AN ABSTRACT OF THE THESIS OF

John D. Kirby for the degree of Doctor of Philosophy in Poultry Science presented on May 4, 1990.

Title: Physiological and Anatomical Factors Associated with Heritable Spermatozoal Degeneration in the Male Domestic Fowl(Gallus domesticus)

Abstract Approved: Redacted for privacy
David P. Froman

The objective of this work was to define the basis of heritable spermatozoal degeneration in the fowl. In previous work (Froman and Bernier, 1987), it was hypothesized that spermatozoa died prematurely due to a dysfunction of the ductus deferens. The objective of the first experiments was to determine if spermatozoal degeneration occurred in the oviduct as well as in the ductus deferens. As expected, intravaginal inseminations of viable spermatozoa from affected males resulted in decreased fertility when compared to that of fertile Leghorns over a 21 day interval, 42±1.3 and 52±1.2 %, respectively. However, unlike the other genotypes observed, fertility was not improved following intramagnal insemination of spermatozoa from affected males (P>.05). Intramagnal heterospermic inseminations revealed a linear decline in the proportion of progeny sired by affected males over nine days. It was concluded that premature spermatozoal degeneration occurred in the ductus deferens and oviduct.

The second group of experiments were designed to: 1) determine if spermatozoa from affected males were inherently defective at spermiation or if the defect was acquired during passage through the excurrent ducts, and 2) to document the morphology of the excurrent ducts. Intramagnal insemination of testicular spermatozoa from affected and high fertility males revealed that these spermatozoa had comparable fertilizing abilities, 47 ± 2.2 and $41\pm3.6\%$, respectively. The inspection of excurrent duct morphology revealed obvious differences between affected and normal males only in the ductuli efferentes proximales. The ductuli efferentes proximales of affected roosters were characterized by a greater luminal cross-sectional area as well as a diminished height and number of epithelial folds ($P<.005$). It was concluded that the decreased viability of spermatozoa from affected males was the result of passage through the excurrent ducts.

Intramagnal inseminations of spermatozoa from affected males resulted in a transient superiority in fertilizing ability. The final experiments investigated the metabolic activity of spermatozoa from affected males, subfertile Wyandottes homozygous for the rose comb allele and fertile Leghorns. The tetrazolium dye reduction assay revealed that spermatozoa from affected males were not characterized by an aberrant metabolism. It was concluded that the defect acquired by passage through the excurrent ducts of affected males was not associated with spermatozoal metabolism.

Physiological and Anatomical Factors Associated with Heritable Spermatozoal Degeneration in the Male Domestic Fowl (*Gallus domesticus*)

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DEDICATION

This Doctoral Dissertation is dedicated to my parents, Jack and Arlene Kirby. I couldn't have done this without your tolerance and loving support. Thank you for everything.

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Professor Paul Bernier, emeritus, provided insight into the Delaware anomaly that only his experience could provide. I wish to thank him for his guidance and hope to some day possess his wisdom and 1/2 of his knowledge.

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PREFACE

Dr. David P. Froman, as major advisor, participated in all phases of the research reported herein. He was active in supervising the design, execution and analysis of each experiment.

Dr. Paul E. Bernier screened and determined the genotype of each male used in this work. Based on these results he also conducted the pedigree mating and hatching programs.

Dr. Harold N. Engel, Jr. served as the supervising surgeon for the intramagnal inseminations completed in Chapters III and IV. He also performed the surgical ligation of the ductus deferens discussed in Chapter IV.

Dr. Rex A. Hess completed the quantitative histology discussed in Chapter IV in his laboratory at the University of Illinois, Urbana, Illinois. He also photographed the tissue presented in Figure IV.3.

Rita M. Lawler classified and quantified the immune cells within the excurrent ducts as described in Appendix 2. She was also instrumental in the initial tissue embedding and histology reported in Chapter IV and Appendix 2.

Physiological and Anatomical Factors Associated with Heritable Spermatozoal Degeneration in the Male Domestic Fowl (*Gallus domesticus*)

CHAPTER I

INTRODUCTION

Successful sexual reproduction can only be possible following sperm and egg fusion. All of the myriad of events, physiological or behavioral, associated with reproduction are moot if fertilization does not occur. At the most basic level, all that is required for fertilization are an ovum and a spermatozoon in an appropriate medium. These seemingly simple requirements belie the complex processes that are required to place the sperm and ovum in proximity. In the domestic fowl, for example, the mature commercial laying hen will produce an ovum nearly every day. As the fowl is oviparous, the ovum and yolk are invested with a thick heterogeneous covering of proteins, the albumen, which in turn is enclosed by membranes and a hard, calcified, shell. These structures enhance the probability of survival for the developing embryo. However, the addition of these structures to the ovum results in a very narrow window in which sperm and egg binding must occur if fertilization is to be successful.

To overcome the difficulties in fertilization associated with oviparity, copulation usually occurs when the oviduct is not obstructed by an egg and many species have developed a capacity for sperm storage within the female's reproductive

tract (Thibault, 1973). The sequestration of sperm within the uterovaginal sperm storage tubules of the hen facilitates the production of fertilized eggs for several days following a single mating (van Drimmelen, 1946; Bobr, et al., 1964). This long-term extragonadal, indeed extra-male, maintenance of spermatozoa is truly unique when contrasted with typical mammalian and invertebrate model systems. Additionally, the excurrent ducts of the fowl's testis are seemingly simple when compared to those of mammals. Fowl also lack the male accessory organs that play a key role in the preparation of spermatozoa for fertilization in mammals. Recent reports (Esponda & Bedford, 1985; Morris, et al., 1987; Froman & Bernier, 1987; Clulow & Jones, 1988) have implied that the excurrent ducts of the fowl testis may play a significant role in sperm maturation and survival and thus are not simply transit and storage organs as previously thought (Munro, 1938; Bedford, 1975). Based on the above, the study of the excurrent ducts of the fowl testis is warranted, particularly in the context of spermatozoal maturation and function.

The research presented in this thesis was designed to define the basis of heritable spermatozoal degeneration in the domestic fowl as described by Froman & Bernier (1987). Experimental approaches included comparative and competitive fertility trials, histological evaluations of excurrent ducts, and measurements of sperm metabolic capacity. A parenthetical result of the fertility work was a reevaluation of statistical

approaches for analyzing fertility data. The results of the entire research effort should confirm the role of the fowl's excurrent ducts in sperm maturation and enhanced survivability, while describing a unique animal model system for the study of male reproduction.

REFERENCES

Bedford JM, 1979. Evolution of the sperm maturation and storage functions of the epididymis. In: The Spermatozoon. DW Fawcett and JM Bedford, Eds. Baltimore: Urban and Schwarzenberg, pp. 7-22.

Bobr LW, Lorenz FW, Ogasawara FX, 1964. Distribution of spermatozoa in the oviduct and fertility in domestic birds. I. Residence sites of spermatozoa in fowl oviducts. J Reprod Fert 8:39-47.

Clulow J, Jones RC, 1988. Studies of fluid and spermatozoal transport in the extratesticular genital ducts of the Japanese quail. J Anat 157:1-11.

Esponda P, Bedford JM, 1985. Surface of the rooster spermatozoon changes in passing through the Wolffian duct. J Exp Zool 234:441-449.

Froman DP, Bernier PE, 1987. Identification of heritable spermatozoal degeneration within the ductus deferens of the chicken (Gallus domesticus). Biol Reprod 37:969-977.

Morris SA, Howarth, B, Jr, Crim JW, Rodriguez de Cordoba S, Esponda P, Bedford JM, 1987. Specificity of sperm-binding Wolffian duct proteins in the rooster and their persistence on spermatozoa in the female host glands. J Exper Zool 242:189-198.

Munro SS, 1938. Functional changes in fowl sperm during their passage through the excurrent ducts of the male. J Exp Zool 79:71-92.

Thibault C, 1973. Sperm transport and storage in vertebrates. J Reprod Fert (Suppl)18:39-53.

van Drimmelen GG, 1946. Sperm nests in the oviduct of the domestic hen. J S African Vet Med Assoc 17:42-52.

CHAPTER II

LITERATURE REVIEW: GENETIC MODELS OF SUBFERTILITY IN THE MALE DOMESTIC FOWL

Significant advances in the study of biological phenomena frequently result from the study of unique genetic models. The study of such genetic diseases as hemophilia and phenylketonuria led to dramatic advances in the understanding of factors promoting blood clotting and phenylalanine metabolism, respectively (Gardner & Snustad, 1984). In contrast, the study of male reproductive function in the domestic fowl has been based primarily on highly selected commercial strains. In many cases these animals have been selected primarily for reproductive capacity. To date heritable subfertility due to factors other than selection for growth rate has only been reported in three types of males: Cornish (Parker, 1961), males homozygous for the rose comb gene (Crawford & Merritt, 1963), and the Delaware (Froman & Bernier, 1987).

Of the three, the Cornish rooster's subfertility was due to body conformation and behavior as normal fertility was observed when hens were artificially inseminated (Parker, 1961). While this condition demonstrated the importance of body conformation and behavior on the male's capacity to mate naturally, it had no effect on reproductive tract or gamete function. The pleiotropy associated with homozygosity of the

rose comb gene on fertility and heritable spermatozoal degeneration in the Delaware male are directly associated with the functionality of the reproductive tract or gametes. Thus, each of these will be reviewed separately.

HOMOZYGOSITY FOR THE ROSE COMB ALLELE AND FERTILITY

The rose comb allele (R) is completely dominant to that for single comb (r) as described by Bateson (1902). It is an autosomal allele with homozygous (RR) and heterozygous (Rr) individuals being indistinguishable by comb type (Bateson, 1902; Crawford, 1963). A second gene associated with comb morphology is pea (P). When the P allele is present with R_ a walnut comb results and with rr a pea comb. Rose comb and single comb individuals are homozygous for p (Bateson & Punnett, 1905).

The effect of rose comb on fertility was initially described by Hindhaugh (1932). He reported that the depressed average fertility of White Wyandottes, where rose comb is a breed standard, was due to the very poor fertility of only a few of the individuals within a flock. Hutt (1940) confirmed the subfertile status of the White Wyandotte by comparing hatching records from pedigree stations. He was able to show that the hatchability of the Wyandottes was consistently lower than that of either Leghorns or Rhode Island Reds, both of which have a single comb. However, as Crawford (1963) observed, these investigators did not make any connection

between comb type and reduced reproductive capacity.

The connection between the rose comb gene and subfertility of White Wyandottes was first reported in 1951, in two separate reports (Cochez, 1951; Ponsignon, 1951). Of these, Cochez (1951) demonstrated poor fertility in White Wyandottes only when they were homozygous for the rose comb. In this report the effects of homozygosity for rose comb were not separated into male and female effects. In contrast to Ponsignon (1951), Cochez (1951) attributed the reduced hatchability of White Wyandottes solely to a decrease in fertility. Cochez (1951) also reported that hens of all three comb genotypes (rr, Rr, RR) had normal fertility following insemination with spermatozoa from rr or Rr males. However, both Cochez (1951) and Ponsignon (1951) attributed the reduced fertility or overall hatchability of rose comb birds, when compared to that of single comb birds, to a lethal factor associated with the rose comb gene.

Crawford & Merritt (1963), using the commercial Ottawa Meat Control strain, reported that the reduced hatchability of eggs from rose comb matings was due to reduced fertility. They also demonstrated that the observed effects were due to the rose comb gene and that it was only expressed in males homozygous for rose comb. Their work (Crawford & Merritt, 1963) confirmed Cochez's (1951) finding that hens of all three comb genotypes appeared to reproduce equally well.

The reduced fertility observed in hens mated to RR males

was investigated further in a series of reports (Crawford, 1963,1965; Crawford & Smyth, 1964a,b,c,d). Crawford & Smyth (1964a) proposed the pleiotropic effect of RR on fertility and presented evidence against linkage. They further demonstrated that the normal fertility of Rr males was due to equally fertile R and r containing spermatozoa. Thus, Crawford & Smyth (1964a) concluded that the decreased fertility of spermatozoa from RR males was not due to a direct action of the R gene in the sperm cell itself, but was mediated through a "host body effect". In a subsequent report (Crawford & Smyth, 1964b), the microscopic evaluation of blastodiscs of eggs macroscopically determined to be infertile provided further evidence that the RR males were truly subfertile. This work also confirmed the observations of Crawford & Merritt (1963), via artificial insemination, that the reduced fertility of RR males was not primarily due to decreased sexual activity.

Crawford & Smyth (1964c) utilized intravaginal, intrauterine, and intraperitoneal insemination to study the effects of sperm transport on fertility. From these experiments they concluded that aberrant sperm transport was not involved in the decreased fertility observed with spermatozoa from RR males. In yet another report (Crawford & Smyth, 1964d), a subjective sperm motility scoring system and the methylene blue reduction time assay were used to estimate sperm metabolism. These authors concluded that the metabolism of spermatozoa from RR males was equivalent to that of

spermatozoa from Rr or rr males. An effect of seminal plasma on the fertilizing capacity of spermatozoa from RR males was discounted by fertility trials utilizing spermatozoa from RR and rr males coincubated in the seminal plasma of the other. The fertilizing capacity of spermatozoa from RR males was neither improved following incubation in seminal plasma from rr males nor did seminal plasma from RR males have any deleterious effect upon spermatozoa from rr males. Crawford (1965) also presented results of heterospermic inseminations of Single Comb White Leghorn (SCWL) hens with spermatozoa from RR and Rr males. In this experiment 75% of the progeny sired were expected to be Rr and 25% rr, if spermatozoa from RR and Rr males had equivalent fertilizing capabilities. However, only 56.6% of the progeny were rose comb leading Crawford (1965) to conclude that the RR males were functionally infertile following heterospermic insemination. Crawford (1965) further concluded that the number of progeny sired by RR males was further reduced in the Ottawa Meat Control Strain due to those males' reduced frequency of copulation when compared to Rr penmates.

The reduced fertilizing ability of spermatozoa from RR males was also confirmed in Ottawa Meat Control Strain males segregating for the P allele (Crawford, 1964). In this study the effects of RR were shown to be independent of the P allele as both walnut and rose comb individuals demonstrated reduced fertility. As the Ottawa Meat Control Strain was derived

partially from White Wyandotte stock, a second, only distantly related breed of rose comb bird, Silver Spangled Hamburg, was investigated (Crawford, 1971). When homozygous for the rose comb allele these birds demonstrated a reduction in fertility similar to that observed with White Wyandottes and Ottawa Meat Control Strain males, which further supported pleiotropy as the cause of reduced fertility.

Fox et al. (1964) investigated the persistence of the r allele in populations where only R_ individuals were allowed to mate. Based on limited selection, a reproductive fitness of 0 for rr individuals, and 60 generations since the formation of the Wyandotte breed, Fox et al. (1964) estimated the frequency of the r allele in a randomly mating population to be 0.016. Additionally, based on the assumption of operational overdominance at the R allele, derived from fertility trials using artificial insemination, they calculated a frequency for r at 0.07. However, in their experimental flocks as well as others, between 10 and 17% of Wyandotte progeny were single comb implying that a true frequency for r should be closer to 0.40 (Fox et al., 1964). Fox and his coworkers attributed the large disparity in predicted and observed frequencies of the r allele to the poor performance of spermatozoa from RR males following heterospermic or sequential matings and reduced libido (as reported in: Crawford & Merritt, 1963; Crawford & Smyth, 1964a,b,c,d; Crawford, 1963,1965).

In an attempt to explain the disparity between Fox et

al.'s (1964) expected frequency and observed frequency for r, Wehrhahn & Crawford (1965) proposed another model. According to Wehrhahn & Crawford (1965) if the RR males in any given randomly mating population, following removal of the rr males whose fitness is 0, were considered to be sterile then an equilibrium of 14.64% rr progeny was rapidly attained. In theory this level of single comb progeny could be found in any flock, following removal of rr individuals from the parent generation, regardless of the flock's breeding history. Thus, Wehrhahn & Crawford (1965) concluded that the large proportion of Rr and rr individuals found in any generation could be readily explained by assuming infertility of the RR males. The frequencies of rr progeny, 10 to 17%, are very close to the equilibrium frequency predicted by Wehrhahn & Crawford (1965) and were easily accounted for through minor adjustments to the model, primarily through allowing for a small proportion of offspring to be sired by RR males.

Petitjean & Cochez (1966) reexamined the causes of subfertility in RR males. These investigators reaffirmed the subfertile status of White Wyandotte RR males. In contrast to the Ottawa Meat Control Strain, they found in their population that the RR and Rr males were behaviorally indistinguishable. In further studies they were unable to find any differences in the physical attributes, e.g., sperm morphology, mortality or initial motility of spermatozoa among genotypes (Petitjean & Cochez, 1966). However, when spermatozoa were placed in a

diluent and stored for 18 or more hours at 3° C, the motility of spermatozoa from RR males was significantly reduced. Petitjean and Cochez (1966) also exchanged the seminal plasma of ejaculates from RR and Rr males, resuspended the mixtures in diluent and evaluated motility following storage at 3° C for 18 hours. The exchange of seminal plasma had no effect on the motility of spermatozoa from either genotype, based on the subjective scoring system used. Based on these experiments Petitjean & Cochez (1966) concluded that the reduced fertility observed in RR males could be due to poor motility and that these effects were independent of the seminal plasma.

Buckland & Hawes (1968) investigated the effects of the R and P alleles on spermatozoal and testicular attributes. They reported no differences in sperm morphology, viability or testicular attributes that could account for the differences observed in fertility. Furthermore, they found no effect of the P allele on reproductive parameters (Buckland & Hawes, 1968). Buckland et al. (1969) assayed the activities of several enzymes associated with the Krebs cycle. These included fumarase, aconitase, isocitric dehydrogenase and malic dehydrogenase. Comparisons were made with extracts of spermatozoa from Macdonald Large White (RR,Rr,rr), Rhode Island Red and Athens Random-bred Control males. The spermatozoa of the RR males were characterized by reduced levels of activity for fumarase and aconitase. However, only fumarase activity was significantly correlated with the

fertilizing ability of spermatozoa. From these results Buckland et al. (1969) concluded that fumarase activity affects the fertilizing ability of fowl spermatozoa. They also postulated that spermatozoa from RR males, characterized by reduced levels of fumarase activity, may have a decreased duration of survival within the oviduct leading to a reduced level and duration of fertility. Petitjean and Servouse (1981) compared the levels of fumarase activity of spermatozoa from RR and rr males. This work failed to identify any genotypic effect on fumarase activity, casting doubt on the findings of Buckland et al (1969).

Etches et al. (1974) used heterospermic intramagnal and intravaginal inseminations with spermatozoa from RR and rr males, as well as those from PoPo (polydactyly) and popo males, to further elucidate the problem of RR subfertility. Following heterospermic intramagnal and intravaginal inseminations, the RR and popo males sired significantly fewer progeny, than did the rr and PoPo males. However, the fertilizing ability of spermatozoa from RR males was dramatically improved as indicated by a near four-fold increase in progeny (8.7 to 31.7% of total sired) following intramagnal insemination. Allen & Grigg (1957) and Shindler et al. (1967) had previously demonstrated that motility was required for spermatozoa to pass through the uterovaginal junction. Therefore, Etches et al. (1974) concluded that the reduced fertility of RR males was attributable to an inability

of spermatozoa to pass through the lower oviduct and thus become sequestered within the uterovaginal sperm storage tubules.

The possibility that poorly motile spermatozoa fail to become sequestered within the uterovaginal sperm storage tubules, is supported by the observations of Howarth (1983). In this work, testicular sperm from fertile males, which were characterized by the nearly total absence of motility, were inseminated either intravaginally or intramagnally. Following intravaginal inseminations, testicular spermatozoa failed to fertilize ova. However, fertility was observed when these spermatozoa were placed into the magnum. Howarth (1983) thus demonstrated that testicular spermatozoa of the fowl are capable of fertilizing ova when placed within the magnum and that testicular spermatozoa are apparently incapable of entering the sperm storage tubules. This work closely parallels the findings of Etches et al. (1974) using spermatozoa from RR males.

A major assumption of the research done to date with spermatozoa from RR males is that there is no direct effect of the rose comb allele on the sperm cell as R carrying spermatozoa from heterozygous males function normally (Crawford & Smyth, 1964a). Thus, most efforts were concentrated on determining an external or host effect on the spermatozoa of RR males. This assumption may not be valid, as Braun et al. (1989) have shown that postmeiotic events in the

haploid spermatid do not necessarily lead to two different classes of sperm cells. Through the use of transgenic mice, the exchange of messenger RNA and proteins via intracellular bridges connecting the developing spermatids has been clearly demonstrated. Thus, though the sperm cell itself may not possess a copy of the allele in question, it may well contain the gene product. This model could possibly answer the question of why spermatozoa from heterozygotes containing the R allele perform so well, if the reduced fertilizing ability of spermatozoa from RR males is due to a direct action of the R allele.

SUBFERTILE DELAWARE MALES

A subfertile line of Delaware roosters was identified in 1964 by Drs. J.E. Parker and P.E. Bernier of the Oregon Agricultural Experiment Station (P.E. Bernier, personal communication). A heritable basis was established and selection for reduced fertility was continued until 1977. Following Dr. Bernier's retirement, selection was discontinued until 1984 at which time a program was established to continue work on the line.

The cause of subfertility in Delaware males was described by Froman & Bernier (1987). In this work, the observed reduction in fertility was attributed to premature death and degeneration of spermatozoa within the ductus deferens. Froman & Bernier (1987) demonstrated that the proportion of

viable spermatozoa, as determined via ethidium bromide exclusion (Bilgili & Renden, 1984), increased due to frequent ejaculation. Thus, they hypothesized that spermatozoal degeneration was due to a dysfunction in the storage capacity of the ductus deferens.

Based on the appearance of degenerating sperm nuclei and axonemal fusion in the micrographs of Froman & Bernier (1987), Kirby & Froman (1988) attempted to reverse spermatozoal degeneration via dietary supplementation with zinc. Feeding affected roosters diets containing up to 25 times the daily requirement of zinc had no apparent effect on spermatozoal integrity or semen production. Neither the concentration of zinc within the seminal plasma nor spermatozoa was affected by supplementation.

Through the use of crossbreeding experiments the genetic basis for heritable spermatozoal degeneration has been described (Kirby et al., 1990). The genetic basis for the trait appears to be due to a single dominant gene. The progeny of affected Delaware x Rhode Island Red males and Single Comb White Leghorn females segregated for the trait at approximately a one to one ratio. The penetrance for the trait appeared to be incomplete as full brothers frequently showed ranges of degenerate spermatozoa per ejaculate of 20 to 80% (unpublished data). The degree of spermatozoal degeneration may be affected by associated modifier genes,which may have been co-selected along with the primary trait (P.E. Bernier,

personal communication).

REFERENCES

Allen TE, Grigg GW, 1957. Sperm transport in the fowl. Aust J Agri Res 8:788-799.

Bateson W, 1902. Experiments with poultry. Repts Evol Comm Roy Soc I:87-124. (Cited from Crawford, 1963).

Bateson W, Punnett RC, 1905. A suggestion as to the nature of the "walnut" comb in fowls. Proc Cambridge Phil Soc 13:165-168.

Bilgili SF, Renden JA, 1984. Fluorometric determination of avian sperm viability and concentration. Poultry Sci 63:2275-2277.

Braun RE, Behringer RR, Peschon JJ, Brinster RL, Palmiter RD, 1989. Genetically haploid spermatids are phenotypically diploid. Nature 337:373-376.

Buckland RB, Hawes RO, 1968. Comb type and reproduction in the male fowl: Semen characteristics and testes structure. Poultry Sci 47:704-710.

Buckland RB, Wilcox FH, Schaffner CS, 1969. Influence of homozygosity for rose comb on fumarase, aconitase, isocitric dehydrogenase and malic dehydrogenase activity in spermatozoa of the domestic fowl (Gallus domesticus). J Reprod Fert 18:89-95.

Cochez LP, 1951. An infertility factor balanced by breeding in White Wyandottes. Proc IXth World's Poultry Cong I:122-128

Crawford RD, 1963. Studies on the relationship between fertility and the gene for rose comb in the domestic fowl. Ph. D. dissertation. Amherst: U. of Mass.

Crawford RD, 1964. Fertility of walnut-combed domestic fowl. Can J Anim Sci 44:184-186.

Crawford RD, 1965. Comb dimorphism in Wyandotte domestic fowl. I. Sperm competition in relation to rose and single comb alleles. Can J Genet Cytol 7:500-504.

Crawford RD, 1971. Rose comb and fertility in Silver Spangled Hamburgs. Poultry Sci 50:867-869.

Crawford RD, Merritt ES, 1963. The relationship between rose comb and reproduction in the domestic fowl. Can J Gen Cytol 5:89-95.

Crawford RD, Smyth, JR, Jr, 1964a. Infertility and action of the gene for rose comb in the domestic fowl. Can J Gen Cytol 6:298-303.

Crawford RD, Smyth, JR, Jr, 1964b. Studies on the relationship between fertility and the gene for rose comb in the domestic fowl. 1. The relationship between comb genotype and fertility. Poultry Sci 43:1009-1017.

Crawford RD, Smyth, JR, Jr, 1964c. Studies on the relationship between fertility and the gene for rose comb in the domestic fowl. 2. The relationship between genotype and duration of fertility. Poultry Sci 43:1018-1026.

Crawford RD, Smyth, JR, Jr, 1964d. Semen quality and the gene for rose comb in the domestic fowl. Poultry Sci 43:1551-1557.

Etches RJ, Buckland RB, Hawes RO, 1974. The effect of the genes for rose comb and polydactyly on sperm transport in the hen's oviduct. Poultry Sci 53:422-424.

Fox TW, Crawford RD, Smyth, JR, Jr, 1964. Rose comb: An example of operational overdominance in the domestic fowl. Genet Res 5:379-383.

Froman DP, Bernier PE, 1987. Identification of heritable spermatozoal degeneration within the ductus deferens of the chicken (Gallus domesticus). Biol Reprod 37:969-977.

Gardner EJ, Snustad DP, 1984. Principles of Genetics, 7th edn. New York: Wiley, pp.230-270.

Hindhaugh WLS, 1932. Some observations on fertility in White Wyandottes. Harper Adams Util Poultry J 17:555-560.

Howarth, B Jr, 1983. Fertilizing ability of cock spermatozoa from the testis, epididymis, and vas deferens following intramagnal insemination. Biol Reprod 28:586-590.

Hutt FB, 1940. A relation between breed characteristics and poor reproduction in White Wyandotte fowls. Am Nat 74:148-156.

Kirby JD, Bernier PE, Froman DP, 1990. Heritable spermatozoal degeneration in the fowl due to a dominant gene. J Androl 11:P49.

Kirby JD, Froman DP, 1988. Effect of dietary supplementation of zinc on the spermatozoal integrity of low-fertility Delaware roosters. Poultry Sci 67(Sup. 1):103.

Parker JE, 1961. Observations on the low fertility problem in Cornish cockerels. Poultry Sci 40:1214-1219.

Petitjean MJ, Cochez LP, 1966. Subfertility of cocks homozygous for the "R" gene (rose comb). Proc XIIIth World's Poultry Cong I:121-127.

Petitjean MJ, Servouse M, 1981. Comparative study of some characteristics of the semen of RR (rose comb) or rr (single comb) cockerels. Reprod Nutr Develop 21:1085-1093.

Schindler HE, Ben-David E, Hurwitz S, Kempinich-Pinto O, 1967. The relation of spermatozoa to the glandular tissue in the storage sites of the hen oviduct. Poultry Sci 46:1462-1471.

CHAPTER III

DECREASED SPERM SURVIVABILITY IN SUBFERTILE DELAWARE ROOSTERS AS INDICATED BY COMPARATIVE AND COMPETITIVE FERTILIZATION[1]

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ABSTRACT

Duration of fertility following intravaginal and intramagnal insemination of hens with viable spermatozoa from subfertile Delaware roosters was compared with that obtained with spermatozoa from fertile Leghorns and subfertile Wyandotte roosters. In contrast to results with Leghorn and Wyandotte birds, duration of fertility was not increased following intramagnal insemination of spermatozoa from Delaware birds. Heritable subfertility in Wyandotte and Delaware birds appears to be attributable to distinct sperm defects.

INTRODUCTION

Froman and Bernier (1987) identified a heritable reproductive disorder in roosters within a line of Delaware chickens maintained by the Oregon Agricultural Experiment Station. The disorder was characterized by sperm degeneration within the mid- and distal ductus deferens which appeared to account for the subfertility that originally drew attention to these roosters. Because seminal quality was improved by changing the frequency of ejaculation from a weekly to a daily basis, the extent of sperm degeneration appeared to depend on the duration of sperm residence within the ductus deferens. Therefore, Froman and Bernier (1987) suggested that a dysfunction of the ductus deferens resulted in sperm degeneration therein.

However, this hypothesis is tenable only if a sperm defect can be discounted. After spermiogenesis, chicken spermatozoa are maintained within the distal ductus deferens for several days before being ejaculated or voided into the cloaca (de Reviers, 1975). After ejaculation, chicken spermatozoa can be maintained within the hen's uterovaginal sperm storage glands for many days before being made available for fertilization (Bobr et al.,1964). Consequently, a diminished duration of fertility would be symptomatic of a sperm defect. Therefore, the objective of the present work was to assess the duration of fertility of viable spermatozoa

ejaculated from roosters characterized by the reproductive disorder described by Froman and Bernier (1987).

MATERIALS AND METHODS

EXPERIMENT 1

Semen was collected from roosters representing 4 genotypes: Single Comb White Leghorn (SCWL; n=5), Silver-Laced Wyandotte heterozygous for rose comb (n=4), subfertile Delaware (n=5), and Silver-Laced Wyandotte homozygous for rose comb (n=5). All roosters had been ejaculated for $\geq$3 weeks before this experiment. With the exception of the Delaware birds, roosters were ejaculated weekly throughout the experiment. Daily ejaculation of Delaware males began 4 days before the first insemination and continued every other day thereafter.

Semen was pooled according to rooster genotype. Percentages of viable spermatozoa as well as sperm concentrations were determined fluorometrically by ethidium bromide uptake (Bilgili and Renden, 1984). Each of the 4 semen samples was diluted to 2.0×10^9 viable spermatozoa/ml with Beltsville Poultry Semen Extender (BPSE; a gift from Dr. T. Sexton, USDA/ARS, Beltsville, MD, USA). Each sperm suspension was used to intravaginally inseminate 30 SCWL hens with 1.0×10^8 viable spermatozoa per hen. Three replicate fertility trials were completed for each rooster genotype.

Egg collection began on the second day after insemination and continued for 21 days. Eggs were set weekly. Fertility was assessed by breaking eggs open after 4 days of incubation and examining the contents for embryonic development. Arc sine

transformations of percentages of fertilized eggs were analyzed by single classification analysis of variance (Sokal and Rohlf, 1981).

Data were pooled when no difference ($p>0.05$) was observed among replicate fertility trials. Percentages of fertilized eggs were plotted by day. Because plots conformed to logistic functions, parameters of

$$y(x) = \frac{\gamma}{1+e^{\beta(\tau-x)}}$$

were estimated by iterative least squares (Freund and Littell, 1986a).

EXPERIMENT 2

Semen was collected and sperm suspensions prepared as above with two exceptions. First, semen from Wyandotte roosters heterozygous for rose comb was not used. Second, each sperm suspension contained 5×10^8 viable spermatozoa/ml. For each sperm suspension, each of 25 to 30 SCWL hens was inseminated intramagnally with 5×10^7 viable spermatozoa via laparotomy (Howarth, 1983). Egg collection, incubation and data analyses were performed as above.

EXPERIMENT 3

Semen was collected from the Delaware roosters used in the preceding experiment as well as from 5 Brown Leghorns. The latter had been characterized as having a fertility equivalent to that of the SCWL roosters ($p>0.05$). Ejaculates were pooled according to breed. After the seminal evaluations described

above, the semen samples from the Delaware and Brown Leghorn birds were diluted with BPSE to 2.0 X 10^9 viable spermatozoa/ml. Equal volumes of these sperm suspensions were mixed, and each of 40 New Hampshire hens was inseminated intravaginally with 1 X 10^8 viable spermatozoa from this mixture.

Eggs were collected and set as above. However, eggs were incubated for 21 days and down color of chicks was assessed at hatch. Down color was also recorded for chicks that failed to hatch. Percentages of chicks sired by Delaware males were plotted by day. Parameters of

$$y(x) = \alpha + \beta_1 x + \beta_2 x^2$$

were estimated by weighted least squares (Freund and Littell, 1986b).

EXPERIMENT 4

Semen was collected and sperm suspensions prepared according to the preceding experiment with one exception: each sperm suspension contained 5 X 10^8 viable spermatozoa/ml. Each of the 40 New Hampshire hens used in the preceding experiment was inseminated intramagnally (Howarth, 1983) with 5 X 10^7 viable spermatozoa. Egg collection, incubation, and chick down color were determined as above. Parameters of

$$y(x) = \alpha + \beta x$$

were estimated by least squares (Freund and Littell, 1986a).

RESULTS

As shown in Table III.1, daily ejaculation of subfertile Delaware roosters for 5 consecutive days and every other day thereafter yielded percentages of viable spermatozoa that were comparable to those of Leghorn and Wyandotte birds. However, as shown in Table III.2, sperm fertilizing ability was not equivalent among rooster genotypes. The means in Table III.2 represent those of pooled data as no differences were observed ($p>0.05$) among replicate fertility trials. The fertilizing ability of spermatozoa from Delaware birds was intermediate ($p<0.001$) between that of Wyandottes homozygous for rose comb, which are known to be characterized by subfertility (Crawford and Merritt, 1963), and that of Wyandottes heterozygous for rose comb, which like Leghorns, are not subfertile.

Fertility after intramagnal insemination of spermatozoa from Leghorn, Delaware or subfertile Wyandotte roosters is shown in Table III.2. When compared to values obtained with intravaginal insemination, fertility of Leghorn and subfertile Wyandotte birds increased by 13 and 30%, respectively, but there was no change for Delaware roosters.

These relationships were more evident after graphical analyses (Figure III.1). Estimates of τ, time at which half-maximal fertility occurred, were 11.8, 9.1 and 6.0 days after intravaginal insemination for Leghorn, Delaware, and subfertile Wyandotte birds, respectively, and 15.2, 9.6 and 13.9 days following intramagnal insemination. For spermatozoa

from Delaware males, the hypothesis that 9.1 and 9.6 were estimates of the same parameter was not rejected ($p>0.05$). Thus, unlike the spermatozoa from the Leghorn and subfertile Wyandotte birds, intramagnal insemination of those from Delaware birds failed to increase duration of fertility.

When New Hampshire hens were inseminated intravaginally or intramagnally with spermatozoa from Delaware and Brown Leghorn roosters, most chicks were sired by Brown Leghorns (Table III.3). As shown by the fitted curves in Figure III.2, percentages of chicks sired by Delaware birds were not constant throughout the egg collection intervals. The hatch of fertile eggs after intramagnal insemination (Table III.3) also shows that the insemination dose of 5×10^7 spermatozoa did not increase the incidence of embryonic mortality.

TABLE III.1 Percentages of viable spermatozoa ejaculated by 4 genotypes of rooster.

Breed of rooster	No. of birds	Comb genotype	Sperm viability (%)[1]
White Leghorn	3	rrpp	100±0.0
Wyandotte	4	Rrpp	100±0.0
Delaware	5	rrpp	98±1.1
Wyandotte	5	RRpp	96±1.0

[1]Each value represents a mean ± SEM for 3 replicates of pooled semen.

TABLE III.2 Fertility of Single Comb White Leghorn hens over a 21-day egg collection interval following a single intravaginal or intramagnal insemination with 1×10^8 or 5×10^7 spermatozoa, respectively.

Insemination route	Breed of semen donor	Comb genotype	No. of eggs	Fertility (%)[1]
Intravaginal	White Leghorn	rrpp	1366	51±1.2*
	Wyandotte	Rrpp	1360	47±1.4*
	Delaware	rrpp	1295	42±1.3
	Wyandotte	RRpp	1348	26±1.4*
Intramagnal	White Leghorn	rrpp	161	64±1.2*
	Delaware	rrpp	202	41±0.4
	Wyandotte	RRpp	176	57±1.0*

[1]Fertility values are mean ± SEM.
*$p<0.001$ compared with that for Delaware roosters.

TABLE III.3 Comparison of fertility, hatchability, and paternity of chicks from eggs laid by New Hampshire hens over a 2-week interval following insemination with spermatozoa from Delaware and Brown Leghorn roosters[1].

Type of Insemination	No. of eggs	Fertility (%)	Hatch of fertilized eggs (%)	Chicks sired by Delawares (%)
Intravaginal	407	69	71	36
Intramagnal	365	70	75	33

[1]Insemination doses were 1 X 10^8 and 5 X 10^7 viable spermatozoa per hen for intravaginal and intramagnal inseminations, respectively. In either case, 50% of the spermatozoa inseminated were from Delaware roosters.

FIGURE III.1 Duration of fertility after intravaginal (●) and intramagnal (△) insemination of Single Comb White Leghorn hens with spermatozoa from (a) Single Comb White Leghorn roosters, (b) Delaware roosters, and (c) Wyandotte roosters homozygous for rose comb. Insemination doses were 1×10^8 and 5×10^7 viable spermatozoa, respectively. Solid lines represent the functions

III.1(a) $y(x) = 97/1+e^{-0.5340(11.8-x)}$ and $y(x) = 94/1+e^{-0.7634(15.2-x)}$,

III.1(b) $y(x) = 100/1+e^{-0.4266(9.1-x)}$ and $y(x) = 100/1+e^{-0.7507(9.6-x)}$,

III.1(c) $y(x) = 94/1+e^{-0.4653(6.0-x)}$ and $y(x) = 97/1+e^{-0.8782(13.9-x)}$.

FIGURE III.1(a).

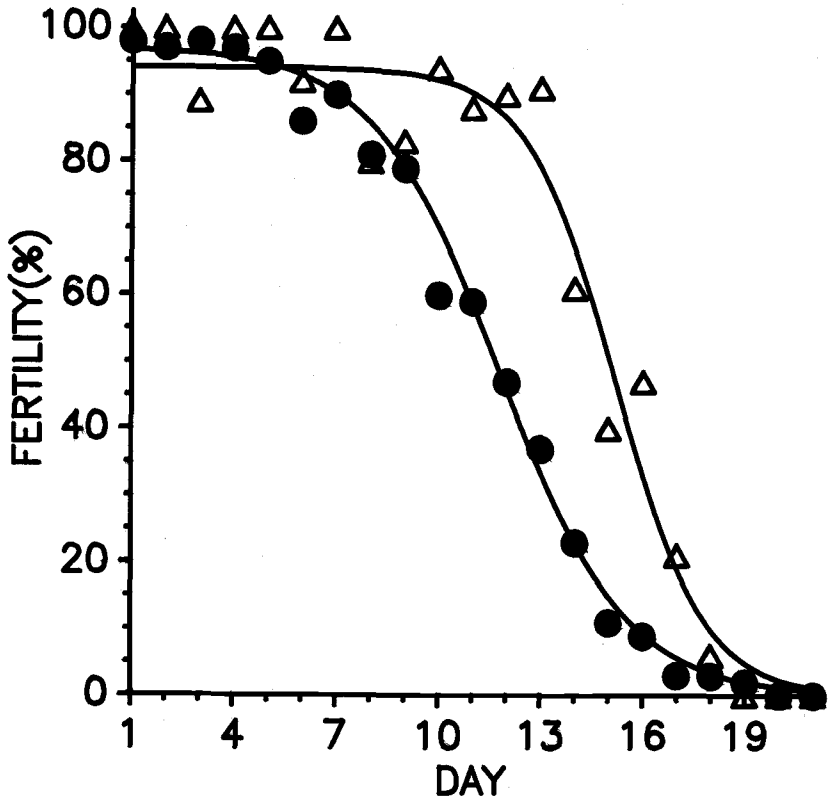


FIGURE III.1(b).

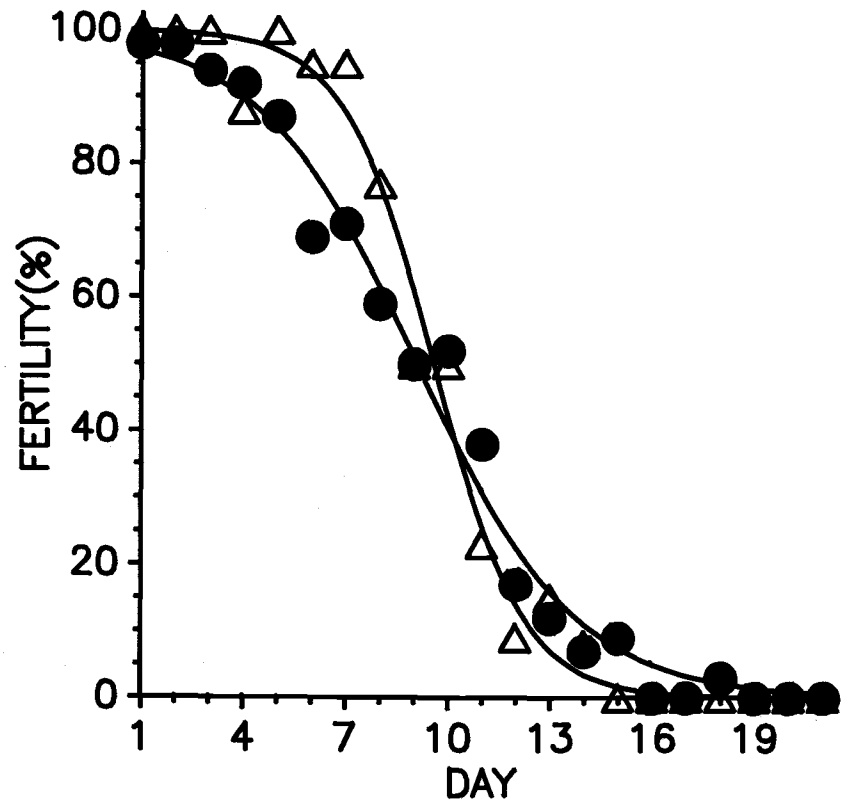


FIGURE III.1(c).

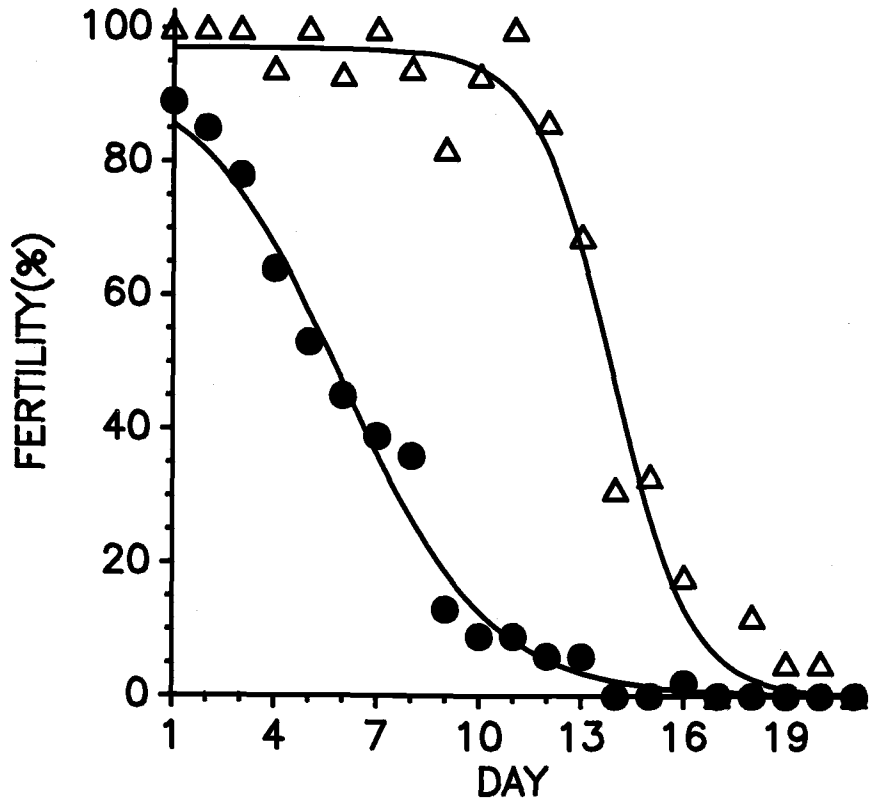


FIGURE III.2 Percentages of chicks sired by Delaware roosters (▲) following (a) intravaginal and (b) intramagnal insemination of New Hampshire hens with equal numbers of spermatozoa from Delaware and Brown Leghorn roosters. In (a) the solid line (R^2=0.87) represents the function

$$y(x) = 37.5 + 1.01(x) - 0.214(x^2)$$

and the datum from Day 11 was excluded from the estimation of the fitted curve as a consequence of outlier detection (Freund and Littell, 1986c). In (b) the solid line (R^2=0.94) represents the function

$$y(x) = 68.9 - 5.6(x).$$

FIGURE III.2(a).

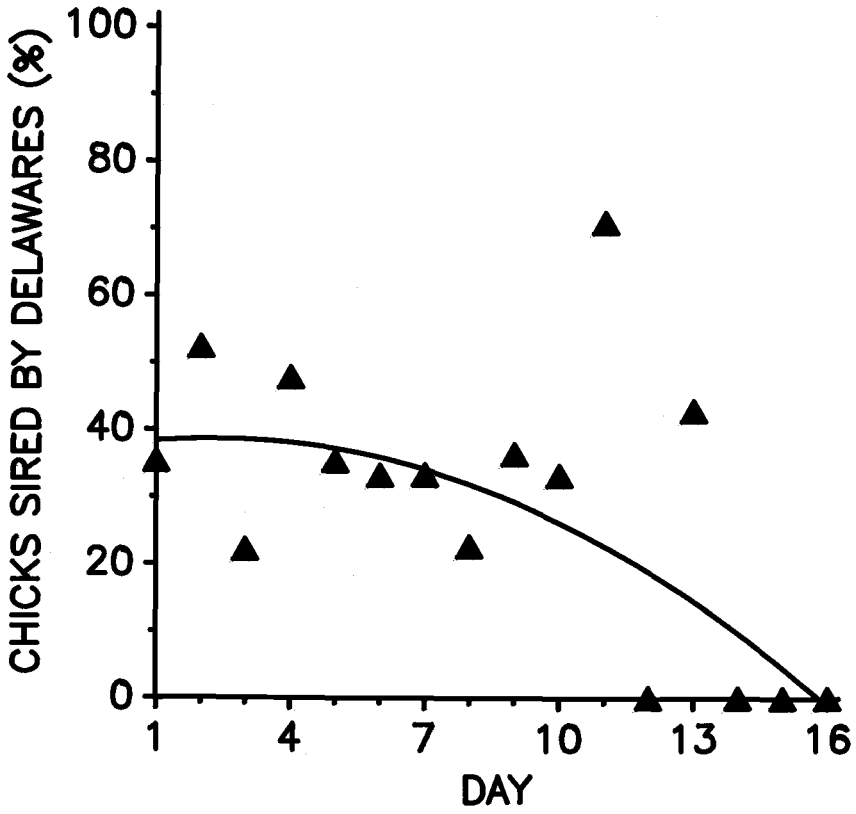
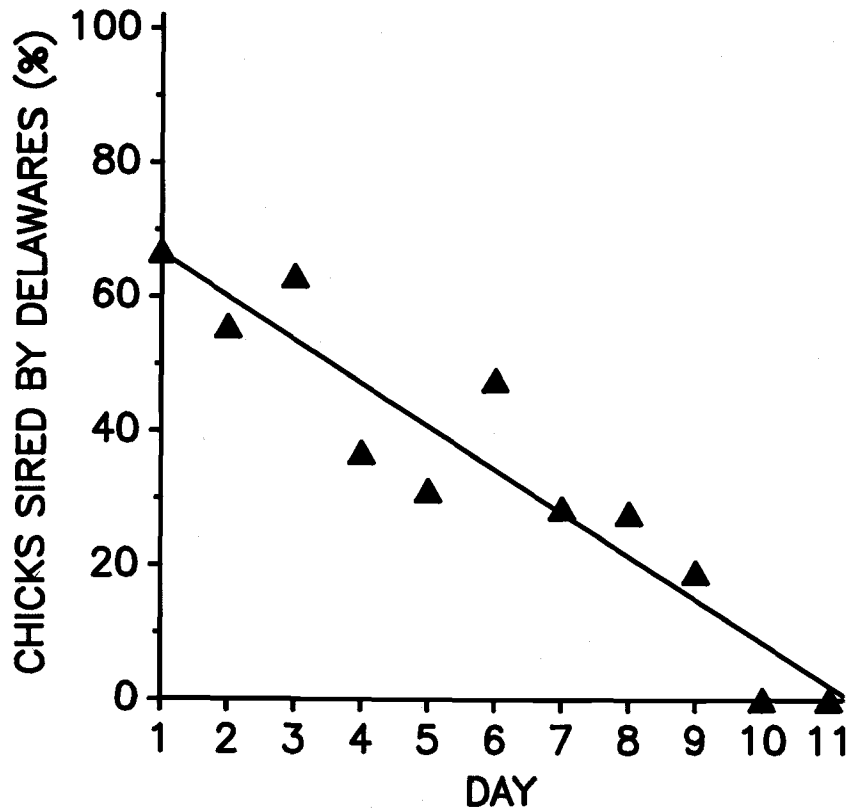


FIGURE III.2(b).



DISCUSSION

Heritable subfertility in roosters has been reported in two instances: roosters homozygous for rose comb (RR; Crawford and Merritt, 1963) and Delaware roosters (Froman and Bernier, 1987). The latter are characterized by a single comb and a Columbian feather color pattern. In each case, subfertility has been attributed to reduced sperm fertilizing ability. However, the mechanisms appear to be distinct.

Buckland et al. (1969) attributed subfertility in RR roosters to a reduced sperm fumarase activity and suggested that this difference might affect sperm survivability within the oviduct. In view of the duration of fertility observed following the intramagnal insemination of RR Wyandotte spermatozoa (Figure III.1c) as well as the inability of Petitjean and Servouse (1981) to detect reduced fumarase activity in extracts of spermatozoa from RR roosters, the hypothesis of Buckland et al. (1969) is untenable. Petitjean and Servouse (1981) did, however, report that spermatozoa from RR roosters were characterized by poor motility. Thus, in agreement with Etches et al. (1974), the subfertility of RR Wyandotte birds after intravaginal insemination (Figure III.1c) may be attributable to inadequate sequestration of spermatozoa within the uterovaginal sperm storage glands.

In contrast, Froman and Bernier (1987) concluded that sperm degeneration accounted for subfertility in Delaware

roosters. Theoretically, sperm degeneration within the ductus deferens could be the consequence of an autoimmune condition, a dysfunction of the ductus deferens, or a sperm defect. The autoimmunity hypothesis has been tested and rejected (unpublished data). Of the two remaining hypotheses, sperm autolysis warranted investigation first because such a phenomenon, were it to occur, would confound any investigation of the sperm maintenance function of the ductus deferens. We proposed that, if an aberrant duration of fertility were observed following the insemination of viable Delaware spermatozoa, then a sperm defect would be likely.

In Experiment 1, the fertilizing ability of spermatozoa from Delaware was compared with those of a genotype characterized by subfertility and two genotypes that are not. As shown in Table III.2, the fertilizing ability of Delaware birds was intermediate ($p<0.001$) between that of the RR and Rr Wyandottes. Even so, the fertilizing ability of Delaware chicken spermatozoa was more comparable to that of the Rr Wyandotte as well as New Hampshire and Barred Plymouth Rock birds (unpublished data) than it was to that of RR Wyandotte roosters. The implicit ranking of fertilizing abilities in Table III.2 is not only typical among breeds but also among individuals within breeds (Allen and Champion, 1955; Martin and Dziuk, 1977). The fertilizing ability of spermatozoa from Delaware roosters in experiment 1 was more typical of

spermatozoa from American breeds than from subfertile roosters *per se*.

However, when intramagnal inseminations were performed, spermatozoa from Delaware birds failed to yield fertility greater than that observed following intravaginal insemination. As was expected , based upon the work of Van Krey *et al*. (1966), intramagnal insemination with spermatozoa of SCWL birds increased fertility (Figure III.1a). This was also true with spermatozoa from RR Wyandottes (Figure III.1c). The latter may be explicable in terms of circumventing sperm sequestration within the uterovaginal sperm storage glands; Howarth (1983) demonstrated that intramagnal insemination of testicular spermatozoa, which are characterized by poor motility and an inability to fertilize ova following intravaginal insemination, yielded fertility equivalent to that obtained with ejaculated spermatozoa similarly inseminated. Because the two estimates of τ appeared to be independent estimates of the same unknown parameter, only in the case of Delaware spermatozoa, we concluded that intramagnal insemination of spermatozoa from Delaware birds revealed an aberrant duration of fertility.

Competitive fertilization was performed to characterize further the fertilizing ability of viable spermatozoa from Delaware roosters. According to Martin *et al*. (1974), the insemination of hens with equal numbers of spermatozoa from Leghorns and Columbian roosters yielded disproportionate

numbers of offspring but the proportions were constant over a 15 day egg collection interval. We anticipated a declining proportion of chicks sired by Delaware roosters under similar conditions. In these experiments, Brown Leghorns were used rather than White Leghorns because the former sire a brown chick when crossed with a New Hampshire hen whereas Delawares and White Leghorns sire yellow chicks. Preliminary work had shown that the fertilizing ability of spermatozoa of Brown Leghorns was equivalent to that of spermatozoa from White Leghorns ($p>0.05$). More importantly, preliminary work had also shown that there was no difference in the percentage of embryonic dead when New Hampshire hens were inseminated with spermatozoa from Brown Leghorn or Delaware roosters ($p>0.05$). As expected, the proportion of chicks sired by Delaware roosters declined as a function of time following intravaginal and intramagnal insemination (Figure III.2). However, the high percentage of chicks sired by Delaware roosters over the first three days following intramagnal insemination was not anticipated.

Froman and Bernier (1987) observed that once the percentage of degenerate spermatozoa in ejaculates from subfertile Delaware roosters had been reduced to $\leq 5\%$ via frequent ejaculation, the amount of sperm degeneration increased after 3 and was maximal after 12 days of sexual rest. In the present work, daily fertility following the intramagnal insemination of spermatozoa from Delaware birds

decreased from 100 to 14% over a 12 day interval. In contrast, when equal numbers of spermatozoa from SCWL and RR Wyandotte birds were inseminated similarly, daily fertility was $\geq$80% over the same interval. It therefore appears that heritable sperm degeneration within the ductus deferens of the chicken may be attributable to a sperm defect. A premature activation of sperm metabolism might account for sperm degeneration within the ductus deferens and the transient initial superiority, following intramagnal insemination, of spermatozoa of Delaware roosters.

REFERENCES

Allen CJ, Champion LR, 1955. Competitive fertilization in the fowl. Poultry Sci 34:1332-1342.

Bilgili SF, Renden JA, 1984. Fluorometric determination of avian sperm viability and concentration. Poultry Sci 63:2275-2277.

Bobr LW, Lorenz FW, Ogasawara FX, 1964. Distribution of spermatozoa in the oviduct and fertility in domestic birds. I. Residence sites of spermatozoa in fowl oviducts. J Reprod Fert 8:39-47.

Buckland RB, Wilcox FH, Shaffner CS, 1969. Influence of homozygosity for rose comb on fumarase, aconitase, isocitric dehydrogenase and malic dehydrogenase activity in spermatozoa of the domestic fowl (Gallus domesticus). J Reprod Fert 18:89-95.

Crawford RD, Merritt ES, 1963. The relation between rose comb and reproduction in the domestic fowl. Can J Genet Cytol 5:89-95.

de Reviers M, 1975. Sperm transport and survival in male birds. In: The Biology of Spermatozoa. ESE Hafez and CB Thibault, Eds. Basel: Karger, pp. 10-16.

Etches RJ, Buckland RB, Hawes RO, 1974. The effect of the genes for rose comb and polydactyly on sperm transport in the hen's oviduct. Poultry Sci 53:422-424.

Freund RJ, Littell RC, 1986a. Observations. In: SAS System for Regression. Cary, NC: SAS Institute Inc., pp. 47-74.

Freund RJ, Littell RC, 1986b. Polynomial models. In: SAS System for Regression. Cary, NC: SAS Institute Inc., PP. 103-126.

Freund RJ, Littell RC, 1986c. Using PROC REG. In: SAS System for Regression. Cary, NC: SAS Institute Inc, pp. 15-46.

Froman DP, Bernier PE, 1987. Identification of heritable spermatozoal degeneration within the ductus deferens of the chicken (Gallus domesticus). Biol Reprod 37:969-977.

Howarth, BH, Jr., 1983. Fertilizing ability of cock spermatozoa from the testis, epididymis, and vas deferens following intramagnal insemination. Biol Reprod 28:586-590.

Martin PA, Dziuk PJ, 1977. Assessment of relative fertility of males (cockerels and boars) by competitive mating. J Reprod Fert 49:323-329.

Martin PA, Reimers TJ, Lodge JR, Dziuk PJ, 1974. The effect of ratios and numbers of spermatozoa mixed from two males on proportions of offspring. J Reprod Fert 39:251-258.

Petitjean M, Servouse M, 1981. Comparative study of some characteristics of the semen of RR (rose comb) and rr (single comb) cockerels. Reprod Nutr Develop 68:1085-1093.

Sokal RR, Rohlf FJ, 1981. Single classification analysis of variance. In: Biometry, second edition. New York: Freeman, pp. 208-270.

Van Krey HP, Ogasawara FX, Lorenz FW, 1966. Distribution of spermatozoa in the oviduct and fertility in domestic birds. IV. Fertility of spermatozoa from infundibular and uterovaginal glands. J Reprod Fert 11:257-262.

CHAPTER IV

DECREASED SPERMATOZOAL SURVIVABILITY ASSOCIATED WITH ABERRANT MORPHOLOGY OF THE DUCTULI EFFERENTES PROXIMALES OF THE CHICKEN (Gallus domesticus)[1]

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[1]OAES Technical Paper No. 8958. A portion of these data were presented at the annual meeting of the Poultry Science Association, Madison, Wisconsin, July 24-28, 1989.

ABSTRACT

The objectives of this research were twofold: 1) to determine if decreased spermatozoal longevity, a previously reported heritable trait in the chicken, was attributable to spermatozoal passage through the excurrent ducts, and 2) to document the morphology of the testicular excurrent ducts from affected roosters. Though spermatozoa were viable at ejaculation as evidenced by their exclusion of ethidium bromide, fertility following intravaginal insemination of spermatozoa from affected roosters was less than ($P<0.001$) that observed with spermatozoa from non-affected controls, 37 ± 2.3 versus $58 \pm 1.5\%$, respectively, over a 21-d egg collection interval. In contrast, fertility following intramagnal insemination of testicular spermatozoa from affected roosters was equivalent ($P>0.05$) to that of non-affected controls, 47 ± 2.2 versus $41 \pm 3.6\%$, respectively. Following intravaginal insemination, neither type of testicular spermatozoa fertilized oocytes. The ductuli efferentes proximales from affected roosters were characterized by a greater luminal cross-sectional area as well as a diminished height and number of longitudinal epithelial folds ($P<.005$). It was concluded that heritable decreased spermatozoal longevity in the chicken is not attributable to an inherent spermatozoal defect. Rather, the defect is acquired during passage of spermatozoa through the extragonadal ducts of the rooster.

INTRODUCTION

Froman and Bernier (1987) identified a heritable reproductive disorder in the rooster. As evidenced by spontaneous uptake of ethidium bromide (Bilgili and Renden, 1984), affected roosters typically ejaculated semen containing 40-60% degenerate spermatozoa when given $\geq$ 7 d of sexual rest between ejaculations. Spermatozoal degeneration became apparent in semen within the mid-ductus deferens and increased in extent towards the distal ductus. It was hypothesized that autoimmunity, a dysfunction of the excurrent ducts, or an inherent spermatozoal defect could account for these observations.

According to Classen (1977), symptoms of autoimmunity to chicken spermatozoa include increased amounts of IgY and IgA as well as numbers of lymphocytes within the ductus deferens. Based upon immunofluorescent evaluation of reproductive tract immunoglobulin content and histological estimation of ductal lymphocyte numbers, Froman et al. (1989) rejected autoimmunity as the basis for heritable spermatozoal degeneration. However, an important *a posteriori* observation was made following this study. Froman et al., (1989), observed that the excurrent ducts appeared abnormal, viz., a lack of symmetry in folding as well as a distended appearance.

As evidenced by comparative and competitive fertility trials, Kirby et al. (1989) demonstrated that viable

ejaculated spermatozoa procured from affected roosters following frequent ejaculation had a decreased life span within the oviduct. Thus, spermatozoal degeneration was not limited to the confines of the ductus deferens. This realization supported the hypothesis that spermatozoal degeneration was due to an inherent defect.

However, a dysfunction of the excurrent ducts could not be precluded. This possibility seemed probable in view of Esponda and Bedford's (1985) demonstration that spermatozoa acquire secretory proteins during passage through the rooster's excurrent ducts and the observations of Froman et al., (1989). Therefore, the objectives of the present research were to determine if spermatozoal passage through the excurrent ducts contributed to spermatozoal degeneration as evidenced by diminished duration of fertility and to study the histology of the excurrent ducts.

MATERIALS AND METHODS

ANIMALS

Cross-bred roosters were obtained by breeding previously identified low-fertility Delaware roosters with Single Comb White Leghorn (SCWL) hens. Eggs were incubated, chicks hatched, reared, and affected progeny identified as described by Froman et al. (1989). When ejaculated weekly, ejaculates from affected (n=6) and non-affected (n=6) roosters contained 44 $\pm$ 7.0 and 0% degenerate spermatozoa, as determined via ethidium bromide uptake (Bilgili and Renden, 1984), respectively.

EXPERIMENT 1

Seminal quality of affected roosters was improved by daily ejaculation for 5 consecutive days. This procedure reduced percentages of ejaculated degenerate spermatozoa to $\leq$ 3%. Pooled semen representing each phenotype was diluted to 2 x 10^9 viable spermatozoa per ml with Beltsville Poultry Semen Extender, pH 7.5 (a gift from Dr. T.J. Sexton, USDA, Beltsville, MD). Each suspension was used to intravaginally inseminate 30 SCWL hens with 1 x 10^8 viable spermatozoa per hen.

Egg collection began on the second day following artificial insemination and continued for 21 d. Eggs were set weekly. Fertility was determined by breaking eggs open after 4 d of incubation and examining contents for embryonic development. Arc sine transformations of percentages of

fertilized eggs were analyzed by single classification ANOVA (Sokal and Rohlf, 1981). Percentages of fertilized eggs were also plotted by day. Because these plots conformed to logistic functions, parameters of $y(x)=\gamma/1+e^{\beta(\tau-x)}$ were estimated by iterative least squares (Freund and Littell, 1986).

EXPERIMENT 2

Inseminations with testicular spermatozoa were made on 4 separate days. On each day, 3 roosters representing a phenotype were sacrificed. Testicular weights were recorded and extracts of testicular spermatozoa prepared with minimum essential medium as described by Howarth (1983). Approximately 12-15 and 15-20 SCWL hens were inseminated intravaginally and intramagnally via laparotomy, respectively. In either case, each hen received 7×10^7 testicular spermatozoa in a volume of 0.3 ml. Egg collection, incubation, and data analyses were performed as above.

EXPERIMENT 3

Epididymides from representative roosters were fixed in 89 mM phosphate buffer, pH 7.35, containing 2.5% (v/v) glutaraldehyde, 2.5% (w/v) paraformaldehyde, 0.05% picric acid, and 2.5% (w/v) polyvinylpyrrolidone (MW 40,000) as described by Forssmann, et al. (1977). Following fixation, tissue samples were washed in running water and then stored in 70% (v/v) ethanol at 4° C. Specimens were dehydrated in

graded ethanol solutions and embedded in Paraplast Plus Tissue Embedding Medium (Lancer, St. Louis, MO) at 56.5^0 C. Serial sections, 8-10 μm thick, were stained with eosin-Y and hematoxylin. Additional representative tissues were embedded in plastic with the JB-4 Embedding Kit (Polyscience, Washington, PA), sectioned a 1.5 μm, stained with eosin-Y and hematoxylin and photographed.

The paraffin embedded sections were used for quantitative histology. Histologic sections of the epididymal region from non-affected (n=4) and affected (n=5) roosters were analyzed with Sigma Scan Image Analysis software (Jandel Scientific, Corte Madera, CA) and a Numonics Graphics Digitizer with an LED cursor (Numonics Inc, Montgomeryville, PA). Regions of the excurrent duct were identified at 400 x following the nomenclature of Budras and Sauer (1975). In the regions analyzed, the luminal area (μm^2) and epithelial periphery (μm) were estimated by tracing the epithelium with the projected cursor point (0.1 μm). Epithelial heights were examined in non-affected (n=3) and affected (n=3) birds as well. From each region 10 measurements were randomly made in areas where the epithelium and basement membrane were distinct. Differences in luminal area, periphery and epithelial height were analyzed by single classification ANOVA (Sokal and Rohlf, 1981).

RESULTS

As shown in Table IV.1, the fertility of affected roosters was 21 percentage units less ($P<0.001$) than that of non-affected roosters, even though hens received identical insemination doses. The difference in duration of fertility is illustrated in Figure IV.1. Estimates of τ, time at which half-maximal fertility occurred were 9.4 and 12.4 d for affected and non-affected roosters, respectively.

Combined testicular weight averaged 24.7 $\pm$ 3.44 and 21.7 $\pm$ 2.86 g for affected and non-affected roosters, respectively. When data were analyzed by single classification ANOVA (Sokal and Rohlf, 1981), no difference ($P>0.05$) was observed between phenotypes. Similarly, no differences were observed ($P>0.05$) between phenotypes with respect to the fertilizing ability of testicular spermatozoa following either intravaginal or intramagnal insemination (Table IV.2; Figure IV.2). However, there was a pronounced difference in fertility due to insemination technique (Table IV.2). Whereas no fertilized eggs were observed following intravaginal insemination of testicular spermatozoa, relatively high fertility was observed when testicular spermatozoa were inseminated surgically into the magnum (Table IV.2).

Preliminary examination of tissue sections from excurrent ducts of affected males revealed gross

morphological abnormalities only within the ductuli efferentes. As shown in Table IV.3, the luminal cross sectional area of the ductuli efferentes proximales was greater ($P<.01$) in affected males. Additionally the epithelial periphery was reduced ($P<.01$) when compared to the non-affected birds. No significant differences were noted for epithelial height, luminal area or periphery within the ductuli efferentes distales ($P>.05$). Figure IV.3 shows the characteristically larger lumen and smaller epithelial folds of affected roosters when compared to such ducts from non-affected males. Phagocytosis of spermatozoa was rare in either case. No differences were observed between phenotypes with respect to the ductuli efferentes distales (Figure IV.3).

TABLE IV.1 Fertility over a 21-d egg collection interval following a single intravaginal insemination[1] with semen from either affected or non-affected roosters.

Rooster Phenotype	Hens (n)	Eggs (n)	Fertility[2] (%)
Affected	29	554	37 ± 2.3^{a}
Non-affected	30	581	58 ± 1.5^{b}

[1]Each hen was inseminated with $1x10^{8}$ viable spermatozoa.

[2]Each value represents a mean $\pm$ SEM.

[a,b]Means bearing different superscripts are different at $P<0.001$.

TABLE IV.2 Fertility over a 21-d egg collection interval following a single intravaginal or intramagnal insemination[1] with testicular spermatozoa from either affected or non-affected roosters.

Rooster Phenotype	Insemination[1]					
	Intravaginal			Intramagnal		
	Hens (n)	Eggs (n)	Fertility[2] (%)	Hens (n)	Eggs (n)	Fertility (%)
Affected	27	501	0	30	560	47 ± 3.2
Non-affected	25	445	0	25	462	41 ± 3.6

[1]Each hen was inseminated with 7×10^7 testicular spermatozoa.

[2]Each value represents a mean ± SEM

TABLE IV.3 Quantitative histology of ductuli efferentes from affected and non-affected roosters.

		Ductuli Efferentes Proximales			Ductuli Efferentes Distales	
Phenotype	Roosters (n)	Luminal cross-sectional[1] Area (μm)	Luminal Periphery (μm)	Epithelial Cell Height (μm)	Cross-sectional Area (μm)	Luminal Periphery (μm)
Affected	(5)	223,421± 19,751[a]	2,571± 117[a]	15.8± 0.41[a]	6,745± 518[a]	380± 17[a]
Non-affected	(5)	110,923± 9,118[b]	3,523± 175[b]	17.1± 0.41[a]	5,915± 573[a]	378± 22[a]

[1]Each value represents a mean ± SEM.

[a,b]Means bearing different superscripts are different at P<.01.

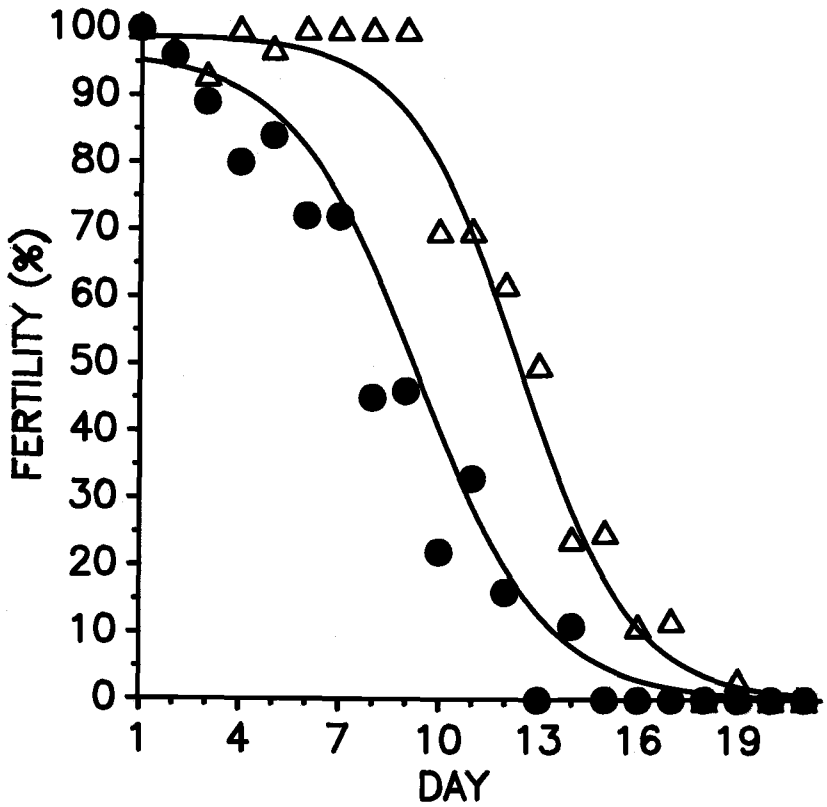


FIGURE IV.1 Duration of fertility following a single intravaginal insemination of leghorn hens with spermatozoa from affected (●) and non-affected (△) roosters. Affected roosters ejaculated semen containing 40-50% degenerate spermatozoa when ejaculated weekly. Prior to insemination, these roosters were ejaculated daily for 5 d. This procedure reduced percentages of degenerate spermatozoa in ejaculates to $\leq$ 3%. Each hen was inseminated with 1 x 10^8 viable spermatozoa in a volume of 50 μl. Solid lines represent the functions $y(x)=96/1+e^{-0.5190\,(9.4-x)}$ and $y(x)=99/1+e^{-0.5884\,(12.4-x)}$ where 9.4 and 12.4 represent the predicted day on which half-maximal fertility occurred.

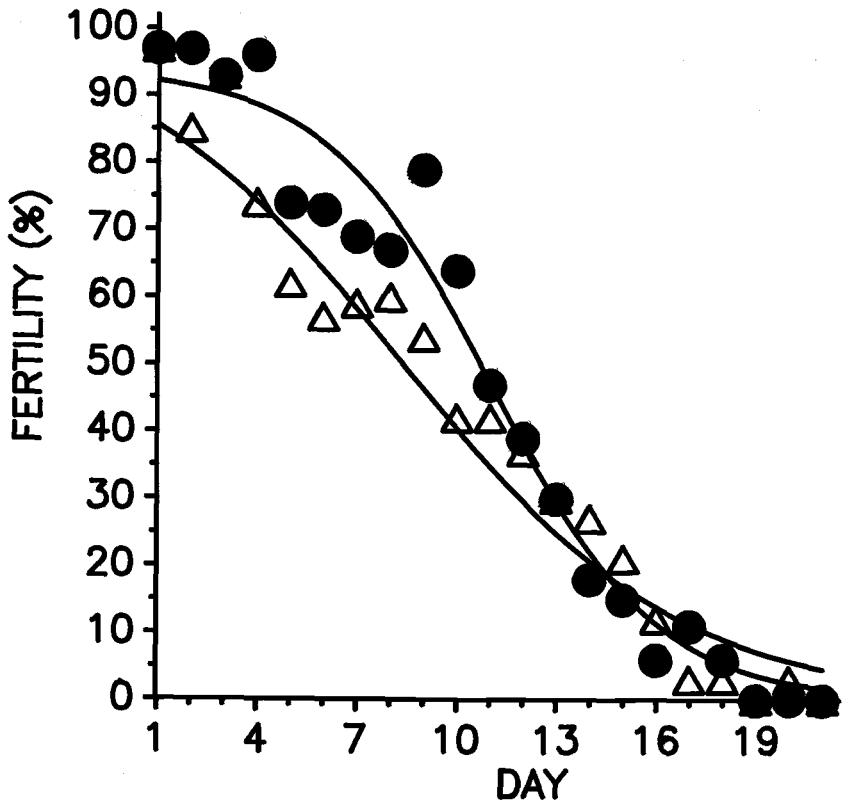


FIGURE IV.2 Duration of fertility following a single intramagnal insemination of leghorn hens with testicular spermatozoa from affected (●) and non-affected (△) roosters. Affected roosters ejaculated semen containing 40-50% degenerate spermatozoa when ejaculated weekly. Each hen was inseminated surgically with 7×10^7 testicular spermatozoa in a volume of 0.3ml. Solid lines represent the functions $y(x)=94/1+e^{-0.4007(11.0-x)}$ and $y(x)=105/1+e^{-0.2416(8.4-x)}$.

FIGURE IV.3

1. Ductulus efferens proximalis (DeP) from a non-affected rooster. The cross-section (95x) is characterized by a highly folded epithelium with numerous crypts.

2. Epithelial fold (385x) within a proximal efferent ductule from a non-affected rooster. Both ciliated (C) and non-ciliated cells (N) are evident.

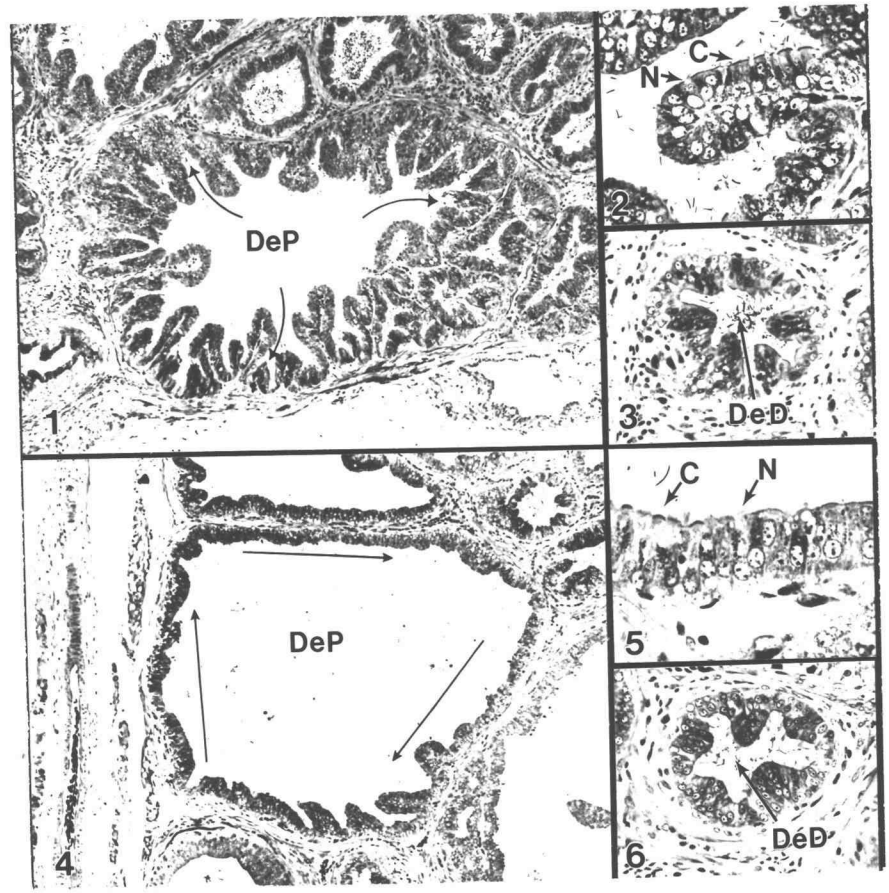
3. Ductulus efferens distalis (DeD) from a non-affected rooster. The cross-section (220x) is characterized by low epithelial folds containing numerous ciliated cells.

4. Ductulus efferens proximalis (DeP) from an affected rooster. The cross-section (95x) is characterized by a relatively even epithelial surface (arrows) with some low folds. Increased luminal area is proportional to decreased epithelial folding.

5. Epithelial cells (500x) adjoining the lumen of a proximal efferent duct from an affected rooster. The epithelium appears normal and contains ciliated (C) as well as non-ciliated (N) cells.

6. Ductulus efferens distalis (DeD) from an affected rooster. The cross-section (220x) is characterized by low epithelial folds similar to those of non-affected roosters.

FIGURE IV.3



DISCUSSION

Froman and Bernier (1987) identified a heritable reproductive disorder in the rooster. Affected roosters were identified originally by subfertility, which was found to be attributable to large percentages, e.g., 30-70%, of degenerate spermatozoa within ejaculated semen. Thereafter, affected roosters were identified by spermatozoal uptake of ethidium bromide (Bilgili and Renden, 1984). By evaluating spermatozoal uptake of ethidium bromide in semen sampled from serial sections of the ductus deferens from affected and non-affected roosters, spermatozoal degeneration appeared to begin within the mid-ductus deferens. Percentages of degenerate spermatozoa within the distal ductus deferens were comparable to those observed in ejaculated semen. Because seminal quality, as evidenced by ethidium bromide uptake, improved due to frequent ejaculation, Froman and Bernier (1987) hypothesized that a dysfunction of the ductus deferens resulted in spermatozoal degeneration therein.

Kirby et al. (1989) tested this hypothesis by implementing the following argument: if spermatozoal longevity within the oviduct were diminished, as evidenced by a decreased duration of fertility, then spermatozoal degeneration could be attributable to a spermatozoal defect. Based upon comparative and competitive fertilization, viable spermatozoa from affected roosters manifested decreased

longevity within the oviduct. Therefore, Kirby et al. (1989) concluded that heritable spermatozoal degeneration was probably due to a spermatozoal defect.

Theoretically, such a defect could be either inherent, i.e., originating during spermatogenesis, or a result of spermatozoal passage through the excurrent ducts. The latter seemed more probable for four reasons. First, Esponda and Bedford (1985) have demonstrated that chicken spermatozoa acquire cell surface proteins while passing through the excurrent ducts. Second, Morris et al. (1987) have demonstrated that these proteins remain upon spermatozoa sequestered within the hen's oviduct and have postulated that such proteins may promote spermatozoal survival. Third, Tingari (1971, 1972), as well as Bakst (1980) reported apical blebbing of excurrent duct epithelial cells, which Bakst (1980) attributed to apocrine secretion. Fourth, Froman et al. (1989) discovered that the excurrent ducts of affected roosters appeared to be swollen and demonstrated a reduced symmetry of folding as compared to non-affected males.

Unlike mammalian testicular spermatozoa, which acquire fertilizing ability via passage through the epididymis (Guraya, 1987), chicken testicular spermatozoa are capable of fertilizing oocytes, providing that they are placed within the oviduct above the utero-vaginal junction (Howarth, 1983). Consequently, we implemented the following

argument: if decreased fertility is observed when testicular spermatozoa from affected roosters are inseminated intramagnally, then an inherent spermatozoal defect may be inferred. However, we first affirmed the subfertile status of affected roosters via intravaginal insemination of ejaculated spermatozoa (Table IV.1; Figure IV.1). We used cross-bred roosters in order to maximize the difference in duration of fertility between rooster phenotypes.

By utilizing a logistic model, the duration of fertility may be denoted by τ, which is an estimate of the time at which half-maximal fertility occurred. As evidenced by τ=12.4 (Figure IV.1), duration of fertility following intravaginal insemination of spermatozoa from non-affected roosters was comparable to that observed previously with SCWL roosters, i.e., τ=11.8 (Kirby et al., 1989). It should be noted that leghorns are one of the most fertile breeds of chickens. In contrast, duration of fertility following intravaginal insemination of spermatozoa from affected roosters was equivalent to that observed previously with affected pure-bred Delaware roosters (Kirby et al., 1989), τ=9.4 and 9.1, respectively. Thus, though the affected crossbred roosters were the progeny of SCWL hens, no heterosis was observed, strengthening the proposed dominant gene basis for this trait (Kirby, et al., 1990).

No difference in duration of fertility was observed

between phenotypes when testicular spermatozoa were placed within the magnum (Table IV.2). When expressed as a function of time (Figure IV.2), fertility following intramagnal insemination of testicular spermatozoa from affected roosters did not decline at a greater rate than that from non-affected roosters. As expected, intravaginal insemination of testicular spermatozoa from either phenotype failed to yield fertilized eggs. In summary, we conclude that spermatozoal passage through the excurrent ducts of affected roosters contributes to subsequent premature spermatozoal degeneration.

The ductuli efferentes proximales of affected roosters were characterized by a greater luminal cross-sectional area and a diminished epithelial surface area when compared to ducts from non-affected roosters (Figure IV.3). In some sections, spermatozoal concentrations appeared greater within the ductuli efferentes of affected roosters. According to Tingari (1971), concentrated semen is not found within the ductuli efferentes but rather within the connective ductules, epididymal ducts, and ductus deferens. Tingari and Lake (1972) showed that ligation of the ductus deferens increased spermatozoal concentrations and decreased epithelial folding within the chicken's ductuli efferentes. Thus, the aberrant morphology could be explicable in terms of a partial obstruction. We repeated (unpublished data) the experiment of Tingari and Lake (1972) with SCWL roosters

and confirmed their observations relative to ductal morphology as well as widespread epithelial phagocytosis of spermatozoa. It is noteworthy that such phagocytosis was rare in affected birds and that they ejaculated more spermatozoa than non-affected controls (Froman et al., 1989). Therefore, any obstruction seems unlikely.

A primary function of the ductuli efferentes is water resorption (Jones and Jurd, 1987). Nakai et al. (1989) have demonstrated an endocytotic activity of epithelial cells within the rooster's ductuli efferentes proximales. Due to its extensive epithelial folding (Tingari, 1971), evidence of apocrine secretion (Tingari, 1971; Tingari 1972; Bakst, 1980), and the presence of ciliated cells (Tingari, 1972; Bakst, 1980), the ductuli efferentes of the rooster appear to be ducts wherein spermatozoa are mixed with epithelial secretions and then concentrated. Because the ductuli efferentes proximales of affected roosters have a diminished epithelial surface, spermatozoa suspended within these ductules may not encounter optimal concentrations of fluid components. We hypothesize that heritable degeneration of chicken spermatozoa may be attributable to a dysfunction of the ductuli efferentes.

REFERENCES

Bakst MR, 1980. Luminal topography of the male chicken and turkey excurrent duct system. Scanning Electron Microscopy 3:419-25.

Bilgili SF, Renden JA, 1984. Fluorometric determination of avian sperm viability and concentration, Poult Sci 63:2275-77.

Budras KD, Sauer T, 1975. Morphology of the epididymis of the cock (Gallus domesticus) and its effect upon the steroid hormone synthesis. I. Ontogenesis, morphology and distribution of the epididymis. Anat Embryol 148:175-196.

Classen HL, 1977. Antigenicity of spermatozoa and its relationship to fertility in domestic fowl. Ph.D. Dissertation, University of Massachusetts, pp. 30-74.

Esponda P, Bedford JM, 1985. Surface of the rooster spermatozoan changes in passing through the Wolffian duct. J Exp Zool 234:441-49.

Freund RJ, Littell RC, 1986. Observations. In: SAS System for Regression. Cary, NC: SAS Institute Inc., pp. 103-26.

Forssmann WG, Ito S, Weihe E, Aoki A, Dym M, Fawcett DW, 1977. An improved perfusion fixation method for the testis. Ant Rev 188:307-14.

Froman DP, Bernier PE, 1987. Identification of heritable spermatozoal degeneration within the ductus deferens of the chicken (Gallus domesticus). Biol Reprod 37:969-77.

Froman DP, Kirby JD, Lawler RM, Bernier PE, 1989. Onset of spermatozoal degeneration in low-fertility Delaware roosters and test for autoimmune basis. J Androl (In press).

Guraya SS, 1987. General considerations. In: Biology of Spermatogenesis and Spermatozoa in Mammals. Berlin: Springer-Verlag, pp. 181-86.

Howarth, B Jr, 1983. Fertilizing ability of cock spermatozoa from the testis, epididymis, and vas deferens following intramagnal insemination. Biol Reprod 28:586-90.

Jones RC, Jurd KM, 1987. Structural differentiation and fluid reabsorption in the ductuli efferentes testis of the rat. Austral J Biol Sci 40:79-90.

Kirby JD, Froman DP, Engel HN Jr., Bernier PE, 1989. Decreased sperm survivability in subfertile Delaware roosters as indicated by comparative and competitive fertilization. J Reprod Fert 86:671-677.

Kirby JD, Bernier PE, Froman DP, 1990. Heritable spermatozoal degeneration in the fowl due to a dominant gene. J Androl (In press).

Morris, SA, Howarth B Jr., Crim JW, Rodriguez de Cordoba S, Esponda P, Bedford JM, 1987. Specificity of sperm-binding Wolffian duct proteins in the rooster and their persistence on spermatozoa in the female host glands. J Exp Zool 242:189-98.

Nakai M, Hashimoto Y, Kitagawa H, Kon Y, Kudo N, 1989. Histological study on seminal plasma absorption and spermiophagy in the epididymal region of domestic fowl. Poult Sci 68:582-89.

Sokal RR, Rohlf FJ, 1981. Single classification analysis of variance. In: Biometry, second edition. New York: Freeman, pp. 208-270.

Tingari MD, 1971. On the structure of the epididymal region and ductus deferens of the domestic fowl (Gallus domesticus). J Anat 109:423-35.

Tingari MD, 1972. The fine structure of the epithelial lining of the excurrent duct system of the testis of the domestic fowl (Gallus domesticus). Q J Exp Physiol 59:271-95.

Tingari MD, Lake PE, 1972. Ultrastructural evidence for resorption of spermatozoa and testicular fluid in the excurrent ducts of the testis of the domestic fowl, (Gallus domesticus). J Reprod Fert 31:373-81.

CHAPTER V

COMPARATIVE METABOLISM OF SPERMATOZOA FROM SUBFERTILE DELAWARE AND WYANDOTTE ROOSTERS[1]

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ABSTRACT

The metabolism of spermatozoa from subfertile Delaware roosters was compared to that of subfertile Wyandotte as well as fertile Leghorn roosters. Sperm metabolism was determined via reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to formazan. When the reaction mixture contained cyanide, which blocks cytochrome oxidase and thus maximizes intermediate electron transfer to INT, and calcium which stimulates fowl sperm motility, the metabolic capacity of Delaware and Wyandotte spermatozoa was 90 and 63% of that of Leghorn spermatozoa. When the assay was performed under physiological conditions, no difference in metabolism was observed between Delaware and Leghorn spermatozoa ($P>0.05$). However, the metabolism of Wyandotte spermatozoa was 66% of that observed with Delaware or Leghorn spermatozoa. This work provides further evidence that heritable subfertility in Delaware and Wyandotte roosters is attributable to distinct sperm defects.

INTRODUCTION

Heritable sperm degeneration within the ductus deferens of the Delaware rooster was described by Froman & Bernier (1987). Affected roosters were characterized by large proportions of degenerate spermatozoa within ejaculated semen or semen removed from the mid- and distal ductus deferens. Seminal quality could be transiently improved by daily ejaculation for five or more days. Froman & Bernier (1987) hypothesized that a dysfunction in the storage capacity of the ductus deferens could account for their observations. In subsequent work (Froman et al., 1990), the nature of the onset of sperm degeneration was described. At puberty, most affected birds ejaculated high proportions of viable spermatozoa. However, by 6-8 weeks post puberty these same roosters were ejaculating large proportions of degenerate spermatozoa.

Kirby et al. (1989) demonstrated via intramagnal and intravaginal inseminations that viable sperm from affected Delaware had reduced fertilizing ability. It was concluded that this was due to sperm degeneration within the oviduct. Sperm competition was used to help clarify this question. While heterospermic inseminations supported this inference, the initial high proportion of chicks sired by Delaware roosters following intramagnal insemination was not anticipated (Kirby, et al., 1989). Additional intramagnal inseminations with testicular spermatozoa from affected and

normal males demonstrated that spermatozoa from affected males do not suffer from an inherent defect (Kirby, et al., 1990). In fact, testicular spermatozoa from affected males actually fertilized a higher percentage of ova (47±3.2%) over a 21 day collection interval than those of the controls (41±3.6%), though this difference was not significant ($P>0.05$).

Chaudhari & Wishart (1988a) have described a simple and objective assay for measuring the metabolic activity of fowl spermatozoa . This assay has been shown to be highly correlated with fowl sperm motility, morphology, ATP content, and fertilizing ability (Chaudhari & Wishart, 1988b). Therefore, the objective of the present work was to assess if the transient superiority of Delaware spermatozoa following intramagnal inseminations could be explained in terms of different sperm metabolic rates as postulated by Kirby et al. (1989).

MATERIALS AND METHODS

EXPERIMENT 1

Semen was collected from roosters representing 3 genotypes: Single Comb White Leghorns (SCWL; n=6), subfertile Delaware (n=6), and Silver-Laced Wyandotte homozygous for the rose comb (n=6). Roosters were handled as described in Kirby et al. (1989) in order to reduce sperm senescence. Semen was pooled according to rooster genotype. Percentage of viable spermatozoa as well as sperm concentration were determined fluorometrically by ethidium bromide uptake (Bilgili & Renden, 1984). Each sperm suspension was diluted to 1.5×10^9 viable spermatozoa/ml with 150 mM NaCl in 20 Mm HEPES (N-[2]hydroxy-ethyl]-piperazine-N'-[2-ethanesulfonic acid]), Ph 7.4, solution.

Following dilution, 0.2 ml of sperm suspension was mixed with 0.84 ml HEPES buffer, with a final concentration of 0.16 mM 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) and 7.7×10^{-3} mM phenazine methosulphate (PMS) and 7.8 Mm glucose to final volume of 1.28 ml. For assays completed at 25° C, 60 μl of HEPES buffer was replaced with 40 μl 100 mM $CaCl_2$ and 20 μl 100 mM KCN, all other constituents remained constant. Sperm metabolism was estimated by reduction of INT to red formazan pigment. Metabolism was stopped by admixture of the reaction mixture with 0.2 ml of 5% (v/v) Triton X-100 in 0.1 M HCl. Following centrifugation the concentration of formazan was

measured spectrophotometrically at 520 nm.

The protocol of Chaudhari & Wishart (1988a) was followed with the following exceptions: 1) 20 mM HEPES was used in lieu of 20 mM TES (N-tris[hydroxymethyl]-methyl, 2-aminoethane sulfonic acid) for the diluent and buffer solution, 2) sperm metabolism was measured at 25° C with KCN and $CaCl_2$ in the reaction solution, and at 40° C without KCN or $CaCl_2$, 3) 3 x 10^8 viable spermatozoa were used in the reaction mixture, and 4) formazan concentration was measured at 0, 2, 4, 8, and 16 minutes of incubation. Five replicates were assayed for each genotype. Data from each trial were pooled and parameters of

$$y(x) = \alpha + \beta(x)$$

were estimated by least squares (Freund & Littell, 1986).

EXPERIMENT 2

Semen was collected from each of the roosters used in the previous experiment. However, individual ejaculates were evaluated in triplicate following dilution to 1.5 x 10^9 viable sperm / ml. The INT reduction assay was completed as above, except that all measurements were made following 16 minutes of incubation. Formazan formation (picomoles/1 X 10^6 spermatozoa) as well as sperm concentration were analyzed for male and genotype effects via analysis of variance (Sokal & Rohlf, 1981).

RESULTS

As shown in Table V.1, daily ejaculation of subfertile Delaware roosters for >5 consecutive days prior to, as well as during, the experimental period yielded percentages of viable spermatozoa equivalent to those of SCWL and Wyandotte males. However, the Delaware roosters produced ejaculates which contained higher concentrations of spermatozoa ($P<0.05$; Table V.1). When standardized sperm suspensions were used to assess formazan formation at 25° C, in the presence of cyanide and calcium, three divergent lines were observed (Figure V.1). In contrast, at near body temperature (40° C), in 150 mM NaCl containing 20 mM HEPES, pH 7.4, formazan formation within Delaware and SCWL spermatozoa was comparable ($P>0.05$) and superior to that of Wyandottes (Figure V.2).

When the metabolic activity of individual ejaculates was evaluated at 25° C (Table V.2) highly significant ($P<0.0001$) differences were found between all three genotypes (SCWL> Delaware> Wyandotte). While at 40° C, as predicted from pooled samples in Figure V.2, the spermatozoa from Wyandotte roosters were less metabolically active ($P<0.0001$) than those from either the Delaware or SCWL males, which were comparable ($P>0.05$; Table V.2). To further elucidate the relative changes in metabolic activity of spermatozoa from a single ejaculate between the two assays, 40° and 25° C, each ejaculate was ranked according to its

relative position, from 1 to 18 (Table V.2). As shown in Table V.2, while the relative ranking of Delaware ejaculates was improved by running the assay at body temperature (40° C), no apparent metabolic advantage was conferred when compared to the SCWL.

TABLE V.1 Sperm concentrations and percentages of viable spermatozoa from 3 genotypes of rooster.

Breed of rooster	Number of birds	Comb genotype	Sperm concentration[1] ($X\ 10^9$ sperm/ml)	Sperm viability[1] (%)
Delaware	6	rrpp	6.49 ± 0.36^a	100 ± 0
Leghorn	6	rrpp	5.69 ± 0.48^b	100 ± 0
Wyandotte	6	Rrpp	5.31 ± 0.38^b	100 ± 0

[1]Mean $\pm$ SEM.
[a,b]Means denoted with different superscripts are different ($p<0.05$).

TABLE V.2 Formazan formation within spermatozoa according to breed of rooster and incubation conditions.

Breed of rooster	Rooster number	Formazan formation (picomoles/10^6 spermatozoa) 25° C Mean[1]	25° C Rank[2]	40° C Mean	40° C Rank
Delaware	1	162.5±3.3	11	86.9±5.3	5
	2	194.4±2.2	6	94.2±6.0	3
	3	157.0±4.2	12	68.6±3.4	12
	4	193.1±5.8	8	83.9±4.1	6
	5	200.4±3.0	5	80.3±3.1	8
	6	194.4±2.3	6	83.0±6.2	7
	Group mean	183.6±5.0[b]	8.0	82.8±2.5[a]	6.8
White Leghorn	1	213.6±1.5	2	96.0±1.6	1
	2	206.7±3.9	4	68.6±5.8	11
	3	207.0±2.5	3	91.1±5.0	4
	4	190.9±2.1	9	74.5±2.1	9
	5	189.9±2.6	10	65.0±2.6	13
	6	217.1±3.9	1	96.0±1.6	1
	Group mean	204.2±2.7[a]	4.8	81.9±2.5[a]	6.5
Wyandotte	1	147.3±2.9	13	73.9±3.3	10
	2	123.3±1.9	17	59.1±1.0	14
	3	89.6±2.9	18	45.9±0.4	18
	4	130.0±3.6	16	47.8±0.8	17
	5	148.0±2.6	13	51.0±1.8	15
	6	140.0±5.2	15	50.5±2.7	16
	Group mean	129.7±5.0[c]	15.5	54.71±2.4[b]	15.0

[1]Mean ± S.E.M. from triplicate samples.
[2]Rank relative to other males within the assay, 1-18.
[a,b,c]Means in a column with different superscript($p<0.0001$).

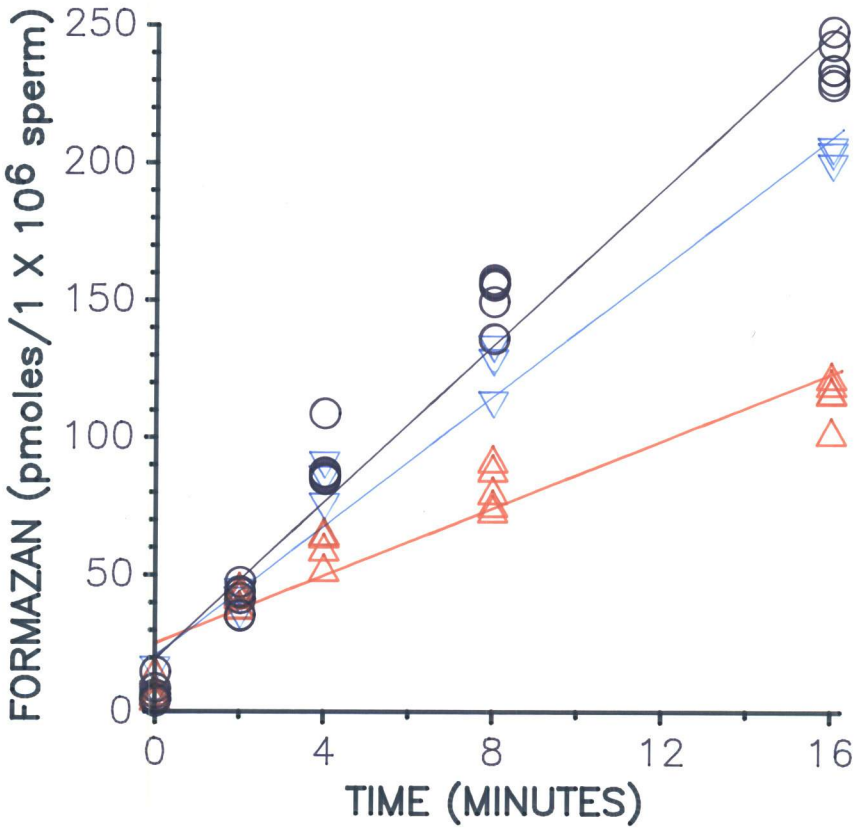

FIGURE V.1 Formazan formation in spermatozoa from Single Comb White Leghorn (SCWL; ◯), subfertile Delaware (▽) and Wyandotte roosters homozygous for the rose comb gene (△) when incubated at 25° C in 150 mM NaCl containing 1.56 mM KCN, 3.12 mM $CaCl_2$, 7.8 mM glucose and 20 mM HEPES, pH 7.4. Solid lines represent the functions:

a) SCWL, $y(x) = 19.85 + 14.24(x)$, $R^2=0.98$,
b) Delaware, $y(x) = 20.91 + 11.80(x)$, $R^2=0.98$,
and c) Wyandotte, $y(x) = 25.07 + 6.13(x)$, $R^2=0.95$.

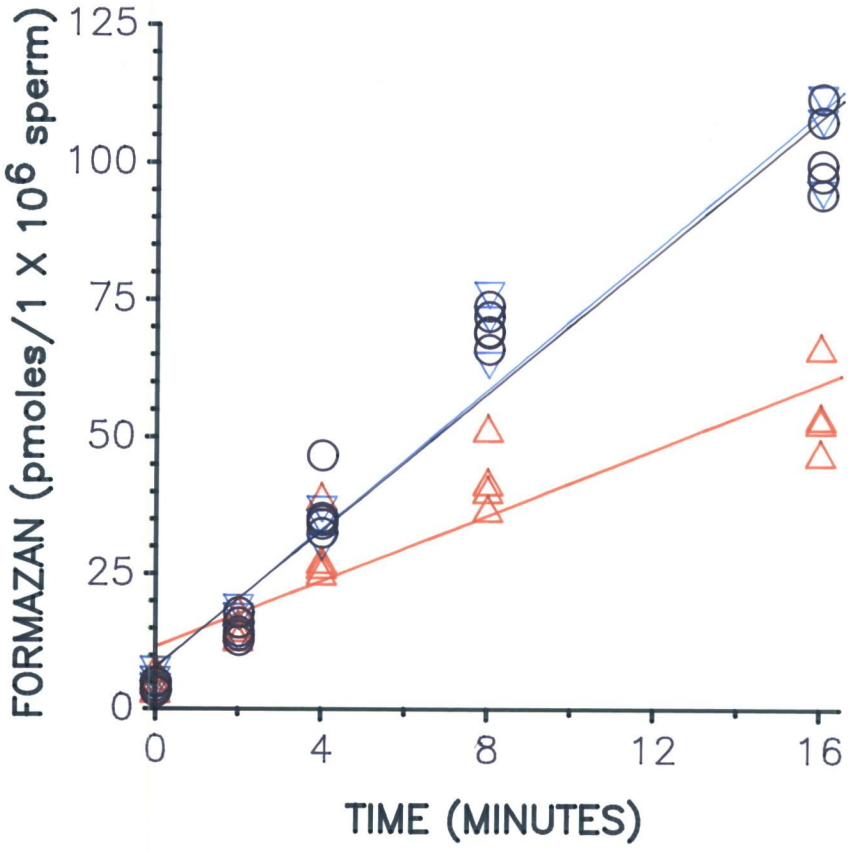

FIGURE V.2 Formazan formation in spermatozoa from Single Comb White Leghorn (SCWL; ◯), subfertile Delaware (▽) and Wyandotte roosters homozygous for the rose comb gene (△) when incubated at 40° C in 150 mM NaCl containing 7.8 mM glucose and 20 mM HEPES, pH 7.4. Solid lines represent the functions:

a) SCWL, $y(x) = 7.98 + 6.27(x)$, $R^2=0.98$,
b) Delaware, $y(x) = 7.94 + 6.37(x)$, $R^2=0.98$,
and c) Wyandotte, $y(x) = 11.58 + 3.01(x)$, $R^2=0.92$.

DISCUSSION

Heritable subfertility in the domestic fowl has been reported in two instances: roosters homozygous for the rose comb gene (RR; Crawford & Merritt, 1963) and Delaware roosters (Froman & Bernier, 1987). The latter are characterized by a single comb and the Columbian feather colour gene (Co). The mechanisms leading to subfertility in these types are different, in that Delaware are subfertile due to sperm death or degeneration as the result of a single dominant gene (Froman & Bernier, 1987; Kirby, et al., 1989, 1990a) whereas spermatozoa from RR roosters are characterized by poor motility (Buckland, et al., 1969; Etches, et al., 1974; Petitjean & Servouse, 1981). Although subfertile, the Delaware has been characterized by a transient superiority in fertilizing ability during the first 3-4 days following intramagnal insemination (Kirby, et al., 1989, 1990b). The possibility that the decreased life span of Delaware spermatozoa could be due to increased metabolism merited further consideration.

However, the metabolic capacity of Delaware spermatozoa was less than that observed in spermatozoa from Single Comb White Leghorns (Figure V.1; Table V.2). The measurements at 25° C reflect relative metabolic capacity as cyanide binds tightly to cytochrome oxidase. This prevents the reduction of oxygen and shunts electrons to the INT dye (Harold, 1986). Additionally the 25° C media contained calcium in

order to enhance motility and accelerate the rate of formazan formation (Wishart & Ashizawa, 1987; Chaudhari & Wishart, 1988a) and thus reflects a capacity for metabolism. The metabolic activity of spermatozoa at 40° C is an indicator of how well the respective cells should function at body temperature, such as encountered following insemination. While this assay was performed under less than optimal conditions for manifesting differences among individual males within a genotype (Chaudhari & Wishart, 1988a), it demonstrated that spermatozoa from affected Delawares are not characterized by heightened metabolism when compared to SCWL males (Figure V.2, Table V.2). Furthermore, an inspection of the rankings of individual ejaculates revealed no dramatic shifts in the relative positions of males between assays.

The results for the metabolic activity of spermatozoa from RR Wyandottes is in agreement with those of previous investigators (Buckland, et al., 1969; Etches, et al., 1974; Petitjean & Servouse, 1981), in that the implied reduction of metabolic capacity from subjective observations of motility are confirmed. The range in rates of formazan production at 25° C would seem to indicate differences in fertilizing ability of spermatozoa from RR males, as well. Although all RR males are subfertile, the range in fertilizing ability appears to be similar to that observed with high fertility roosters (unpublished data).

The majority of fowl spermatozoa acquire the capacity for motility within the epididymis (Munro, 1938; Howarth, 1983). Additionally, the excurrent ducts of the fowl's testis secrete several unique proteins that bind to spermatozoa and then persist during storage in the oviduct (Esponda & Bedford, 1985; Morris, et al., 1987). In the bird and many mammals, efferent ductules, located between the testicular rete and the epididymis, are a site of water resorption and possible protein secretion (Bakst, 1980; Jones & Jurd, 1987; Clulow & Jones, 1988). In the subfertile Delaware the proximal efferent ductules are abnormal, in that they are characterized by a reduced surface to volume ratio (Kirby et al., 1990b). We hypothesized that aberrant sperm metabolism could possibly be indicative of abnormal maturation within the Delaware's excurrent ducts. However, if malformed efferent ductules do lead to abnormal sperm maturation, sperm metabolism is unaffected (Figure V.2). Therefore, the decreased survivability of Delaware sperm may be related to sperm surface properties.

REFERENCES

Bakst MR, 1980. Luminal topography of the male chicken and turkey excurrent duct system. Scanning Electron Microsc 3:419-426.

Bilgili SF, Renden JA, 1984. Fluorometric determination of avian sperm viability and concentration. Poultry Sci 63:2275-2277.

Buckland RB, Wilcox FHK, Shaffner CS, 1969. Influence of homozygosity for rose comb on fumarase, aconitase, isocitric dehydrogenase and malic dehydrogenase activity in spermatozoa of the domestic fowl (Gallus domesticus). J Reprod Fert 18:89-95.

Chaudhari D, Wishart GJ, 1988a. Predicting the fertilising ability of avian semen: The development of an objective colourimetric method for assessing the metabolic activity of fowl spermatozoa. Brit Poultry Sci 29:837-845.

Chaudhari D, Wishart GJ, 1988b. Predicting the fertilising ability of avian semen: Comparison of a simple colourimetric test with other methods for predicting the fertilising ability of fowl semen. Brit Poultry Sci 29:847-851.

Clulow J, Jones RC, 1988. Studies of fluid and spermatozoal transport in the extratesticular genital ducts of the Japanese quail. J Anat 157:1-11.

Crawford RD, Merritt ES, 1963. The relation between rose comb and reproduction in the domestic fowl. Can J Genet Cytol 5:89-95.

Esponda P, Bedford JM, 1985. Surface of the rooster spermatozoon changes in passing through the Wolffian duct. J Exp Zool 234:441-449.

Etches RJ, Buckland RB, Hawes RO, 1974. The effect of the genes for rose comb and polydactyly on sperm transport in the hen's oviduct. Poultry Sci 53:422-424.

Freund RJ, Littell RC, 1986. Using PROC REG. In: SAS System for Regression. Cary, N.C.: SAS Institute, Inc, pp.15-46.

Froman DP, Bernier PE, 1987. Identification of heritable spermatozoal degeneration within the ductus deferens of the chicken (Gallus domesticus). Biol Reprod 37:969-977.

Froman DP, Kirby JD, Lawler RM, Bernier PE, 1990. Onset of spermatozoal degeneration in low-fertility Delaware roosters and test for autoimmune basis. J Androl 11:(in press).

Harold MH, 1986. The Vital Force: A Study of Bioenergetics. New York: Freeman, pp.197-250.

Howarth, B, Jr, 1983. Fertilizing ability of cock spermatozoa from the testis, epididymis, and vas deferens following intramagnal insemination. Biol Reprod 28:586-590.

Jones RC, Jurd KM, 1987. Structural differentiation and fluid reabsorption in the ductuli efferentes testis of the rat. Aust J Biol Sci 40:79-90.

Kirby JD, Froman DP, Engel, HN, Jr, Bernier PE, 1989. Decreased sperm survivability in subfertile Delaware roosters as indicated by comparative and competitive fertilization. J Reprod Fert 86:671-677.

Kirby JD, Bernier PE, Froman DP, 1990a. Heritable spermatozoal degeneration in the fowl due to a dominant gene. J Androl 11:(in press).

Kirby JD, Froman DP, Engel, H, Jr, Bernier PE, Hess RA, 1990b. Decreased spermatozoal survivability associated with aberrant morphology of the ductuli efferentes proximales of the chicken (Gallus domesticus). Biol Reprod 42:383-389.

Morris SA, Howarth, B, Jr, Crim JW, Rodriguez de Cordoba S, Esponda P, Bedford JM, 1987. Specificity of sperm-binding Wolffian duct proteins and their persistence on spermatozoa in the female host glands. J Exp Zool 242:189-198.

Munro SS, 1938. Functional changes in fowl sperm during their passage through the excurrent ducts of the male. J Exp Zool 79:71-92.

Petitjean M, Servouse M, 1981. Comparative study of some characteristics of the semen of RR (rose comb) or rr (single comb) cockerels. Reprod Nutr Develop 6B:1085-1093.

Sokal RR, Rohlf FJ, 1981. Single classification analysis of variance. In: Biometry, 2nd. edn. New York: Freeman, pp. 208-270.

Wishart GJ, Ashizawa K, 1987. Regulation of the motility of fowl spermatozoa by calcium and cyclic AMP. J Reprod Fert 80:607-611.

CHAPTER VI

CONCLUSIONS

This research has demonstrated that heritable spermatozoal degeneration is most likely due to an aberrant function of at least one region of the excurrent ducts of the fowl's testis. In the first series of experiments, reported in Chapter III, fertility trials were used to determine if spermatozoal degeneration was due to a storage dysfunction of the ductus deferens. The failure of viable Delaware spermatozoa to persist within the oviduct following intramagnal insemination was indicative of spermatozoal death. The nearly linear reduction in progeny sired by Delaware males following intramagnal heterospermic inseminations strengthened that inference. From these experiments it was concluded that the spermatozoa of affected Delaware males suffer from a decreased capacity for survival, whether within the ductus deferens or the oviduct.

In the next set of experiments, Chapter IV, the primary objectives were twofold. First, based on the previous experiments, to evaluate if the decreased duration of fertility observed with spermatozoa from Delaware males was due to an inherent defect or if passage through the excurrent ducts was required. Second, to document the morphology of the excurrent ducts if the fertilizing

capacity of testicular spermatozoa from affected males was comparable to that of high fertility males. The inseminations of testicular spermatozoa resulted in fertilized eggs only when the spermatozoa were placed within the magnum of the oviduct. Overall fertility and the duration of fertility of affected males were comparable to those obtained with normal males. The histological evaluation of the excurrent ducts revealed abnormal morphology only in the proximal efferent ducts. Based on these results it was concluded that spermatozoa from affected males appear to be without defect at spermiation. However, upon leaving the testis, they acquire a defect which leads to premature cell death and degeneration. This most likely occurs as they pass through the proximal efferent ducts. The reduced surface to volume ratio of the proximal efferent ducts may limit spermatozoal acquisition of secretory products from this region.

The final experiments, Chapter V, compared the metabolic rates of spermatozoa from Single Comb White Leghorns, subfertile males homozygous for the rose comb allele, and subfertile single comb Delaware males. In previous experiments, viable spermatozoa from affected males had demonstrated a transient superiority in fertilizing ability to spermatozoa from normal males following intramagnal inseminations. Consequently, experiments were designed to evaluate if spermatozoa from

affected males could be characterized by a heightened metabolic rate or capacity. However, spermatozoa from affected males were not characterized by an aberrant metabolic rate. Therefore, it was concluded that the observed early superiority in fertilizing ability of spermatozoa from affected males, following intramagnal insemination, was not due to a metabolic defect.

In summary, affected males produce spermatozoa that die prematurely if allowed to pass through the excurrent ducts of the testis. Premature spermatozoal death was attributed neither to a dysfunction in the storage capacity of the ductus deferens nor in spermatozoal metabolism. Rather, premature spermatozoal death is most likely related to a malformation of the proximal efferent ducts. This is a metabolically active region of the excurrent ducts in which spermatozoa are mixed with secretions and concentrated. Therefore, premature spermatozoal death is attributed to aberrant spermatozoal maturation. This work is the first to demonstrate that spermatozoal maturation within the fowl's excurrent ducts is related to spermatozoal survival, either within the excurrent ducts or within the oviduct.

BIBLIOGRAPHY

Allen CJ, Champion LR, 1955. Competitive fertilization in the fowl. Poultry Sci 34:1332-1342.

Allen TE, Grigg GW, 1957. Sperm transport in the fowl. Aust J Agri Res 8:788-799.

Bakst MR, 1980. Luminal topography of the male chicken and turkey excurrent duct system. Scanning Electron Microsc 3:419-426.

Bateson W, 1902. Experiments with poultry. Repts Evol Comm Roy Soc I:87-124. (Cited from Crawford, 1963).

Bateson W, Punnett RC, 1905. A suggestion as to the nature of The "walnut" comb in fowls. Proc Cambridge Phil Soc 13:165-168.

Bedford JM, 1979. Evolution of the sperm maturation and storage functions of the epididymis. In: The Spermatozoon. DW Fawcett and JM Bedford, Eds. Baltimore: Urban and Schwarzenberg, pp. 7-22.

Bilgili SF, Renden JA, 1984. Fluorometric determination of avian sperm viability and concentration. Poultry Sci 63:2275-2277.

Bobr LW, Lorenz FW, Ogasawara FX, 1964. Distribution of spermatozoa in the oviduct and fertility in domestic birds. I. Residence sites of spermatozoa in fowl oviducts. J Reprod Fert 8:39-47.

Braun RE, Behringer RR, Peschon JJ, Brinster RL, Palmiter RD, 1989. Genetically haploid spermatids are phenotypically diploid. Nature 337:373-376.

Buckland RB, Hawes RO, 1968. Comb type and reproduction in the male fowl: Semen characteristics and testes structure. Poultry Sci 47:704-710.

Buckland RB, Wilcox FH, Schaffner CS, 1969. Influence of homozygosity for rose comb on fumarase, aconitase, isocitric dehydrogenase and malic dehydrogenase activity in spermatozoa of the domestic fowl (Gallus domesticus). J Reprod Fert 18:89-95.

Budras KD, Sauer T, 1975. Morphology of the epididymis of the cock (Gallus domesticus) and its effect upon the steroid hormone synthesis. I. Ontogenesis, morphology and distribution of the epididymis. Anat Embryol 148:175-196.

Chaudhari D, Wishart GJ, 1988a. Predicting the fertilising ability of avian semen: The development of an objective colourimetric method for assessing the metabolic activity of fowl spermatozoa. Brit Poultry Sci 29:837-845.

Chaudhari D, Wishart GJ, 1988b. Predicting the fertilising ability of avian semen: Comparison of a simple colourimetric test with other methods for predicting the fertilising ability of fowl semen. Brit Poultry Sci 29:847-851.

Classen HL, 1977. Antigenicity of spermatozoa and its relationship to fertility in domestic fowl. Ph.D. Dissertation, University of Massachusetts, pp. 30-74.

Clulow J, Jones RC, 1988. Studies of fluid and spermatozoal transport in the extratesticular genital ducts of the Japanese quail. J Anat 157:1-11.

Cochez LP, 1951. An infertility factor balanced by breeding in White Wyandottes. Proc IXth World's Poultry Cong I:122-128

Crawford RD, 1963. Studies on the relationship between fertility and the gene for rose comb in the domestic fowl. Ph. D. dissertation. Amherst: U. of Mass.

Crawford RD, 1964. Fertility of walnut-combed domestic fowl. Can J Anim Sci 44:184-186.

Crawford RD, 1965. Comb dimorphism in Wyandotte domestic fowl. I. Sperm competition in relation to rose and single comb alleles. Can J Genet Cytol 7:500-504.

Crawford RD, 1971. Rose comb and fertility in Silver Spangled Hamburgs. Poultry Sci 50:867-869.

Crawford RD, Merritt ES, 1963. The relationship between rose comb and reproduction in the domestic fowl. Can J Gen Cytol 5:89-95.

Crawford RD, Smyth, JR, Jr, 1964a. Infertility and action of the gene for rose comb in the domestic fowl. Can J Gen Cytol 6:298-303.

Crawford RD, Smyth, JR, Jr, 1964b. Studies on the relationship between fertility and the gene for rose comb in the domestic fowl. 1. The relationship between comb genotype and fertility. Poultry Sci 43:1009-1017.

Crawford RD, Smyth, JR, Jr, 1964c. Studies on the relationship between fertility and the gene for rose comb in the domestic fowl. 2. The relationship between genotype and duration of fertility. Poultry Sci 43:1018-1026.

Crawford RD, Smyth, JR, Jr, 1964d. Semen quality and the gene for rose comb in the domestic fowl. Poultry Sci 43:1551-1557.

de Reviers M, 1975. Sperm transport and survival in male birds. In: The Biology of Spermatozoa. ESE Hafez and CB Thibault, Eds. Basel: Karger, pp. 10-16.

Esponda P, Bedford JM, 1985. Surface of the rooster spermatozoon changes in passing through the Wolffian duct. J Exp Zool 234:441-449.

Etches RJ, Buckland RB, Hawes RO, 1974. The effect of the genes for rose comb and polydactyly on sperm transport in the hen's oviduct. Poultry Sci 53:422-424.

Forssmann WG, Ito S, Weihe E, Aoki A, Dym M, Fawcett DW, 1977. An improved perfusion fixation method for the testis. Ant Rev 188:307-14.

Fox TW, Crawford RD, Smyth, JR, Jr, 1964. Rose comb: An example of operational overdominance in the domestic fowl. Genet Res 5:379-383.

Freund RJ, Littell RC, 1986a. Observations. In: SAS System for Regression. Cary, NC: SAS Institute Inc., pp. 47-74.

Freund RJ, Littell RC, 1986b. Polynomial models. In: SAS System for Regression. Cary, NC: SAS Institute Inc., PP. 103-126.

Freund RJ, Littell RC, 1986c. Using PROC REG. In: SAS System for Regression. Cary, NC: SAS Institute Inc, pp. 15-46.

Froman DP, Bernier PE, 1987. Identification of heritable spermatozoal degeneration within the ductus deferens of the chicken (Gallus domesticus). Biol Reprod 37:969-977.

Froman DP, Kirby JD, Lawler RM, Bernier PE, 1990. Onset of spermatozoal degeneration in low-fertility Delaware roosters and test for autoimmune basis. J Androl 11:(in press).

Gardner EJ, Snustad DP, 1984. Principles of Genetics, 7th edn. New York: Wiley, pp.230-270.

Guraya SS, 1987. General considerations. In: Biology of Spermatogenesis and Spermatozoa in mammals. Berlin: Springer-Verlag, pp. 181-86.

Harold MH, 1986. The Vital Force: A Study of Bioenergetics. New York: Freeman, pp.197-250.

Hindhaugh WLS, 1932. Some observations on fertility in White Wyandottes. Harper Adams Util Poultry J 17:555-560.

Howarth, B Jr, 1983. Fertilizing ability of cock spermatozoa from the testis, epididymis, and vas deferens following intramagnal insemination. Biol Reprod 28:586-590.

Hutt FB, 1940. A relation between breed characteristics and poor reproduction in White Wyandotte fowls. Am Nat 74:148-156.

Jones RC, Jurd KM, 1987. Structural differentiation and fluid reabsorption in the ductuli efferentes testis of the rat. Aust J Biol Sci 40:79-90.

Kirby JD, Bernier PE, Froman DP, 1990. Heritable spermatozoal degeneration in the fowl due to a dominant gene. J Androl 11:P49.

Kirby JD, Froman DP, 1988. Effect of dietary supplementation of zinc on the spermatozoal integrity of low-fertility Delaware roosters. Poultry Sci 67(Sup. 1):103.

Kirby JD, Froman DP, Engel, HN, Jr, Bernier PE, 1989. Decreased sperm survivability in subfertile Delaware roosters as indicated by comparative and competitive fertilization. J Reprod Fert 86:671-677.

Kirby JD, Froman DP, Engel, H, Jr, Bernier PE, Hess RA, 1990b. Decreased spermatozoal survivability associated with aberrant morphology of the ductuli efferentes proximales of the chicken (Gallus domesticus). Biol Reprod 42:383-389.

Martin PA, Dziuk PJ, 1977. Assessment of relative fertility of males (cockerels and boars) by competitive mating. J Reprod Fert 49:323-329.

Martin PA, Reimers TJ, Lodge JR, Dziuk PJ, 1974. The effect of ratios and numbers of spermatozoa mixed from two males on proportions of offspring. J Reprod Fert 39:251-258.

Morris SA, Howarth, B, Jr, Crim JW, Rodriguez de Cordoba S, Esponda P, Bedford JM, 1987. Specificity of sperm-binding Wolffian duct proteins in the rooster and their persistence on spermatozoa in the female host glands. J Exper Zool 242:189-198.

Munro SS, 1938. Functional changes in fowl sperm during their passage through the excurrent ducts of the male. J Exp Zool 79:71-92.

Nakai M, Hashimoto Y, Kitagawa H, Kon Y, Kudo N, 1989. Histological study on seminal plasma absorption and spermiophagy in the epididymal region of domestic fowl. Poult Sci 68:582-89.

Parker JE, 1961. Observations on the low fertility problem in Cornish cockerels. Poultry Sci 40:1214-1219.

Petitjean MJ, Cochez LP, 1966. Subfertility of cocks homozygous for the "R" gene (rose comb). Proc XIIIth World's Poultry Cong I:121-127.

Petitjean MJ, Servouse M, 1981. Comparative study of some characteristics of the semen of RR (rose comb) or rr (single comb) cockerels. Reprod Nutr Develop 21:1085-1093.

Schindler HE, Ben-David E, Hurwitz S, Kempinich-Pinto O, 1967. The relation of spermatozoa to the glandular tissue in the storage sites of the hen oviduct. Poultry Sci 46:1462-1471.

Sokal RR, Rohlf FJ, 1981. Single classification analysis of variance. In: Biometry, second edition. New York: Freeman, pp. 208-270.

Thibault C, 1973. Sperm transport and storage in vertebrates. J Reprod Fert (Suppl)18:39-53.

Tingari MD, 1971. On the structure of the epididymal region and ductus deferens of the domestic fowl (Gallus domesticus). J Anat 109:423-35.

Tingari MD, 1972. The fine structure of the epithelial lining of the excurrent duct system of the testis of the domestic fowl (Gallus domesticus). Q J Exp Physiol 59:271-95.

Tingari MD, Lake PE, 1972. Ultrastructural evidence for resorption of spermatozoa and testicular fluid in the excurrent ducts of the testis of the domestic fowl, (Gallus domesticus). J Reprod Fert 31:373-81.

van Drimmelen GG, 1946. Sperm nests in the oviduct of the domestic hen. J S African Vet Med Assoc 17:42-52.

Van Krey HP, Ogasawara FX, Lorenz FW, 1966. Distribution of spermatozoa in the oviduct and fertility in domestic birds. IV. Fertility of spermatozoa from infundibular and uterovaginal glands. J Reprod Fert 11:257-262.

Wishart GJ, Ashizawa K, 1987. Regulation of the motility of fowl spermatozoa by calcium and cyclic AMP. J Reprod Fert 80:607-611.

APPENDICES

APPENDIX 1

ANALYSIS OF POULTRY FERTILITY DATA[1,2]

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ABSTRACT

Single Comb White Leghorn (SCWL) hens were inseminated intravaginally with spermatozoa from either SCWL or subfertile Delaware roosters in three replicate fertility trials. Overall fertility was analyzed with a log odds model following logit transformation. Duration of fertility was analyzed by iterative least squares. Typically, these methods are not used to analyze poultry fertility data. However, these methods have advantages over more traditional methods. The advantages, sample programs, and a recommendation for the presentation of fertility data are discussed.

INTRODUCTION

To date, investigators have tended to use linear parametric tests such as analysis of variance to evaluate fertility data. These data are derived from eggs that typically are collected throughout an arbitrary interval following insemination. There are two drawbacks to this stereotypical approach. First, fertility data in general do not meet the assumptions of the analysis of variance even when arc sine transformed prior to analysis (Chaudhary et al., 1984). Second, when the fertility of hens within a similarly treated group is plotted as a function of time, the resultant pattern of data points denote a logistic relationship between these two variables. This pattern is observed following both intravaginal (Froman and Bernier, 1987; Froman and Engel, 1989; Froman et al., 1990) and intramagnal insemination (Kirby et al., 1989), providing that an adequate amount of time exists between consecutive inseminations.

When choosing a method of statistical analysis, familiarity should not be equated with applicability; for an experimenter's choice may not only cause the experimenter to err when making inferences from data but may mislead others as well. Commonly used analyses may limit the information available from a fertility trial. Furthermore, the format with which fertility data are presented may preclude independent analysis. Therefore, the purpose of this work

is threefold: 1) to describe a model statement that compensates for the inherent lack of additivity and homogeneity of variance that characterize fertility data, 2) to propose a standardized method for estimating the duration of fertility, and 3) to suggest a simple format for reporting fertility data that would afford independent analysis.

MATERIALS AND METHODS

For the purpose of comparison, fertility data were obtained from three replicate trials in which Single Comb White Leghorn (SCWL) hens (n = 20 to 25) were inseminated intravaginally with either SCWL or Delaware spermatozoa. The latter were characterized by heritable subfertility as previously described (Froman and Bernier, 1987; Froman et al., 1989; Kirby et al., 1989; Kirby et al., 1990). Semen was collected from roosters by abdominal massage (Burrows and Quinn, 1937). Spermatozoal concentration and percentage of live spermatozoa were determined according to Bilgili and Renden (1984). Ejaculates from six roosters were pooled and then diluted with Beltsville Poultry Semen Extender (a gift from Dr. Tom Sexton, USDA, Beltsville, MD) to 2.0×10^9 live spermatozoa per mL. The insemination dose was 1×10^8 live spermatozoa per hen.

Eggs were collected throughout a 21-d interval, which commenced on the second day following insemination. Eggs were identified by hen and date, set at weekly intervals, and broken open after 4 d of incubation. Fertility was assessed by examining contents for embryonic development.

Fertility data were transformed to logits as a preliminary step in the analysis. Each logit, L_i, was calculated, with a continuity correction, as follows:

$$L_i = \log_e[(r_i - 0.5)/(n_i - r_i - 0.5)],$$

where r_i and n_i represent the numbers of fertilized and total eggs laid by a group throughout the egg collection interval. A weighting variable was also calculated as follows:

$$w_i = (r_i)(n_i - r_i)/(n_i - 1).$$

Transformed data were analyzed with a log odds model:

$$p(x) = 1/[1 + e^{-l(x)}],$$

where $0 \leq p \leq 1$ and $l(x) = \mu + \alpha_i + \beta_j + e_{ij}$. The parameters α and β represented breed and replicate effects, respectively. Parameters were estimated with the General Linear Models procedure of SAS (SAS Institute, 1986; see Appendix A.1(A.1)).

When nontransformed data from replicate treatment groups were plotted as functions of time, plots conformed to curves predicted by logistic functions. Therefore, parameters of

$$y(x) = \gamma \; / \; 1 + e^{\beta(\tau - x)}$$

were estimated by iterative least squares (Freund and Littell, 1986; see Appendix A.1(A.2). A test was made in order to determine if estimates of τ, which represents the time of half-maximal fertility, were actually estimates of a parameter common to both breeds of roosters. By imposing this hypothetical condition upon the model, i.e., $\tau_{SCWL} = \tau_{Delaware}$, a conditioned sum of squared residual errors (CSSE) was generated in addition to the sum of squared residual errors obtained unconditionally (SSE). An extra sums of

squares F test was performed as follows:

$$f_{r,(n-p)} = [SSH/r]/[SSE/(n-p)],$$

where SSH is the difference between CSSE and SSE, r denotes the number of independent parametric statements implied by the condition, n denotes the number of observations, and p denotes the number of parameters within the observational model.

RESULTS

As shown in Table A1.1, there was a significant difference ($P<0.05$) in overall fertility between SCWL (64.3 $\pm$ 4.6%) and Delaware roosters (47.7 $\pm$ 2.2%). Percentages of eggs fertilized by spermatozoa of each breed in each replicate trial are shown in Table A1.2. No replicate effect was observed ($P>0.05$). An inspection of residual errors confirmed that errors were independent and randomly distributed. Therefore, neither the logit transformation nor weighting induced confounding.

The τs predicted via iterative least squares for SCWL and Delaware spermatozoa were 13.6 and 10.3 days, respectively. These τs were significantly different ($P<0.0001$). An inspection of residual errors confirmed that errors were independent and distributed randomly. The disparity in duration of fertility between breeds is depicted in Figure A1.1.

TABLE A1.1 Analysis of variance table for the average fertility of hens mated to either Delaware or Single Comb White Leghorn roosters.

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob>F
Model	3	63.869	21.290	24.18	0.040
--Sire	(1)	(49.793)	49.793	56.55	0.0172
--Block	(2)	(14.076)	7.038	7.99	0.1112
Error	2	1.761	0.880		
Corrected total:	5	65.630			

R^2=.973

TABLE A1.2 Summary of overall fertility following insemination[1] of Single Comb White Leghorn (SCWL) hens with spermatozoa from either SCWL or Delaware roosters.

Fertility trial	Breed of semen donor	Hens (n)	Eggs (n)	Fertilized eggs[2] (n)	Fertility[3] (%)
1	SCWL	19	269	166	61.2 ± 3.3
	Delaware	21	312	146	46.8 ± 3.5
2	SCWL	21	285	201	67.8 ± 2.1
	Delaware	16	207	102	48.1 ± 3.0
3	SCWL	24	434	252	58.3 ± 2.4
	Delaware	25	442	198	44.0 ± 2.8
Total	SCWL	64	988	619	64.3 ± 4.6
	Delaware	62	961	446	47.7 ± 2.2

[1] Each hen was inseminated intravaginally with $1x10^8$ live spermatozoa.
[2] Eggs were collected over a 21-d interval.
[3] Each value represents the mean ± SEM for the fertility of (n) hens over the 21-d egg collection interval.

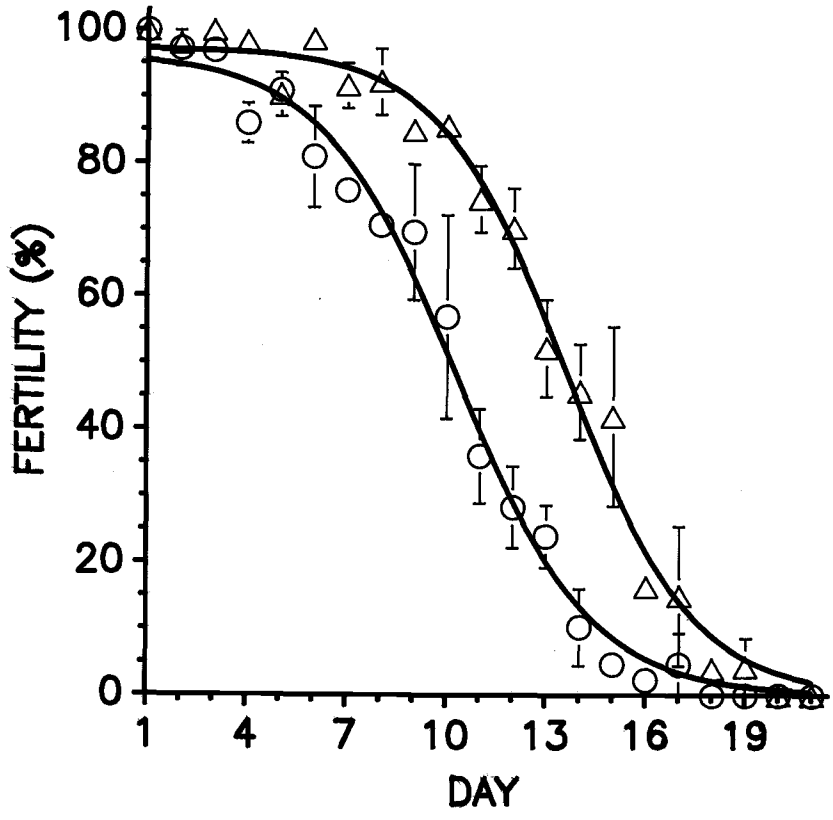

FIGURE A1.1 Duration of fertility following intravaginal insemination of Single Comb White Leghorn (SCWL) hens with $1x10^8$ live spermatozoa from SCWL (△) or Delaware (◯) roosters. Solid lines represent the functions:

$$y(x) = 97.27/\ 1 + e^{-0.5224(13.61-x)}$$
$$y(x) = 96.37/\ 1 + e^{-0.4926(10.32-x)}$$

for hens inseminated with SCWL and Delaware spermatozoa, respectively.

DISCUSSION

Female poultry are nondeterminate layers and have sperm-host glands within their oviducts (Gilbert, 1979). Consequently, these birds may lay fertilized eggs over a period of weeks following a single insemination. In the case of chickens, fertility is typically in excess of 90% for a number of days prior to an abrupt decrease, which in turn precedes a gradual decline in fertility to 0% (Fig. A1.1). Thus, unless inseminations are made frequently, the unit change in fertility is not proportional to the unit change in time, i.e., the assumption of additivity is inappropriate. Furthermore, while some experimental schemes utilize repeated inseminations at a frequency comparable to that used in commercial breeder flocks, such a procedure reduces the amount of information that may be obtained from a fertility trial. Therefore, it is often advantageous to analyze data collected throughout an interval in which fertility is non-linear. In such cases, additivity may be obtained with the logit transformation. This method has found wide application in the biological sciences (Weisberg, 1985; Freeman, 1987; Govindarajulu, 1988; Hosmer and Lemeshow, 1989) because it affords analysis with a linear model. However, with few exceptions (Froman and Thurston, 1984; Tajima et al., 1989), the logit transformation is rarely used to analyze poultry fertility data.

In some cases, the temporal aspects of poultry

fertility are either ignored (Brillard et al., 1989) or are analyzed with complex, multiple regression models. Unfortunately, the parameters estimated with the latter type of model are frequently difficult to interpret, and following interpretation of limited utility (Weisberg, 1985). In contrast, utilization of logistic models (Fig. A.1) provides information that is interpreted easily. For example, the parameter γ is an asymptotic value that denotes the initial level of fertility following insemination, whereas the parameter τ is an estimate of the time at which fertility is half-maximal. We have found such parameters to be instrumental in evaluating subfertility stemming from either genetic causes (Froman and Bernier, 1987; Kirby et al., 1989; Kirby et al., 1990) or from manipulation of spermatozoal attributes (Froman and Engel, 1989). However, the logistic model could also be useful in cryopreservation research or in selecting for duration of fertility. In the latter case, the model would compensate for the dilution of selection pressure arising from the lack of normality associated with fertility (Brah et al., 1982; Chaudhary et al., 1984) and would also provide via τ a meaningful criterion for selection (Falconer, 1981). When using the logistic model, data need not be transformed in order to provide a normal and homoscedastic distribution of errors or implied linearity. Nonetheless, the method affords independent testing of lack of fit via the extra sums of

squares F-test, which is based on the assumption of least squares regression (Weisberg, 1985).

Another problem sometimes encountered with fertility data is the manner in which they are presented. When fertility data do not include numbers of eggs, independent analyses of data cannot be made, e.g., a calculation to determine if sample size was adequate to detect a reported difference (Sokal and Rohlf, 1981) or the use of the Mantel-Haentzel statistic (Mantel and Haentzel, 1959). Thus, egg numbers along with the number of spermatozoa inseminated per hen are essential to the independent analysis and interpretation of fertility data. In conclusion, a thorough presentation of data, logit transformation of percentages prior to analysis with a log odds model, as well as generation of descriptive functions via iterative least squares can be used to enhance the interpretation and utility of poultry fertility data.

REFERENCES

Bilgili SF, Renden JA, 1984. Fluorometric determination of avian sperm viability and concentrations. Poultry Sci 63:2275-2277.

Brah GS, Lanza GM, Potts PL, Washburn KW, 1982. Effects of deviations from normality on selection intensities for shell deformation and egg weight in chickens. Poultry Sci. 61:424-428

Brillard JP, McDaniel GR, de Reviers M, Drane JW, 1989. Expression of several traits of fertility in young and old dwarf broiler breeder hens inseminated with duplicate doses of semen. Poultry Sci 68:558-563.

Burrows WH, Quinn JP, 1937. The collection of spermatozoa from the domestic fowl and turkey. Poultry Sci 16:19-24.

Chaudhary ML, Sandhu JS, Brah GS, 1984. Normality of fertility and hatchability of egg-type chickens. Indian J Anim Sci 54:913-916.

Falconer DS, 1981. Introduction to quantitative genetics, 2nd edition. Hong Kong: Longman, pp. 340.

Freeman, DH, Jr, 1987. Applied categorical data analysis. New York: Marcel Dekker, Inc., pp. 318.

Freund RJ, Littell RC, 1986. SAS system for regression. Cary, N. C.: SAS Institute, Inc., pp. 165.

Froman DP, Bernier PE, 1987. Identification of heritable spermatozoal degeneration within the ductus deferens of the chicken (Gallus domesticus). Biol Reprod 37:969-977.

Froman DP, Engel, HN, Jr, 1989. Alteration of the spermatozoal glycocalyx and its effect on duration of fertility in the fowl (Gallus domesticus). Biol Reprod 40:615-621.

Froman DP, Thurston RJ, 1984. Decreased fertility resulting from treatment of fowl spermatozoa with neuraminidase or phospholipase c. Poultry Sci 63:2479-2482.

Froman DP, Elaghbary AM, Kirby JD, 1990. Effect of immunization of sexually immature pullets against poly-α-L-glutamic acid on long term fertility. J Reprod Fert 88:405-409.

Gilbert AB, 1979. Female genital organs. In: Form and Function in Birds. A.S. King and J. McLelland, ed. New York: Academic Press, pp. 237-260.

Govindarajulu Z, 1988. Statistical Techniques in Bioassay. Basel: Karger, pp. 166.

Hosmer DW, Lemeshow S, 1989. Applied Logistic Regression. New York: Wiley, pp 307.

Kirby JD, Froman DP, Engel, HN, Jr, Bernier PE, 1989. Decreased sperm survivability in subfertile Delaware roosters as indicated by comparative and competitive fertilization. J Reprod Fert 86:671-677.

Kirby JD, Froman DP, Engel, HN, Jr, Bernier PE, Hess RA, 1990. Decreased spermatozoal survivability associated with aberrant morphology of the ductuli efferentes proximales of the chicken (Gallus domesticus). Biol Reprod 42:383-389.

Mantel N, Haentzel W, 1959. Statistical aspects of the analysis of data from retrospective studies of disease. J Natl Cancer Inst 22:719-748.

SAS, 1986. Version 6.03. Cary, North Carolina: SAS Institute, Inc..

Sokal RR, Rohlf FJ, 1981. Biometry, 2nd Edition. New York: Freeman, pp 400-429.

Tajima A, Graham EF, Hawkins DM, 1989. Estimation of the relative fertilizing ability of frozen chicken spermatozoa using a heterospermic competition method. J Reprod Fert 85:1-5.

Weisberg S, 1985. Applied Linear Regression, 2nd edn. New York: Wiley, pp.324.

APPENDIX A.1(A.1) Program analyzing LOGIT transformed fertility data using the SAS General Linear Models Procedure (SAS Inst., 1986).

```
DATA FERTILITY;
INPUT SIRE BLOCK N R ;
Y=R/N;
Z=(R-0.5)/(N-R-0.5); IF Z<0 THEN Z=.; L=LOG(Z);
W=R*(N-R)/(N-1);
CARDS;
(ENTER DATA, I.E. -- 1 1 100 60, etc.)
PROC GLM;
CLASSES SIRE BLOCK;
MODEL  L=BLOCK  SIRE/SS1;
WEIGHT W;
MEANS SIRE / TUKEY;
OUTPUT  OUT=RESULTS PREDICTED=PL;
RUN;
PY=1/[1+EXP(-PL)];
PROC PRINT; VARIABLES SIRE BLOCK Y PY;
RUN;
```

SYMBOLS

N=NUMBER OF EGGS FOR A GIVEN SIRE AND BLOCK
R=NUMBER OF FERTILE EGGS FOR A GIVEN SIRE AND BLOCK;
Y=PROPORTION OF FERTILE EGGS - PY=MODEL PREDICTION OF Y
L=LOGIT TRANSFORMED VALUE - PL=MODEL PREDICTED LOGIT
W=IS A WEIGHTING VARIABLE TO ADJUST FOR DIFFERENCES IN SAMPLE SIZE

APPENDIX A.1(A.2) Program for analyzing daily fertility using the SAS Non-Linear Regression Procedure (SAS Inst., 1986).

```
DATA FERT;
INPUT SIRE DAY FERTILITY; Z1=0;Z2=0;
IF SIRE=1 THEN Z1=1;
IF SIRE=2 THEN Z2=1;
CARDS;
1  1  100
:  <(INCLUDES DATA FROM THREE REPLICATE TRIALS.)
2  21  0
PROC  NLIN  DATA=FERT  BEST=1;
PARMS  G1=99 G2=99 B1=-0.5 B2=-0.5 T1=10.0 T2=10.0;
E1=1/(1+EXP[B1*(T1-X)]);
E2=1/(1+EXP[B2*(T2-X)]);
MODEL Y=Z1*G1*E1+Z2*G2*E2;
DER.G1=Z1*E1;
DER.G2=Z2*E2;
DER.B1=Z1*G1*(X-T1)*E1*(1-E1);
DER.B2=Z2*G2*(X-T2)*E2*(1-E2);
DER.T1=Z1*(-G1)*B1*E1*(1-E1);
DER.T2=Z2*(-G2)*B2*E2*(1-E2);
OUTPUT OUT=FERT1 P=PY R=RY;
RUN;
PROC PLOT DATA=FERT1;
PLOT RY*PY;
RUN;
PROC NLIN DATA=FERT  BEST=1;
PARMS  G1=98 G2=98 B1=-0.5 B2=-0.5 T=11;
E1=1/(1+EXP[B1*(T-X)]);
E2=1/(1+EXP[B2*(T-X)]);
MODEL Y=G1*Z1*E1+G2*Z2*E2;
DER.G1=Z1*E1;
DER.G2=Z2*E2;
DER.B1=Z1*G*(X-T)*E1*(1-E1);
DER.B2=Z2*G*(X-T)*E2*(1-E2);
DER.T=Z1*[G1*B1*E1*(1-E1)] + Z2*[-G2*B2*E2*(1-E2)];
OUTPUT  OUT=CONFERT  P=CPY  R=CRY;
RUN;
PROC  PLOT  DATA=CONFERT;
PLOT  CRY*CPY;
RUN;
```

SYMBOLS:

SIRE=TREATMENT (BREED)
DAY=DAY OF EGG COLLECTION (1-21)
FERTILITY=PERCENTAGE OF FERTILE EGGS BY DAY
Z_1 AND Z_2=DUMMY VARIABLES
G=γ; T=τ; B=ß
R=RESIDUAL ERRORS
P=PREDICTED VALUES OF Y (FERTILITY)

APPENDIX 2

ONSET OF SPERMATOZOAL DEGENERATION IN LOW-FERTILITY DELAWARE ROOSTERS AND TEST FOR AUTOIMMUNE BASIS[1,2]

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[1]OAES Technical Paper No. 8698

ABSTRACT

The objectives of this study were twofold: 1) to determine the onset of a heritable reproductive disorder in the rooster, which is characterized by extensive spermatozoal degeneration within the ductus deferens, and 2) to determine if autoimmunity was associated with spermatozoal degeneration. Seventy-five percent of affected roosters did not ejaculate large percentages of degenerate spermatozoa at 20 weeks of age, approximately the age of sexual maturity. Rather, seminal quality gradually declined over the next 6 weeks, as both ejaculate volume and number of spermatozoa ejaculated increased. The evaluation of testicular and excurrent duct tissues via immunofluorescence failed to reveal either IgY or IgA associated with spermatozoa. While histological examination revealed greater lymphocyte numbers ($P<.05$) in the proximal ductus deferens, these cells were not associated with spermatozoa nor spermatozoal clumping. While spermatozoal degeneration tends to be latent at the onset of semen production, it does not appear to be due to spermatozoal autoimmunity.

INTRODUCTION

In 1987, Froman and Bernier described the symptoms of a previously unreported heritable reproductive disorder in the rooster. Affected roosters were identified originally by low fertility, which was found to be attributable to large percentages of degenerate spermatozoa within ejaculates. Electron microscopy revealed that such spermatozoa were characterized by vesiculated membranes, decondensed nuclei, as well as disintegrated axonemes and mitochondria. Thereafter, affected roosters have been identified routinely via differential spermatozoal uptake of ethidium bromide (Bilgili and Renden, 1984). Using this technique, Froman and Bernier (1987) demonstrated that spermatozoal degeneration occurred within the ductus deferens. When deferent ducts of affected roosters were sectioned serially and seminal quality determined therein, degenerate spermatozoa were detected in the mid-ductus deferens and the extent of degeneration increased within the distal ductus and receptaculum.

We hypothesized that this condition was explicable in terms of autoimmunity, especially in view of the maxim of Pavia et al. (1987) that autoimmunity against seminal antigens does not necessarily adversely affect spermatogenesis. Until the present study was conducted, our work was limited to roosters 30 weeks of age and older, whereas semen production generally commences at

approximately 20 weeks of age (Parker and McClusky, 1961). Therefore, we anticipated that spermatozoal degeneration would be latent at the onset of semen production, would become manifest in the weeks thereafter, and once apparent, the deferent ducts of such roosters would be found to contain increased amounts of immunoglobulin and numbers of lymphocytes as described by Classen (1977). Consequently, the objectives of the present research were to determine the onset of spermatozoal degeneration and test for symptoms of autoimmunity to spermatozoa.

MATERIALS AND METHODS

ANIMALS

Seven affected Delaware roosters and 42 Delaware hens were selected as breeding stock. Birds were 45 weeks of age when breeding began. Using the technique of Burrows and Quinn (1937), each rooster was ejaculated daily for 4 days prior to the first insemination. Previous work (Froman and Bernier, 1987) had shown this to be an effective means of decreasing the number of degenerate spermatozoa in ejaculates from 50-60% to less than 5%. Following the first insemination, roosters were ejaculated every other day over the course of 4 weeks in order to maintain minimal numbers of degenerate spermatozoa in ejaculates. Each of 6 hens was bred every other day with semen from a designated rooster.

Four batches of eggs were set at weekly intervals. Fertility and hatchability were determined for each hatch. After hatching, chicks were brooded and reared in floor pens until 18 weeks of age. At this time, roosters were transferred to a caged-layer house.

EXPERIMENT 1

Weekly ejaculations began at 20 weeks of age. Each rooster was ejaculated for 11 consecutive weeks after the first ejaculate was obtained. The volume of each ejaculate was recorded and the percentage of viable spermatozoa as well as the spermatozoal concentration were determined by

ethidium bromide uptake (Bilgili and Renden, 1984).

By evaluating consecutive estimates of viable spermatozoa, each rooster was assigned to one of three seminal quality (SQ) categories: consistently good (+), initially good but eventually poor (±), or consistently poor (-). To compensate for the variability observed in age when the first ejaculate was obtained, data were normalized to week of the first ejaculate. Mean percentage of viable spermatozoa, ejaculate volume, and total spermatozoa per ejaculate were plotted against time for each SQ category. Weekly differences in the latter two variables among SQ categories were determined by the Kruskal-Wallis test (Sokal and Rohlf, 1981a).

EXPERIMENT 2

Immunofluorescent evaluation of reproductive tract IgY and IgA content from each of 5 SQ+ and 5 SQ± 36 week old roosters was performed according to Classen (1977) with the following modifications. Both testicular tissue and whole excurrent ducts were fixed in 89 mM phosphate buffer, pH 7.35, containing 2.5% (v/v) glutaraldehyde, 2.5% (w/v) paraformaldehyde, 0.05% picric acid, and 2.5% (w/v) polyvinylpyrrolidone (MW 40,000) as described by Forssmann et al. (1977). Following fixation, tissue samples were washed in running water and stored in 70% (v/v) ethanol at 4° C. Specimens were dehydrated in graded ethanol solutions, cleared in toluene, and then embedded in

Paraplast Plus Tissue Embedding Medium (Lancer, St. Louis, MO) at 56.5° C.

Serial sections, 8-10 um thick, were mounted on gelatin-coated slides at 37° C. Sections were deparaffinized in toluene and then rehydrated by passage through a 1:1 mixture of toluene and ethanol followed by graded ethanol solutions into water. Sections were then covered with a 1:500 dilution of either mouse monoclonal anti-chicken IgY or IgA (Fisher Scientific, Orangeburg, NY) in a humid chamber for 2-4 hours. During this time, sections assessed for IgY and IgA were incubated at 25 and 4° C, respectively. Following repeated washings in 50 mM phosphate buffer, pH 7.5, sections were covered with a 1:150 working dilution of fluorescein isothiocyanate (FITC) conjugated to goat anti-mouse IgG antibody (Sigma Chemical Co., St. Louis, MO) and incubated at 25° C for 1-2 hours. Following repeated washings and prior to mounting, sections were treated with 0.25 M n-propyl gallate in glycerol to reduce the rate of photobleaching (Giloh and Sedat, 1982).

Control tissues were generated to evaluate the efficacy of the monoclonal antibodies against fixed antigen. Briefly, a partially purified IgA solution was obtained from the bile of chickens via extensive dialysis and $(NH_4)_2SO_4$ precipitation as described by Classen (1977). The resultant solution was then added to sheep red blood cells (SRBC) previously fixed in Alsevier's solution and treated with

0.0005% (w/v) tannic acid, and incubated for 15 minutes at 35°C (Harlow and Lane, 1988). The antigen coated cells were then fixed as described above for whole tissues. IgY was obtained by intravenously injecting 6 cockerels with SRBC at 16 and 20 weeks of age. The birds were bled 7 days following the secondary injection. Serum was separated from the clot within 4 hours of bleeding. Serum was pooled and mixed 1:1 with a 1% solution of SRBC in phosphate buffered saline. The agglutinated cells were then resuspended by shaking, washed twice and centrifuged at 1450 x g for 30 minutes. The washed cells were then resuspended in the fixative described above for whole tissues. Control cells were created by utilizing tanned SRBC incubated in bovine serum albumen, 0.5% (w/v) in phosphate buffered saline (pH 7.4), and processed in the absence of antibody.

Post fixation procedures were carried out as for the whole tissue except the SRBC were not embedded in paraffin. Rather, the cells were suspended in the appropriate solution, centrifuged and resuspended in the subsequent solution. Following rehydration 0.5 ml samples of control, IgA and IgY treated cells were incubated with either the mouse monoclonal anti-chicken IgA or IgY. The washed cells were then incubated with the FITC conjugated goat anti-mouse IgG antibody, washed, fixed in ethanol, and placed on glycerol treated slides, air dried, treated with n-propyl gallate and fluorescence observed.

EXPERIMENT 3

Numbers of immune cells within the proximal ductus deferens were estimated in tissues from 4 SQ+ and 5 SQ± roosters as follows. This tissue was used because preliminary work showed negligible numbers of immune cells in both the middle and distal regions of the ductus deferens. Sections 8-10 um thick were stained with hematoxylin and eosin. Four different cross-sections of the ductus deferens were evaluated per bird. Intra-lumenal immune cells were categorized as lymphocytes, macrophages, or granulocytes. Data were analyzed by a single classification ANOVA (Sokal and Rohlf, 1981b).

RESULTS

Frequent ejaculation of affected roosters and insemination yielded fertility >90% (Table A2.1). While the hatch of fertilized eggs was relatively low (Table A2.1), these values are typical of this inbred line. When the same roosters are ejaculated similarly and bred to leghorn hens, fertility is comparable to that shown in Table 1, but hatch of fertilized eggs is >90% (Froman and Bernier, 1987; unpublished data). Consequently, the "low-fertility" designation, as explained in the Introduction, is appropriate.

Based upon an ANOVA performed with a randomized complete block design (Sokal and Rohlf, 1981c), there was no difference in the number of roosters assigned to any given seminal quality (SQ) category among hatches (Table A2.1). Therefore, data were pooled according to SQ categories. Of the 140 roosters studied, 86 or 61% were categorized as SQ± roosters (Table A2.1). These birds initially ejaculated semen containing a large percentage of viable spermatozoa but within 6 wk of the first ejaculate were producing semen containing only 60% viable spermatozoa (Figure A2.1). Twenty-nine roosters or 21% of those studied were assigned to the SQ- category, and 25 (18%) were categorized as SQ+ roosters (Table A2.1). Thus, by the end of the experiment, 82% of the roosters were ejaculating degenerate spermatozoa.

Evaluation of the control tissues revealed intense immunofluorescence associated with SRBC incubated in the presence of chicken IgA and IgY and the monoclonal anti-chicken IgA and IgY, respectively. All of the untreated control cells, the IgA-coupled cells incubated with anti-chicken IgY, and IgY-treated cells incubated with anti-chicken IgA, as well as the IgA-coupled and IgY-treated cells incubated only with FITC conjugated goat anti-mouse IgG, demonstrated only low levels of background fluorescence.

There was no apparent difference in fluorescence in either the SQ± or SQ+ groups. Immunofluorescence revealed IgY- and IgA-positive cells scattered through the subepithelial and surrounding tissue layers of the testis and excurrent ducts of all the males observed. These cells appeared to be isolated and were external to the excurrent duct epithelium at all levels of the reproductive tract. It should be noted that fluorescence was not associated with spermatozoa at any level of the excurrent ducts nor within the lumina of the seminiferous tubules for any rooster. IgY positive cells were most frequently observed within blood vessels.

Differences in ejaculate volume and total spermatozoa per ejaculate were found among SQ categories (Figures A2.2 and A2.3). As shown in Figure A2.2, while mean ejaculate volume for SQ+ and SQ- roosters followed parallel tracks,

the former was typically 1.3 times greater than the latter. In contrast, the mean ejaculate volume of SQ± roosters followed an intersecting track (Figure A2.2). Initially, these values approximated those of the SQ+ roosters, but at 6 wk after the first ejaculate and thereafter, values approximated those of SQ- roosters. Similar patterns were observed with respect to total spermatozoa ejaculated (Figure A2.3). Spermatozoal concentration was analyzed by single classification ANOVA at week 6 and no difference ($P>0.05$) was found among SQ categories.

TABLE A2.1 Summary of fertility, hatchability, and categorization[1] of male progeny by hatch.

Hatch	Eggs (n)	Fertility (%)	Hatch of Fertilized Eggs (%)	Male Progeny per Category (n)		
				SQ +	SQ ±	SQ -
1	127	94	59	3	22	4
2	156	97	67	5	24	2
3	169	92	60	8	18	12
4	173	94	69	9	22	11
				25	86	29

[1]Seminal quality (SQ) categories were as follows: consistently good (+), initially good but eventually poor (±) or consistently poor (-) over the course of a 12-wk experiment.

TABLE A2.2 Immune cells within 8-10 um thick cross sections of the proximal ductus deferens according to seminal quality (SQ) category from 36 week old roosters.[1]

Category	Roosters (n)	Immune Cells		
		Lymphocytes	Macrophages	Granulocytes
SQ+	4	5.4 ± 0.90[a]	0.8 ± 0.40	0
SQ±	5	14.8 ± 1.79[b]	1.9 ± 0.39	1.4 ± 0.96

[1]Categories: consistency good (+) and initially good but eventually poor (±) over the course of a 12-wk experiment. SQ± males were selected as representative of affected males.
[2]Each value represents a mean ± SEM.
[a,b] Means within a column bearing different superscripts are different ($P<0.05$).

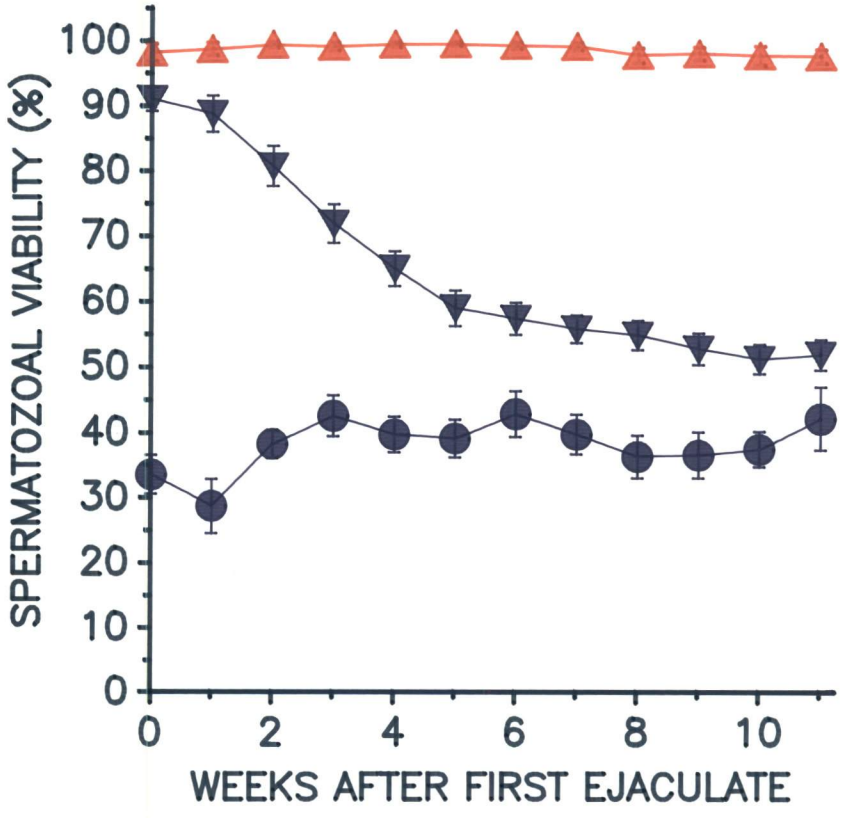

FIGURE A2.1 Percentages (mean ± SEM) of viable spermatozoa ejaculated by roosters categorized by seminal quality: consistently good (▲ ; n=25), initially good but eventually poor (▼ ; n=86) or consistently poor (● ; n=29).

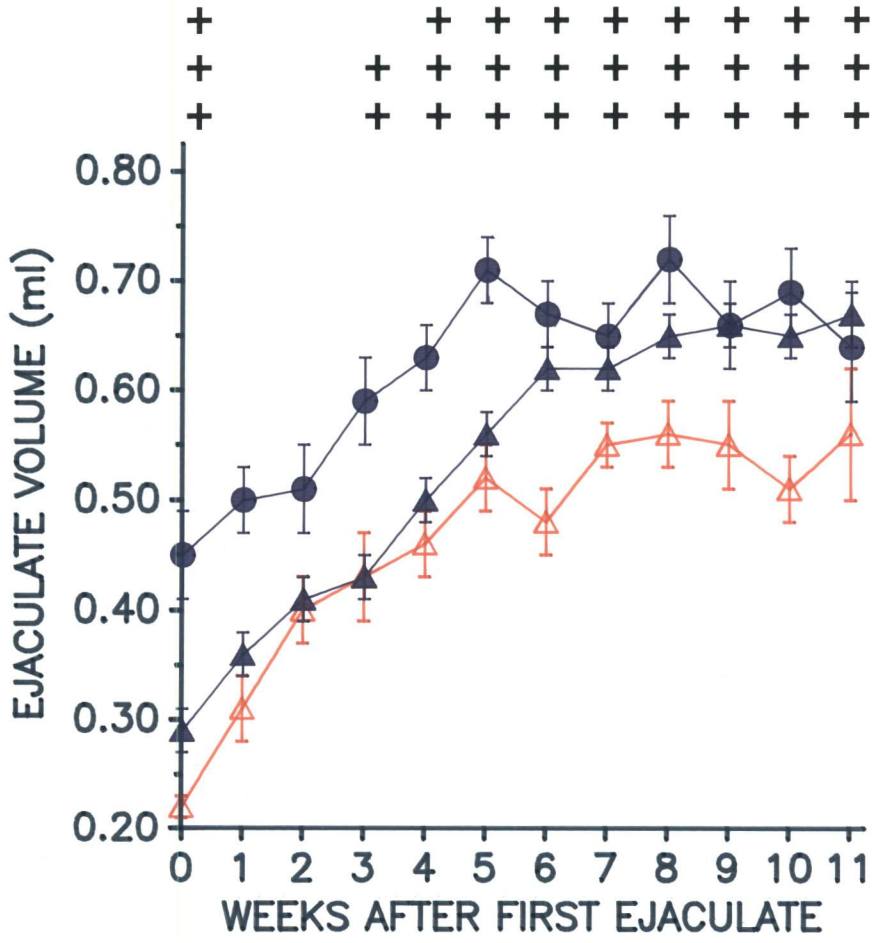

FIGURE A2.2 Ejaculate volumes (mean ± SEM) from roosters characterized by seminal quality: consistently good (△ ; n=25) initially good but eventually poor (▲ ; n=86), or consistently poor (● ; n=29). The crosses along the top edge of the graph denote significant differences among seminal quality categories by week, where ++ and +++ denote $P<0.01$ and 0.0001, respectively.

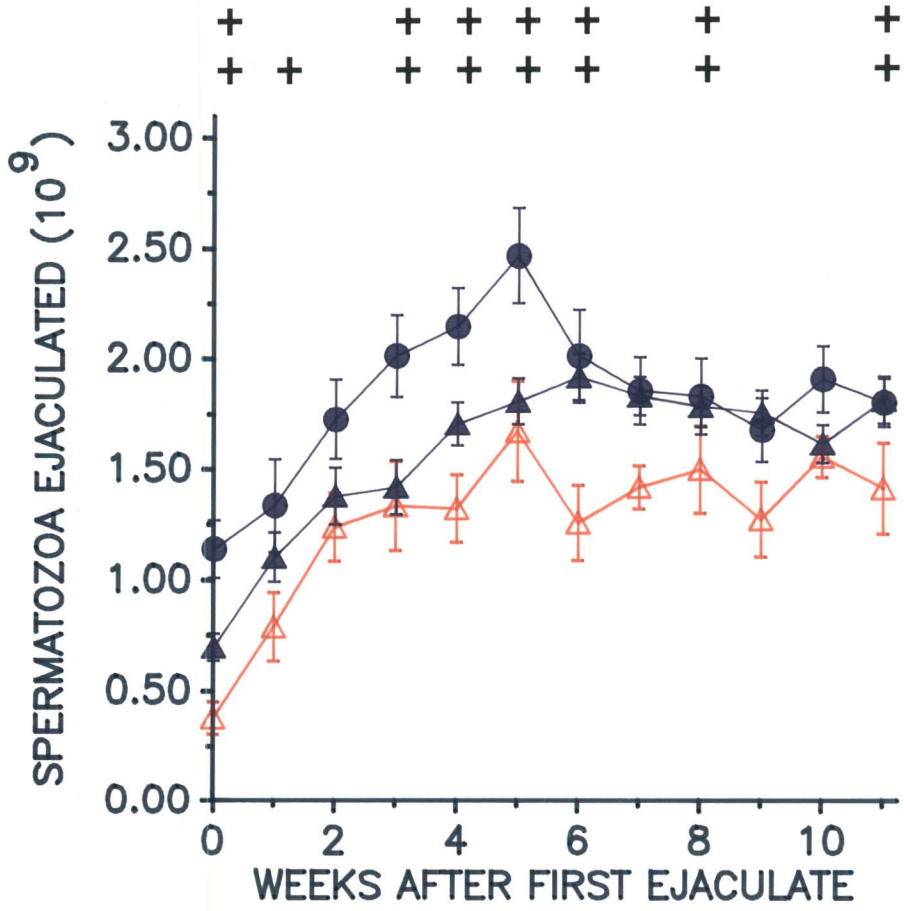

FIGURE A2.3 Total spermatozoa per ejaculate (mean ± SEM) from roosters characterized by seminal quality: consistently good (△ ; n=25), initially good but eventually poor (▲ ; n=86), or consistently poor (● ; n=29). The crosses along the top edge of the graph denote significant differences among seminal quality categories by week, where + and ++ denote $P<0.05$ and 0.01, respectively.

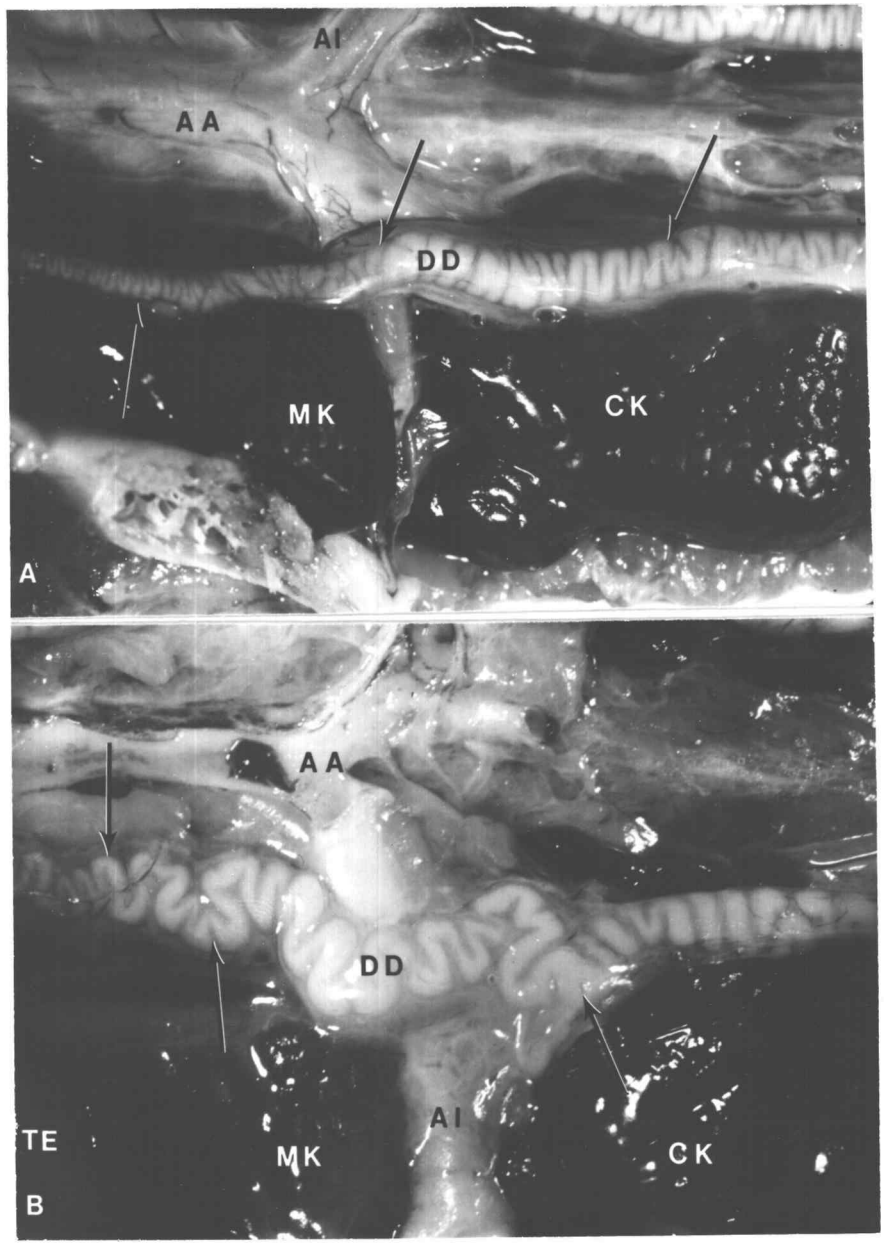

FIGURE A2.4 Proximal and middle regions of a ductus deferens (DD), photographed *in situ* of (a) normal (SQ+) and (b) affected (SQ±) roosters. Note the swollen appearance and decreased coiling of the ductus deferens in the affected male (b) as compared to the normal (a). The other structures include: abdominal aorta (AA); ischiadic artery (AI); caudal (CK) and middle (MK) lobes of the kidney; Testis (TE). Approximately 5 x magnification.

DISCUSSION

Froman and Bernier (1987) provided an explanation for the low fertility associated with certain roosters within the OSU Delaware flock over the course of 25 years, viz., extensive spermatozoal degeneration within the ductus deferens. However, the onset of this unusual phenomenon and its possible causes were not studied. A number of similarities existed among the phenomenon described by Froman and Bernier (1987), the symptoms of rooster autoimmunity to spermatozoa described by Classen (1977), and what is known generally of male autoimmunity to spermatozoa (Pavia et al., 1987). Therefore, we hypothesized that heritable spermatozoal degeneration within the rooster's ductus deferens would be latent at the onset of semen production and, once manifest, would be associated with an autoimmune condition.

While the majority of first ejaculates contained <10% degenerate spermatozoa (Figure A2.1), 82% of the 140 roosters studied were classified eventually as affected roosters (Table A2.1). It should be noted that approximately 75% of the SQ+ roosters eventually were identified as affected following the 12-wk experiment. In some cases this did not occur for several additional months. These results corroborate the conclusion of Bernier (unpublished data) that this disorder is attributable to a simple dominant gene. While 21% of the roosters studied had

≥50% degenerate spermatozoa in their first ejaculates, it is probable that many of these roosters were sexually mature prior to 20 weeks of age when routine semen collection began (Parker and McClusky, 1961). We conclude that the trait is latent at the onset of semen production.

According to Classen (1977), a prominent symptom of autoimmunity to spermatozoa in roosters is an increased amount of IgY and IgA within the ductus deferens. According to Meinertz (1987), the presence of antispermatozoal IgA is measured best in situ and least effectively via serum titers. For these reasons as well as the availability of monoclonal anti-chicken IgY and IgA antibodies, immunofluorescence was the technique of choice for evaluating immunoglobulin content within the ductus deferens. However, no differences in either IgY or IgA content were observed between SQ+ and SQ± roosters. The SQ± roosters were selected as representative affected individuals for three reasons. First, preliminary cross breeding experiments indicated no differences in the extent of spermatozoal degeneration in the progeny of SQ- and SQ± roosters (unpublished data). Second, the percentages of degenerate spermatozoa in the SQ± roosters were comparable with those from SQ- roosters by the end of the initial experiment (Figure A2.1). Third, birds producing a first ejaculate at 21 weeks of age or older were never classified as SQ- males. Based on these points, as well as the range

of 12 to 24 weeks of age until first ejaculate reported for this line of Delaware roosters by Parker and McClusky (1961), we feel the SQ- roosters represent early maturing birds.

A second symptom of autoimmunity to spermatozoa in the rooster is increased lymphocyte numbers within the ductus deferens (Classen 1977). As shown in Table A2.2, such a difference ($P<0.05$) was observed. The SQ± roosters had 2.7 times more lymphocytes within 8-10 *um* sections of the proximal ductus deferens as compared to SQ+ roosters. However, Classen (1977) reported averages of 86.8 to 111.3 lymphocytes within a 10 *um* thick sections of ductus deferens when autoimmunity was experimentally induced. In contrast, Classen (1977) observed an average of 11.3 lymphocytes in 10 *um* thick sections of the ductus deferens from non-immunized control roosters, which is comparable to that observed in tissue from SQ± roosters (Table A2.2). Finally, a third but less frequent symptom of spermatozoal autoimmunity in the rooster as reported by Classen (1977) is the existence of large aggregates of lymphocytes and spermatozoa within the ductus deferens. No such aggregates were observed in similar tissue from either SQ+ or SQ± roosters. Therefore, while a difference in lymphocyte counts was observed, we do not believe this observation was symptomatic of cell-mediated autoimmunity. In summary, while heritable spermatozoal degeneration within the ductus deferens of the

rooster was latent at the onset of semen production, it does not appear to be associated with autoimmunity to spermatozoa. Consequently, an alternative explanation must be sought.

It is noteworthy that both seminal volume and total spermatozoa per ejaculate differed between affected and non-affected roosters (Figures A2.2 and A2.3). Froman and Bernier (1987) compared semen production between affected and non-affected roosters and did not observe a difference in the cumulative number of spermatozoa ejaculated over the course of a 5 week experiment. In contrast, the present study clearly depicts a relationship between ejaculate size, spermatozoal content, and seminal quality. This is especially true in the case of the 86 SQ± roosters, whose apparent rate of semen production increased concomitantly with a decline in seminal quality (Figures A2.1, A2.2, and A2.3).

An alternative explanation must account for the time-dependent manifestation of this disorder and the apparent increase in semen production with which it is associated. Based upon *a posteriori* evaluations of gross morphology, the deferent ducts of affected birds appeared distended (Figure A2.4). The distal ductus deferens contains 65% of a rooster's extra-gonadal spermatozoal reserve (Reviers, 1975), and spermatozoal transit through this organ is comparatively rapid. Thus, delayed spermatozoal transit

might account for the anatomical observations, increased ejaculate volume, and number of spermatozoa therein.

Based upon *a posteriori* evaluations of excurrent duct histology, two morphological abnormalities have been identified in the ductuli efferentes proximales of affected roosters (Hess et al., 1989). These roosters had significantly larger luminal cross-sectional area and a decreased epithelial periphery than observed in non-affected Delaware roosters (Hess et. al., 1989). Esponda and Bedford (1985) have shown that chicken spermatozoa acquire cell surface proteins as they pass through the excurrent ducts. Furthermore, Morris et al., (1987) have shown that such proteins remain adsorbed to spermatozoa resident within the oviduct and have postulated that such proteins may play a role in spermatozoal survival. In view of these observations, as well as the discovery that viable spermatozoa from affected roosters have a decreased functional life span within the oviduct (Kirby et al., 1989), we suspect that spermatozoal degeneration may be attributable to a dysfunction of the excurrent ducts. Such a dysfunction may not be critical until a certain threshold of semen production is reached.

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REFERENCES

Bilgili SF, Renden JA, 1984. Fluorometric determination of avian sperm viability and concentration. Poultry Sci 63:2275-2277.

Burrows WH, Quinn JP, 1937. The collection of spermatozoa from the domestic fowl and turkey. Poultry Sci 16:19-24.

Classen HL, 1977. Antigenicity of spermatozoa and its relationship to fertility in domestic fowl. Ph.D. Dissertation, University of Massachusetts, 1977.

Esponda P, Bedford JM, 1985. Surface of the rooster spermatozoon changes in passing through the Wolffian duct. J Exp Zool 234:441-449.

Forssmann WG, Ito S, Weihe E, Aoki A, Dym M, Fawcett DW, 1977. An improved perfusion fixation method for the testis. Anat Rec 188:307-314.

Froman DP, Bernier PE, 1987.Identification of heritable spermatozoal degeneration within the ductus deferens of the chicken (Gallus domesticus). Biol Reprod 37:969-977.

Giloh H, Sedat JW. Fluorescence microscopy: Reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl gallate. Science 217:1252-1255.

Harlow E, Lane DP, 1988. Immunizations. In: Antibodies: A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Laboratory, pp.53-138.

Hess RA, Froman DP, Kirby JD, Bernier PE, 1989. Morphometric analysis of ductuli efferentes from low-fertility Delaware roosters. Poultry Sci 68(Suppl.1):66.

Kirby JD, Froman DP, Engel HN, Jr., Bernier PE, 1989. Decreased sperm survivability in subfertile Delaware roosters as indicated by comparative and competitive fertilization. J Reprod Fert 86:671-677.

Meinertz H, 1987. Indirect mixed antiglobulin reaction (MAR) as a screening procedure for antisperm antibodies. II. Clinical studies. Amer J Reprod Immunol Micro 15:101-105.

Morris SA, Howarth B, Jr., Crim JW, Rodriguez de Cordoba S, Esponda P, Bedford JM, 1987. Specificity of sperm-binding wolffian duct proteins in the rooster and their persistence on spermatozoa in the female host glands. J Exp Zool 242:189-198.

Parker JE, McClusky WH, 1961. The effect of length of daily light periods on the sexual development of chickens. Poultry Sci 40:1441.

Pavia CS, Stites DP, Bronson RA, 1987. Reproductive immunology. In: Stites DP, Stobo JD, Wells JV, eds. Basic and Clinical Immunology; sixth edn., East Norwalk: Appleton & Lange, pp. 619-633.

Reviers M de, 1975. Sperm transport and survival in male birds. In: Hafez ESE, Thibault GG, eds. The biology of spermatozoa. Basel: Karger, pp. 10-16.

Sokal RR, Rohlf FJ, 1981a. Assumptions of analysis of variance. In: Biometry, 2d edn. New York:Freeman, pp. 400-453.

Sokal RR, Rohlf FJ, 1981b. Single classification analysis of variance. In: Biometry, 2d edn. New York:Freeman, pp. 208-262.

Sokal RR, Rohlf FJ, 1981c. Two-way analysis of variance. In: Biometry; 2d edn. New York: Freeman, pp. 321-371.