AN ABSTRACT OF THE THESIS OF

WILLIAM LOGAN WIGLE for the degree of MASTER OF SCIENCE

in POULTRY SCIENCE presented on June 8, 1976

Title: SHORT-TERM STORAGE AND POOLING OF CHICKEN SEMEN AS RELATED TO FERTILITY AND HATCHABILITY

Abstract approved: Redacted for privacy

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Three separate trials were conducted to observe the effects of short-term in vitro storage and pooling of chicken semen on fertility and hatchability. Semen was collected and samples were prepared for storage by placing either semen from an individual male into a glass vial or by pooling and mixing semen from several males and placing this pooled sample into a similar container. Individually caged Single Comb White Leghorn hens were artificially inseminated with either .03 cc. or .05 cc. of fresh semen or semen which had been stored for intervals of from 1 to 24 hours. In each trial, eggs were saved and incubated for two periods, days 2-8 post-artificial insemination (A.I.) and days 9-15 post-A.I.

In the first trial, percent fertility for hens inseminated with semen which had been stored for periods longer than 4 hours was significantly lower ($P < .05$) than percent fertility for hens inseminated with semen which had been stored for shorter periods.

In subsequent trials, conducted later in the egg-production year, *in vitro* storage of semen had a more severe effect in reducing percent fertility. Duration of fertility in days following a single insemination was affected by semen storage time in the same manner as percent fertility. Individual male semen samples and pooled semen samples did not differ significantly in either percent fertility or duration of fertility. Increasing the insemination dosage from .03 cc. to .05 cc. did not significantly increase percent fertility or duration of fertiltiy when using stored semen.

Hatchability of eggs fertilized by stored semen was not significantly different from hatchability of eggs fertilized by fresh semen. Hatchability of fertile eggs laid during the period 2 to 8 days following artificial insemination tended to be higher than hatchability of eggs laid 9 to 15 days following artificial insemination. No statistically significant interaction existed between the effects of *in vitro* and *in vivo* semen storage time as measured by the amount of reduction in second week hatchability. Hatchability was not affected by pooling of semen.

Short-term Storage and Pooling of Chicken Semen as Related to Fertility and Hatchability

by

William Logan Wigle

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed June 1976

Commencement June 1977

APPROVED:

Redacted for privacy

Professor of Poultry Science

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Date thesis is presented June 8, 1976

Typed by Clover Redfern for William Logan Wigle

ACKNOWLEDGMENT

Grateful acknowledgment is made to Dr. J.E. Parker for his direction and assistance in the preparation of this thesis.

Thanks are extended to staff members of the Department of Poultry Science and Poultry Research Farm for their assistance, their suggestions and their encouragement.

Recommendations for the statistical analysis of data from Dr. R.G. Petersen and S.T. Chester of the Department of Statistics are appreciated.

Financial assistance provided by the Chester M. Wilcox Memorial Scholarship and the Pacific Egg and Poultry Association Scholarship is gratefully acknowledged.

Acknowledgment is made to Skylane Farms of Woodburn, Oregon, Babcock Poultry Farm, Inc. of Ithaca, New York and Jenks' Hatchery of Tangent, Oregon for supplying the birds, in the form of chicks, used in this investigation.

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SHORT-TERM STORAGE AND POOLING OF CHICKEN SEMEN AS RELATED TO FERTILITY AND HATCHABILITY

I. INTRODUCTION

Since 1935, when Burrows and Quinn first reported a manual massage technique for obtaining semen from the male fowl, researchers have been interested in the *in vitro* storage of cock semen. The utility of holding semen for extended periods for its later use in artificial insemination has been illustrated by successful application in the dairy industry. However, similar application in the poultry industry has been denied by the lack of a suitable technique for long-term storage of fowl semen.

The rapid decline observed in the fertilizing ability of avian spermatozoa after *in vitro* storage must be prevented if the full potential of artificial insemination of poultry is ever to be exploited. This problem has been approached from various directions by numerous investigators. In most of these endeavors, pooled semen samples have been utilized as a matter of convenience. Very few experiments have been conducted using semen samples from individual males and none have compared the fertility resulting from individual male versus pooled semen after storage. The purpose of this study is to make such a comparison and to determine whether pooled or unpooled semen results in higher fertility following *in vitro* storage.

Evidence exists that extended in vivo storage of spermatozoa within the hen's oviduct following insemination has a detrimental effect upon hatchability of eggs fertilized with such "stale" sperm. The present investigation attempts to determine if there is a similar effect on hatchability of fertile eggs by in vitro storage of semen and if there is some type of interaction between in vitro and in vivo aging of spermatozoa.

II. REVIEW OF LITERATURE

A complete breeding program utilizing artificial insemination is dependent to some degree upon a satisfactory technique for the *in vitro* storage of semen. The most dramatic success with semen storage has been with cattle where calves have been obtained from semen which had been stored for 14 years (Herman, 1968). The longest reported period of storage for fown semen where live chicks were hatched has been 33 days (Polge, 1951).

Fertility and Fertilization in the Fowl

Lake (1967) points out that the definition for successful fertilization differs between domestic fowl and cattle. Fertility in the hen is often determined as the percentage of fertile eggs laid over a period of approximately one week following a single insemination. On the other hand, fertility in cattle is measured as the percentage of cows becoming pregnant following insemination. For satisfactory levels of fertility in the fowl, spermatozoa must survive *in vivo* and retain their fertilizing capacity within the hen's oviduct for the entire "fertile period". Fresh fowl spermatozoa can remain viable *in vivo* and fertilize eggs for up to 35 days after insemination (Crew, 1926; Dunn, 1927; Curtis and Lambert, 1929: Nicolaides, 1934; Nalbandov and Card, 1943); however, following *in vitro* storage of several hours

duration, subsequent in vivo fertilizing capacity decreases in duration (Garren and Shaffner, 1952; Hunsaker et al., 1956). Fresh bovine spermatozoa can survive for approximately 28 hours within the female tract (Parkes, 1960), but extended in vivo survival is not required to achieve a high fertility rate as it is determined in this species.

For fertilization to occur, the spermatozoa must be viable and vigorous enough following storage and artificial insemination to ascend the oviduct of the hen and penetrate the vitelline membrane of the ovum. Vigor, represented as motility, is required for sperm transport within the oviduct, for penetration of the sperm storage sites (Schindler et al., 1967), and possibly for the penetration of the vitelline membrane during the actual process of fertilization. Motility may not be required in the transportation of sperm within the oviduct between the uterovaginal junction and the infundibulum. The movement of spermatozoa between these two regions is facilitated by muscular contractions of the oviduct according to Allen and Grigg (1957).

The uterovaginal junction serves as a barrier beyond which immobile spermatozoa cannot progress (Allen and Grigg, 1957) and as a possible site of spermatozoa storage (Bobr et al., 1962, 1964; Fujii and Tamura, 1963; Schindler et al., 1967). The infundibulum also serves as a site of spermatozoa storage (Van Drimmelen, 1946;

Schindler et al., 1967) in addition to being the site of fertilization (Olsen and Neher, 1948). Numerous "tubular glands" exist in both the uterovaginal and infundibular regions of the oviduct and serve as residence sites for the spermatozoa during in vivo storage (Schindler et al., 1967). The mechanism by which these glands sustain the spermatozoa appears to be unknown. Munro (1938) reported on reversible immobilization of spermatozoa at body temperature in certain suspending media including oviduct extracts. He considered this phenomenon to be instrumental in the in vivo survival of spermatozoa within the hen's oviduct.

Intrauterine insemination of dead sperm, a means employed to bypass the uterovaginal junction, has shown that these spermatozoa can ascend the oviduct (Allen and Grigg, 1957). Ogasawara et al. (1966) reported an improvement in fertility following intrauterine insemination of semen from males of proven low fertility. Intrauterine insemination of spermatozoa which had been treated with glycerol (Allen and Bobr, 1955) and spermatozoa injected intramagnally following storage (Ogasawara et al., 1969) gave higher fertility than did intravaginal insemination of such semen.

Motility of spermatozoa, although apparently not necessary to traverse all parts of the female reproductive tract, is correlated with fertility (Parker et al., 1942; Allen and Champion, 1955; Cooper and Rowell, 1958; McDaniel and Craig, 1959). However, spermatozoa which have a severely reduced capacity to fertilize ova may still have

excellent motility (Shaffner, 1942; Clark and Shaffner, 1960; Morgan, 1969).

The actual process of fertilization requires that the spermatozoa be capable of penetrating the vitelline membrane of the ovum, transforming into a male pronucleus, and uniting with the female pronucleus (Romanoff, 1960). Vitelline membrane penetration may involve a trypsin-like enzyme located in the acrosome of the spermatozoa (Ho and Meizel, 1970; Palmer and Howarth, 1972). The transformation process includes the detachment of the tail and the formation of an ovoid nucleus from the head and midpiece (Olsen, 1942). Changes occurring during in vitro storage which would interfere with any of the above processes would result in reduced fertility. As storage time passes, the outer layer surrounding the sperm cell disrupts allowing exposure of the internal nuclear material to the suspending chemical environment. Damage to the spermatozoal head results from such exposure and is a cause of infertility (Lake, 1954).

Metabolic and Chemical Changes in Fowl Semen During Storage

Reduction in the fertilizing capacity of spermatozoa following in vitro storage also may be a result of an exhaustion of the cells' energy supply, an accumulation of toxic materials such as spermatozoal metabolic end products, bacterial growth, or the presence of other substances unfavorable to spermatozoal viability in the

suspending medium (Garren and Shaffner, 1952; Lake, 1956, 1966).

Both the anaerobic glycolytic pathway and the aerobic citric acid cycle are present in fowl spermatozoa for energy metabolism (Lorenz, 1964). Fructose, glucose and mannose may serve as energy sources for fowl spermatozoa (Lorenz, 1959; Harris and Wilcox, 1962). Improved in vitro survival, as measured by motility, has been reported when fructose was added to the stored semen (Shaffner et al., 1941). Improved fertility also resulted when fructose was added after storage immediately prior to insemination according to Wilcox and Shaffner (1958) leading them to conclude that conditions during storage which differ from those during insemination may be required for optimum results. They suggested that conditions be such that metabolism is slowed without damaging the spermatozoa during storage; but, just prior to insemination, the activity of the sperm cells should be increased. Whether sugars via the blood are available as a source of energy to spermatozoa during in vivo storage within the hen's oviduct is not known (Aitken, 1971). Little or no endogenous fructose is present in fowl semen which is due to the lack of seminal vesicles in the fowl (Lake, 1966). Most free carbohydrate in fowl semen is glucose which is found in the "transparent fluid" fraction (Lake, 1966). This glucose would be available as an energy source during in vitro storage. It is converted to fructose by the spermatozoa (Lorenz, 1958).

Carbohydrate disappearance and pH decline were found to be greatest during the first hour of in vitro storage and the rate of change decreased with increasing storage time (Harris and Wilcox, 1962). Lactic acid is an end product of glycolysis and its accumulation is a contributing factor to declining pH values. Schindler et al. (1958) found little lactate production from fowl semen stored at 10°C. for 24 hours and concluded that cock semen lacked glycolytic processes. Wilcox (1958a) however compared pH changes after storage at different temperatures and found a marked lowering of pH at 25°C. and 40°C. which he attributed to production of "acidic substances". In the same series of experiments, Wilcox added antibiotics to the semen and the results suggested that bacterial contamination was not responsible for the pH change but that the sperm themselves were responsible. Metabolic rate, based upon methylene blue reduction time, was increased by alkaline conditions and retarded by acid conditions but highest fertility was obtained with semen stored in acid conditions (pH = 6) for 4 hours (Bogdonoff and Shaffner, 1954). Little difference in fertility resulted when semen was stored over a range of pH from 6.47 to 7.95 (Wilcox and Shaffner, 1957; Wilcox, 1959a).

Spermatozoa collected from the vas deferens of the male fowl is sterile; but when it is collected by the massage technique it is contaminated with bacteria indigenous to the cloaca. Coliform bacilli, staphylococci, diphtheroid bacilli and hemolytic streptococci are the

major types found in the cloaca (Smith, 1949). Either competition from bacteria for the energy source of the spermatozoa or production of enzymes and toxins by bacteria may be responsible for their deleterious effect on fertility. De Muelenaere and Quicke (1958) reported that glucose utilization by fowl semen suspensions was reduced when penicillin or sulfa drugs were added to control bacterial growth, however these agents were suspected of adversely affecting spermatozoal metabolism and fertilizing capacity. The antibiotics oxytetracycline (OTC) and dihydrostreptomycin added to stored fowl semen have the ability to improve fertility of stored semen (Wilcox and Shorb, 1958). Wilcox (1958b) suggested that the improvement brought about by OTC might be due to chelation of divalent cations such as Mg^{++} and Ca^{++}. The binding of Mg^{++} would remove a cofactor from enzyme systems thereby slowing metabolism (Lardy and Phillips, 1943; Lehninger, 1970), while the removal of Ca^{++} might prevent agglutination (Wales and White, 1958).

The anatomy of the male fowl is not conducive of allowing the collection of pure uncontaminated semen. The vas deferns, ureters, lymph folds and vascular bodies are all closely associated within the cloaca. When semen is collected by the massage technique (Burrows and Quinn, 1939a), it may contain, in addition to spermatozoa and fluid from the vas deferens, the following: fluid containing urates and uric acid from the ureters, epithelial secretions and blood transudates

from the vascular bodies and lymph folds, expressed red blood cells and feces (Lake, 1957).

Wilcox and Shaffner (1957) suggested that urine contamination might be a factor affecting pH variability in semen samples. The epethelial secretions and blood transudates, the so called "transparent fluid" (Nishiyama, 1951), has been alleged to cause clumping and agglutination of spermatozoa (Nishiyama, 1955; Lake, 1956). Chloride ions which are found in relatively high concentrations in the transparent fluid as compared to the vas deferens fluid (Lake, 1966) have a detrimental effect on semen during in vitro storage (Wilcox and Wilson, 1961). Boone and Hughes (1970) showed a severe depression in fertility when fresh semen was experimentally contaminated with feces.

In Vitro Storage Techniques

In order to maintain spermatozoa in their optimum condition, storage conditions must be such that harmful effects upon the sperm cells are minimized. Low temperatures, dilution, various additives, and careful collection techniques have been employed to achieve this goal.

How long semen is to be stored often dictates the means of storage. Long-term storage for periods of several years must involve freezing the semen. Such a technique might be desirable when the

semen of a truly superior sire was to be stored. Intermediate length storage extending over several days to one week requires special semen diluents. Successful storage of semen for this length of time seems to require dilution of semen and low holding temperatures that are above freezing. Transportation of semen from its point of collection to another location for insemination is an example of where storage for such a length of time might be necessary (Lake et al., 1959). Short-term storage of semen for periods of several hours to one day can be successfully accomplished with unfrozen, undiluted semen (Garren and Shaffner, 1952; Schindler et al., 1955; Hunsaker et al., 1956).

Low temperatures tend to slow metabolic processes and retard bacterial growth. The relative absence of cold shock exhibited by fowl spermatozoa (Wales and White, 1959; De Silva, 1963) would suggest that freezing would be a feasible method of storage. Although long-term survival of fowl spermatozoa, as measured by motility after thawing, has been observed with semen stored by freezing, unsatisfactory levels of fertility have resulted following the insemination of such spermatozoa (Shaffner et al., 1941; Shaffner, 1942; Smith and Polge, 1950; Polge, 1951; Clark and Shaffner, 1960; Harris, 1968).

More recent investigations concerning freezing fowl spermatozoa have focused upon freeze-thaw damage to the sperm cells (Harris et al., 1971), methods to avoid the damaging effect of glycerol (Allen

and Bobr, 1955; Brown and Harris, 1963; Brown et al., 1963; Neville et al., 1971), and the use of different cryoprotective agents (Harris, 1965, 1966, 1968; Sexton, 1972a, b, 1974).

While the development of a freezing method for long-term storage of fowl semen would be desirable, a satisfactory one has not yet been perfected. Successful storage of unfrozen diluted fowl semen has been reported for periods of up to 5 days (Harris et al., 1960, 1961). The diluent used in these investigations contained the metabolic inhibitor 2-ethyl-5-methyl-benzimidazole (EMB). Blackwood and Harris (1960) showed that the mechanism by which the 2,5-alkyl benzimidazoles enhance spermatozoal longevity and fertilizing capacity following storage is an inhibition of motility without a concomitant reduction of fructolysis. Metabolism of glucose by stored cock spermatozoa was shown to be independent of motility and to continue after cessation of motility (Nevo et al., 1963).

Diluents not containing metabolic inhibitors should provide osmotic balance, buffering action, chelation and possibly nutrition for the spermatozoa (Lorenz, 1959). These qualities would tend to negate the adverse effects of sperm metabolism and seminal contaminants during storage. Using diluents of this nature, acceptable fertility has been observed following storage of semen for periods of 1 to 3 days (Wilcox, 1958a, 1959a, b, 1960; Wilcox and Shaffner, 1958; Wilcox and Clark, 1962; Lake, 1960; Hobbs and Harris, 1963a, b; Harris and

Hobbs, 1964, 1968; Phillip et al., 1974). Synthetic diluents have proven to be more successful in extending the functional life of spermatozoa than the "natural" diluent, seminal plasma (Wilcox, 1958a, Sexton, 1973). The optimum temperature for storing diluted fowl semen appears to be 1°-2°C. (Lorenz, 1964). Wilcox (1960) suggested that dilution may somehow mitigate the harmful effects of temperatures lower than 10°C. upon semen.

Storage of undiluted fowl semen, the easiest method so far, is also the least successful in maintaining the fertilizing capacity of the spermatozoa and cannot be used for periods exceeding 24 hours duration. This method relies upon relatively low temperatures to maintain the spermatozoa in optimum condition. Burrows and Quinn (1939b) reported that although undiluted semen would retain motility for as long as 10 days when held at temperatures of 2°-4°C., no fertility resulted after storage of semen at these temperatures for as short a period as 2 hours. Some fertility resulted after storage for 2 to 8 hours at 20°C.

After holding fowl semen for 24 hours at 50°-60°F., 6% of eggs laid during the first week following insemination were fertile. Semen held for 5 hours at these temperatures gave 66% fertility (Warren and Gish, 1943).

In 1952, Garren and Shaffner studied the effects of temperatures between 0°C. and 40°C. upon stored fowl semen. They concluded that

10°C. was the optimum temperature for storage of up to 7.5 hours duration. Similar experiments (Hunsaker et al., 1956) indicated 15°C. to be the best temperature for semen storage for up to 9 hours. Fertility of 35% during days 2-6 post artificial insemination was reported after storing undiluted fowl semen for 24 hours at 10°C. (Schindler et al., 1955). These last results however were based on a rather small sample size (24 eggs).

Research involving the atmosphere under which undiluted fowl semen can be stored was conducted by Proudfoot (1966, 1972) and Proudfoot and Stewart (1967). Oxygen under normal pressures and air or nitrogen under high pressure proved to be beneficial.

Pooling of Semen Samples

In the above studies involving undiluted semen, all investigators used semen from several males which was pooled and mixed. It is conceivable that the process of pooling semen from several males might affect fertility following storage. Grodzinski and Marchlewski (1938) suggested that an agglutinogen was present in seminal plasma when they observed clumping of spermatozoa following dilution of semen with blood plasma. Clumping and bending of spermatozoa was more pronounced in semen diluted with the secretion of the lymph folds than in semen diluted with a citrate-glucose-saline buffer; and significantly lower fertility following 20 minutes of storage at

20°-22°C. resulted from semen samples containing a greater amount of the lymph fold and vascular body secretions (Lake, 1956).

Chickens have at least six series of blood group antigens as determined by agglutination reactions (Gilmour, 1960). The mixing of semen samples from male fowl of different blood groups could possibly result in agglutination of spermatozoa and therefore, lower fertility than would result from the use of individually stored semen samples. This effect might be more apparent following *in vitro* storage. Parker *et al*. (1942) found that slightly better fertility resulted from the use of unmixed rather than mixed fowl semen. Working with turkeys, Holleman and Biellier (1969) had similar results. These two investigations were conducted with insemination immediately following collection of semen with no intervening storage period.

Semen Storage and Hatchability

Wilcox (1960) summarized hatchability data from a number of investigations and concluded that a slight decrease in hatchability resulted from increasing the length of *in vitro* semen storage. Other investigators have proposed that extended *in vivo* residence of sperm cells within the hen's oviduct tends to lower hatchability of fertile eggs. Dunn (1927) reported hatchability values of 87%, 80% and 67% for eggs laid during the first, second, and third weeks, respectively

after natural mating. Nalbandov and Card (1943) found not only a reduction in hatchability of eggs fertilized by aging sperm but also observed that as sperm became more "stale", embryonic development tended to cease at an earlier age. Kosin (1947) published two sets of data; one of which supports the aging sperm theory and another which contradicts it.

III. EXPERIMENTAL PROCEDURE

General Procedure

Three separate experiments were conducted in the course of these investigations. The same groups of chickens were used for all three experiments. Both males and females were individually caged Single Comb White Leghorns maintained in the same building and subjected to 14 hours of light per day. Females were of the Babcock-300 strain while males were of the Hy-Line W36 strain.

Semen was collected and artificially inseminated by a procedure modified from that described by Burrows and Quinn (1939a). Semen was inseminated as soon as possible after collection or after specified storage periods as described below. Insemination equipment was similar to that described by Parker et al. (1942). Semen was stored in sterile, stoppered round-bottom glass tubes 50 cm. long and 13 cm. in diameter. Each semen sample was divided into several portions so that prior inseminations would not contaminate semen being stored for longer periods.

Hens were assigned to treatments randomly. However, their time of oviposition was recorded and only those which had oviposited at least two hours previously or those without a hard-shelled egg in the uterus were inseminated. This was done to avoid the deleterious effect on fertility of insemination within two hours after oviposition

which was reported by Hughes (1970).

Eggs laid 2 to 15 days following artificial insemination were collected, marked for identification with hen number and date, and stored at 55° to 60°F. These eggs were then set in a forced draft incubator.

All eggs were candled on the 18th day of incubation to determine fertility and fertile eggs were transferred to hatching trays. Those eggs appearing infertile were broken open and examined macroscopically for embryonic development. Eggs failing to hatch by the 22nd day of incubation were also broken open to determine fertility and hatchability. Percentages of fertility or of hatchability for all hens inseminated with one semen sample per treatment were averaged and these values were considered as replicates. Analysis of variance was used to determine if differences between treatments were statistically significant (Snedecor and Cochran, 1967). Standard error values were pooled for all treatments within each experiment.

Experiment I

Experiment I was exploratory in nature and was designed to determine the expected fertility and hatchability of eggs which were fertilized with semen held for short periods of time at 20°C. Treatments included different dosages of either individual male or pooled semen samples held for periods of up to 6.5 hours.

Semen was collected from a number of males and six of these samples which exceeded 0.75 cc. in volume were selected for the individual male treatments. Two pooled semen samples were made by mixing together the semen of 3 males to make one pooled sample and the semen of 4 other males to make the other pooled sample. Assignment of males to either the individual male or pooled semen samples was done on the basis of volume of semen produced so as to insure that enough semen would be available to conduct all treatments in the experiment. Shaffner and Andrews (1948) and McDaniel and Craig (1959) reported that the correlation between semen volume produced and percent fertility was non-significant so assignment of males to the treatments on this basis should not have affected the resultant fertility.

Hens were inseminated with either 0.03 cc. or 0.05 cc. of undiluted semen from one of the eight semen samples. Inseminations were made at intervals of 0, 1, 2.5, 4, 5.5 and 6.5 hours of semen storage. The semen was maintained at a temperature of 20°C. in a box equipped with an electric bulb for a heat source since the ambient temperature was below 20°C. A total of 168 hens were inseminated and 1861 eggs were laid during this experiment.

Experiment II

In Experiment II, the semen storage period was extended to 24 hours and a comparison was made between semen samples which were composed of semen contributed by varying numbers of males. Because of the extended storage time involved, it was decided to hold the semen at the more favorable temperature of 10°C. (Garren and Shaffner, 1952).

Again males were assigned to either individual male or pooled semen samples on the basis of volume of semen produced. Semen from 4 males producing large volumes were designated as the individual male semen samples. Two pooled samples containing semen from 3 males each and two other pooled samples containing semen from 6 males each constituted the other treatments. No attempt was made to use exactly equal volumes or equal numbers of sperm cells from each male contributing to a pooled semen sample.

All hens were inseminated with a 0.03 cc. dosage. Inseminations were made after intervals of 0, 4, 8, 12 and 24 hours of semen storage. The semen was stored in a portable electric refrigerator. A total of 160 hens were inseminated and 1645 eggs were laid during this experiment.

Experiment III

Experiment III was designed to eliminate any bias introduced into the results of the first two experiments by way of individual male variability in fertility or variability in semen collection technique. The pooled semen samples, containing semen from a greater number of males than was represented by the individual male semen samples, might have a higher probability of containing semen from a superior male. Such a superior male might then be responsible for higher fertility from that pooled semen sample to which he contributed. This must be considered in light of the fact that relatively few replications were used in these experiments. Furthermore, since a smaller volume of semen was required from each male contributing to a pooled semen sample, less emphasis may have been placed on extracting the maximum volume of semen from such males after sufficient individual male semen samples had been collected. Less pressure might have been applied to males designated for pooled semen samples during collection and their semen would have been less diluted with "transparent fluid" (Lake, 1957; Nishiyama, 1961).

In Experiment III, semen was collected from 12 individual males. From each of these 12 samples, 0.20 cc. of semen was taken and three pooled samples were made, each containing the semen from 4 of the males. Therefore, pooled sample A contained semen from males 1

through 4, pooled sample B contained semen from males 5 through 8, and pooled sample C contained semen from males 9 through 12. Percentages of fertility or of hatchability for hens inseminated with semen from pooled samples A, B and C were compared with average values for the hens inseminated with semen from males 1 through 4, 5 through 8, and 9 through 12, respectively. Thus there were three replications in this experiment.

Hens were inseminated with 0.03 cc. of undiluted semen either immediately after collection or after a period of 8 hours of semen storage. Semen was stored at a temperature of 10°C. inside a walk-in refrigerator. A total of 120 hens were inseminated and 1261 eggs were laid during this experiment.

IV. RESULTS AND DISCUSSION

Effect of Semen Storage Time on Percent Fertility

The effect of semen storage time on percent fertility is presented on Tables 1, 2 and 3. These tables correspond to the three experiments which involved different lengths of time of semen storage.

As the period of storage was increased, a generally smooth decline in percent fertility was observed (Tables 1 and 2). Also, as the egg-production year progressed, the length of storage which the semen would tolerate became shorter. This effect can be seen by a comparison of the data on Tables 1, 2 and 3. Data from Table 1 was collected in mid-February, that from Table 2 in late April and that from Table 3 in early July. In the first experiment, for the period 2-8 days following artificial insemination, the first week of egg collection, a significant decline ($P < .05$) in fertility was observed when semen had been stored for 5.5 hours. However, in the second experiment, fertility during the first week of egg collection was significantly lower ($P < .01$) when semen had been stored for a period of 4 hours. The decline in fertility resulting from similar periods of semen storage also became more pronounced as the egg-production year progressed.

Table 1. Effect of semen storage time on percent fertility. Experiment I.

Eggs laid	Storage time (hours) at 20°C. 0	1	2.5	4	5.5	6.5	LSD	SE
Days 2-8 post-A.I.[1]	94.85a	80.82ab	84.35ab	83.06ab	71.39b	65.74b	16.83	±5.98
Days 9-15 post-A.I.[2]	57.50a	38.91ab	35.53b	21.38b	17.39b	17.98b	20.55	±5.50
Days 2-15 post-A.I.[2]	76.44a	59.90ab	59.42ab	52.34b	44.20b	41.33b	19.13	±5.13

[1]Means within a row having a different letter are significantly different ($P < .05$).

[2]Means within a row having a different letter are significantly different ($P < .01$).

Table 2. Effect of semen storage time on percent fertility. Experiment II.

Eggs laid	Storage time (hours) at 10°C. 0	4	8	12	24	LSD	SE
Days 2-8 post-A.I.	98.82a[1]	66.47b	62.13b	14.33c	3.51c	20.91	±4.97
Days 9-15 post-A.I.	62.09a	14.93b	15.91b	0.43c	0c	14.39	±3.42
Days 2-15 post-A.I.	79.19a	41.05b	39.27b	7.83c	1.63c	13.97	±3.32

[1]Means within a row having a different letter are significantly different ($P < .01$).

Table 3. Effect of semen storage on percent fertility. Experiment III.

Eggs laid	Fresh	Stored 8 hours at 10°C.	SE
Days 2-8 post-A.I. [1]	88.79a	38.23b	±6.10
Days 9-15 post-A.I.	38.37a	4.04b	±2.90
Days 2-15 post-A.I.	64.35a	21.76b	±4.16

[1] Means within a row having a different letter are significantly different ($P < .01$).

Such a reduction in the ability of fowl semen to withstand in vitro storage may coincide with a reduced ability to withstand in vivo storage in the hen's oviduct. Fertility of eggs laid 9-15 days following insemination, the second week of egg collection, was substantially lower than fertility during the first week of egg collection. This is to be expected since fertility generally declines from a peak reached two to three days following artificial insemination (Moore and Byerly, 1942; Parker et al., 1942). Semen which had been stored in vitro for greater periods of time appeared to suffer a greater decline in fertilizing capacity during the second week of egg collection. As the length of semen storage time increased, fertility during days 9-15 post-A.I. constituted a smaller proportion of the fertility observed during days 2-8 post-A.I. This effect was found to be statistically significant ($P < .01$) and is in agreement with the findings of Garren and Shaffner (1952).

Extension of the semen storage period to as long as 24 hours was attempted in Experiment II and these results are presented in Table 2. Semen storage for a period of 4 hours resulted in a highly significant ($P < .01$) drop in fertility. This level of fertility was maintained when semen was stored for 8 hours. With semen which had been held for 12 hours, fertility was significantly lower ($P < .01$) than with semen held for shorter periods. Very little fertility was obtained with semen stored for 24 hours.

Fertility obtained in this investigation was considerably higher than that observed by Garren and Shaffner (1952) for both fresh and stored semen. Schindler et al. (1955) reported higher fertility after 4 and 24 hours storage of undiluted semen than that obtained in the present investigation but their data were based on eggs laid on days 2 to 6 following artificial insemination.

Effect of Pooling Semen on Percent Fertility

The results of the investigation in regard to the effect of pooling semen on percent fertility are presented in Tables 4, 5 and 6. Values found on these tables are averages for both fresh and stored semen. These tables contain data which may appear contradictory at first glance but these differences can be explained.

In Experiment I, semen from individual males gave slightly, but non-significantly higher fertility than did pooled semen. Males

Table 4. Effect of pooling semen on percent fertility. Experiment I.

Eggs laid	Semen sample[1] Individual	Pooled	SE
Days 2-8 post-A.I.	80.47	78.60	±3.52
Days 9-15 post-A.I.	31.99	29.77	±2.70
Days 2-15 post-A.I.	56.13	54.11	±3.17

[1]Data based on both fresh and stored semen samples.

Table 5. Effect of pooling semen on percent fertility. Experiment II.

Eggs laid	Number of males contributing to semen sample[1] 1	3	6	LSD	SE
Days 2-8 post-A.I.	51.49a[2]	36.77b	57.70a	16.20	±3.85
Days 9-15 post-A.I.	19.60	16.26	20.15	11.14	±2.67
Days 2-15 post-A.I.	35.66a	26.25b	39.46a	10.82	±2.57

[1]Data based on both fresh and stored semen samples.

[2]Means within a row having a different letter are significantly different ($P < .01$).

Table 6. Effect of pooling semen on percent fertility. Experiment III.

Eggs laid	Semen sample[1] Individual	Pooled	SE
Days 2-8 post-A.I.	59.20	67.81	±6.10
Days 9-15 post-A.I.	19.24	23.17	±2.90
Days 2-15 post-A.I.	40.11	46.01	±4.16

[1]Data based on both fresh and stored semen samples.

were assigned to either the individual or pooled treatments with regard to volume only. The relative fertility of the males contributing to the pooled samples was unknown.

Males were assigned to the individual or pooled treatments in a similar fashion for Experiment II. The data in Table 5 indicate significantly lower ($P < .01$) fertility from the pooled semen samples containing semen from three males than from the other two treatments during days 2-8 post-A.I. This difference was not statistically significant during the second week of egg collection. Since only two replications were utilized in this experiment, it is possible that those males contributing to the three-male pooled semen samples were of generally poor fertility. It should be noted that the highest fertility observed in this experiment was obtained with the six-male pooled semen samples and that one of these samples gave the only fertility obtained with semen which had been stored for 24 hours. It is conceivable that the six-male pooled semen samples had a greater probability of containing semen from a single male of high fertility and that such a male was responsible for the improved fertility obtained with those samples.

In Experiment III, where the same males were used for both the individual male semen samples and the pooled semen samples, the pooled samples gave slightly higher fertility. This difference in

fertility was not statistically significant. These results are found in Table 6.

In general, these results show that the pooling process itself does not seem to be as responsible for the different levels of fertility observed as does the quality of semen contributed to the samples by the particular males. No statistically significant interaction was found to exist between pooling of semen and semen storage time.

Effect of Insemination Dosage on Percent Fertility and Duration of Fertility

The effect of insemination dosage on percent fertility is shown by the data in Table 7. These fertility values and data in Table 8 are averages for results from both fresh and stored semen. Increasing the dosage of semen used from 0.03 cc. to 0.05 cc. resulted in no significant increase in fertility. When considered separately, fresh semen consistently gave slightly improved fertility with the larger dosage while fertility results from varying the dosage of stored semen were unpredictable. No statistically significant interaction existed between semen dosage and semen storage time. These results disagree with those of Garren and Shaffner (1952) who reported an increase in both fertility and duration of fertility when 0.20 cc. of semen was inseminated as compared to a 0.10 cc. dosage. The actual volumes inseminated in the present investigation were

considerably less than those employed by Garren and Shaffner and this may account for the difference in results.

Table 7. Effect of semen dosage on percent fertility.

Eggs laid	Dosage[1] .03 cc.	.05 cc.	SE
Days 2-8 post-A.I.	79.84	80.24	±3.45
Days 9-15 post-A.I.	29.57	33.31	±3.18
Days 2-15 post-A.I.	54.72	56.48	±2.96

[1]Data based on both fresh and stored semen samples.

Table 8. Effect of semen dosage on duration of fertility in days following a single insemination.

Eggs laid by	Dosage[1] .03 cc.	.05 cc.	SE
All hens	9.56	9.74	±0.41
Fertile hens only	10.08	10.15	±0.38

[1]Data based on both fresh and stored semen samples.

Data in Table 8 indicate that insemination dosage had no significant effect on duration of fertility, however when using fresh semen the larger dosage resulted in consistently longer duration of fertility while mixed results were obtained with stored semen, as was the case with percent fertility.

Effect of Semen Storage Time on Duration of Fertility

Duration of fertility in days following a single insemination as a measure of fertility was first used by Gowe and Hutt (1949). It was used as the response variable by Hunsaker et al. (1956) and was employed by Garren and Shaffner (1952) in these two studies on semen storage.

Duration of fertility for both all hens inseminated and for fertile hens only was observed and results for both were quite similar. The mean values for duration of fertility were slightly higher when considering fertile hens only. Results closely paralleled those for percent fertility. The coefficient of correlation between percent fertility for two weeks and for duration of fertility was found to be .992. As with percent fertility, semen storage time had a statistically significant effect on reducing the duration of fertility as shown in Tables 9, 10 and 11.

Data in Tables 9, 10 and 11 were collected at the same time as data in Tables 1, 2 and 3, respectively. Again as the laying cycle progressed, storage of semen had a more detrimental effect upon duration of fertility just as it had upon percent fertility.

In this investigation eggs were collected for only the first 15 days following artificial insemination. Had they been collected for a

Table 9. Effect of semen storage time on duration of fertility in days following a single insemination. Experiment I.

Eggs laid by	Storage time (hours) at 20°C.							
	0	1	2.5	4	5.5	6.5	LSD	SE
All hens	12.50a[1]	10.34ab	10.44ab	9.05bc	8.04bc	7.52c	2.48	±0.67
Fertile hens only	12.50a	10.56ab	10.44ab	9.39bc	9.28bc	8.51bc	2.61	±0.70

[1]Means within a row having a different letter are significantly different ($P < .05$).

Table 10. Effect of semen storage time on duration of fertility in days following a single insemination. Experiment II.

Eggs laid by	Storage time (hours) at 10°C.						
	0	4	8	12	24	LSD	SE
All hens	12.67a[1]	7.94b	8.00b	1.94c	0.54c	3.50	±0.83
Fertile hens only	12.67a	9.24b	8.50b	4.04c	--	3.28	±0.78

[1]Means within a row having a different letter are significantly different ($P < .01$).

longer period, differences between duration of fertility from fresh versus stored semen may have been greater.

Table 11. Effect of semen storage on duration of fertility in days following a single insemination. Experiment III.

Eggs laid by	Fresh	Stored 8 hours at 10°C.	SE
All hens[1]	10.70a	5.02b	±0.67
Fertile hens only	10.70a	6.23b	±0.68

[1] Means within a row having a different letter are significantly different ($P < .01$).

Effect of Pooling Semen on Duration of Fertility

Since the process of pooling semen was suspected of having some detrimental effect on percent fertility as suggested in the review of literature, its effect on duration of fertility was observed and these results are presented in Tables 12, 13 and 14. In each experiment, duration of fertility responded to pooling of semen in an unpredictable manner as did percent fertility. While small differences did exist between duration of fertility resulting from individual male semen samples and from pooled semen samples, these differences were neither consistent nor statistically significant.

Duration of fertility proved to be a slightly less sensitive variable than did percent fertility. In Experiment II pooling of semen did cause a statistically significant ($P < .01$) reduction in percent

Table 12. Effect of pooling semen on duration of fertility in days following a single insemination. Experiment I.

Eggs laid by	Semen sample[1]		
	Individual	Pooled	SE
All hens	9.77	9.31	±0.45
Fertile hens only	10.27	9.64	±0.44

[1]Data based on both fresh and stored semen samples.

Table 13. Effect of pooling semen on duration of fertility in days following a single insemination. Experiment II.

Eggs laid by	Number of males contributing to semen sample[1]			
	1	3	6	SE
All hens	6.29	5.05	7.34	±0.64
Fertile hens only	8.88	7.84	9.12	±0.67

[1]Data based on both fresh and stored semen samples.

Table 14. Effect of pooling semen on duration of fertility in days following a single insemination. Experiment III.

Eggs laid by	Semen sample[1]		
	Individual	Pooled	SE
All hens	7.05	8.67	±0.67
Fertile hens only	8.26	8.67	±0.68

[1]Data based on both fresh and stored semen samples.

fertility in one treatment as noted in Table 5; whereas duration of fertility in this case was not significantly reduced as indicated by data in Table 14. In this particular instance, the differences in results from the three-male pooled semen samples probably were due to the semen quality of the contributing males as stated before.

Effect of Semen Storage Time and Pooling of Semen on Percent Hatchability of Fertile Eggs

The percent hatchability of fertile eggs as affected by semen storage time and by pooling of semen is shown in Tables 15 through 17 and Tables 18 through 20, respectively. In no instance were results found to be significantly different.

In nearly all instances, hatchability of fertile eggs was observed to be lower during days 9-15 post-A.I. than during days 2-8 post-A.I. Total overall hatchability of fertile eggs was found to be 88.67% during the first week of egg collection and 81.47% during the second week. These figures are in close agreement with those published by Dunn (1927). Even though this difference was statistically non-significant, the consistency of results suggested that eggs fertilized by "stale" sperm, sperm cells which have undergone *in vivo* storage within the female tract, had a reduced capacity to complete embryonic development and produce viable chicks. This idea was studied earlier by Nalbandov and Card (1943) who found this effect to be present to a

Table 15. Effect of semen storage time on percent hatchability of fertile eggs. Experiment I.

Eggs laid	Storage time (hours) at 20°C.						
	0	1	2.5	4	5.5	6.5	SE
Days 2-8 post-A.I.	94.87	86.51	90.54	96.74	86.68	78.64	±5.83
Days 9-15 post-A.I.	83.39	82.10	85.28	98.04	59.72	84.17	±7.59
Days 2-15 post-A.I.	90.27	84.66	87.18	96.43	84.55	79.09	±6.02

Table 16. Effect of semen storage time on percent hatchability of fertile eggs. Experiment II.

Eggs laid	Storage time (hours) at 10°C.			
	0	4	8	SE
Days 2-8 post-A.I.	84.07	85.30	94.78	±3.69
Days 9-15 post-A.I.	77.46	80.95	77.98	±9.88
Days 2-15 post-A.I.	81.24	84.54	93.09	±4.69

Table 17. Effect of semen storage on percent hatchability of fertile eggs. Experiment III.

Eggs laid	Fresh	Stored 8 hours at 10°C.	SE
Days 2-8 post-A.I.	87.12	90.14	±1.95
Days 9-15 post-A.I.	82.31	86.08	±8.10
Days 2-15 post-A.I.	86.31	88.76	±1.85

Table 18. Effect of pooling semen on percent hatchability of fertile eggs. Experiment I.

Eggs laid	Semen sample[1]		SE
	Individual	Pooled	
Days 2-8 post-A.I.	91.14	86.85	±3.36
Days 9-15 post-A.I.	83.99	80.24	±4.38
Days 2-15 post-A.I.	88.34	85.72	±3.47

[1]Data based on both fresh and stored semen samples.

Table 19. Effect of pooling semen on percent hatchability of fertile eggs. Experiment II.

Eggs laid	Number of males contributing to semen sample[1]			SE
	1	3	6	
Days 2-8 post-A.I.	89.83	88.03	86.28	±3.69
Days 9-15 post-A.I.	83.48	80.55	72.36	±9.88
Days 2-15 post-A.I.	89.25	86.76	82.86	±4.69

[1]Data based on both fresh and stored semen samples.

slight degree during the first two weeks following copulation and to a greater extent thereafter.

Table 20. Effect of pooling semen on percent hatchability of fertile eggs. Experiment III.

Eggs laid	Semen sample[1]		
	Individual	Pooled	SE
Days 2-8 post-A.I.	88.20	89.06	±1.95
Days 9-15 post-A.I.	89.16	79.23	±8.10
Days 2-15 post-A.I.	87.88	87.19	±1.85

[1]Data based on both fresh and stored semen samples.

It was thought that in vitro storage of semen might simulate in vivo storage of sperm cells in the hen's oviduct as far as the effect on hatchability is concerned; however, experimental results did not support this view since in many instances, hatchability of eggs fertilized with stored semen was as high or higher than hatchability of eggs fertilized with fresh semen. No statistically significant interaction was found to exist between in vitro semen storage time and the amount of reduction in second week hatchability.

V. SUMMARY AND CONCLUSIONS

Several experiments were conducted in this investigation to determine the effects of short-term in vitro storage and pooling of chicken semen upon fertility and hatchability of hen's eggs. The following conclusions were drawn from this study:

1. Undiluted fowl semen can be stored for periods of up to 4 hours prior to insemination without significantly lowering the percent fertility if done early in the egg-production year.
2. As the production year progresses, fowl semen tends to lose its ability to withstand the deleterious effects of in vitro storage.
3. Hens inseminated with semen subjected to in vitro storage are fertile for fewer days following insemination than are hens inseminated with fresh semen.
4. Increasing insemination dosage size from 0.03 cc. to 0.05 cc. does not significantly increase percent fertility or duration of fertility when using stored semen.
5. The process of pooling semen collected from different cockerels does not adversely affect the semen's ability to withstand the harmful effects of in vitro storage.
6. Percent fertility or duration of fertility resulting from the insemination of pooled semen is not significantly different

from that resulting from the insemination of individual-male semen.

7. Hatchability of eggs fertilized by semen subjected to in vitro storage is not significantly different from hatchability of eggs fertilized with fresh semen.
8. Hatchability of fertile eggs laid during the second week after artificial insemination tends to be lower than hatchability of fertile eggs laid during the first week after insemination.
9. No statistically significant interaction exists between in vitro semen storage time and in vivo storage effects as measured by amount of reduction in second week hatchability.
10. Hatchability of fertile eggs is not affected by pooling of semen.

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