSE, respectively. The differences between studies are most likely attributable to the following factors. First, Ca\(^{+2}\) has been shown to promote spermatozoal motility (Ashizawa and Wishart, 1987; Wishart and Ashizawa, 1987). Beltsville Poultry Semen Extender is not only Ca\(^{+2}\)-free but contains citrate, which can chelate Ca\(^{+2}\). Second, the seminal plasma used by Lake and Ravie (1987) did not contain transparent fluid whereas the seminal plasma used in the present study did. Third, depth of insemination in the present study was only half that used by Lake and Ravie (1987), i.e. 2 versus 4 cm.

In conclusion, while the present study did not show that the fertility enhancing ability of seminal plasma is limited to the large molecular weight fraction, it did demonstrate that this fraction contains a component that enhances fertility. Based upon the work of Weakley and Shaffner (1951), Wales and White (1961), Fewlass et al. (1975), Lake and Ravie (1987), and Blesbois and Mauger (1987), the effect cannot be attributed to macromolecules in general and is species specific. Based upon the work of Blesbois and Hermier (1990), it is unlikely that
lipoproteins are responsible for this activity. Therefore, we attribute this activity to other seminal plasma proteins.
TABLE A1.1  
Fertility over a 21-day egg collection interval following a single intravaginal insemination\(^1\) of Single Comb White Leghorn hens with spermatozoa from fertile roosters suspended beforehand in Beltsville Poultry Semen Extender (BPSE) or BPSE containing seminal plasma.

<table>
<thead>
<tr>
<th>Extender</th>
<th>Hens (n)</th>
<th>Eggs (n)</th>
<th>Fertility(^2) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPSE</td>
<td>55</td>
<td>1004</td>
<td>14 ± 0.9(^a)</td>
</tr>
<tr>
<td>Seminal Plasma</td>
<td>54</td>
<td>1008</td>
<td>32 ± 0.1.6(^b)</td>
</tr>
</tbody>
</table>

\(^1\)Each hen was inseminated with 1 x 10\(^7\) viable spermatozoa.  
\(^2\)Each value represents a mean ± SEM.  
\(^a,b\)Means within a column bearing different superscripts are different (P<0.0001).
TABLE A1.2  Fertility over a 21-day egg collection interval following a single intravaginal insemination\(^1\) of Single Comb White Leghorn hens with spermatozoa from fertile roosters suspended beforehand in Beltsville Poultry Semen Extender (BPSE), or BPSE supplemented with the high molecular weight fraction of seminal plasma.

<table>
<thead>
<tr>
<th>Extender</th>
<th>Hens (n)</th>
<th>Eggs (n)</th>
<th>Fertility(^2) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPSE</td>
<td>57</td>
<td>1117</td>
<td>31 ± 1.8(^a)</td>
</tr>
<tr>
<td>Supplemented BPSE</td>
<td>58</td>
<td>1149</td>
<td>41 ± 1.5(^b)</td>
</tr>
</tbody>
</table>

\(^1\)Each hen was inseminated with \(1 \times 10^8\) viable spermatozoa.  
\(^2\)Each value represents a mean ± SEM.  
\(^{a,b}\)Means within a column bearing different superscripts are different (\(P<0.001\)).
FIGURE A1.1 Duration of fertility following intravaginal insemination of Single Comb White Leghorn hens with spermatozoa suspended either in Beltsville Poultry Semen Extender (BPSE; ○ ), or seminal plasma ( ● ). Each suspension contained $2 \times 10^8$ viable spermatozoa per ml. Each hen was inseminated with $10 \times 10^6$ spermatozoa. Solid lines represent the functions

$$y(x) = \frac{66.7}{1+e^{-5579(5.01-x)}}$$

and

$$y(x) = \frac{85.5}{1+e^{-4736(8.35-x)}}.$$
FIGURE A1.2 Duration of fertility following intravaginal insemination of Single Comb White Leghorn hens with spermatozoa suspended either in Beltsville Poultry Semen Extender (BPSE; ○), or BPSE supplemented with the high molecular weight fraction of seminal plasma (●). Each suspension contained 2 x 10⁶ viable spermatozoa per ml. Each hen was inseminated with 10x10⁶ spermatozoa. Solid lines represent the functions

\[ y(x) = \frac{85.9}{1 + e^{-419(7.81-x)}} \],
and
\[ y(x) = \frac{86.8}{1 + e^{-4663(10.00-x)}} \].
REFERENCES


APPENDIX 2

ANALYSIS OF THE COMBINED EFFECT OF THE Sd ALLELE AND HOMOZYGOSITY OF THE ROSE COMB ALLELE (R) ON THE FERTILITY OF ROOSTERS (Gallus domesticus)

DAVID P. FROMAN\textsuperscript{2}, JOHN D. KIRBY, AND ABDULWALI M. AL-AGHBARI

Department of Animal Sciences
Oregon State University
Corvallis, Oregon 97331
USA

Running Head: Heritable subfertility

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\textsuperscript{2}To whom correspondence should be addressed.
ABSTRACT

Subfertility associated with homozygosity for the rose comb allele (R/R) is attributable to a spermatozoal defect that is distinct from subfertility stemming from heritable spermatozoal degeneration, which is an effect of the dominant spermatozoal degeneration allele (Sd). The objective of the present research was to see if these effects were cumulative. Domestic fowl bearing these alleles were bred in order to obtain the following genotypes: R/r+ sd+/sd+, Rr+ Sd/sd+, R/R sd+/sd+, and R/R Sd/sd+. When Single Comb White Leghorn hens were inseminated with equivalent numbers of viable spermatozoa from roosters representing one of those genotypes, fertility over a 21-d egg collection interval was 53 ± 2.1, 36 ± 1.6, 21 ± 2.1, and 11 ± 1.2%, respectively. Therefore, the effect of homozygosity for the R allele and the presence of the Sd allele was cumulative.
INTRODUCTION

Heritable male subfertility in the domestic fowl has been well-documented with two distinct genetic models. Since the 1960s (Crawford and Merritt, 1963; Crawford and Smyth, 1964) roosters homozygous for the rose comb allele (R/R) have been known to be subfertile. While the mechanism responsible for spermatozoal dysfunction is as yet unknown, spermatozoal motility is suspect (Petitjean and Cochez, 1966; Petitjean and Servouse, 1981; Kirby et al., 1989; Kirby and Froman, 1991). In contrast, Froman and Bernier (1987) reported heritable subfertility in single comb (r+/r+) roosters. Subfertility was attributed to spermatozoal degeneration (Froman and Bernier, 1987; Kirby et al., 1989). Spermatozoal degeneration was not a consequence of autoimmunity against spermatozoa (Froman et al., 1990), but rather passage of spermatozoa through malformed excurrent ducts of the testis (Kirby et al., 1990a). Kirby et al. (1990b) demonstrated that the trait was most likely due to a single dominant gene. The symbol Sd is proposed to denote this allele. The objective of the present research was to see if the effect of homozygosity for the rose comb allele and the presence of the Sd allele were cumulative.
MATERIALS AND METHODS

Silver-laced Wyandotte hens were artificially inseminated with semen from a purebred Delaware rooster homozygous for the Sd allele. Single comb, or r+/r+, progeny were discarded. Thus, the genotype of the remaining F₁ birds was R/r⁺ Sd/sd⁺. Female F₁ sibs were artificially inseminated with semen pooled from male F₁ sibs. At sexual maturity, two F₂ genotypes were identified as follows. Homozygosity of the R allele in males was determined by breeding each rose comb male to Single Comb White Leghorn (SCWL) females, incubating eggs, and observing the comb type of chicks at hatch. Heterozygosity for the R allele in females was done similarly except that the hens under investigation were inseminated with semen from SCWL roosters. The seminal quality of R/R males was determined by ethidium bromide exclusion (Bilgili and Renden, 1984) as implemented by Froman and Bernier (1987) to identify males bearing an Sd allele. The absence of an Sd allele in R/r⁺ females was determined by inseminating each R/r female with semen from SCWL roosters, incubating eggs, rearing male progeny to sexual maturity, and determining seminal quality as above. In summary, R/R Sd/sd⁺ males and R/r⁺ sd+/sd⁺ females were identified for breeding. The comb genotype and seminal phenotype of F₃ males were determined as above. Five roosters were selected to represent each of the
following genotypes: $R^+/r^+\,sd^+/sd^+,\;R^+/r^+\,Sd^+/sd^+,\;R/R\,sd^+/sd^+$, and $R/R\,Sd^+/sd^+$.

Two replicate fertility trials were performed. In each trial, semen was pooled according to genotype. Spermatozoal viability and concentration were determined according to Bilgili and Renden (1984). Semen was diluted with Beltsville Poultry Semen Extender (a gift from Dr. Tom Sexton, USDA, Beltsville, MD) to a concentration of $2 \times 10^9$ viable spermatozoa per ml. Then 30 SCWL hens were inseminated with each spermatozoal suspension. Each hen received $1 \times 10^8$ viable spermatozoa in a volume of 50 μl. Eggs were collected, fertility determined, and data analyzed according to Kirby and Froman (1990).
RESULTS AND DISCUSSION

As shown in Table A2.1, profound differences (P<0.0001) in fertility were observed among the four genotypes of rose comb roosters. When fertility was evaluated over a 21-d interval, mean fertility was 53, 36, 21, and 11 for R/r+ sd+/sd+, R/r+ Sd/sd+, R/R sd+/sd+, and R/R Sd/sd+ roosters, respectively. Kirby et al. (1989) reported a mean fertility of 51% for SCWL roosters over an equivalent egg collection interval. Consequently, the fertility of R/r+ se/se roosters in the present study was equivalent to that of SCWL roosters, which are characterized by high fertility. Kirby et al. (1989) also reported a mean fertility of 42 and 26% for r+/r+ Sd/Sd and R/R sd+/sd+ roosters, respectively. Consequently, the effect of the Sd allele and homozygosity for the R allele was comparable to that observed previously.

As shown in Figure A2.1, distinct patterns of fertility were observed following a single intravaginal insemination. The time of half-maximal fertility, or \( \tau \), for the R/r+ sd+/sd+, R/r+ Sd/sd+, and R/R sd+/sd+ genotypes was 12.1, 8.6, and 5.4 days, respectively. Each estimate was different (P<0.0001) than the others. Tau could not be estimated for the R/R Sd/sd+ genotype because the data did not conform to a logistic function when fertility was plotted as a function of time (Fig. A2.1). Rather, these data conformed to an exponential function, viz. \( y(x) = -0.48 \)
Thus, the $R/R\ Sd/sd^+$ genotype yielded an uncharacteristic pattern of fertility for the domestic fowl.

In the case of $R/R$ roosters, fertility is actually dependent upon the site of spermatozoal deposition in the oviduct. When intravaginal insemination is employed (Crawford, 1965; Etches, et al., 1974; Kirby et al., 1989; Froman et al., unpublished data), the fertilizing ability of spermatozoa from $R/R$ males is much less than that of $R/r^+$ or $r^+/r^+$ males. However, when spermatozoa from $R/R$ roosters are placed within the magnum of the oviduct (Etches et al., 1974; Kirby et al., 1989; Froman et al., unpublished data), subfertility is not observed. Etches et al. (1974) suspected that spermatozoal behavior within the vagina was related to aberrant spermatozoal metabolism.

Based upon the reduction of $2-(p$-iodophenyl$)-3-(p$-nitrophenyl$)-5$-phenyl tetrazolium chloride to formazan (Chaudhuri and Wishart, 1988), Kirby and Froman (1991) demonstrated that the metabolic capacity of spermatozoa from $R/R$ roosters is indeed less than that from either $r^+/r^+\ Sd/sd^+$ or $r^+/r^+\ sd^+/sd^+$ roosters. Due to the correlation between rate of formazan production and spermatozoal motility ($r = 0.88$; Chaudhuri et al., 1988), as well as the fact that the metabolism of spermatozoa from $R/r^+$ roosters is comparable to that of $r^+/r^+$ roosters (Kirby and Froman, unpublished data), differences in motility may be inferred
for spermatozoa from \( R/R \) and \( R/r^+ \) roosters in the present study. Consequently, the poor motility of sperm from \( R/R \) roosters probably contributes to suboptimal filling of the uterovaginal glands.

Froman and Bernier (1987) discovered that spermatozoa degenerate within the deferent ducts of roosters bearing the \( Sd \) allele. These authors also demonstrated that frequent ejaculation can increase the percentage of viable spermatozoa ejaculated from such roosters to levels comparable to those observed for fertile roosters. If these viable spermatozoa from \( Sd \) roosters are placed within the oviduct, they nonetheless die prematurely (Kirby et al., 1989). Consequently, subfertility associated with the \( R/R \) \( Sd/sd^+ \) genotype is attributed to inadequate filling of the uterovaginal glands and premature degeneration of spermatozoa that are sequestered within these glands.
TABLE A2.1  Fertility over a 21-day egg collection interval following a single intravaginal insemination of Single Comb White Leghorn hens with spermatozoa from one of four genotypes of rose comb roosters.

<table>
<thead>
<tr>
<th>Rooster Genotype$^2$</th>
<th>Hens (n)</th>
<th>Eggs (n)</th>
<th>Fertility$^3$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R/r$^+$ sd$^+$/sd$^+$</td>
<td>60</td>
<td>999</td>
<td>53 ± 2.1$^a$</td>
</tr>
<tr>
<td>R/r$^+$ Sd/sd$^+$</td>
<td>60</td>
<td>1080</td>
<td>36 ± 1.6$^b$</td>
</tr>
<tr>
<td>R/R sd$^+$/sd$^+$</td>
<td>60</td>
<td>1098</td>
<td>21 ± 2.1$^c$</td>
</tr>
<tr>
<td>R/R Sd/sd$^+$</td>
<td>60</td>
<td>1051</td>
<td>11 ± 1.2$^d$</td>
</tr>
</tbody>
</table>

$^1$Each hen was inseminated with 1 x 10$^8$ viable spermatozoa.
$^2$Sd denotes the sperm degeneration allele.
$^3$Each value represents a mean ± SEM.

$^a$,$^b$,$^c$,$^d$Means within a column bearing different superscripts are different (P<0.001).
FIGURE A2.1 Duration of fertility following intravaginal insemination of Single Comb White Leghorn hens with spermatozoa R/r+ sd+/sd+ (□), R/r+ Sd/sd+ (△), R/R sd+/sd+ (○) or R/R Sd/sd+ (△) roosters. Each hen (n = 60 per treatment) was inseminated with 1x10⁸ spermatozoa. Solid lines represent the functions

\[ y(x) = \frac{94.7}{1 + e^{-5228(12.1-x)}}, \]
\[ y(x) = \frac{93.8}{1 + e^{-4675(8.8-x)}}, \]
\[ y(x) = \frac{73.9}{1 + e^{-3379(5.04-x)}}, \]
and
\[ y(x) = -0.4775 + [59.68]e^{(-0.2829x)}. \]
REFERENCES


