STUDIES ON REPRODUCTIVE CHARACTERISTICS AND ARTIFICIAL INSEMINATION IN CAPTIVE MALLARD DUCKS (*Anas platyrhynchos*)

by

CAROLYN ELIZABETH STUNDEN

B.Sc., The University of British Columbia, 1993

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Animal Science)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

March 1996

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Animal Science

The University of British Columbia
Vancouver, Canada

Date March 6, 1996

ABSTRACT

Several studies were conducted to evaluate the use of artificial insemination (AI) as a means to improve the reproductive success of hand-reared wild-strain Mallards (*Anas platyrhynchos*). The effects of age, mate choice and isolation on reproductive parameters of captive Mallards were studied.

Eighteen adult and yearling hens were allowed physical, visual-auditory or auditory only contact with drakes. In addition, another 28 yearling hens were placed (17 random, 15 self-chosen) with yearling drakes. Age of males was a key determinant in Mallard reproductive success, specifically egg fertility. Self-chosen pairs performed better than randomly assigned pairs and isolation of hens from drakes did not affect egg production. The data suggests that artificial breeding is needed to supplement natural mating and increase egg fertility in yearlings.

Since egg fertility was generally low in captive held yearling Mallards, comparisons between captive and free-flying Mallard drakes were made to assess differences in gonadal development. Measurements of numerous parameters indicated that, gonadal development was suppressed in captive drakes of both age classes. For the captive drakes, seasonal profiles of testosterone (T) levels and semen characteristics were determined in yearlings and adults. Plasma T concentrations increased from basal levels in March, peaked in April and decreased to basal levels in May. The decrease in T concentration to basal level occurred two weeks earlier in yearlings compared to that of adults. Surprisingly,

semen volume and semen concentration were not different between the two age groups. Mallards were artificially inseminated by modifying a technique developed for domesticated poultry. Egg fertility obtained with AI was 73%. Thus, these results suggest that AI may indeed be used successfully in propagating wild Mallards.

Two poultry semen extenders [Beltsville Poultry Semen Extender (BPSE) and Lake's Poultry Semen Extender (LAKE)] were compared with respect to their effects on sperm survival and sperm fertilizing capacity. BPSE and LAKE worked equally well to maintain the semen fertilizing capacity during short-term storage. Last of all, effects of various concentrations of cryoprotectants [glycerol, ethylene glycol (EG), propylene glycol (PG) and sucrose] on the fertilizing capacity of unstored chicken spermatozoa were tested. The addition of sucrose to EG or PG on the viability of frozen-thawed chicken semen was determined. Fertility declined with increasing concentrations of glycerol and sucrose, but not with increasing concentrations of EG and PG. The addition of sucrose (0.2 M) did not improve freeze-thaw protection of EG and PG.

TABLE OF CONTENTS

LIST OF TABLES

Table **Page**

LIST OF FIGURES

LIST OF APPENDICES

Appendix **Page**

ACKNOWLEDGEMENTS

This project was made possible by funding from the North American Wildlife Foundation through the Delta Waterfowl Research Station and I am grateful for their financial support. I would like to thank my supervisor, Dr. R. Rajamahendran for his guidance of this project; Dr. K. Cheng for assistance with statistics (which was always carried out with good humour); and the other members of my supervisory committee, Drs. C. Bluhm and R. Fitzsimmons for their comments and guidance. Additionally I would like to thank: the staff of the Delta Waterfowl Research Station, the San Rafael Research Aviary and the University of British Columbia Avian Research Center, particularly C. Nichols and K. Shekhtman, for their exceptional care and management of the birds; the Department of Animal Science laboratory technicians and graduate students who gave of their time; M. MacCluskie for sharing self-chosen Mallard pair data; and Dr. F. Rohwer for harvesting drakes. Finally I would like to acknowledge my family whose support, patience and encouragement have been paramount in the completion of this project.

In Memoria

Kathleen Marie Stunden

"An obstacle is often an unrecognized opportunity."

GENERAL INTRODUCTION

The Mallard duck (*Anas platyrhynchos*) is a popular game bird which is widely distributed in North America. Loss of its natural habitat has lead to a slow decline in the population of this species (Gottschalk and Studholm, 1970; Johnson and Shaffer, 1987; Greenwood et al., 1995). Although the large scale release of game-farm Mallards have been proposed as a means to replenish wild stocks (Cheng et al., 1978), there is concern that game-farm Mallards are genetically different from the wild population (Cheng et al., 1980). To help reestablish the wild population, the Delta Waterfowl and Wetlands Research Station began in 1990 an extensive program of captive propagation and release of wild-strain Mallards. However, wild-strain ducks often do not achieve their full reproductive potential under captive conditions (Cheng et al., 1980; Phillips and van Tienhoven, 1960; Phillips, 1964; Bluhm et al., 1983). The primary focus of this thesis, therefore, is to evaluate the use of artificial insemination (AI) as a means to improve the reproductive success of captive Mallards.

AI is a technique by which semen is collected from the male, evaluated, processed, and deposited in the female's reproductive tract. Since the development of a simple and effective semen collection technique by Burrows and Quinn (1937), AI has been practiced in the commercial breeding of domesticated fowl such as the turkey, chicken and duck. Reports of AI in wild ducks are currently not available.

The initial concerns related to designing an AI program for

hand-reared wild-stain ducks were: 1) determining the age of maximum response, 2) the effect of isolation of the sexes on egg production, 3) pair bonding, and 4) the effect of captivity on reproduction. Mallards will breed as yearlings (Batt and Prince, 1978; Krapu and Doty, 1979), but productivity of yearlings is less than that of adults (C. Bluhm personal communication). Poor fertility in yearling pairs is often attributed to copulatory inexperience (Curio, 1982; Bruggers et al., 1981), which could be over come by the use of AI.

The monogamous relationship of pair-bonding is a feature of the Mallard mating system (McKinney, 1985; Bluhm, 1985). When the present study was initiated, it was not known what effect isolation of the sexes would have on egg production. In Canvasback ducks, hens only lay eggs after they have formed a strong pair-bond (Bluhm, 1985) and domesticated turkey hens will lay fewer eggs when isolated from males (Jones and Leighton, 1987). For this work therefore, it was hypothesized that females who are only visually exposed to or completely isolated from drakes would lay fewer eggs than hens with physical exposure to drakes. As well, hens that were placed with self-chosen drakes would be more productive than hens placed randomly with drakes.

Failure of Pintail and Mallard hens to reproduce in captivity has previously been reported (Phillips and van Tienhoven, 1960). Phillips and van Tienhoven (1960) found a lack of ovarian development in captive hens, which was related to inhibited pituitary gonadotropin output. No work was done on Mallard drakes.

In the present study, it was hypothesized that captivity also prevents the gonads of Mallard drakes from maturing normally.

AI in birds has been restricted to the use of freshly diluted semen. This is primarily because the current methods of freezing semen result in poor sperm survival. Experience with domesticated poultry has shown that undiluted, fresh semen held at ambient temperatures will deteriorate within the first hour of semen collection depending on initial quality (Garren and Shaffner, 1952; Bootwalla and Miles, 1992). The physio-chemical nature of extenders are designed to ensure an ideal environment *in vitro* that will maintain semen fertilizing ability. In general, some basic criteria for extenders include: proper pH, adequate buffering, and an energy source for the survival of spermatozoa (Renganathan, 1982). Despite the fact that extensive work has been carried out towards perfecting an ideal poultry semen extender (Sexton et al., 1980; Tajima et al., 1989; Lake and Ravie, 1979), little work has been reported on direct comparisons of different extenders.

The application of AI makes possible the use of frozen semen. Cryopreservation, the freezing of semen at extremely low temperatures, provides greater flexibility in handling of semen, allows the creation of gene banks, and facilitates breeding between geographically separated individuals. These aspects of cryopreservation would be beneficial to a program of captive propagation. Many researchers have reported research into the most suitable cryoprotectant for poultry semen (Sexton, 1975; Lake and Ravie, 1984; Maeda, 1984; Phillips, 1993). The selection of a

suitable cryoprotectant is the most critical aspect towards the survival of spermatozoa during the process of cryopreservation. The use of glycerol at a concentration of 8% is most common and provides good freeze protection. However, the concentration of glycerol must be reduced to less than 2% after thawing and before insemination otherwise fertility is significantly reduced (Lake and Ravie, 1984). The constraints of time and equipment make the reduction of glycerol concentration impractical under field conditions. A cryoprotectant is needed that provides equal freezing protection, but doesn't lower sperm fertilizing capacity. Propylene glycol (PG) and ethylene glycol (EG) have been suggested as alternatives to glycerol, but they do not protect as well against freeze damage (Lake and Ravie, 1984; Maeda, 1984). Many reports have claimed that the addition of certain carbohydrates, such as sucrose, protect biological membranes of cryoprotected mammalian embryos (Schneider and Mazur, 1984; Takeda et al., 1987; Saito et al., 1994). The effect of sucrose in combination with ethylene glycol or propylene glycol on the freezing of avian semen has not previously been investigated.

The general layout of this scheme of research is as follows: Chapter one focuses on factors related to the design of an AI program for ducks, specifically age, mate choice and isolation of the sexes. Comparative gonadal development between captive and free-flying wild Mallard drakes is outlined in chapter two, with an objective to determine whether captivity affects reproduction. Chapter three centres on plasma testosterone profiles and semen

characteristics of the captive drakes. This chapter also investigates the use of AI as an alternative management technique in the rearing of captive Mallards. Chapter four compares two commonly used poultry semen extenders, Beltsville Poultry Semen Extender and Lake's Poultry Semen Extender, with respect to their effects on sperm survival and sperm fertilizing capacity. It is hoped that one of these extenders could be modified for future use with wild-type duck semen. Finally, chapter five examines the effect of various concentrations of glycerol, EG, PG and sucrose on the fertilizing ability of chicken spermatozoa and the effect of sucrose addition to PG or EG in BPSE on the survival of frozen-thawed chicken spermatozoa.

1.0 FACTORS AFFECTING REPRODUCTIVE PERFORMANCE IN CAPTIVE MALLARD DUCKS

1.1 ABSTRACT

Propagation of wild-strain Mallards (*Anas platyrhynchos*) in captivity is hindered by low egg fertility. Therefore, the effects of age, mate choice and isolation on reproductive parameters of captive Mallards were studied. Eighteen adult and yearling hens were allowed physical, visual-auditory or auditory only contact with drakes. In addition, 28 yearling hens were placed (17 random, 15 self-chosen) with yearling drakes. Yearling hens weighed less than adult hens at the beginning of the breeding season, but no differences were found between their initial clutch size (13.2 ± 1.9, 12.9 ± 1.9), initial egg volume (49.46 ± 0.40, 49.24 ± 0.34) and clutch number (1.1 ± 0.2, 1.5 ± 0.2). Yearling pairs had lower egg fertility (7%) than adult pairs (80%). Egg fertility was higher (51% vs. 21%) in self-chosen pairs than in randomly assigned pairs. Such random pairs did not reconcile their differences over the reproductive season. Isolation of hens did not influence egg production. The data suggest that artificial insemination is needed to supplement natural mating and increase egg fertility in yearlings.

1.2 INTRODUCTION

The North American Mallard duck population has been declining in recent years, due to loss of habitat and/or human activities

(Greenwood et al., 1995; Johnson and Shaffer, 1987). In the early 1980's, the Mallard breeding population was at its lowest level since population surveys began in 1955 (Greenwood et al., 1995). This prompted the Delta Waterfowl and Wetlands Research Station in 1990 to begin an extensive program of captive propagation and release of wild Mallards onto vacant restored or enhanced habitat. In contrast to domesticated farm animals, most of the emphasis on reproducing wild birds in captivity has been on natural breeding. Unfortunately, even in carefully controlled breeding environments where birds achieve full reproductive condition, many fail to reproduce (Gee and Temple, 1978). Failure to reproduce in captivity is often linked to the failure of wild-caught females held in captivity to lay eggs (Phillips and van Tienhoven, 1960). However, in the case of hand-reared ducks hatched from eggs taken in the wild, the failure is linked to poor male fertility (Cheng et al., 1980). Often a pair will engage in all aspects of courtship behaviour, but still fail to lay fertile eggs (Gee and Temple, 1978).

Although age can play a role in reproductive performance (Batt and Prince, 1978), low fertility in yearling pairs is often attributed to lack of experience or delayed physical maturation (Curio, 1982). Bruggers et al. (1981) found the pre- and post-copulatory behaviour of yearling Mandarin ducks was more variable than adults. As well, he found that the mounting time was twice as long for yearling drakes due to their inability to position themselves properly. It was therefore postulated that reproductive

performance would be improved by pairing experienced adult drakes with inexperienced yearling hens. In addition, it was hypothesized that birds that are allowed to choose their own mate would be more successful than randomly assigned pairs. In captive Canvasback ducks, it has been found that females are highly discriminatory in their mate preferences and egg-laying is dependent on allowing hens free choice of mate (Bluhm, 1985).

Artificial insemination (AI) has been suggested as a means to improve wild-strain Mallard egg fertility. AI is a technique by which semen is collected from the male, evaluated, processed and deposited within the female's reproductive tract. AI is used in the commercial production of poultry, especially turkeys, allowing females to be housed separately from males. Wild Mallards are monogamous (McKinney, 1985) and visual and auditory displays are an important component of avian courtship (Lehrman, 1959). However, it was not known what effect isolation of the sexes would have on reproductive performance. Studies concerning the influence of social displays on ovarian development in captive Mallards are limited. Desforges (1971) found the sight of a drake was not necessary for ovulation to occur; however, all Mallard hens used had previously been through at least one laying cycle. Other studies on the effects of isolation of the sexes in highly domesticated species also have conflicting results. One study found that egg production in turkeys was significantly higher for females under natural mating conditions than for those visually exposed to or completely isolated from males (Jones and Leighton, 1987).

However, in two other reports studying chickens, the presence of sexually active males did not affect egg production (Tarapovski, 1977 and Renden et al., 1982). Thus, if AI is to be practiced in wild Mallards, one should know the effect of the separation of hens from drakes during the breeding season would have on egg laying and nesting behaviour. Age effects, if present, and incompatible temperaments could also be overcome by AI.

In summary, this study examined whether certain factors, potentially important to the implementation of an AI program, influence reproductive performance in captive Mallards. Specific objectives of this study were to determine if:

1. Reproductive performance of yearling hens is improved by pairing with adult drakes.
2. Self-chosen pairs are more successful than randomly assigned pairs.
3. Physical, visual-auditory, or auditory only contact with drakes affects reproductive performance of hens.

1.3 MATERIALS AND METHODS

1.3.1 Housing and Management

Genetically wild Mallards used in this study were hatched and hand-reared from eggs salvaged from agricultural fields in south central Manitoba. Birds were reared and maintained using standard procedures outlined by Bluhm et al. (1993) at the Delta Waterfowl Research Station (50°11'N, 98°19'W) in Delta, Manitoba. All birds were kept over the winter within same age groups with exposure to

natural daylight.

Two weeks before the expected onset of egg laying (May 1), thirty-six hens (18 yearlings and 18 adults) were randomly assigned to physical, visual-auditory or auditory only treatment groups. Pairs were further divided into adult(♀)-adult(♂), adult(♀)-yearling(♂), yearling(♀)-adult(♂), and yearling(♀)-yearling(♂) pairs to examine age effects on reproduction (Appendix 1). Ducks were weighed to the nearest 10 grams at: (1) the start of the trial, (2) the end of each clutch and (3) the end of the trial using a Pesola spring scale.

Hens in the physical contact group were placed in individual breeding pens with a drake. The individual breeding pens were approximately 2 m x 2.5 m; one-third swimming water and two-thirds dry concrete base. Individual pens for visual-auditory contact groups were further divided with an inner wall of nylon mesh. This allowed the hen and drake to see and hear each other, but prevented physical contact. Pens for the auditory only groups were divided with solid wood preventing physical and visual contact between the hen and drake. All ducks were fed *ad libitum*, a high protein commercially available duck chow, supplemented with wheat, grit and oyster shell. Hens were given access to straw-filled wooden nest boxes. At the time of breeding, many adult hens had laid eggs the previous year. In contrast, yearling hens had no previous breeding experience.

1.3.2 Egg collection and Incubation

Nest boxes were checked each morning for new eggs. New eggs within a clutch were numbered and the length and diameter measured with a Mututoyo vernier metric caliper then returned to the nest. Egg volume was calculated using the formula from Romanoff and Romanoff (1949) where egg volume equals $0.526(\text{length})(\text{diameter})^2$. Clutches were left in the nest box until no new eggs were laid for five consecutive days. At this time, the eggs were removed and the nest box material was replaced. Some hens were continuous layers. In such cases, when more than 15 eggs were laid, the newest six eggs were left in the nest and the remaining eggs removed. The appearance of down within the nest was also monitored and recorded as an indication of nesting behaviour. After completion of the first clutch, all hens were allowed physical access to a drake.

Eggs from hens with physical contact with drakes, were washed in a chlorinated egg washing solution (Kleenegg), candled and incubated horizontally in a Humidaire forced draft incubator. Eggs were candled on day 7, 14 and 23 to determine whether development was proceeding. On Day 23, viable eggs were transferred to individual hatching compartments of a Humidaire hatcher. At the time of first candling, eggs lacking development were opened and classified as early dead or infertile, using the technique outlined by Fant (1957). This method is based on the observation that the yolks of infertile eggs incubated for seven days remain clear and unblemished, while a dead embryo in the gastrulation stage causes the yolk to mottle. Dead embryos detected during additional

candlings were recorded, aged by the technique of Caldwell and Snart (1974) and classified as early-dead (<7 days), mid-dead (8-18 days) or late-dead (>19 days). Mortalities were also classified into one of seven malposition groups (Fant, 1957).

1.3.3 Self-chosen versus Random Pairs

An additional 64 yearlings were placed in individual breeding pens as self-chosen pairs (15) or randomly assigned pairs (17). Eggs were removed from the nest boxes daily and replaced with plastic dummy eggs to maintain the normal laying pattern. Pulled eggs were used by another researcher for a different study. Therefore, only date to first egg, clutch size and percent fertility were analyzed.

1.3.4 Statistical Analyses

Data for drake contact and pairs of different ages (date to first egg from start of trial, clutch size, and down deposited as a percent of clutch size) were analyzed by two-way analysis of variance for unbalanced data sets (Littell, 1991). The least-squares method in the General Linear Models, Statistical Analysis 6.04 (Littell, 1991) was used to test for differences between main effects. The two-way classification model was:

$$Y_{ijk} = \mu + T_i + A_j + TA_{ij} + \epsilon_{ijk}$$

where

$i = 1...3$; $j = 1..4$
Y_{ijk} = equals the k^{th} observation for the $(i,j)^{th}$ cell.
μ = population mean.
T_i = effect of drake contact.

A_j = effect of pair age.
TA_{ij} = interaction of main effects.
ϵ_{ijk} = random error associated with individual observations.

T-tests were used to compare trait means between self-chosen and random pairs, and adult and yearling Mallards.

Percent fertility was defined as the number of fertile eggs divided by the total number of eggs incubated. Percent hatchability was defined as the number of eggs hatched divided by the number of fertile eggs set. Contingency tables in chi-square analysis were used to test effects on fertility. Due to the limited number of observations, hatchability and embryo mortalities were not statistically analyzed. Unless otherwise stated, all comparisons were made at the 5% level of significance ($P<0.05$).

1.4 RESULTS

At the start of the breeding season, adult hens weighed significantly ($P<0.05$) more than yearling hens; adult hens averaged 100g heavier than yearlings (Table 1). This trend was also present when comparing only laying yearling hens with laying adult hens. By the end of the first clutch, the weight difference was no longer significant and remained so through to the end of the breeding season. Yearling drakes weighed the same as adult drakes (Table 2).

The average number of clutches for yearling and adult pairs was 1.1 and 1.5 clutches, respectively (Table 3). Initial clutch size, down deposited and duckling hatch mass did not differ significantly between yearling and adult pairs.

Egg volume (Table 4) did not differ between yearling and adult

hens ($P>0.05$). However, pair-wise comparisons between clutches revealed that second clutch (B) eggs were significantly bigger than first clutch (A) and third clutch (C) eggs.

LSMeans and analysis of variance on clutch size are presented in Table 5 and 7 and Appendix 3. Initial clutch sizes laid by pairs of different ages were not significantly different ($P>0.05$). All hens that failed to lay eggs were yearlings. Egg production for hens with physical contact with drakes was not significantly different from hens with visual-auditory or auditory only contact ($P> 0.05$). LSMeans and analysis of variance on reproductive parameters of Mallard pair combinations of different ages with physical, visual-auditory or auditory only contact are presented in Tables 5 and 7, Figure 1 and, Appendix 4 and 5. Adult hens did not lay earlier than yearling hens. In addition, hens with physical contact did not initiate egg laying sooner than hens with visual-auditory or auditory only contact. No significant differences were observed between treatment groups for down deposited as a percentage of clutch size. Figure 1 summarizes fertility data on 193 individual eggs from different pair combinations. Chi-square tests performed on data indicated significant differences between expected and observed values ($\chi^2 = 52.28$, $N = 193$). Yearling pairs had lower fertility than adult pairs; however, fertility could be improved dramatically by pairing a yearling hen with an adult drake.

Figure 2 summarizes egg fertility data for self-chosen and randomly assigned yearling pairs. Yearling hens placed with self-

chosen mates had fertility rates twice as high as random pairs (χ^2 = 27.09, N = 295). Random pairs did not reconcile their differences over the reproductive season. Egg fertility was significantly lower in second clutches (χ^2 = 21.29, N = 160). Initial body weights, days to first egg, clutch sizes and total eggs laid were not significantly different (P>0.05)(Table 6).

TABLE 1. Body weights of yearling and adult Mallard hens over the breeding season

Time	Yearling	Adult
Start of trial* (all hens)	958 ± 20 (18)[a]	1058 ± 18 (18)[b]
Start trail* (laying hens)	960 ± 25 (13)[a]	1058 ± 18 (18)[b]
End of first clutch	938 ± 14 (13)[a]	950 ± 22 (18)[a]
End of trial^ (laying hens)	999 ± 21 (13)[a]	1021 ± 16 (18)[a]

Values are presented as mean ± SEM (n)
* Trial started May 1
^ Trail ended July 9
Means in the same row with no common superscripts differ significantly ($P < 0.05$).
Differences in n are due to mortalities or hens that did not lay.

TABLE 2. Body weights of yearling and adult Mallard drakes over the breeding season

Time	Yearling	Adult
Start of trial*	1097 ± 32 (18)[a]	1137 ± 32 (18)[a]
End of trial^	1081 ± 20 (16)[a]	1134 ± 17 (15)[b]

Values are presented as mean ± SEM (n)
* Trial started May 1
^ Trail ended July 9
Means in the same row with no common superscripts differ significantly ($P < 0.05$).
Differences in n are due to mortalities, or drakes paired to hens that did not lay.

TABLE 3. Comparison of reproductive characteristics of yearling and adult captive Mallard hens

Parameter	Age	
	Yearling	Adult
Number of clutches	1.1 ± 0.2 (18)[a]	1.5 ± 0.2 (18)[a]
Initial clutch size"	13.2 ± 1.9 (13)[a]	12.9 ± 1.9 (18)[a]
Down deposited*	65.7 ± 5.5 (10)[a]	64.0 ± 5.5 (16)[a]
Duckling hatch wt.(g)	35.91 ± 0.74 (12^)[a]	36.16 ± 0.40 (45^)[a]

Values are presented as means ± SEM (n).
Means within rows with common superscripts do not differ significantly (P > 0.05).
"laying hens only
* as a percentage of clutch size.
^n = hatched duckling sample size.

TABLE 4. Comparison of egg volume (ml) for yearling and adult captive Mallards

	Age	
Clutch	Yearling	Adult
Clutch A	49.46 ± 0.40(171)[a]	49.24 ± 0.34(233)[a]
Clutch B	52.46 ± 0.64(59)[b]	53.18 ± 0.56(58)[b]
Clutch C	51.91 ± 0.90(12)[a]	52.60 ± 0.88(13)[a]

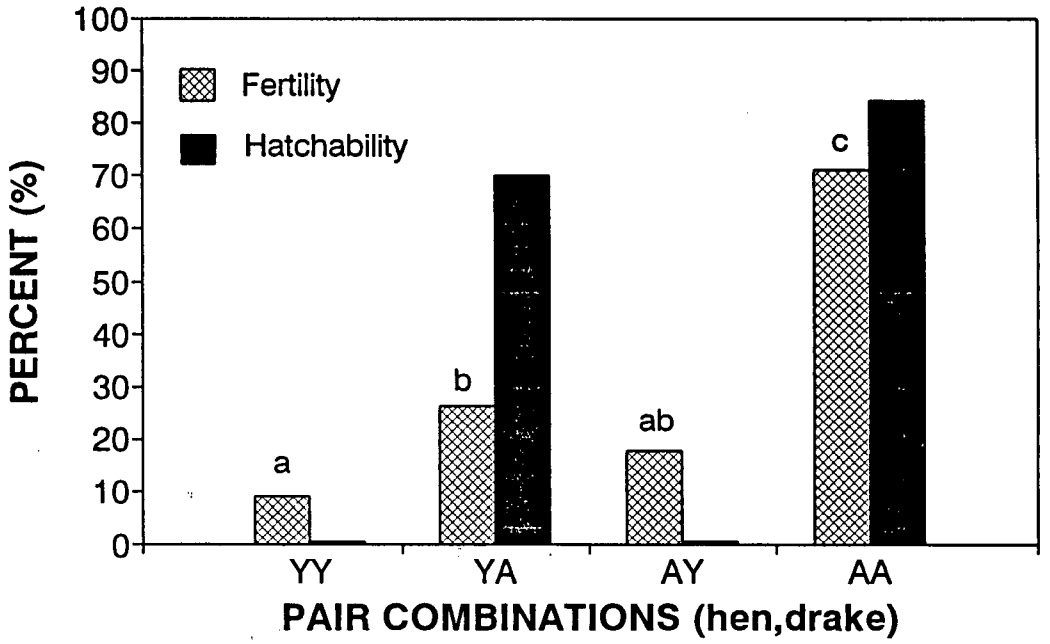
Values are presented as means ± SEM (n).
Means within rows and columns with common superscripts do not differ significantly ($P > 0.05$).

TABLE 5. Comparison of reproductive parameters of different Mallard pair combinations

Parameter	Group			
	Yearling ♀ & Yearling ♂	Yearling ♀ & Adult ♂	Adult ♀ & Yearling ♂	Adult ♀ & Adult ♂
Days to 1st egg*	19.8 ± 2.5 (7)[a]	15.1 ± 2.9 (6)[a]	15.7 ± 2.2 (9)[a]	15.0 ± 2.2 (9)[a]
Initial clutch size	9.1 ± 2.4 (9)[a]	9.9 ± 2.4 (9)[a]	13.7 ± 2.4 (9)[a]	12.2 ± 2.4 (9)[a]
% Fertile	9.3 (5/54)![a]	26.3 (10/38)[b]	17.9 (10/56)[ab]	71.1 (32/45)[c]
% Hatched	0.0 (0/5)	70.0 (7/10)	0.0 (0/10)	84.3 (27/32)
% Embryonic mortality:				
Early-dead	100.0 (1/1)^	66.7 (2/3)	100.0 (10/10)	0.0 (0/4)^
Mid-dead	0.0 (0/1)	0.0 (0/3)	0.0 (0/10)	25.0 (1/4)
Late-dead	0.0 (0/1)	33.3 (1/3)	0.0 (0/10)	75.0 (3/4)

Days to 1st egg and clutch size values are presented as LSMeans ± SEM (n).
* Days from start of trial (May 1).
Means within rows with common superscripts do not differ significantly (P>0.05).
! Five out of 54 eggs were classified as fertile.
^ Discrepancies between fertile egg number and embryo mortality number due to cracked eggs.

FIGURE 1. Egg fertility and hatchability of yearling-yearling (YY), yearling-adult (YA), adult-yearling (AY) and adult-adult (AA) pairs.



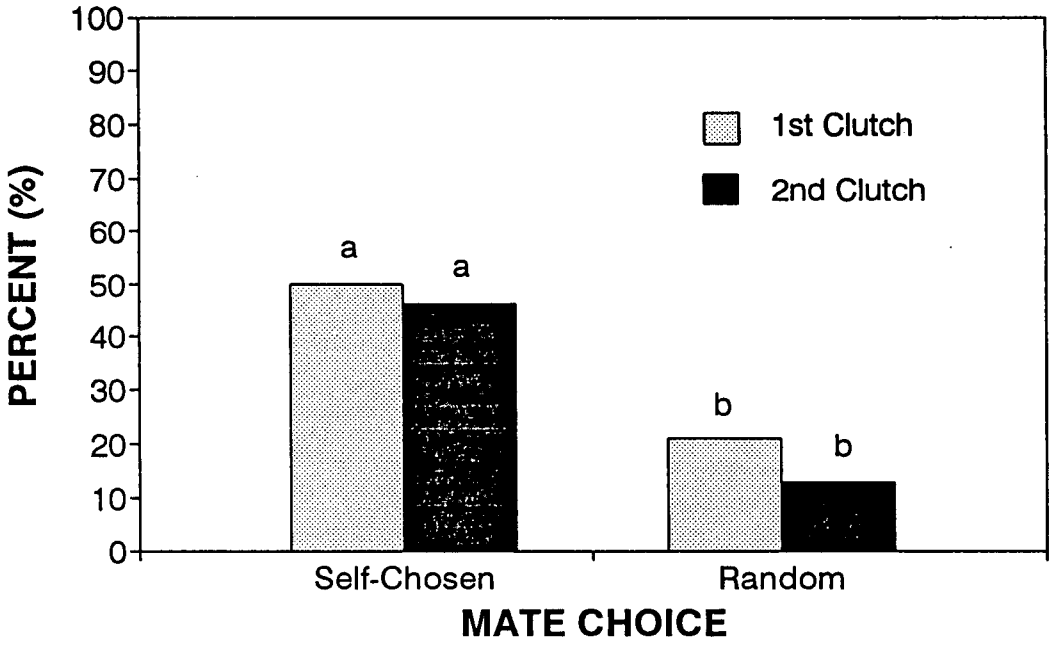
Bars with different letters are different ($P<0.05$)
Hatchability = fertilie eggs only

TABLE 6. Effect of mate choice on reproductive parameters of captive Mallard hens

Parameter	Random	Self-chosen
Hen initial body weight (g)	991 ± 17 (17)[a]	994 ± 18 (15)[a]
Days to first egg*	14.4 ± 1.7 (14)[a]	13.10 ± 2.1 (10)[a]
Number of clutches	1.4 ± 0.2 (17)[a]	1.2 ± 0.3 (15)[a]
First clutch size	10.1 ± 1.7 (17)[a]	8.3 ± 1.7 (15)[a]
Total eggs laid	16.8 ± 3.0 (17)[a]	13.5 ± 3.0 (15)[a]

Values are presented as group means ± SEM (n).
* Days from start of trial.
Means within rows with common superscripts do not differ significantly ($P > 0.05$).

FIGURE 2. Egg fertility of captive Mallards (15 self-chosen and 17 randomly assigned pairs)



Bars with different letters are different ($P<0.05$)

TABLE 7. Effect of contact with drake on reproductive parameters of captive Mallard hens

Parameter	Group		
	Physical	Visual	Auditory
Days to 1st egg*	18.9 ± 2.0 (11)[a]	15.9 ± 2.0 (11)[a]	14.4 ± 2.4 (9)[a]
Clutch size"	9.9 ± 2.1 (12)[a]	14.6 ± 2.1 (12)[a]	9.2 ± 2.1 (12)[a]
Down deposited^	61.0 ± 7.4 (10)[a]	69.1 ± 7.4 (10)[a]	63.5 ± 5.4 (6)[a]

Values are presented as group LSMeans ± SEM (n).
Means within rows with common superscripts do not differ significantly (P>0.05).
* Days from start of trial (May 1).
^ as a percentage of clutch size.
" Includes laying and non-laying hens.

1.5 DISCUSSION

Mallards usually breed as yearlings (Batt and Prince, 1978; Krapu and Doty, 1979). However, several studies have reported that yearlings do not reproduce as well as older more experienced birds (Batt and Prince, 1978; Blohm, 1979). The findings of this study confirm that age is a key determinant to reproductive success, specifically egg fertility.

In the present study, both adult hens and yearling hens paired with yearling drakes exhibited low fertility. Reproductive performance was improved dramatically by pairing yearling hens with adult drakes; however, fertility rates for yearling hens paired with adult drakes did not increase to the degree obtained with adult hens. Poor reproductive output therefore, in yearling birds cannot be contributed solely to the yearling drake. Conventionally, poor reproductive performance by yearlings is attributed to inexperience, delayed physical development, or both (Curio, 1982; Bruggers et al., 1981; Blohm, 1979; Hamann et al., 1987). If lack of experience and failure to copulate contributed to the poor reproductive performance of yearlings, then under conditions of AI, fertility rates of yearling hens inseminated with viable semen should be improved. Alternatively, if sexual maturity is delayed in yearlings, this should be reflected in circulating androgen levels as the timing and extent of drake gonadal development is correlated to circulating testosterone concentrations (Paulke and Haase, 1978). A delay in sexual maturity may help optimize lifetime reproduction (Curio, 1982; Hamann et al., 1987). This aspect of the

reproductive physiology in Mallards warrants further consideration.

Body condition of the hen is an important factor influencing reproductive success. Blohm (1979) found the tendency of free-flying female Gadwalls to renest is greater in adults. He speculated that this tendency to renest was related to initial differences in breeding condition. Exhaustion of energy reserves during the initial laying period may have discouraged further breeding attempts in yearling hens. The results of the present study do not support Blohm's hypothesis in captive Mallards. At the beginning of the breeding season, adult hens averaged 100g heavier than yearling hens, but no difference in clutch number or clutch size were observed. The results, however, do agree with the findings of Batt and Prince (1978) who found that under captive conditions where food is available *ad libitum*, yearling Mallard females were capable of the same reproductive potential as older birds weighing significantly more at the onset of breeding. The number of eggs laid by captive Mallards was found by Krapu and Doty (1979) to be directly related to the quantity and quality of food available during the laying period. In the field, yearling hens entering the breeding season in suboptimal physical condition may be less able to compete with older birds for prime nesting habitat and high quality food sources (Batt and Prince, 1978; Dzubin, 1969). Thus the reproductive potential of yearling hens may be reduced under these conditions.

In the present study, all hens failing to lay eggs during the breeding season were yearlings. Similar observations have been made

by Hamann et at. (1987) and Curio (1982) who speculated that some yearlings may refrain from entering the breeding population to be in better condition upon their first breeding attempt and to enhance their prospects of future reproduction.

Earlier nesting by adults has been observed in Mallards (Coulter et al., 1968), Wood ducks (Bellrose et al., 1964; Rohwer et al., 1991) and Gadwalls (Blohm, 1979) and is thought to contribute to larger clutch size. No differences in the date to first egg or clutch size were observed between adult and yearling hens in this study. It cannot be certain that adult hens did not begin egg laying sooner than yearlings as egg laying by individual birds was not monitored until after birds were placed in individual outdoor breeding pens. This make comparisons of clutch size between adult and yearling hens suspect.

Down generally appear in the nest after 65% of a clutch was laid. This agrees with work done by Caldwell and Cornwell (1975) who found that Mallards hens will incorporate down plucked from their breast into the nest bowl between the fourth and sixth egg. They found that down was an important insulating source for maintaining egg temperature when the nest was unattended.

Ducklings hatched from eggs laid by yearling hens weighed the same as those hatched from eggs laid by adult hens. This is in contrast to the results of Calogeros (1996) who found that ducklings hatched from eggs laid by yearling hens weighed less than those hatched from eggs laid by adults, even after correction for egg size.

The results demonstrate that self-chosen pairs perform better than randomly assigned pairs during the breeding season in terms of egg fertility. Although, Bluhm (1985) found that only Canvasback females of self-chosen pairs laid eggs, in Mallards initiation of egg laying was not dependent on allowing free-choice of mate.

There was no effect of physical, visual-auditory or auditory only hen contact with drakes on egg production. This is consistent with results reported by Desforges (1971) for Mallards, and Renden and Pierson (1982) for chickens. These observations suggest that hens can be housed separately from drakes to simplify AI, without any adverse effects on egg production. This is in contrast to Canvasbacks, where it was found that courtship of hens by their self-chosen mates was important in stimulating ovarian development and egg laying (Bluhm, 1985). In view of the ability to readily reproduce in captivity without the need for male courtship, it is easy to understand why the Mallard is the ancestor of all 17 breeds of domesticated duck except the Muscovy (*Cairina moschata*) (Batt and Prince, 1978).

In summary, data presented in this chapter suggests that there is a need for the use of AI to supplement natural mating in yearling pairs and that hens can be housed separately from drakes to facilitate easier handling of birds under an AI program.

2.0 COMPARATIVE GONADAL DEVELOPMENT BETWEEN CAPTIVE AND FREE-FLYING MIGRATORY MALLARD DRAKES

2.1 ABSTRACT

Comparisons between captive and free-flying Mallard drakes (*Anas platyrhynchos*) were made to assess differences in gonadal development. Body weight, testes size, testes weight, sperm number and gonad histology were determined in yearling and adult drakes. Captive drakes had smaller testes, lower sperm numbers and histologically immature testes. This study provides further support that gonad development is suppressed in captive drakes.

2.2 INTRODUCTION

Reproduction in wild ducks is often inhibited by captivity (Phillips and van Tienhoven, 1960; Phillips, 1964). Phillips and van Tienhoven (1960) found that the gonads of female Mallards held in captivity failed to mature normally. They speculated that constant anxiety or fear was the main cause of the inhibition. Surprisingly, little or no work was done on gonadal development in captive drakes. In preliminary studies of captive Mallard semen characteristics (Table 8), it was apparent that sperm counts for the captive Mallards were lower than comparable figures available for poultry and domesticated ducks (Lake and Stewart, 1978a; Gvaryahu et al., 1984; Gee, 1983). Low fertility in captive Mallards, especially yearling pairs, is a major problem that retards progress in duck raising. It is important, therefore, to

determine if the gonads of Mallard drakes held in captivity are inhibited by manipulated environmental or behavioral restraints.

2.3 MATERIALS AND METHODS

2.3.1 Drake Collection

Twenty wild, free-flying Mallard drakes were harvested in Minnedosa, Manitoba between May 26 and June 7, 1994 to get quantitative information on gonadal development and determine an appropriate benchmark for captive Mallards. Six captive Mallard drakes were used for comparisons.

2.3.2 Age Determination

All collected carcasses were brought back to the laboratory for examination and recording of various data. Harvested birds were categorized as yearling or adults based on air-dried feather measurements (Gatti, 1983). Measurements taken on feathers were: shaft diameter of primary V (DPV); total length of primary IX (LPIX); total length of greater secondary covert nine (L9C); bottom vane width of greater secondary covert five (BW5C) and top vane width of greater secondary covert five (TW5C). Lengths of primaries and greater secondary coverts (to 0.5 mm) were measured from the tip of the inferior umbilicus to the tip of the rachis, while straightened. Primary shaft diameter (to 0.02 mm) was measured with a vernier caliper taken in the plane of the feather vane with the superior umbilicus centred between the instrument tips (Gatti, 1983). The equation (D) used to discriminate between yearling and

adults was:

$$D = 0.684(TW5C) + 2.527(DPV) + 0.073(LPIX) - 0.221(L9C) + 0.268(BW5C) - 24.335.$$

where adult is $D \geq 0.03$ and yearling is $D < 0.03$.

2.3.3 Histology

Drakes were weighed using a Pesola spring scale. Birds were dipped in a solution of soapy water before dissection to keep feathers down. Testicular length and width (measured in the dorso-ventral plane) in millimetres were obtained with vernier calipers. Combined weight of testes was determined on a Sartorius type 2205 scale. Testes used for histological work were fixed in 10% formalin and imbedded in paraffin. Preparations (3 microns thick) were stained with Gill III haematoxylin and counterstained with acidified 1.0% eosin. Sections were viewed under low magnification (400x) and classified, based on Johnson's (1961) histological development of the testes as follows:

Stage 1 = (inactive testis), lumen bordered by clear cytoplasmic material, peripheral row of spermatogonia and few spermatocytes.

Stage 2 = increased number of spermatocytes, many of which are in synapsis.

Stage 3 = majority of spermatocytes in synapsis, and a few secondary spermatocytes present.

Stage 4 = secondary spermatocytes, some spermatids and immature spermatozoa.

Stage 5 = large number of spermatids, and moderate numbers of spermatozoa.

Stage 6 = (full breeding condition) large number of mature spermatozoa, some present in large central lumen.

Stage 7 = (regression) large numbers of degenerating spermatozoa and cells in tubular lumen.

2.3.4 Sperm Count

The phallus was removed by cutting along its line of attachment adjacent to the cloacal wall and weighed. The right and left vas deferens were surgically excised from their point of entry at the cloaca to the inferior edge of the kidneys. The vas deferens was flushed with a warmed Lakes solution (Lake and Ravie, 1979) toward the cloacal end. The contents were collected in to a small test tube and stored at room temperature. Test tubes were centrifuged at 2500 rpm for five minutes in a Damon IEC HN-S centrifuge. The supernatant was removed and replaced with 250 ul of Lakes solution. Sperm were resuspended. A sample of sperm solution was taken and diluted appropriately with spermicide (Lakes plus a few drops of 90% ethyl alcohol) to get an accurate sperm count using a haemocytometer (see Materials and Methods Chapter Three).

2.3.5 Statistical Analysis

T-tests were used to compare trait means between wild and captive drakes and, yearling and adult drakes (Littell, 1991). Unless otherwise stated, all comparisons were made at the 5% level of significance ($P<0.05$).

2.4 RESULTS

Comparisons of captive and free-flying migratory Mallard drake gonadal development is summarized in Table 9 and Table 10. Testis

and phallus weight were significantly higher in free-flying Mallard drakes than in captive drakes ($P<0.01$). However, it should be noted that the captive Mallard sample size was small. As well, testis size was significantly larger in free-flying Mallard drakes ($P<0.01$). In both free-flying and captive drakes, the left testis was larger. Captive drakes tended to be in the transitory stages (3 and 4) between the inactive condition and full breeding condition. Very little variability was found in the maturity of testes of free-flying drakes.

TABLE 8. Results of preliminary laboratory evaluation of captive Mallard semen characteristics

Parameter	Mean ± SEM (n)
Volume (ml)	0.05 ± 0.02 (12)
Concentration (sperm/ml)	$8.2 \times 10^7 \pm 2.4 \times 10^7$ (3)
Appearance	3.0 ± 0.3 (15)
Motility	1.0 ± 0.3 (12)

TABLE 9. Comparison of gonadal characteristics between free-flying and captive Mallard drakes

Parameter	Free	Captive
Body weight (g)	1140 ± 13^{a} (20)	1108 ± 66^{a} (6)
Testes weight* (g)	21.9 ± 1.0^{a} (20)	8.3 ± 3.2^{b} (6)
Distribution of spermatogenic stages	6(20)	3(2)), 4(2), 6(2)
Right testis length (mm)	44.9 ± 1.0^{a} (20)	27.2 ± 3.7^{b} (6)
Right testis width (mm)	22.6 ± 0.5^{a} (20)	15.4 ± 2.4^{b} (6)
Left testis length (mm)	46.8 ± 1.2^{a} (19)	29.1 ± 3.5^{b} (6)
Left testis width (mm)	23.1 ± 0.5^{a} (19)	15.5 ± 2.2^{b} (6)
Phallus weight (g)	2.1 ± 0.1^{a} (20)	1.3 ± 0.2^{b} (6)
Total sperm number	$5.6\text{x}10^{8} \pm 1.3\text{x}10^{8a}$ (12)	$8.6\text{x}10^{7} \pm 7.6\text{x}10^{7\,b}$ (5)

Data presented as mean ± SEM (n).
Values within rows with no common superscripts differ significantly ($P<0.05$).
* Combined weight of right and left testis.

TABLE 10. Comparison of characteristics between yearling and adult free-flying and captive Mallard drakes

Parameter	Yearling	Adult
Free-flying:		
Body weight (g)	1150 ± 36[a] (5)	1137 ± 13[a] (15)
Testes weight* (g)	21.9 ± 1.1[a] (5)	21.8 ± 1.3[a] (15)
Captive:		
Body weight (g)	1175 ± 100[a] (2)	1075 ± 91[a] (4)
Testes weight* (g)	2.3 ± 0.8[a] (2)	11.3 ± 4.2[a] (4)

Data presented as mean ± SEM (n).
Values within rows with common superscripts do not differ significantly (P>0.05).
* Combined weight of right and left testis.

2.5 DISCUSSION

Comparisons of testicular weight and size between captive and free-flying Mallards suggest that gonadal development is suppressed in captive drakes. Several factors can influence gonad development, primarily daylength (Haase, 1983; Donham, 1979), ambient temperature and nutrition (see review by Bluhm, 1992). These factors appear to be adequate for reproductive development in this case, as Mallard hens held under the same conditions laid eggs. Phillips and van Tienhoven (1960) found that wild-caught Pintail ducks held in captivity failed to show ovarian development and bioassays of their pituitary glands revealed a lack of gonadotropin activity. It was speculated that captivity in some way inhibited normal gonadotropin secretion. It is highly probable that this is also the case in Mallard drakes held in captivity. Further work is needed in this area.

3.0 PLASMA TESTOSTERONE PROFILES, SEMEN CHARACTERISTICS AND ARTIFICIAL INSEMINATION IN CAPTIVE MALLARDS

3.1 ABSTRACT

Testosterone (T) profiles and semen characteristics were determined on yearling and adult hand-reared wild Mallard (*Anas platyrhynchos*) drakes. Mallard hens were artificially inseminated by modifying a technique developed for domesticated poultry. In both adult and yearling drakes, there was a change in the concentration of circulating plasma T during the reproductive season. T concentrations increased from basal levels in March, peaked in April and decreased to basal levels in May. The decrease in T concentration to basal level was two weeks earlier in yearlings compared to adults. The decrease in T concentration was associated with the onset of postnuptial molt. Semen volume (0.04 to 0.08 ml) and semen concentration (~1.32×10^9 spermatozoa per ml) were not different between adult and yearling drakes. Fertility obtained with artificial insemination was 73%. These results suggest that artificial insemination may be used successfully in propagating wild Mallards.

3.2 INTRODUCTION

Artificial insemination (AI) is a technique by which semen is collected from the male, evaluated, processed and deposited within the female's reproductive tract. Collected semen can be processed for short-term (fresh; < 24 hours) or long-term (frozen;

indefinite) storage *in vitro* (Hafez, 1987). AI has been practiced routinely in the commercial breeding of domesticated fowl, such as the turkey, since the development of a simple and effective procedure by Burrows and Quinn (1937). AI has also been successfully applied to the breeding of domesticated ducks such as the Muscovy (Tan, 1980; Watanabe, 1957), Peking (Tai, 1983) and Golden (Kim, 1980). Several researchers have noted the periodic use of AI in the breeding of wild ducks, but no detailed studies have been reported.

Many domesticated ducks are polygamous and drakes will mount any hen to the point of ejaculation (Gvaryahu et al., 1984). In such cases it is possible to use an artificial vagina for the collection of semen. Wild Mallards are monogamous and, as was shown in Chapter one, hens are very tenacious in their choice of a mate. It can be assumed, therefore, that wild Mallards would not copulate in the presence of attendants to enable the use of an artificial vagina. However, it may be possible to collect semen by adapting the forced or massage method commonly used in the poultry industry.

Due in recent years to unprecedented population declines, efforts to breed endangered species in captivity have increased (Ballou, 1992). This is a worthy cause since breeding individuals of some waterfowl are at critically low levels (NAWF, 1991) It is therefore a matter of urgency that we seek to improve our understanding of the basic breeding biology of waterfowl and to increase our control over the breeding process by the application of special techniques of artificial breeding. AI may be one such

technique.

Failure to reproduce in captivity is often linked to a failure of wild caught females held in captivity to lay eggs (Phillips, 1960). However, in the case of hand-reared birds hatched from eggs taken from nests in the wild, the failure to reproduce tends to be due to low egg fertility (personal observation). Often a pair will engage in all aspects of courtship behaviour but still fail to lay fertile eggs (Gee and Temple, 1978). In cases where failure to copulate is due to inexperience, incompatible temperaments or lack of a mate, it may be worthwhile to use AI to supplement natural mating.

Reduced genetic viability due to inbreeding is a problem in any small wild or captive population. For example, the New Zealand Brown Teal, when bred in captivity at the Wildfowl Trust had shown loss of reproductive efficiency; in particular in male infertility (Kear, 1978). Consequently, outbreeding became necessary to maintain the population. With the advent of AI it would be possible to introduce genetic variability into small captive or wild populations by transporting semen from a distant population. Accordingly, the risk of introducing parasites and diseases associated with foreign birds and the problems connected with the transport of live birds is reduced.

Work has been done to characterize the annual reproductive cycle in Mallards (Donham, 1979; Paulke and Haase, 1978; Haase, 1983); however, virtually no studies have compared the relationship between age and blood testosterone levels in drakes. The major

functions of the testes are the production of spermatozoa and the secretion of testosterone. Testosterone stimulates development and maintenance of the reproductive tract, spermatogenesis and sexual displays (Phillips and McKinney, 1962; Bluhm, 1992). In Mallards exposed to natural lighting, the breeding season is restricted to the spring. During the winter birds become photosensitive and in this state respond to increasing photoperiod with gonadal growth and increasing plasma testosterone levels (Haase, 1983). Circulating testosterone level peak and the testes are fully developed during the breeding season in April and May (Paulke and Haase, 1978; Haase et al., 1985). In late spring and early summer, birds become photorefractory as reproductive activity is not maintained by long days. This corresponds to a rapid decline in circulating concentrations of testosterone and regression of the testes (Haase et al., 1985). Decline in testosterone is correlated with postnuptial molt at the end of which drakes are rendered flightless for two to three weeks due to loss of their primary flight feathers (Johnson, 1961). After postnuptial molt, the drake can no longer be distinguished from the hen on the basis of plumage colour since the drakes characteristic green head feathers are replaced by brown feathers. When the growth of new flight feathers is completed, the prenuptial molt begins (Johnson, 1961). The testes appear to be completely regressed by the time that complete prenuptial plumage is acquired (Johnson, 1961) and the drakes have returned to their characteristic breeding plumage.

A second peak in testosterone occurs in late fall, but unlike

the first is not accompanied by an increase in testes size; rather, it is thought to be related to pair bond formation (Paulke and Haase, 1978). Blood levels of avian testosterone have also been shown to exhibit a diurnal rhythm and pulsatile release (Johnson, 1986).

Although Mallard drakes breed as yearlings, they show reduced productivity compared with their older counterparts. This reproductive inferiority of young birds is often attributed to lack of reproductive experience, lack of physical development or both (Curio, 1982). In a study on Gadwalls (Blohm, 1982) adult males were involved in 74% of pair bonds while yearling males were involved in only 26%. It was suggested that age related differences in neuroendocrine development or courtship behaviour may have bestowed a competitive advantage to the older more experienced males during early courtship, thereby explaining the preponderance of adult drakes in pair bonds.

Circulating androgen levels are a reflection of the timing and extent of gonadal development. Therefore, a comparison of testosterone profiles of yearling and adult drakes may determine if the poor reproductive success of yearling drakes is due to inexperience or lack of testicular development.

Specific objectives of this study were to:

1. Compare semen characteristics of yearling and adult Mallard drakes
2. Compare the plasma testosterone profiles of yearling and adult Mallard drakes.

3. Determine if AI could be used as an alternative management technique in the rearing of captive wild Mallards

3.3 MATERIALS AND METHODS

3.3.1 Breeding Stock

Genetically wild Mallards used in this study were reared from eggs salvaged from agricultural fields in south central Manitoba. Eggs were hatched, and birds were reared and maintained using standard procedures outlined by Bluhm et al. (1993) at the Delta Waterfowl Research Station in Delta, Manitoba.

In January 1995, adult (two years old) and yearling (hatched summer 1994) Mallards were transported by air from Delta, Manitoba to the University of British Columbia's San Rafael Research Aviary in Surrey, British Columbia. Although Delta has facilities for housing captive birds, the San Rafael location offered the advantage of being close to specialized equipment (such as a gamma counter), and due to warmer climate and earlier spring, the period available for the training of drakes for semen collection was extended.

3.3.2 Care and Management of Birds

On Feb. 14, 1995 twenty-three drakes were assigned to outdoor cluster pens (siblings and brood mates were separated). Drakes were separated by age and kept approximately six to a pen. Each pen (approximately 2.4 m by 3 m) contained areas of open water and dry

land (gravel). A 1.2 m plywood wall around the base of the pen, shielded the birds from outside disturbances. On March 28, hens were individually assigned to pens (approximately 2.4 m x 1.5 m) adjacent to drakes. Birds in separate pens had visual and auditory exposure to each other, but were physically separated by chain-link fencing. All birds were fed *ad libitum*, with commercially available duck pellets, supplemented with wheat and oyster shell. Hens were given access to straw filled wooden nest boxes. Before introduction to their respective pens, hens were weighed with a spring scale.

3.3.3 Semen Collection

Collections were done between 1300 and 1500h, within drake pens. Each drake was caught by hand. The method of semen collection follows the massage procedure outlined by Lake and Stewart (1978a) with the following modifications. Semen was collected by a single seated technician, with the drake held with his head toward the technician and his wings held between the technicians knees. The tube, used for catching the semen, was held between the fingers of the hand used to massage the abdomen. The opposite hand gently stroked the back toward the tail ending with the thumb and forefinger on each side of the cloaca. Gentle pressure was sometimes applied around the cloacal region to extrude the phallus. It was found preferable to obtain the semen before complete extrusion, in which case the semen was lost as it flowed down the spiral-shaped phallus. It was also sometimes helpful to clip the

feathers around the vent.

Twenty-three drakes used for AI were trained twice a week for about three weeks before inseminations were begun. This training involved handling and attempting to ejaculate the birds. After that, drakes were routinely collected from twice a week. Care was taken to minimize contamination of semen with uric acid waste. Semen was pooled by age group.

3.3.4 Semen Evaluation

The parameters to assess and compare semen characteristics of individual yearling and adult Mallard drake semen samples included volume, concentration, visual appearance, motility, live sperm percentage, and abnormal sperm percentage.

3.3.5 Laboratory Analyses

The following laboratory procedures were performed to determine semen quality and concentration.

(1) Appearance - A small drop of undiluted semen was placed on a microscope slide. Semen appearance and colour were scored as follows:

1 = watery or clear semen

2 = watery with white streaks

3 = medium

4 = thick white

5 = very viscous and chalky white

(2) Motility - After evaluating semen appearance, the drop of semen was covered with a coverslip and viewed under low magnification (100x) to award a score as follows:

0 = No motility.

1 = 1- 20% motile, little movement.

2 = 20-40% motile, no waves

3 = 40-60% motile, slow eddies or waves.

4 = 60-80% motile, waves and eddies of movement.

5 = 80-100% motile, extremely rapid eddies and movement.

(3) Semen volume (cc) - Vials were pre-weighed before semen collection and again after collection using an electronic scale. The specific gravity of semen is approximately equal to one; therefore, one gram of semen is approximately equal one ml of semen.

(4) Concentration - Ten ul of undiluted semen was added to 1990ul of 3% NaCl solution (200x dilution). Two counting chambers of a clean dry haemocytometer were covered with a coverslip and filled with approximately 10ul of sample. Spermatozoa were allowed to settle and then five squares of the large central grid were counted. Sperm concentration per ml was calculated as follows:

5 squares x 200 dilution x 10,000 x average number of spermatozoa in 2 chambers.

3.3.6 Insemination

A one ml tuberculin syringe was used for inseminations directly into the oviduct of laying birds. About 0.1 ml of air was first drawn into the syringe to be able to push out the whole volume of semen at the time of insemination. To prevent injury, insemination was not attempted until each hen had laid at least one egg. It was virtually impossible to evert the oviduct, as is commonly done with domestic chickens and turkeys; therefore, the syringe was gently inserted on the left side of the cloaca and somewhat dorsally until the oviduct was discovered. The syringe was then gently inserted one or two centimetres and the semen deposited.

3.3.7 Egg Collection and Incubation

Nest boxes were examined once a day. Eggs were removed from the nest boxes as they were laid and replaced with dummy chicken eggs to maintain the normal egg laying pattern. Dummy clutches were removed five days after the last egg was laid. Collected eggs were stored for one week in a cool cupboard during which they were rotated daily. Eggs were washed with an antibacterial solution just before incubation in a Humidaire Model 20 incubator at 37.6°C and 21.1°C wet bulb temperature. Eggs were automatically rotated hourly through 90°.

Eggs were candled at seven days to determine whether development was proceeding. Questionable eggs were removed from the incubator and opened to ascertain whether they were fertile, using

the technique outlined by Fant (1957). Two days before the expected hatch date, fertile eggs were placed into a hatching tray at the bottom of the incubator. At hatch, ducklings were weighed and sexed by cloacal exposure (Ward and Batt, 1973). Dead embryos detected during additional candlings were recorded, aged by the technique of Caldwell and Snart (1974) and classified as early-dead (<7 days), mid-dead (8-18 days) or late-dead (>19 days). Mortalities were also classified into one of seven malposition groups (Fant, 1957).

3.3.8 Blood Collection

Blood was collected every two weeks between February and May 1995 from the twenty-three drakes (10 yearlings, and 13 adults) used for AI. Blood samples (1-1.5 ml) were collected between 1200 and 1600 hours from the medial wing vein using sterile 3 cc syringes and 22 gauge needles. Samples were transferred to individual 3 ml heparinized vacutainer tubes (Becton Dickson, Vacutainer Systems, Rutherford, New Jersey, USA) and centrifuged at 1250 X g for five minutes within 30 minutes of collection; the plasma was aspirated and stored at -20°C until assayed for testosterone.

3.3.9 Radioimmunoassay (RIA)

A commercial radioimmunoassay kit (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, USA) was used for testosterone assay. Samples were run in duplicate. The kit uses a solid-phase radioimmunoassay based on polypropylene tubes coated

with antibodies to testosterone. ^{125}I-labelled testosterone competes for a fixed period of time with testosterone in the sample for antibody sites. Once the supernatant is decanted (to separate bound testosterone from free), the tube is counted in a gamma counter to yield counts per minute. These counts were converted to the concentration of testosterone in the sample by way of a calibration curve. The Coat-A-Count kit is equipped with human serum-based calibrators ready to use having testosterone values ranging from 0 to 16 ng/ml. The assay can detect as little as 0.04 ng/ml

3.3.10 Statistical Analyses

T-tests were used to compare trait means between yearling and adult drakes. Plasma testosterone values were analyzed as repeated measures analysis for a split-plot design. Differences in treatment means were separated by least squares analysis of variance procedure using General Linear Models procedure of SAS Institute Inc. (Littell, 1991). The model used was:

$$Y_{ijk} = \mu + A_i + D_j(A_i) + T_k + AT_{ik} + \epsilon_{ijk}$$

$i = 1...2$ (age); $j = 1...8$ (time)
Y_{ijk} = equals the k^{th} observation for the $(i,j)^{th}$ cell.
μ = population mean.
A_i = effect of drake age.
$D_j(A_i)$ = drake nested within age group.
T_k = effect of sampling time.
AT_{ik} = interaction of age with sampling time.
ϵ_{ijk} = random error associated with individual observations.

The drake(age) mean square was used as error term to test age mean square. The other effects [drake(age), sampling time and interaction of age with sampling time] were tested using the random

error mean square.

Chi-square analysis with Yates correction for continuity on 2 x 2 contingency tables was used to test for drake effects on egg fertility. Fertility was expressed as the percentage of viable embryos on day seven of incubation. Hatchability was defined as the percentage of eggs hatched per fertile eggs incubated. Due to the limited number of observations, hatchability and embryo mortalities were not statistically analyzed. Unless otherwise stated, all comparisons were made at the 5% level of significance.

3.4 RESULTS

Semen was collected from 18 (nine yearlings and nine adults) out of 24 drakes. The ability to obtain semen from individual birds was highly variable. If semen did not appear after 60 seconds of massage of any drake, it was unlikely that further massage would result in an ejaculation. In which case, he was released in his pen and tried another day. Mean semen characteristics of adult and yearling captive Mallard drakes are presented in Table 11. There were no significant ($P>0.05$) differences with respect to volume, appearance, motility or concentration between the two age groups.

LSMeans, profiles and analysis of variance of plasma testosterone concentrations are presented in Table 12, Figure 3 and Appendix 6. Testosterone LSMean concentrations ranged from 0.02 to 1.59 ng/ml during the 4-month collection period. Testosterone concentrations were not significantly different between yearling and adult drakes ($P>0.05$), but there was considerable variation in

testosterone concentrations between individual birds. Testosterone concentrations were significantly influenced by time ($P<0.01$), increasing from a low of 0.13 ng/ml in February to a high of 1.26 ng/ml in early April. In May, testosterone concentrations declined.

On April 25, yearling drakes had significantly lower plasma testosterone concentrations than adult drakes ($P<0.01$). The respective LSMeans for plasma testosterone concentrations were 0.55 ± 0.28 ng/ml and 1.59 ± 0.25 ng/ml for yearling and adult drakes. By May 9, plasma testosterone concentrations for yearling (0.61 ± 0.29 ng/ml) and adult (0.99 ± 0.27 ng/ml) drakes were not different ($P>0.05$).

The fertility and hatchability data is presented in Table 13 and Table 14. The mean fertility was 72.6%, ranging from 25 to 100%. This was comparable to fertility of matings between adult Mallards in 1994 (Figure 1). The mean hatchability for 1995 was 53%, ranging from 0 to 67%. Fertility of eggs from hens inseminated with pooled yearling or adult drake semen was 59% and 76%, respectively. There was no significant ($P>0.05$) difference between egg fertility rates for the two age groups.

TABLE 11. Semen characteristics of adult and yearling Mallard drakes

Parameter	Yearling	Adult
Volume (ml)	0.04 ± 0.01[a] (23)	0.08 ± 0.02[a] (26)
Appearance	3.1 ± 0.2[a] (22)	3.3 ± 0.2[a] (20)
Motility	2.8 ± 0.3[a] (21)	3.5 ± 0.3[a] (19)
Concentration ($x10^9$/ml)	1.32 ± 0.29[a] (18)	1.32 ± 0.31[a] (21)

Values are means of nine yearling and nine adult drakes subjected to nine collections periods.
Values within rows with common superscripts do not differ significantly (P>0.05).

Table 12. Effect of Mallard drake age on plasma testosterone concentrations*

	Age	
Date	Yearling	Adult
February 14	0.09 ± 0.28	0.18 ± 0.24
February 28	0.32 ± 0.28	0.42 ± 0.24
March 14	1.01 ± 0.28	0.77 ± 0.27
March 28	1.28 ± 0.31	0.92 ± 0.24
April 11	1.34 ± 0.28	1.19 ± 0.24
April 25	0.55 ± 0.28[a]	1.59 ± 0.25[b]
May 9	0.61 ± 0.29	0.99 ± 0.27
May 23	0.19 ± 0.29	0.02 ± 0.28

Values are presented as LSMeans ± SEM.
Values within a row with different superscripts differ significantly ($P<0.05$).

FIGURE 3. Plasma testosterone concentration in relation to drake age

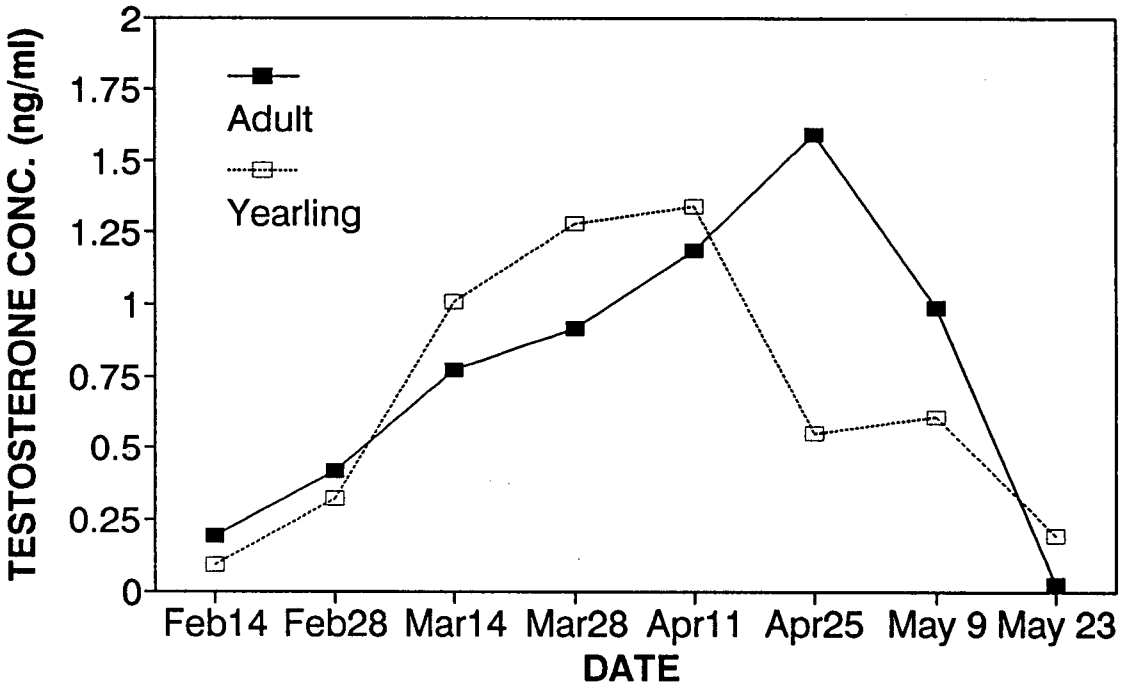


TABLE 13. Fertility and hatchability of individual Mallard hens after artificial insemination

Hen	Hen age	Drake age	Laying sequence after first and subsequent inseminations*	% Fertile	% Hatch of fertile
B	1	2	AI -O-FFF-F	80.0	0.0
B	1	2	AI NFOO-F-F	66.7	50.0
C	1	2	AI --FFFFF-	100.0	n/a
F	2	1	AI ---FO-OO	25.0	0.0
F	2	1	AI NFFFOOO-	57.0	50.0
J	2	2	AI ---FF-F-	100.0	n/a
J	2	2	AI O-FF--FO	60.0	66.7
J	2	2	AI O-FOFF--	60.0	0.0
K	2	2	AI OOFF-FF-	66.7	50.0
L	2	2	AI ---FF---	100.0	0.0
M	1	1	AI OF-FF-FF	83.0	0.0
Mean				72.6	53.3

*0 = infertile egg; F = fertile egg; - = no egg laid; n/a = fertile eggs not incubated to hatch; N = eggs likely fertilized by previous insemination.

TABLE 14. Effect of drake age (yearling verse adult) on fertility, hatchability and embryo mortality rates of Mallard eggs

Parameter	Yearling	Adult	Total
% Fertile	58.8 (10/17)[†a]	75.7 (28/37)[a]	70.4 (38/54)
% Hatched	20.0 (2/10)[a]	33.3 (6/18)[a]	28.6 (8/28)
% Mortality:			
Early-dead[*]	50.0 (4/8)	33.3 (4/12)	40.0 (8/20)
Late-dead[^]	50.0 (4/8)	66.7 (8/12)	60.0 (12/20)

[†] Ten out of 17 eggs fertile.
Values within rows with common superscripts do not differ significantly (P>0.05).
[*] Mortalities <7 days of incubation.
[^] Mortalities >19 days of incubation.

3.5 DISCUSSION

This is the first known report of successful AI in hand-reared wild Mallards. The overall percentage fertility obtained with AI was comparable with that reported for naturally mated pairs (Chapter 1; Lo, 1994; Batt and Prince, 1978). The production of live ducklings strongly suggests that AI can be used successfully to supplement natural mating in captive Mallards.

A feature of the hens reproductive tract is the ability to store semen in a viable state for extended periods of time after copulation or insemination. This allows for the fertilization of consecutively laid eggs between inseminations. Fertile eggs were obtained as much as eleven days after a single insemination. This agrees with results reported by others (Elder and Weller, 1954; Lake, 1983). The results indicate that the period included from the third to seventh day after an insemination is most likely to contain fertile eggs. It is apparent that with the spermatozoa concentration used in this study, hens need to be inseminated at weekly intervals to maintain maximum fertility using AI.

One disadvantage of AI might be the failure to fertilize those eggs laid before insemination. The irregularity of the Mallard hens reproductive cycle makes it difficult to predict the date upon which an egg will be laid. Often hens will skip a day between egg laying, or stop laying completely for up to two weeks between clutches. It may be possible to over come this problem by inseminating before egg-laying begins, as is done with several other avian species (Berry, 1972). Nest-building often occurs a

few days before ovulation of the first egg and has been correlated to a readiness to copulate in many avian species (Lehrman, 1959). Therefore, nest-building could possibly be used as an indication of reproductive maturation.

It is unlikely that the poor hatchability rates obtained with AI is a result of the use of inferior semen or eggs. Postmortem examinations, revealed that the bulk of mortalities occurred during the final stages of incubation and the majority of these dead embryos were in abnormal positions. Typically the duckling was either rotated so that its beak was away from the air cell or the beak was over the right wing instead of under the wing. Deaths caused by malpositions that make hatching extremely difficult are usually attributed to unfavourable incubation conditions such as abnormal temperature or an excess of atmospheric carbon dioxide (Romanoff, 1949). No such conditions were noted in this study, although incubator humidity readings did fluctuate.

The results of this study do not support a hypothesis that differences in semen characteristics or neuroendocrine development contribute to the low reproductive success of yearling drakes. Semen collected from yearlings and adults did not differ with respect to the number of spermatozoa per ml, sperm motility or semen volume. It is generally accepted that semen characteristics affect fertility (Allen and Champion, 1955).

Comparisons of ejaculates from wild Mallards with those reported by various authors for domesticated duck species (Gvaryahu et al., 1984; Kamar, 1962), suggest that quantitative

characteristics are lower for semen obtained from wild Mallards. Kamar (1962) recorded a mean value of $3.63x10^9$ spermatozoa per ml in Sudani drakes and $5.85x10^9$ spermatozoa per ml in Pekin drakes. In contrast, Gvaryahu et al. (1984) reported a concentration of $1.35x10^9$ sperm cells per ml for Muscovy drakes, which is similar to the concentration obtained in this study. It is possible that the lower concentration found in the present study maybe due to the addition of ejaculatory-groove region fluid during collection. This region is considered an accessory reproductive organ in the drake (Fujihara et al., 1976). At copulation, fluid similar in composition to lymph, is released and mixes with the semen ejected from the vas deferens. Such secretions have been found to have favourable effects on the fertility of fresh spermatozoa from the drake (Fujihara et al., 1976). As well, domesticated ducks have been selected for high reproductive performance and this may have altered the semen characteristics. Differences in degree of adaptation to captivity, breed, and method of collection between this experiment and that of the others, cannot be ruled out as sources of variation.

Seasonal changes in the concentration of testosterone in the peripheral blood of Mallard drakes observed in this study agree with previous findings (Paulke and Haase, 1978; Donham, 1979). Mallard drakes exhibited a steep decrease in the concentration of testosterone in May, associated with the onset of the photorefractory period. It should be appreciated that testosterone concentrations in drakes tended to be quite variable.

One of the most striking features of these results was the pattern of circulating testosterone concentrations decreasing to basal levels two weeks earlier in yearling drakes than in adult drakes. It appears that adulthood delayed the onset of the photorefractory period. The results showed no trend for an overall increase in circulating testosterone concentrations in older birds, which is a pattern observed in many farm animals (Gunarajasingam et al., 1985).

Results presented provide evidence that AI may be used to supplement natural mating in Mallard pairs not willing or unable to mate naturally in captivity. Furthermore, the practice of semen collection and insemination should provide valuable insights into the reproductive physiology of wild Mallards. Finally, a logical extension of an AI program would be to preserve semen to allow the exchange of genetic material between distant populations and the establishment of gene banks.

4.0 COMPARISON OF TWO POULTRY SEMEN EXTENDERS

4.1 ABSTRACT

Little work has been reported on direct comparisons of different poultry semen extenders. Beltsville Poultry Semen Extender (BPSE) and Lake's Poultry Semen Extender (LAKE) were compared. Leghorn hens (6-7 per treatment) were inseminated weekly with approximately 1.2×10^8 spermatozoa before and after 24 hours storage at 5°C. No significant difference was found in egg fertility when semen was freshly diluted and inseminated (BPSE 83%; LAKE 90%; undiluted 92%). The fertilizing capacity of stored semen was significantly reduced; however, semen stored in either BPSE (30%) or LAKE (32%) performed better than the undiluted control (3%) ($P<0.05$). From these results it appears that BPSE and LAKE work equally well to maintain the semen fertilizing capacity during short term storage.

4.2 INTRODUCTION

Short-term storage of semen is a practice by which semen is collected from the male, diluted with a buffered salt solution (referred to as an extender or diluent), and then held at 5°C until insemination. The ability to store semen for a several hours offers flexibility in the timing of inseminations and may allow the transport of semen between farms and even countries.

Semen extenders serve two main purposes: First, extenders provide an ideal environment *in vitro* to sustain sperm viability

during short-term storage. The capability of maintaining semen at temperatures above freezing has been crucial to the success of artificial insemination in farm animals. Secondly, extenders maximize the number of hens that can be inseminated from a single ejaculate. Avian semen has low volume, but is highly concentrated. Therefore, it is common for extenders to be used to increase semen volume and enable a proper dose to be delivered during insemination.

The extender used for domesticated ducks is an egg yolk sodium citrate buffer developed by Watanabe (1961). However, this extender has a holding time of less than 30 minutes and Sexton and Fewless (1978) found that the addition of egg yolk to sperm always resulted in a decline in fertilizing capacity. Due to disease prevention protocols, egg yolk acquired from chicken or domesticated duck eggs would not be a desirable component of an extender for wild-strain Mallard semen. Egg yolk could be a potential vector in the spread of salmonella and other avian diseases to wild populations.

Early work to develop diluents to maintaining poultry sperm fertilizing capacity concentrated on the use of simple diluents containing inorganic ions (Schindler et al., 1955; Wilcox et al., 1958). Since then several complex diluents have been developed (see reviews by Bootwalla et al., 1992; Bakst, 1990). In contrast to simple extenders, complex extenders provide (a) buffers to protect against changes in pH, (b) agents to maintain osmotic balance, (c) an energy source and (d) chelating agents to protect against toxic

ions.

Glutamate and potassium phosphate are components common to most poultry semen extenders. Glutamic acid has been reported to be a major non-protein nitrogen constituent of poultry seminal plasma and it may help to maintain the osmotic pressure of semen (Lake and McIndole, 1959) and/or serve as a chelator (Ogasawara and Ernst, 1975). The role of glutamate in Beltsville Poultry Semen Extender was examined by Sexton and Fewlass (1978), who reported a significant decline in the fertilizing capacity of spermatozoa when it was removed from the extender. High potassium levels are necessary for semen survival since potassium is readily lost from the spermatozoa during stressful conditions such as dilution or storage (Renganathan, 1982).

Sugar additives are commonly used as energy sources in poultry semen extenders. Sexton (1974) found that spermatozoa utilize fructose much more efficiently than glucose and rely primarily on the glycolytic pathway for survival *in vitro*. Hence replacing fructose with glucose could reduce sperm metabolic activity and fertilizing capacity. Thus, it is hypothesized that extenders containing different energy sources may differ in their ability to maintain sperm fertilizing ability.

Experience with domesticated species has shown that undiluted (neat) semen held at ambient temperatures will deteriorate within 30 to 35 minutes of collection; however, extended semen can be maintained for up to 24 hours at 5°C without a significant loss in fertilizing capacity (Garren and Shaffner, 1952; Bootwalla and

Miles, 1992). Extensive work has been carried out perfecting the ideal extender. Many have been developed and used successfully by researchers and commercial poultry breeders (Austin and Natarajan, 1991; Chaudhuri and Lake, 1988; Lake and Ravie, 1979). The most widely accepted and commercially available extender is Beltsville Poultry Semen Extender (BPSE) (Sexton, 1977a). High fertility levels (>88%) have been achieved by Sexton in hens inseminated weekly with semen diluted in BPSE and containing as few as 20 million sperm. Lake and Ravie (1979) also developed an acceptable poultry semen extender (LAKE) by modifying a simple unbuffered salt solution. They have also reported high fertility (>87%). Long-term preservation of diluted semen is achieved through freezing and thawing of a semen sample suspended in an extender containing a cryoprotectant. The successful cryopreservation of avian semen would enable the transport of semen, and the establishment of gene banks (gene pools). Little effort has been made to formulate an extender especially for use with frozen semen; yet, choice of an extender is one component critical to the survival of spermatozoa during cryopreservation. The problem of finding a feasible method of freezing poultry semen is therefore dependent on choosing the "best" extender available. There are, however, few reports of direct comparisons between different poultry semen extenders. This study was conducted to compare two commonly used poultry semen extenders (BPSE and LAKE) with respect to their effects on sperm survival and sperm fertilizing capacity. Adaption of one of these inorganic poultry semen extenders for future use with captive

Mallard semen may be possible.

4.3 MATERIALS AND METHODS

4.3.1 Breeding Stock

Limitations imposed by the short breeding season of wild-strain Mallards for studies on semen extenders led to the use of chickens as a model for developing a technique of semen preservation. The stock used was a commercial White Leghorn strain. All birds were housed individually, fed an 18% protein breeders ration *ad libitum* and maintained on 15 hours light and 9 hours dark after 18 weeks of age. Handling of roosters began at 23 weeks of age to habituate the birds to ejaculation procedures. Semen was collected routinely three time per week (Monday, Tuesday and Friday) between 1230 and 1330h by abdominal massage (Lake and Stewart, 1978a). Trials commenced when roosters and hens were 33 weeks of age. Approximately eight males from a population of 11 males, were used for semen collection.

4.3.2 *In Vitro* Sperm Survival

This study was conducted to test for differences in the ability of each extender to maintain spermatozoa *in vitro*. After collection, individual semen samples, free of contamination with uric acid wastes and dust etc., were pooled and divided into three aliquots: neat, dilution (1:1) with BPSE or dilution (1:1) with LAKE (Appendix 2). Both semen and extenders were at room temperature (17-20°C) before mixing. After pipetting gently to mix

samples, 200 ul of diluted semen (or 200 ul of neat semen) was placed in separate tubes for 0, 8, 24, or 48 hours storage at 5°C. Tubes were lightly covered with paraffin. Laboratory analyses were performed to determine sperm quality before and after storage.

4.3.3 Laboratory Analyses

The following laboratory procedures were performed to determine semen quality and concentration.

(1) Appearance - A small drop of undiluted semen, placed on a microscope slide. Semen appearance and colour was scored as follows:

1 = watery or clear semen

2 = watery with white streaks

3 = medium

4 = thick white

5 = very viscous and chalky white

(2) Motility - After evaluating semen appearance, the drop of undiluted semen was covered with a coverslip and viewed under low magnification (100x) to award a score as follows:

0 = No motility.

1 = 1- 20% motile, little movement.

2 = 20-40% motile, no waves

3 = 40-60% motile, slow eddies or waves.

4 = 60-80% motile, waves and eddies of movement.

5 = 80-100% motile, extremely rapid eddies and movement.

(3) Live Percentage - The method for preparing the stain and examining the smears was as described by Lake and Stewart (1978). Nigrosin and eosin were dissolved in a sodium glutamate solvent. Two drops of semen were added to nigrosin/eosin stain at the end of a glass slide and gently mixed. After two minutes a smear was made and allowed to dry. A total of 200 spermatozoa were examined and differentiated into live (unstained) or dead (partially or totally stained) using an oil immersion objective. Two slides were viewed per sample and the percentage live sperm calculated.

(4) Morphological Abnormalities - stained smears were prepared as above. A total of 200 spermatozoa were counted and differentiated into normal or abnormal (absent head, coiled, bent, etc.) under the oil immersion lens. Two slides were viewed per sample and the percentage total abnormal sperm calculated.

(5) Concentration - Ten ul of undiluted pooled semen was added to 2990 ul of 3% NaCl solution (300x dilution). Two counting chambers of a clean dry haemocytometer were covered with a coverslip and filled with 10 ul of sample. After five minutes, five squares of the large central grid were counted. Sperm concentration per millilitre was calculated as follows: 5 squares x 300 dilution x 10,000 x average number of spermatozoa in two chambers.

4.3.4 *In Vivo* Sperm Fertilizing Capacity

The purpose of this study was to test for differences in the ability of each extender to preserve sperm fertilizing capacity. After collection, individual semen samples free of contamination were pooled and transferred into three vials: (1) no dilution (2) diluted 1:1 with BPSE, and (3) diluted 1:1 with LAKE. Both semen and extender were at room temperature (17-20°C) when mixed. Some of each aliquot was removed for storage in covered three ml vials at 5°C for 24 hours. Each separate pooled sample represented a replicate for each treatment. Sperm quality before and after storage was determined by laboratory analysis as above.

The remaining volume was inseminated within one hour of collection. Forty-two hens (six per treatment) were randomly placed in individual laying cages assigned to one of six treatments (BPSE-0, BPSE-24, LAKE-0, LAKE-24, NEAT-0, NEAT-24). Hens were inseminated weekly for four weeks with approximately 1.2×10^8 spermatozoa (0.1 ml diluted or 0.05 ml neat). Different semen treatments were inseminated alternately at each session, to eliminate any possible effect of different holding times.

Just before insemination, semen was loaded into one ml tuberculin syringes. Stored samples were mixed gently before use. Before drawing semen into the syringe, the plunger was withdrawn slightly to provide an air space so that greater pressure could be applied to help force the semen out of the syringe and up the reproductive tract. Hens were inseminated by eversion of the oviduct. Hens were held between the operators thighs allowing both

hands to be free to evert the oviduct by applying gentle pressure to each side of the vent and the abdominal muscles. As a second operator inserted the syringe into the oviduct, the pressure on the abdominal muscles was released and the plunger depressed to deliver the sperm. This helped to transfer the semen up the reproductive tract.

4.3.5 Egg Collection and Incubation

Individual records were kept on each hen. The eggs laid on the day of insemination and the day after were discarded. Eggs laid from day 2 to 15 were collected daily, identified by hen number and date and placed in an egg cooler at 13°C and 36% relative humidity. Eggs, having been allowed to acclimatize to room temperature, were set once a week in a conventional forced draft Robbins incubator at 37.2°C dry bulb temperature and 22.2°C wet bulb temperature.

Fertility was assessed on the seventh day of incubation by candling. All apparently infertile and questionable eggs were broken open and examined macroscopically for evidence of embryonic development using the method of Fant (1957). Percent fertility was defined as the number of fertile eggs divided by the total number of eggs incubated. Fertility was computed for three periods: (1) initial fertility as determined by the percentage of fertile eggs laid during Days 2-8, (2) final fertility as determined by the percentage of fertile eggs laid during Days 9-15 and (3) duration of fertility as indicated by the percentage of fertile eggs laid during Days 9-15.

4.3.6 Statistical Analyses

The *in vitro* study data was analyzed by two-way Analysis of Variance using the General Linear Models procedure of Statistical Analysis System (Littell, 1991). Percentage data was transformed to arc sine $\sqrt{\%}$ prior to analysis to normalize the distribution (Steele and Torrie, 1960). Main effects were conducted with respect to extender (NEAT, LAKE, and BPSE) and storage time (0h and 24h) and extender-storage time interaction. Significant differences between means for extender and storage time were separated by least squares means.

Univariate Repeated Measures Analysis of Variance (Littell, 1991) was used to compare *in vivo* study fertility data over four one week periods. Percentage data was transformed to arc sine $\sqrt{\%}$ prior to analysis to normalize the distribution (Steele and Torrie, 1960). Significant differences in treatment means were separated by least squares analysis of variance procedure using General Linear Models procedure of SAC Institute Inc. The model used was:

$$Y_{ijkl} = \mu + T_i + S_j + TS_{ij} + \epsilon_{ij} + W_k + TW_{ik} + SW_{jk} + TSW_{ijk} + \epsilon_{ijkl}$$

where:

$i = 1...3$ treatment; $j = 1...2$ storage time; $k = 1...4$ week.
Y_{ijk} = equals the observation for the l^{th} hen, on i^{th} treatment, on the j^{th} storage time, on the k^{th} week.
μ = population mean.
T_i = effect of treatment.
S_j = effect of storage time.
TS_{ij} = interaction of treatment with storage time.
W_k = effect of weekly insemination.
TW_{ik} = interaction of treatment with week.
SW_{jk} = interaction of storage time with week.
TSW_{ijk} = interaction of treatment with storage time with week.
ϵ_{ijkl} = random error associated with individual observations.
ϵ_{ij} = sampling error associated repeated measures.

The sampling error mean square was used to test repeated measures effects of weekly insemination. The between subject effects (treatment, storage time and interaction of treatment with storage time) were tested using the experimental error. Statistical results were the same for transformed and non-transformed fertility data, so values are expressed as non-transformed least square means. Unless otherwise stated, all comparisons were made at the 5% level of significance ($P<0.05$).

4.4 RESULTS

4.4.1 *In Vitro* Sperm Survival

4.4.1.1 Morphological Abnormalities:

Maintenance of semen morphology was affected ($P<0.01$) by treatment, storage time and the interaction of treatment by storage time (Appendix 7). The initial mean percentage of abnormal spermatozoa was 3.1% (Table 15). Dilution of semen in BPSE and LAKE maintained semen morphology during the first eight hours of storage. Regardless of storage time, neat semen rapidly degenerated when compared to extended semen ($P<0.01$)(Figure 4).

4.4.1.2 Live Sperm Percentage:

The profiles, LSMeans and analysis of variance for semen viability are presented in Table 16, Figure 5, and Appendix 8, respectively. Storage time and treatment significantly affected the viability of spermatozoa. Treatment by storage time was not significant. The percentage of dead spermatozoa increased after

eight hours of storage without extension ($P<0.01$). There was no significant difference between semen stored in BPSE and LAKE over the 48 hour storage period ($P>0.05$).

4.4.1.3 Sperm Motility:

Maintenance of semen motility was affected ($P<0.01$) by treatment, storage time and the interaction of treatment by storage time (Appendix 9). Initial motility (0h) averaged 3.6 for fresh semen. At 24 hours of storage, mean scores for motility for NEAT, BPSE, and LAKES were 0.8, 3.0 and 2.8, respectively (Table 17). It was evident that dilution of semen in BPSE or LAKE, resulted in consistently better maintenance of motility than neat semen (Figure 6). Motility of neat semen significantly decreased during the first 8 hours of storage ($P<0.05$).

4.4.2 *In Vivo* Sperm Fertilizing Capacity

The LSMeans and analysis of variance for egg fertility are presented in Table 18 and 19, and Appendix 10. Fertility was affected by storage time, week and the interactions of treatment by storage time and week by treatment. Treatment effects were not significant. Fertility following AI with fresh neat semen was 81% or higher (Table 19). In contrast, fertility following AI with stored neat semen was less than 7%. Regardless of extender treatments, inseminations with unstored semen resulted in superior fertility to stored semen ($P<0.01$).

TABLE 15. Effect of Beltsville Poultry Semen Extender (BPSE) and Lake's Poultry Semen Extender (LAKE) on percentage of abnormal sperm as a function of storage time at 5 °C

Time (h)	n*	NEAT	BPSE	LAKE
0	5	3.1 ± 1.7[a]	3.1 ± 1.7[a]	3.1 ± 1.7[a]
8	3	54.2 ± 2.2[d]	5.1 ± 2.2[ab]	6.7 ± 2.2[ab]
24	3	85.2 ± 2.2[e]	9.4 ± 2.2[b]	15.0 ± 2.2[c]
48	3	90.7 ± 2.2[e]	11.3 ± 2.2[b]	16.2 ± 2.2[c]

Values are presented as nontransformed LSMeans ± SEM (n).
*n = number of replicates per treatment.
Statistical analyses were based on least square means of arc sine transformed data.
Non-transformed LSMeans with no common superscripts differ significantly ($P<0.05$).

FIGURE 4. Percentage abnormal sperm as a function of storage time at 5C.

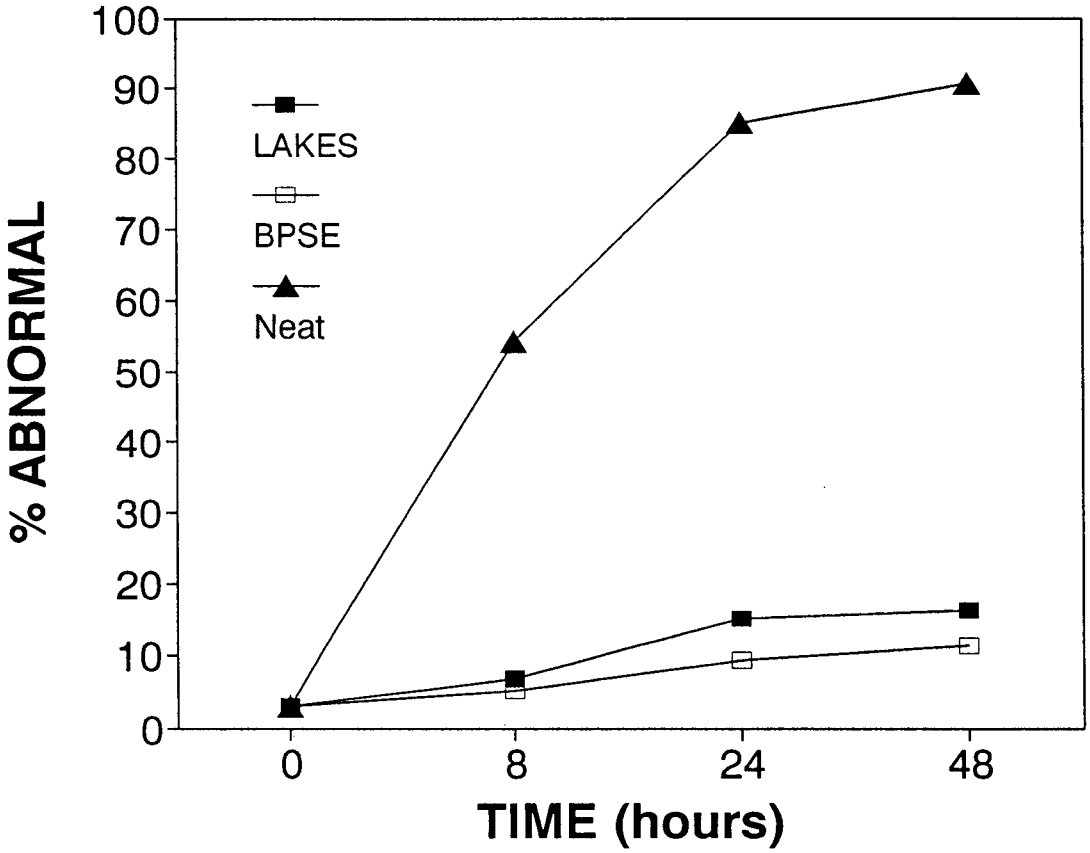


TABLE 16. Effect of Beltsville Poultry Semen Extender (BPSE) and Lake's Poultry Semen Extender (LAKE) on percentage of dead sperm as function of storage time at 5 °C

Variable	n*	Dead (%)
Time (h)		
0	15	4.0 ± 0.7[a]
8	15	5.0 ± 0.7[ab]
24	15	6.5 ± 0.7[b]
48	15	8.7 ± 0.7[c]
Treatment		
NEAT	20	7.7 ± 0.6[a]
BPSE	20	5.1 ± 0.6[b]
LAKES	20	5.2 ± 0.6[b]

Values are presented as nontransformed LSMeans ± SEM (n).
*n = number of replicates per treatment.
Statistical analyses were based on least square means of arc sine transformed data.
Non-transformed LSMeans with no common superscripts differ significantly (P<0.05).

FIGURE 5. Percentage dead sperm as a function of storage time at 5C.

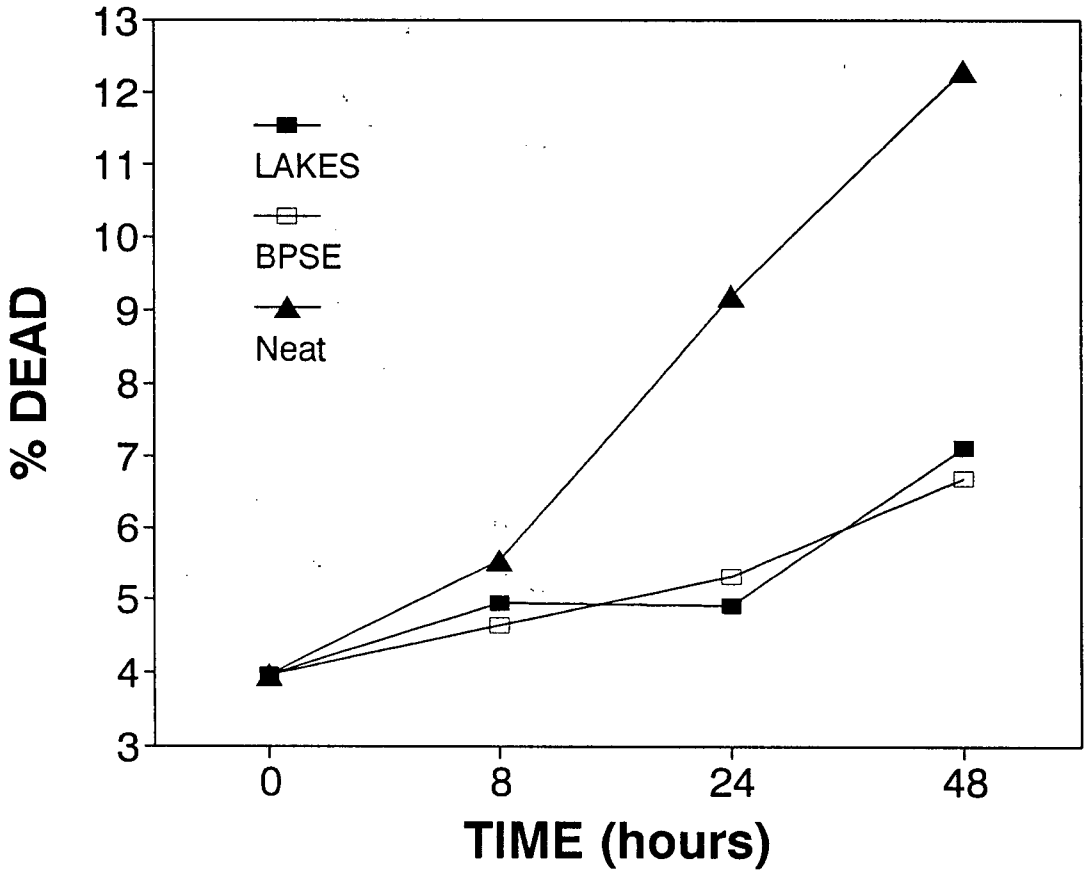


TABLE 17. Effect of Beltsville Poultry Semen Extender (BPSE) and Lake's Poultry Semen Extender (LAKE) on sperm motility score as a function of storage time at 5 °C

Time (h)	n*	NEAT	BPSE	LAKE
0	5	3.6 ± 0.3^{a}	3.6 ± 0.3^{a}	3.6 ± 0.3^{a}
8	5	2.0 ± 0.3^{c}	3.0 ± 0.3^{ab}	3.0 ± 0.3^{ab}
24	5	0.8 ± 0.3^{d}	3.0 ± 0.3^{ab}	2.8 ± 0.3^{abc}
48	5	0.2 ± 0.3^{d}	2.2 ± 0.3^{bc}	2.8 ± 0.3^{abc}

Values are presented as LSMeans ± SEM (n).
*n = number of replicates per treatment.
LSMeans with no common superscripts differ significantly ($P<0.05$).

FIGURE 6. Sperm motility score as a function of storage time at 5C.

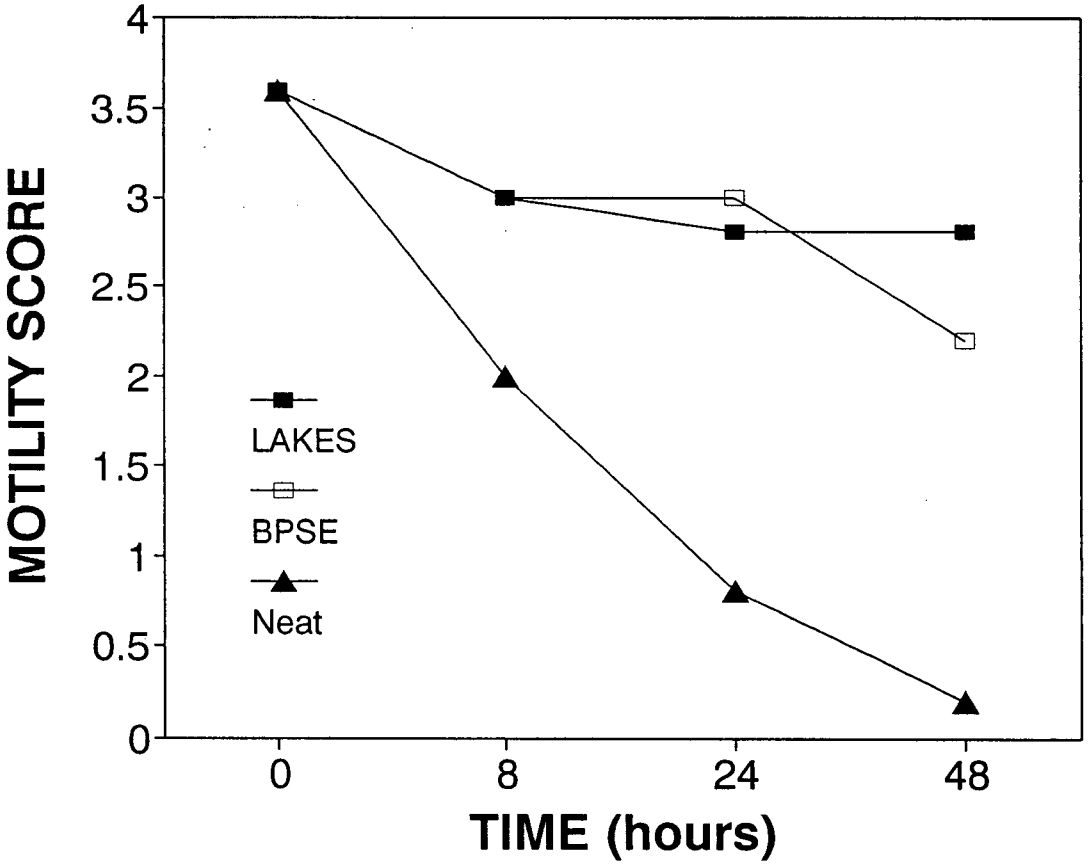


TABLE 18. Percent fertility over 4 weeks of fresh (0h) or stored (24h) chicken semen diluted in Beltsville Poultry Semen Extender (BPSE) or Lakes Poultry Semen Extender (LAKE)

Extender	Storage Time	
	0 Hours	24 Hours
NEAT	92 ± 4.8[a] (6)	3 ± 4.8[b] (7)
BPSE	83 ± 4.8[a] (6)	30 ± 4.5[c] (7)
LAKE	90 ± 4.5[a] (7)	32 ± 4.6[c] (6)

Values are presented as nontransformed LSMeans ± SEM (n) .
n = number of hens per 0 hour and 24 hour groups, respectively. Differences in n are due to mortalities and hens which where not inseminated for the full four week period.
[a-c] Statistical analyses were based on least squared means of arc sine transformed data.
Non-transformed LSMeans followed by different superscripts differ significantly ($P<0.05$).

Table 19. Effect of Beltsville Poultry Semen Extender (BPSE) and Lake's Poultry Semen Extender (LAKE) on fertility after inseminations with fresh (0h) or stored (24h) chicken semen

Week*	Storage	Diluents		
		NEAT	BPSE	LAKE
1	0	81 ± 8.4[a] (6)	88 ± 8.3[a] (6)	75 ± 7.7[a] (7)
2	0	95 ± 8.3[a] (6)	79 ± 8.3[a] (6)	94 ± 7.7[a] (7)
3	0	94 ± 10.4[a] (6)	93 ± 10.4[a] (6)	93 ± 9.6[a] (7)
4	0	98 ± 11.7[a] (6)	72 ± 11.7[ac] (6)	96 ± 10.8[a] (7)
1	24	7 ± 8.4[b](6)	25 ± 7.7[b] (7)	6 ± 8.3[b](7)
2	24	3 ± 8.3[b](6)	24 ± 7.7[b](7)	14 ± 8.3[b](7)
3	24	0 ± 10.4[b](6)	49 ± 9.6[c](7)	54 ± 10.4[c](6)
4	24	0 ± 11.7[b](6)	24 ± 10.8[b](7)	60 ± 11.7[c](7)

*Week represents days 2-8 following each AI.
Values are presented as nontransformed LSMeans ± SEM (n)
n = number of hens per groups.
Differences in n are due to mortalities and hens which did not lay any eggs.
[a-b] Statistical analyses were based on least squared means of arc sine transformed data.
Non-transformed LSMeans for weeks followed by different superscripts differ significantly (P<0.05)

4.5 DISCUSSION

On the study slides, 97% of all cells appeared to have normal morphology and 96% were alive. These are similar to values reported in the literature for good quality samples (Lake and Stewart, 1978a; Allen and Champion, 1955). There was an increased number of coiled spermatozoa in smears of stored neat semen in comparison to smears of unstored neat semen. Spermatozoa morphological abnormalities can be classified according to their origin. Primary abnormalities occur during spermatogenesis and secondary abnormalities occur after the sperm has left the testes, ie. those defects that arise during or after semen collection. As soon as semen is collected from the male, a proportion of the spermatozoa begin to naturally lose their integrity and the process continues during storage *in vitro* (Lake, 1983). Coiling, a secondary abnormality, is an indication that sperm are in the late stages of disintegration (Lake and Stewart, 1978a). It is unlikely that such spermatozoa would have a chance to ascend the oviduct to effect fertilization. Saeki (1960) found that an increase in the proportion of abnormal spermatozoa during the storage of undiluted semen resulted in low fertility. In his study, the incidence of secondary abnormalities reached a rate of 49% after nine hours of storage.

Neat semen could not be stored (even for 24 hours) without significant loss in motility. This is similar to the findings reported by Ax and Lodge (1975). The decline in motility is likely associated with sperms limited metabolic pathways and the

production of metabolic-by-products that may accumulate and reach toxic levels (Wishart, 1989). Dilution of chicken semen with both BPSE and LAKE prolonged sperm motility during storage. The addition of extenders stabilizes pH and osmolality, protect against toxic ions and in other ways extends the life of the semen samples (Bottwalla and Miles, 1992).

One noticeable feature in a comparison between neat semen before and after 24 hours storage, is the decline in egg fertility (from 92% to 3%). Similar observations have been documented by other researchers (Schindler et al., 1955; Sexton, 1977b). Stored neat semen's decline in fertility reflects its poor keeping quality and the need for extenders.

In the present study, the fertilizing capacity of unstored semen was not different when inseminated after dilution in BPSE or LAKE (83% and 90%, respectively). As well, the fertilizing capacity of stored semen was not different when inseminated after dilution and storage for 24 hours in BPSE or LAKE (30% and 32%, respectively). These results are in general agreement with those reported by others for semen diluted in BPSE and LAKE (Sexton, 1977b; Lake and Ravie, 1979). Sexton (1977b) stored poultry semen for 24 hours in BPSE and found the fertilizing capacity of semen was significantly lower than that of unstored controls. Sexton (1974) presented evidence that fructose, present in BPSE (Appendix 2), is used much more efficiently by spermatozoa as an energy source than glucose *in vitro*. It was hypothesized in the present study, therefore, that differences may occur in sperm fertilizing

capacity due to differences in the energy source of each extender. The results did not support this hypothesis.

The highest stored semen fertility rate obtained in this study was 60% for semen diluted in LAKE. Lake and Ravie (1979) reported fertility rates of up to 87% for semen stored for 24 hours in LAKE. The high fertility obtained by Lake and Ravie may be a result of a higher insemination dose, 0.06 ml in contrast to 0.05 ml for the present study, or the selection of hens producing a succession of fertile eggs over a period of several weeks. It is highly probable in the present study that if the insemination dose had been increased or inseminations occurred more frequently, fertility would have be improved, perhaps to the level of unstored samples.

In this study, the sperm number was not taken into consideration at time of insemination. This potentially could have led to reduced fertility because the number of spermatozoa in a semen sample is likely to vary during the course of a breeding period. Variations in sperm numbers can arise from variations in the amount of transparent fluid present in the collected semen samples. During ejaculation, lymphatic folds within the cloaca of the fowl become swollen in response to the massage stimulus and from these folds a variable a mount of transparent fluid is obtained when the copulatory organ is squeezed to obtain semen (Lake, 1983). Significant week effects on fertility might reflect undetected procedural differences or "learning" rather than difference in sperm numbers between inseminations as there appeared to be no correlation between the fertility trends and the number of

sperm inseminated. As well, according to Lake and Stewart (1978a) the minimum number of sperm inseminated (9.6×10^7) should have been sufficient to ensure maximum fertility.

It is frustrating that while chicken semen diluted in extenders and cooled to 5°C to slow catabolic and degradation processes, may only retain their fertilizing capacity for 24 hours *in vitro*, spermatozoa can survive in the oviduct of the hen at body temperature for at least five to 11 days before they fertilize an egg (Lake, 1983). Clearly, future work in this area needs to try to mimic the environment to which the sperm are exposed in the sperm storage tubules. The sperm storage tubules (SST) are located near the junction of the uterus and vagina. After each egg is laid, sperm are released from the SST and are transported to the infundibulum where fertilization takes place. In commercial breeding, it is important to maintain a constant level of high fertility. To achieve this, a sufficient number of fully viable sperm must be inseminated (Bakst, 1989). The fertility of hens inseminated with stored semen in the present study, was much lower than that obtained with fresh semen which indicates that the number of spermatozoa remaining viable after *in vitro* storage is small. It has been suggested that the SST are sperm-selective (Zavaleta, 1987). Thus, the non-viable population of spermatozoa within the inseminate are unlikely to enter the sperm storage tubules, thereby decreasing the number of spermatozoa available for fertilization. Subsequent inseminations may augment the spermatozoa populating the SST. This is reflected in the increase in weekly fertility rate

for spermatozoa stored in LAKE. Also, the presence of dead sperm in the inseminate has been shown to have detrimental effects on fertility, possibly due to the release of intracellular proteolytic enzymes and toxic organic peroxidases (Sexton, 1988). With increased storage time, the number of spermatozoa that degenerate and become non-viable increases. The magnitude and speed of this degeneration depends on dilution of the semen. Therefore, a dose of unstored semen containing sufficient sperm may have to be increased after storage in an undiluted state.

In general, the results of this study indicate that sperm fertilizing capacity is reduced after storage *in vitro*, and extension of semen helps to maintain sperm viability. Both BPSE and LAKE worked equally well to maintain poultry sperm fertilizing capacity after short-term storage *in vitro*. As BPSE is available commercially, selection of the "best" extender can be made based on cost and availability of extender components and personal preference. More work is needed to determine if one of these extenders can be adapted for future use with wild Mallard semen.

5.0 EVALUATION OF VARIOUS CRYOPROTECTANTS FOR THE CRYOPRESERVATION OF AVIAN SEMEN

5.1 ABSTRACT

Several studies were conducted to determine the effect of: a) various concentrations of glycerol, ethylene glycol (EG), propylene glycol (PG) and sucrose on the fertilizing capacity of unstored chicken spermatozoa and b) the addition of sucrose to EG or PG on the viability of frozen-thawed semen. Levels of 4% glycerol, 16% EG or PG and 0.3, 0.4, and 0.5M sucrose significantly reduced fertility ($P<0.05$). No differences were observed between EG and PG. The treatment with the highest post-thaw motility score was glycerol. The presence of sucrose during freezing did not improve avian semen freeze-thaw recovery after cryopreservation.

5.2 INTRODUCTION

Cryopreservation of semen is a process by which a sample is suspended in a diluent containing a cryoprotectant and maintained frozen at -196°C in a viable state for long periods of time. The main obstacle to the success of developing a technique to freeze semen is identifying a cryoprotectant that will maintain good semen fertilizing ability. Simply freezing and thawing sperm in buffered salt solutions, results in cell death.

The lethality of freeze-thawing is associated with the formation of intracellular ice and increasing solute concentration, rather than their ability to endure storage at sub-zero

temperatures (Saacke, 1982). These damaging processes have two opportunities to occur in the cell, once during freezing and once during thawing. As freezing begins, the water component of the extender is frozen out of solution and transformed into ice. This leaves the sperm bathed in increasing concentrations of the soluble components of the extender. The increasing extracellular solute concentration causes water to be drawn out of the sperm. If freezing is slow, this water contributes to the exclusive formation of extracellular ice (Saacke, 1982). But if cooling is too rapid, the cell is not able to osmotically lose water fast enough to prevent the formation of damaging intracellular ice (Szell and Shelton, 1986; Mazur, 1984).

Cooling rates slow enough to prevent the formation of intracellular ice can also be damaging. The cause of this damage is still unclear. As cells lose water during the freeze process the become dehydrated and shrink. Maryman (1974) suggested that the cells may shrink below a minimum volume where structural integrity is jeopardized. In his theory cryoprotectants protect by decreasing the amount of the cell's water that needs to leave during freezing. In contrast, Mazur (1980) effects injury to changes in size of the unfrozen portion and suggests that freezing becomes damaging whenever the unfrozen percentage of the sample drops below 8 to 12%. As ice expands outside the cell, it puts constraints on the shapes that can be assumed by the cells, resulting in cell deformation and damage (Mazur, 1984).

Cryoprotectants are compounds that are added to extenders to

help reduce the detrimental effects of freezing and thawing. Cryoprotectants can be classified as permeating or non-permeating based on their ability to enter the cell. Permeating cryoprotectants such as glycerol, ethylene glycol, propylene glycol and other alcohols penetrate into the cell and lower the temperature at which water initiates ice crystal formation (Mazur, 1984). As a result, there is a decrease in the amount of ice formed and a decrease in the solute concentration to which the cells are exposed at the same sub-zero temperature (Saacke, 1982).

Non-permeating cryoprotectants such as sucrose, glucose, and other sugars are added to the cryoprotectant medium to regulate water movement across the cell membranes. Sucrose, which is impermeable to cells, has been found to have a stabilizing effect on biological membranes by minimizing dramatic changes in osmotic pressure (Takeda et al., 1987; Schneider and Mazur, 1984). This helps lower the amount of cryoprotectant that penetrates the cell, thus reducing possible toxic effects (Szell and Shelton, 1987) and helps prevent rapid swelling as the permeating cryoprotectant moves out of the cell and water flows in during thawing (Takeda et al., 1987).

Many researchers have investigated the use of cryoprotectants for fowl semen (see reviews by Graham et al. 1984; Lake, 1986; Hammerstedt et al., 1992; Bellagamba et al., 1993). Good fertility has been obtained under certain circumstances with semen that has been frozen and stored in the presence of glycerol (Lake and Stewart, 1978b). However, their success was probably augmented by

using only males that produced high quality semen at insemination and depositing the frozen-thawed semen into the hen by deep intra-vaginal inseminations near the sperm storage tubules. The consensus is that under current protocols rooster semen survives poorly with only 1.6% of spermatozoa retaining their fertilizing ability after freezing and thawing (Wishart, 1985). Unlike mammalian spermatozoa, which must fertilize a single ova within 24 hours of insemination, avian spermatozoa must survive in the oviductal sperm storage tubules to fertilize multiple ova over a period of several days.

One concern with glycerol, is its contraceptive effect in the female. Glycerol concentration must be reduced after thawing and before insemination (Lake and Ravie, 1984). Glycerol concentrations can be lowered from the cryoprotectant level (8%) to a more tolerable level (less than 2% v/v) by slow dilution or dialysis (to minimize osmotic-induced swelling). However, these techniques frequently cause damage to already weakened cells and are impractical under routine field conditions. The mechanism by which glycerol exerts the contraceptive action has not yet been identified (Bellagamba et al., 1993).

Alternative cryoprotectants that do not depress sperm fertilizing ability when inseminated at concentrations necessary to protect spermatozoa during freezing are needed. Ethylene glycol and propylene glycol have been examined as alternative cryoprotectants (Lake and Ravie, 1984; Maeda, 1984; Bellagamba, 1993) but neither has been found better than glycerol at protecting against freeze injuries. It may be possible to improve the

protective ability of propylene glycol (PG) or ethylene glycol (EG) by the addition of sucrose to the cryoprotectant. To determine the effectiveness of sucrose addition on freeze-thaw recovery, it is first necessary to know the effect these cryoprotective agents have on the fertilizing ability of fresh semen.

Objectives:

To determine the effect of:

1) Various concentrations of glycerol, propylene glycol, ethylene glycol, and sucrose on the fertilizing capacity of fresh semen.
2) Sucrose addition to the cryoprotectant medium on motility and fertilizing capacity of frozen-thawed semen.

5.3 MATERIALS AND METHODS

To test these concepts, Beltsville Poultry Semen Extender (BPSE)(Sexton, 1977) was selected as a base to which cryoprotectants were added. The following general protocol was used:

5.3.1 Semen Collection and Evaluation

Roosters and hens were maintained as described under Materials and Methods of Chapter four. Semen was collected from roosters by the abdominal massage method (Lake and Stewart, 1978a), inspected to eliminate samples contaminated with debris and pooled for use.

5.3.2 Cryoprotectant Concentration

Pooled semen was divided into equal quantities and placed into three ml glass tubes containing treatments outlined below. Semen was diluted at a rate of 1:1 with treatment solutions at ambient temperature.

(1) Glycerol concentration - Four concentrations of glycerol were used for fertility trials. The four concentrations of the glycerol were 0, 1, 2 or 4% by volume of BPSE (final concentration 0.5%, 1.0% or 2%, respectively).

(2) Ethylene glycol and propylene glycol concentration - The effect of ethylene glycol and propylene glycol on fertility of rooster semen were tested. There were five treatment groups of 0% EG and PG, 8% EG, 16% EG, 8% PG or 16% PG by volume of BPSE (final concentration 0% EG and PG, 4% EG, 8%EG, 4% PG and 8% PG, respectively).

(3) Sucrose concentration - The effect of sucrose concentration (0, 0.1, 0.2, 0.3, 0.4, 0.5m; final concentration 0.0, 0.5, 1.0, 1.5, 2.0, 2.5M sucrose, respectively) in BPSE on fertility of rooster semen were tested.

5.3.3 Sucrose Addition

Pooled semen was taken to a cold room (13°C) and allowed to equilibrate for ten minutes. Semen (500 ul) was placed in a test tube and mixed with BPSE containing either: (1) 0% EG and PG, (2) 8% PG, (3) 8% EG, (4) 8% PG + 0.2M sucrose, (5) 8% EG + 0.2M sucrose or (6) 8% glycerol. Volumes were adjusted to give an

insemination dose of 1.0 x 10^8 sperm/0.15 ml. The aliquot containing 0% EG and PG was inseminated immediately. The remaining aliquots of diluted semen were frozen and then inseminated as outlined below.

5.3.4 Freezing

The procedure for preparing, freezing and thawing the semen was similar to that of Lake and Stewart (1978b). Sperm were allowed to equilibrate for 10 minutes before being drawn up into 0.5 cc straws. Loaded straws were sealed with polyvinyl propylene powder (PVP) and placed in a programmable freezing chamber (Bio-cool II Model 1A000, FTS Systems Ltd., Stone Ridge, NY, USA) at 5°C. The temperature was allowed to drop to -40°C at 1°C per minute. Straws were held at -40°C for five minutes, removed from the freezing chamber and quickly plunged into liquid nitrogen. Each hen was inseminated once with 0.15 ml (1.0 x 10^8 sperm) of frozen-thawed semen. Sperm motility was scored before and after freezing using the procedures outlined in Chapter four.

5.3.5 Thawing and Cryoprotectant Removal

After storage for one week in liquid nitrogen, straws were thawed in a 13°C water bath. The contents of two straws were emptied and pooled. Straws requiring deglycerolization, were emptied into a test tube. The glycerol concentration was reduced by the addition of 0.16, 0.44, 0.80, 1.46, 3.0, and 3.8 ml of BPSE at three minute intervals. Samples were held at 13°C. Samples were

centrifuges at 1000 x g for 15 minutes. The supernatant was decanted and the spermatozoa resuspended to the original volume by gently mixing with BPSE.

5.3.6 Insemination

White Leghorn hens (approximately 26 weeks of age) were randomly assigned to single level laying cages. Hens for each treatment were inseminated once (at approximately 1400h) with 0.01 ml of diluted semen unless otherwise stated. Each treatment consisted of two replicates of six hens each. AI was carried out as described under Materials and Methods of Chapter four.

5.3.7 Egg Collection and Incubation

Egg collection and incubation was carried out as outlined in Materials and Methods of Chapter four.

5.3.8 Statistical Analyses

Fertility was computed for three periods: (1) initial; fertility as determined by the percentage of eggs laid during days 2-8 after insemination, (2) final; fertility as determined by the percentage of eggs laid during days 9-15 after insemination and, (3) duration of fertility as determined by the percentage of fertile eggs laid during days 2-15 of a given trail. The percentage of fertilized eggs laid by each hen was transformed to arc sine $\sqrt{\%}$ prior to analysis to normalize the distribution (Steele and Torrie, 1960).

Data for Days 2-8, 9-15 and 2-15 were subjected to analysis of variance testing main effects of cryoprotectant concentration and replicate (glycerol addition - 4 x 2, EG and PG addition - 5 x 2 and sucrose addition - 6 x 2) using the General Linear Models (GLM) procedure of SAS (Littell, 1991). Each daily pooled semen sample (n = 15) constituted a replicate for each treatment. After obtaining a significant F-value, means were separated by least squares mean at the 5% level of significance ($P<0.05$).

5.4 RESULTS

5.4.1 Glycerol Concentration

The fertility results obtained with semen freshly diluted in BPSE containing various concentrations of glycerol are presented in Table 20, Figure 7 and Appendix 11. Fertility was affected by glycerol concentration (0, 1, 2 or 4%) and replicate (1 or 2). Day 2 to 8 LSMeans were 95.3 ± 7.3, 90.3 ± 7.3, 76.8 ± 7.3, and 31.4 ± 7.3 for sperm treated with 0, 1, 2 or 4% glycerol, respectively. Fertility was significantly ($P<0.01$) reduced with a final glycerol concentration of 4%. There was no significant difference in initial fertility (day 2-8) among the 0, 1, and 2% glycerol treatment groups. The number of sperm per insemination dose ranged from 2.9 x 10^8 for replicate one and 2.0 x 10^8 for replicate two. There was no significant treatment by replicate interaction.

5.4.2 Ethylene Glycol and Propylene Glycol Concentration

The fertility results of hens inseminated with semen diluted in various concentrations of propylene glycol or ethylene glycol are presented in Table 21, Figure 8 and Appendix 12. A concentration of 8% PG or EG had little influence on initial egg fertility when compared to the control. PG and EG at any concentration tested, significantly decreased duration of fertility from a high of 92.6 ± 8.8 percent (0% EG and PG) to a low of 39.3 ± 8.2 percent (16% PG).

5.4.3 Sucrose Concentration

The LSMeans, profiles and analysis of variance for egg fertility after inseminations with various concentrations of sucrose are presented in Table 22, Figure 9 and Appendix 13. Fertility was affected by sucrose concentration (0.0, 0.1, 0.2, 0.3, 0.4, or 0.5 M) and replicate (1 or 2). Dilution with BPSE containing greater than 0.2M sucrose resulted in significantly ($P<0.01$) lower egg fertility than for lower sucrose concentrations. Only 4.1% of eggs laid between day 2-8 were fertile when 0.4M sucrose was used. The number of sperm per insemination dose was approximately 1.0×10^8 for replicate one and two. There was no significant treatment by replicate interaction.

5.4.4 Sucrose Addition

Motility data are summarized in Table 23. Sperm in freshly collected semen samples had a motility score of 4.5 (based on an

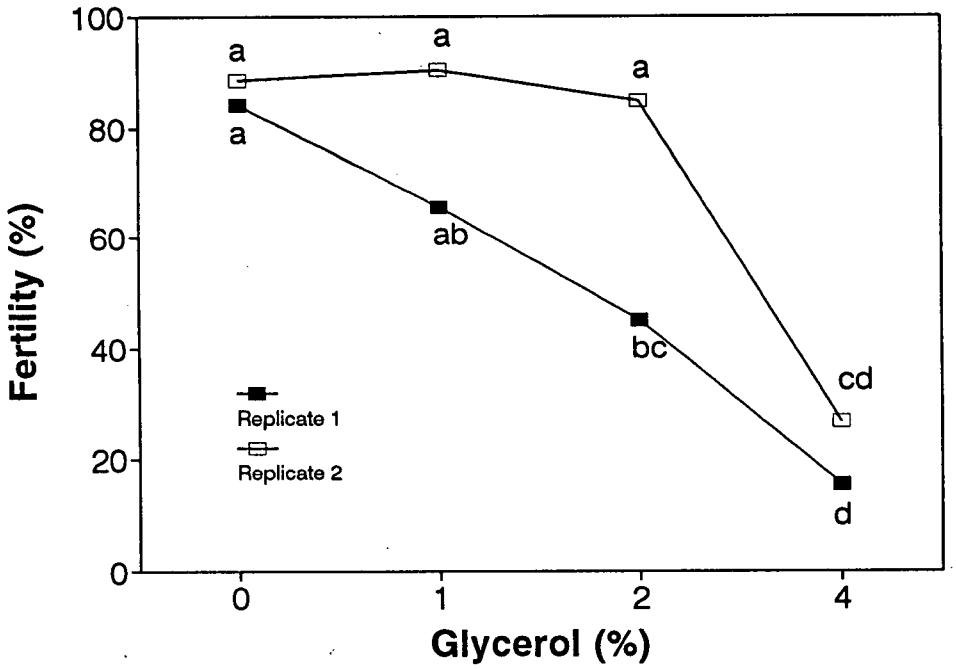
arbitrary scale of 0-5). Dilution in BPSE containing EG or PG did not affect motility. It was evident that the prefreeze motility of semen was decreased by the addition of sucrose to cryoprotectant solutions. Freezing always reduced spermatozoa motility when compared with motility of neat or pre-freeze semen. Post-thaw, the motility score of semen stored with various cryoprotectants ranged between 0.5 and 2. The treatment with the highest post-thaw motility was glycerol. No fertile eggs were obtained post-insemination with frozen-thawed semen stored with any cryoprotectant combinations tested (not presented).

TABLE 20. Effect of different concentrations of glycerol in BPSE on the fertilizing capacity of chicken spermatozoa

Variable	Fertility (%)		
	Days 2-8	Days 9-15	Days 2-15
Glycerol			
0%	95.3 ± 7.3[a]	75.2 ± 7.3[a]	86.4 ± 6.3[a]
1%	90.3 ± 7.3[a]	66.7 ± 6.9[ab]	78.0 ± 6.3[ab]
2%	76.8 ± 7.3[a]	54.2 ± 6.9[b]	64.9 ± 6.3[b]
4%	31.4 ± 7.3[b]	10.5 ± 6.9[c]	21.1 ± 6.3[c]
Replicate			
1	65.1 ± 5.2[a]	39.0 ± 5.0[a]	52.6 ± 4.4[a]
2	81.8 ± 5.2[b]	64.3 ± 4.9[b]	72.6 ± 4.4[b]

[a-c] LSMeans in columns and variables with no common superscripts differ significantly ($P < 0.05$).
Data for glycerol group are LSMeans for 12 hens and replicate for 48 hens.

FIGURE 7. Effect of different concentrations of glycerol on the fertilizing capacity of unstored chicken spermatozoa (days 2-15 post-AI)



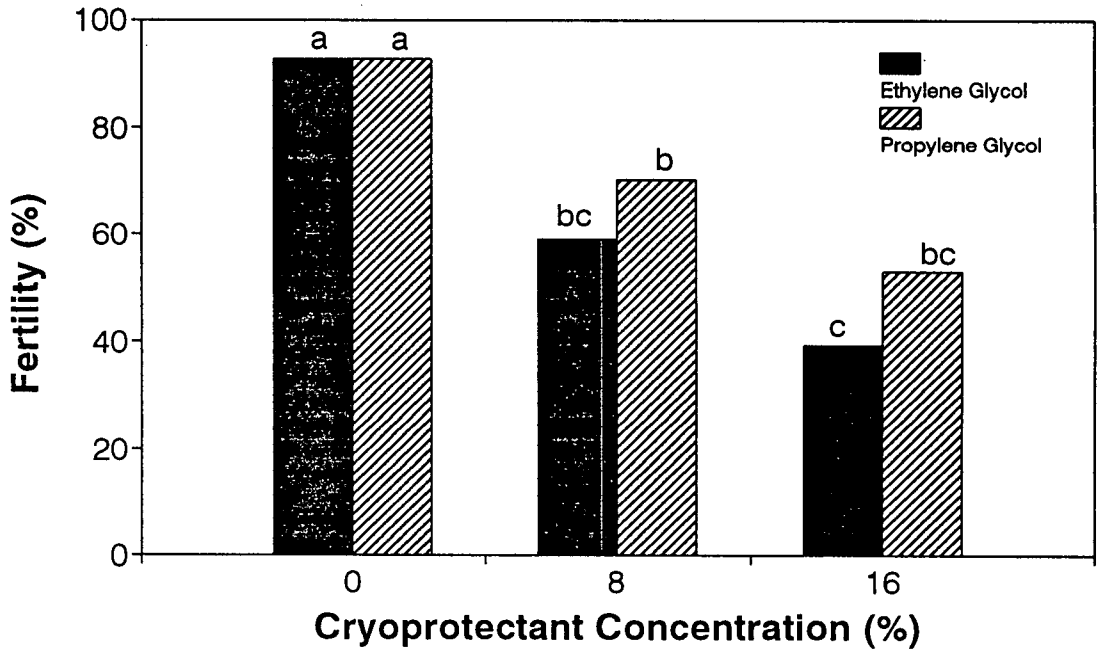
Points with different letters are different (P<0.01)

TABLE 21. Effect of different concentrations of propylene glycol (PG) and ethylene glycol (EG) in BPSE on the fertilizing capacity of chicken spermatozoa

Variable	Fertility (%)		
	Days 2-8	Days 9-15	Days 2-15
Cryoprotectant			
0% EG & PG	97.4 ± 10.0[a]	79.0 ± 7.0[a]	92.6 ± 8.8[a]
8% EG	72.6 ± 10.0[ab]	41.3 ± 7.0[bc]	58.9 ± 8.2[bc]
8% PG	84.7 ± 10.0[a]	55.5 ± 7.0[b]	70.1 ± 8.2[b]
16% EG	49.9 ± 10.0[b]	14.9 ± 7.0[d]	39.3 ± 8.2[c]
16% PG	67.7 ± 10.0[ab]	29.3 ± 7.0[c]	52.9 ± 8.2[bc]
Replicate			
1	77.0 ± 6.3	46.9 ± 4.4	67.0 ± 5.6
2	71.9 ± 6.3	41.1 ± 4.4	58.5 ± 5.0

[a-d]Non-transformed LSMeans for columns and variables with no common superscripts differ significantly ($P < 0.05$).
Data for glycerol group are LSMeans for 12 hens and replicate for 60 hens.

FIGURE 8. Effect of different concentrations of EG and PG on the fertilizing capacity of unstored chicken spermatozoa (days 2-15 post-AI)



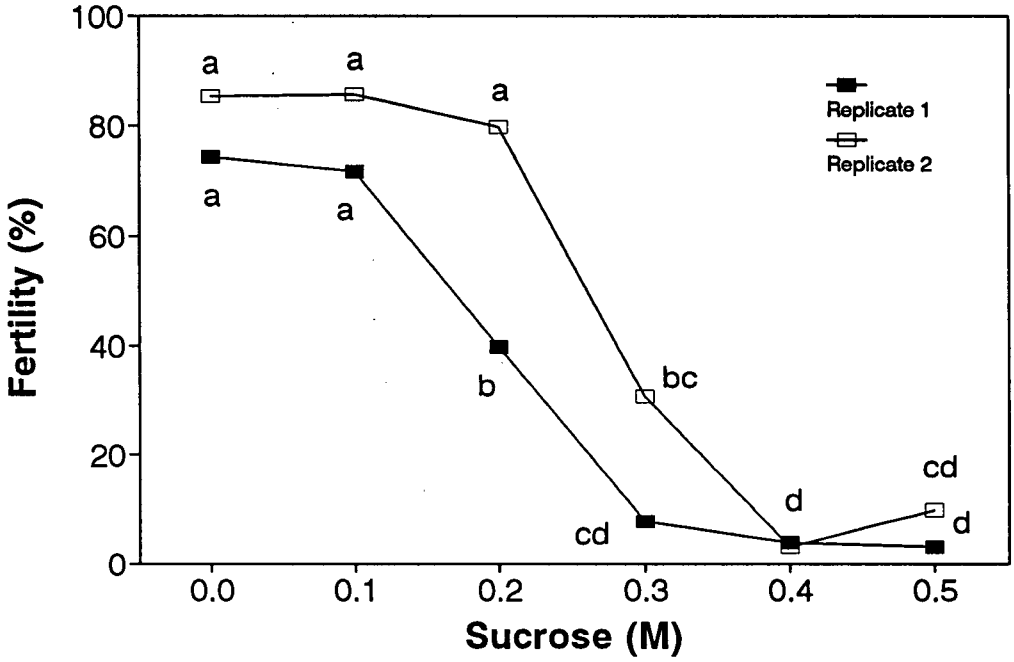
Bars with different letters are different ($P<0.01$)

TABLE 22. Effect of different concentrations of sucrose in BPSE on the fertilizing capacity of chicken spermatozoa

Variable	Fertility (%)		
	Days 2-8	Days 9-15	Days 2-15
Sucrose			
0.0M	91.7 ± 7.1[a]	68.0 ± 6.3[a]	79.8 ± 5.9[a]
0.1M	94.1 ± 7.1[a]	64.7 ± 6.3[a]	78.7 ± 5.9[a]
0.2M	70.8 ± 7.1[a]	49.8 ± 6.3[a]	59.7 ± 5.9[a]
0.3M	31.9 ± 7.1[c]	7.3 ± 6.3[b]	19.1 ± 5.9[b]
0.4M	4.1 ± 7.1[d]	2.8 ± 6.3[b]	3.5 ± 5.9[b]
0.5M	20.3 ± 7.1[cd]	2.4 ± 6.3[b]	6.4 ± 5.9[b]
Replicate			
1	40.3 ± 4.1[a]	26.7 ± 3.6[a]	33.3 ± 3.4[a]
2	64.0 ± 4.1[b]	38.3 ± 3.6[b]	49.0 ± 3.4[b]

[a-d]Non-transformed LSMeans for within columns and variables with no common superscripts differ significantly ($P < 0.05$).
Data for glycerol group are LSMeans for 12 hens and replicate for 48 hens.

FIGURE 9. Effect of different concentrations of sucrose on the fertilizing capacity of unstored chicken spermatozoa (days 2-15 post-AI)



Points with different letter are different (P<0.01)

TABLE 23. Effect of different combinations of cryoprotectants in BPSE and various stages of the freezing process on the motility score of chicken semen

Variable	Motility Score		
	Neat	Pre-freeze	Post-thaw
8% Propylene glycol(PG)	4.5	4.5	0.5
8% Ethylene glycol (EG)	4.5	4.5	1.5
8% PG + 0.2M Sucrose	4.5	1.0	0.5
8% EG + 0.2M Sucrose	4.5	1.0	1.0
8% Glycerol	4.5	3.0	2.0

[a-d]Non-transformed LSMeans for within columns and variables with no common superscripts differ significantly ($P < 0.05$).
Data for glycerol group are LSMeans for 12 hens and replicate for 48 hens.

5.5 DISCUSSION

From the results presented, it is apparent that glycerol concentrations greater that 2% by volume of semen extender were detrimental to the fertilizing capacity of unstored rooster semen. These results are in agreement with those reported by Polge (1951), Neville et al.(1971) and Sexton, (1973). Neville et al. (1971) determined that egg hatchability was not reduced by glycerol concentration, which suggested that glycerol does not exert harmful effects of the developing embryo. In contrast to the methods of Neville et al. (1971) fertile eggs in this study were terminated after seven days of incubation, rather than taken to hatch. However, there was no evidence of early embryonic development being affected by glycerol concentration. The number of early dead embryos was less than 5% of the fertile eggs observed per treatment group.

The present study confirms the need to find an alternative cryoprotectant if cryopreservation of frozen avian semen is going to be practiced routinely in the field. Despite the acceptable fertility results obtained after insemination with fresh semen diluted in BPSE containing less than 2% glycerol, glycerol must be present at a concentration of 8% to successfully protect spermatozoa during the freeze-thaw process (Lake and Ravie, 1984). Post-thaw reduction of glycerol concentration using currently available methods would be impractical under field conditions.

Increasing EG and PG concentrations were found not to reduce fertility in comparison to glycerol. This is in agreement with

results reported by Lake and Ravie (1984), but in contrast to results reported by Sexton (1973). Sexton (1973) found that the fertilizing capacity of washed spermatozoa resuspended in a potassium phosphate buffer (KPB) was reduced by 4 and 8% EG when compared to the control. In the present study, the lack of reduced semen fertilizing capacity in 8% EG might be explained by the use of BPSE as an extender, rather than KPB.

Motility of spermatozoa measured soon after thawing was reduced compared with the fresh sample. This finding is supported by other authors who have demonstrated declines in motility of frozen-thawed semen by 30 to 60% compared to fresh (Westfall and Harris, 1975; Bakst and Sexton, 1979; Scott et al., 1980). Although EG and PG are known to act as cryoprotectants during the freezing of embryos and oocytes, it was shown that in comparison to glycerol they have reduced freeze-thaw protection as determined by thawed spermatozoa motility. Lake and Ravie (1984), and Westfall and Harris (1975) made similar observations. Motility after freezing of spermatozoa in BPSE containing glycerol, however, was not a good indication of fertilizing ability. Although motile, the revived population did not appear to be fully functional since the insemination of motile thawed spermatozoa did not result in any fertile eggs.

Fertility was found to decline with increasing concentrations of sucrose. This decline in fertility agrees with the findings of Sexton (1975), who reported a significant loss in the motility and fertilizing capacity of spermatozoa diluted in 4, 8, and 12%

sucrose. Although higher sucrose concentrations were found to affect fertility, the author choose to use the maximum non-toxic concentration (0.2M) for freezing spermatozoa on the assumption that it may be more efficient at cryoprotectant removal than lower concentrations. There was no profound beneficial effects in spermatozoa freeze-thaw protection observed with the addition carbohydrate in the form of sucrose (0.2M) to the different cryoprotectants. The presence of carbohydrate during freezing does not appear to be a key element to improving avian semen cryopreservation.

Under the conditions employed, glycerol still provides the best freeze protection for avian spermatozoa. While research on avian semen preservation has focused primarily on seeking alternative cryoprotectants to glycerol (Maeda et al, 1984; Lake and Ravie, 1978) or on techniques of glycerol removal before fertilization (Buss, 1993; Lake and Stewart, 1978b), it may be beneficial to direct future work towards identifying the cause of glycerols contraceptive action in the female. Once the action of glycerol on the spermatozoa is identified, corrective measures can be adopted, enabling the use of cryopreservation of avian semen to be routinely applied by game-farm breeders and the poultry industry.

CONCLUSIONS

Propagation of Mallards in captivity was found to be hindered by low egg fertility, especially in yearling pairs. Comparisons of testicular weight and size between captive and free-flying Mallards suggest that gonadal development is suppressed in Mallard drakes under captive conditions. This suppression of gonadal development may partially account for the poor fertility in captive ducks.

With regard to propagating wild avian species in captivity by artificial means, successful preliminary work has been done. This thesis has demonstrated the need for the use of AI to supplement natural mating in yearling Mallard pairs and that AI can be used successfully. To facilitate easier handling of birds held under an AI program, it was found that hens could be housed separately from drakes without any adverse effects on egg production.

A logical extension of an AI program would be to preserve semen to allow the exchange of genetic material between distant populations and the establishment of gene banks. In general, the results of this thesis indicate that sperm fertilizing capacity is reduced after storage *in vitro*, and dilution of semen with extenders helps to maintain sperm viability. Beltsville Poultry Semen Extender (BPSE) and Lake's Poultry Semen Extender were compared and were found to work equally well to maintain poultry sperm fertilizing capacity after short-term storage. As BPSE is available commercially, selection of the "best" extender can be made based on cost and availability of extender components and

personal preference. More work is needed to determine if one of these extenders can be adapted for future use with wild-strain Mallard semen.

The presence of carbohydrate during freezing was found not to be the key element for improving long-term avian semen preservation. In this thesis, glycerol was found to still provide the best protection during cryopreservation of spermatozoa. While research on avian semen preservation has focused primarily on seeking alternative cryoprotectants to glycerol or techniques of glycerol removal before fertilization, it may be beneficial to direct future work towards identifying the cause of glycerols contraceptive action in the female rather than making observations of the spermatozoa reactions to cryoprotective agents. Once the action of glycerol on the spermatozoa is identified, corrective measures can be adopted, enabling the use of cryopreservation of avian semen to be routinely applied by game-farm breeders and the poultry industry.

BIBLIOGRAPHY

Allen, C. and L. Champion (1955) Competitive fertilization in the fowl. Poultry Science 34:1332-1342

Austin, A.J.S. and N. Natarajan (1991) Effect of buffers and dilution rates on motility percent of chicken spermatozoa Indian J. Poultry Science 26:34-38

Ax, R.L. and J.R. Lodge (1975) Rooster spermatozoon motility, forward progression, and fertility at 25, 5, or -196°C in various extenders. Cryobiology 12:93-97

Bakst, M.R. (1990) Preservation of avian cells. In "Poultry Breeding and Genetics. Developments in Animal and Veterinary Sciences" (R.D. Crawford ed.), Elsevier, New York 22:91-108

Bakst, M.R. (1989) Oviductal storage of spermatozoa in the turkey:its relevance to artificial insemination technology. British Poultry Science 30:423-429

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

Batt, B.D.J. and H.H. Prince (1978) Some reproductive parameters of Mallards in relation to age, captivity and geographic origin. J. Wildlife Management 42:834-842

Ballou, J.D. (1992) Potential contribution of cryopreserved germ plasm to the preservation of genetic diversity and conservation of endangered species in captivity. Cryobiology 29:19-25

Bellagamba, F. ,S. Carillon and L.G. Cavalchini (1993) Cryopreservation of poultry semen: a review. World's Poultry Science Journal 49:157-166

Bellrose, F.C, K.L Johnson and T.U. Meyers (1964) Relative value of natural cavities and nesting houses for Wood ducks. J. Wildlife Management 28:661-676

Berry, R.B. (1972) Reproduction by artificial insemination in captive American Goshawks. J. of Wildlife Management 36:1283-1288

Blohm, R.J. (1982) Differential occurrence of yearling and adult male Gadwalls in pair bonds. Auk 99:378-379

Blohm, R.J. (1979) The breeding of the Gadwall in southern Manitoba. Unpublished Ph.D. Thesis. University of Wisconsin-Madison

Bluhm, C.K., A. Calogeros, C.E. Stunden, C. Lo (1993) Gaylord Avicultural Laboratory Manual. Unpublished. Delta Waterfowl Research Station, Delta, MB

Bluhm, C.K. (1992) Environmental and endocrine control of waterfowl reproduction. In "Ecology and Management of Breeding Waterfowl" (Bruce Batt et al. eds) University of Minnesota Press, Minneapolis pp.323-364

Bluhm, C.K. (1985) Mate preferences and mating patterns of Canvasbacks (*Aythya valisineria*). In "Avian Monogamy" (P.A. Gowaty and D.W. Mock, eds) Ornithological Monographs No. 37, American Ornithologists' Union, Washington, D.C. pp.45-56

Bluhm, C.K. R.E. Phillips and W.H. Burke (1983) Serum levels of luteinizing hormone, prolactin, estradiol, and progesterone in laying and nonlaying Mallards (*Anas platyrhynhos*). Biology of Reproduction 28:295-305

Bruggers, R. and W.B. Jackson (1981) Age-related differences in reproductive behaviour of male Mandarin ducks. Bird Study 28:107-114

Bootwalla, S.M. and R.D. Miles (1992) Development of diluents for domestic fowl semen. World's Poultry Science 48:121-128

Burrows, W.H. and J.P. Quinn (1937) The collection of spermatozoa from the domestic fowl and turkey. Poultry Science 16:19-24

Buss, E.G. (1993) Cryopreservation of rooster semen. Poultry Science 72:994-954

Caldwell, P.J. and G.W. Cornwell (1975) Incubation behaviour and temperatures of the Mallard duck. The Auk 92:706-731

Caldwell, P.J. and A.E. Snart (1974) A photographic index for aging Mallard embryos. J. Wildlife Management 38:298-310

Calogeros, A. (1996) Genetics of captive-reared Mallards: Consequences to breeding success. Unpublished M.Sc. thesis, Department of Animal Science, University of British Columbia

Chaudhuri, D. and P.E. Lake (1988) A new diluent and methods of holding fowl semen for up to 17 hr at high temperature. Proceedings of 18th World's Poultry Congress held in Nagoya, Japan. pp.591-593.

Cheng, K.M, R.N. Shoffner, R.E. Phillips and F.B. Lee (1980) Reproductive performance in wild and game-farm Mallards. Poultry Science 59:1970-1976

Cheng, K.M, R.N. Shoffner, R.E. Phillips and F.B. Lee (1978) Mate preference in wild and domesticated (game-farm) Mallards (*Anas platyrhynchos*) I. Initial preference. Animal Behaviour 26:996-1003

Coulter, M.W. and W.R. Miller (1968) Nesting biology of Black Ducks and Mallards in northern New England. Vermont Fish and Game Dept. Bulletin No.68-2, 74pp. cited by Blohm (1979)

Curio, E. (1982) Why do young birds reproduce less well? Ibis 125:400-404

Desforges, M.F. (1971) Observations on the influence of social displays on ovarian development in captive Mallards (*Anas platyrhynchos*). Ibis 114:256-257

Donham, R.S. (1979) Annual cycle of plasma luteinizing hormone and sex hormones in male and female Mallards (*Anas platyrhynchos*). Biology of Reproduction 21:1273-1285

Dzubin, (1969) Assessing breeding populations of ducks by ground counts. In "Saskatoon Wetlands Seminar" Canadian Wildlife Service Report Ser.6 pp.138-160

Elder, W.H. and M.W. Weller (1954) Duration of fertility in the domestic Mallard hen after isolation from the drake. J. Wildlife Management 18:495-502

Fant, R. (1957) Criteria for aging pheasant embryos. J. Wildlife Management 21:324-328

Fijihara, N. and Nishiyama H. (1976) Studies on the accessory reproductive organs in the drake. 5. Effects of the fluid from the ejaculatory groove region on the spermatozoa of the drake. Poultry Science 55:2415-2420

Hafez, E.S.E. (1987) Reproduction in farm animals. Fifth edition. Lea and Febiger, Philadelphia, PA. 649pp.

Hamann, J. and F. Cooke (1987) Age effects on clutch size and laying dates of individual female Lesser Snow Geese *Anser caerulescens*. Ibis 129:527-532

Hammerstedt, R.H. and J.K. Graham (1992) Cryopreservation of poultry semen:the enigma of glycerol. Cryobiology 29:26-38

Haase, E. (1983) The annual reproductive cycle in Mallards. J. Steroid Biochemistry 19:731-737

Haase, E., P.J. Sharp and E. Paulke (1985) Seasonal changes in the concentrations of plasma gonadotropins and prolactin in wild Mallard drakes. Journal of Experimental Zoology 234:301-305

Garren, H.W. and C.S. Shaffner (1952) The effect of temperature and time of storage on the fertilizing capacity of undiluted fowl semen. Poultry Science 31:137-145

Gatti, R.C. (1983) Spring and summer age separation techniques for the Mallard. J. Wildlife Management 47:1054-1062

Gee, G.F. (1983) Avian artificial insemination and semen preservation. Proceedings of the Jean Delacour/IFCB Symposium on Breeding Birds in Captivity. pp 375-398

Gee, G.F. and S.A. Temple (1978) Artificial insemination for breeding non-domesticated birds. Symposia of the Zoological Society of London. Artificial Breeding of Non-domesticated Animals. The proceedings of a Symposium held at the Zoological Society of London on September 7-8 (P.F. Watson ed.) 43:51-72

Gottschalk, J.S. and A.T. Studholm (1970) Waterfowl management in the seventies. Transcript North American Wildlife and National Resources Conference 35:297-304.

Graham, E.F., M.L. Schmehl and R.C.M. Deyo (1984) Cryopreservation and fertility of fish, poultry and mammalian spermatozoa. Proceedings of the Tenth Technical Conference on Artificial Insemination and Reproduction. Sponsored by the National Association of Animal Breeders and Naab Technical Committee April 12-14. Milwaukee, Wisconsin pp.4-24

Greenwood, R.J., A.B. Sargeant, D.H. Johnson, L.M. Cowardin, and T.L. Shaffer (1995) Factors associated with duck nest success in the prairie pothole region of Canada. Wildlife Monographs 128:1-57

Gunarajasingam, D., R. Rajamahendran, B.R. Downey and P.C. Laguë (1985) Testosterone secretion in young and adult buffalo bulls. Theriogenology 24:185-195

Gvaryahu, G., A. Robinzon, A. Meltzer, M. Perek and N. Snapir (1984) An improved method for obtaining semen from Muscovy drakes and some of its quantitative and qualitative characteristics. Poultry Science 63:548-553

Jones, M.C. and A.T. Leighton (1987) Research Note: Effect of presence of the opposite sex on egg production and semen quality of breeder turkeys Poultry Science 66:2056-2059

Johnson, A.L. (1986) Reproduction in the male. In "Avian Physiology 4th edition (Sturkie ed.), Springer-Verlag, New York 444pp.

Johnson, D.H. and T.L. Shaffer (1987) Are Mallards declining in North America? Wildlife Society Bulletin 15:340-345

Johnson, O.W. (1961) Reproductive cycle of the Mallard. Condor 63:351-364

Kamar, G.A.R. (1962) Semen characteristics of various breeds of drakes in the subtropics. J. Reproduction and Fertility 3:405-409

Kear, J. (1978) Captive propagation of waterfowl. In "Endangered birds". Proceedings of the Symposium on Management Techniques for Preserving Endangered Birds held at the University of Wisconsin, Madison pp.243-249

Kim, H.K., S. Kim and K. Kim (1980) A diluent for deep freezing preservation of drake spermatozoa. Proceedings from the 9th International Congress on Animal Reproduction and A.I. held June 16-20 in Madrid, Spain 3:418

Krapu, G.L. and H.A. Doty (1979) Age-related aspects of Mallard reproduction. Wildfowl Trust Reports 30:35-39

Lake, P.E. (1986) The history and future of cryopreservation of avian germ plasm. Poultry Science 65:1-15

Lake, P.E. (1983) Artificial Insemination. World's Poultry Science 39:106-117

Lake, P.E. and W.M. McIndoe (1959) The glutamic acid and creatinine content of cock seminal plasma. Biochemistry J. 71:303-306

Lake, P.E. and O. Ravie (1979) Effect on fertility of storing fowl semen for 24h at 5°C in fluids of different pH. J. Reproduction and Fertility 57:149-155

Lake, P.E. and O. Ravie (1984) An exploration of cryoprotective compounds for fowl spermatozoa. British Poultry Science 25:145-150

Lake, P.E. and J.M. Stewart (1978) Artificial insemination in poultry, Bulletin 213. Ministry of Agriculture, Fisheries and Food Her Majesty's Stationary Office, London. 34pp.

Lake, P.E. and J.M. Stewart (1978) Preservation of fowl semen in liquid nitrogen - an improved method. Poultry Science 19:187-194

Lehrman, D.S. (1959) Hormonal responses to external stimuli in birds. Ibis 101:478-496

Littell, R.C., R.J. Freund and P.C. Spector (1991) SAS Systems for Linear Models, Third edition. SAS series in Statistical Applications. Cary, NC:SAS Institute Inc. 329pp.

Lo, C. (1994) The effect of egg removal on clutch size in Mallard hens *Anas platyrhynchos*. Unpublished ANSC 499 thesis, Department of Animal Science, University of British Columbia

Maeda, T., T. Terada and Y. Ysutsumi (1984) Comparative study of the effects of freezing of various cryoprotectants in preserving the morphology of frozen and thawed fowl spermatozoa. British Poultry Science 25:547-553

Mazur, P. (1984) Freezing of living cells: mechanisms and implications. American J. Physiology 247:c125-c142

Mazur, P. (1980) Fundamental aspects of the freezing of cells, with emphasis on mammalian ova and embryos. 9th International Congress on Animal Reproduction and A.I. held June 16-20 in Madrid, Spain 1:99-114

Maryman, H. (1974) Freezing injury and its prevention in living cells. Annual Review of Biophysics 3:341-363

McKinney, F. (1985) Primary and secondary male reproductive strategies of dabbling ducks. In "Avian Monogamy" (P.A. Gowaty and D.W. Mock, eds) Ornithological Monographs No. 37, American Ornithologists' Union, Washington, D.C. pp.57-67

NAWF (North American Wildlife Foundation)(1991) Waterfowl recovery action plan bulletin #408-102 Wilmot Road Deerfield, Illinois

Neville, W.J., J,W. MacPherson and B. Reinhart (1971) The contraceptive action of glycerol in chickens. Poultry Science 50:1411-1415

Ogasawara, F.X. and R.A. Ernst (1975) Recent developments in the artificial insemination of turkeys. Feedstuffs 47:23-35.

Paulke, E. and E. Haase (1978) A comparison of seasonal changes in the concentrations of androgens in the peripheral blood of wild and domestic ducks. General and Comparative Endocrinology 34:381-390

Phillips, J.J. (1993) Cryopreservation of rooster spermatozoa using methyl cellulose. Biology of Reproduction, Society for the Study of Reproduction 26th Annual Meeting held Aug 1-4 at Fort Collins, Colorado 48:427

Phillips, R.E. (1964). "Wildness" in the Mallard duck; effect of brain lesions and stimulation on "social behaviour" and reproduction. J. Comparative Neurology 122:139-155.

Phillips, R.E. and F. McKinney (1962) The role of testosterone in the displays of some ducks. Animal Behaviour 10:244-246

Phillips, R.E. and A. van Tienhoven (1960) Endocrine factors involved in the failure of Pintail ducks *Anas acuta* to reproduce in captivity. J. Endocrinology 21:253-261

Polge, C. (1951) Functional survival of fowl spermatozoa after freezing at -79°C. Nature 167:949-950

Renden, J.A. and M.L. Peirson (1982) Production of hatching eggs by dwarf broiler breeders maintained in cages or floor pens. Poultry Science 61:991-993

Renganathan, P. (1982) Avian semen extenders. Proceedings of First National Workshop on Artificial Insemination in Poultry held September 21-23, Izatnagar, India pp.62-79

Rohwer, F.C. and H.W. Heusmann (1991) Effect of brood size and age on survival of female Wood ducks. Condor 93:817-824

Romanoff, A.L. and A.J. Romanoff (1949) The Avian Egg. John Wiley and Sons, Inc. New York

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

Saake, R.G. (1982) What happens when a sperm is frozen and thawed? Proceeding of the Ninth Technical Conference on Artificial Insemination and Reproduction held April 30 - May 1 in Milwaukee, Wisconsin. National Association of Animal Breeders and the NAAB. pp.6-12

Saeki, Y. (1960) Crooked-neck spermatozoa in relation to low fertility in the artificial insemination of fowl. Poultry Science 39:1354-1361

Saito, N., K. Imai and M. Tomizawa (1994) Effect of sugars-addition on the survival of vitrified bovine blastocysts produced *in vitro*. Theriogenology 41:1053-1060

Schneider, U. and P. Mazur (1984) Osmotic consequences of cryoprotectant permeability and its relation to the survival of frozen-thawed embryos. Theriogenology 21:68-79

Schindler, H., S. Weinstein, E. Moses and L. Gabriel (1955) The effect of various diluents and storage times on the fertilizing capacity of cock semen. Poultry Science 34:1113-1117

Scott, T., R.B. Buckland and B.W. Kennedy (1980) The effect of selection for fertility of frozen-thawed semen on spermatozoon oxygen uptake, motility and concentration, and ejaculate volume in the chicken. Theriogeniology 14:281-298

Sexton, T.J. (1988) Research note: Influence of damaged spermatozoa on the fertility of turkey semen stored 24 hours at 5 C. Poultry Science 67:1483-1485

Sexton, T.J. (1977a) A new poultry semen extender 1. Effect of extension on the fertility of chicken semen. Poultry Science 56:1443-1446

Sexton, T.J. (1977b) A new poultry semen extender. 3. Effect of storage conditions on the fertilizing capacity of chicken semen stored at 5°C. Poultry Science 57:285-289

Sexton, T.J. (1975) Comparison of various cryoprotective agents on washed chicken spermatozoa. 5. Effect of glucose, sucrose and polyvinylpyrrolidone. Poultry Science 54:1297-1299

Sexton, T.J. (1974) Observation on the washing of turkey spermatozoa. Proceeding of the 15th World's Poultry Congress held in New Orleans pp.391-392

Sexton, T.J. (1973) Effect of various cryoprotective agents on the viability and reproductive efficiency of chicken spermatozoa. Poultry Science 52:1353-1357

Sexton, T.J, and T.A. Fewlass (1978) A new poultry semen extender. 2. Effect of the diluent components on the fertilizing capacity of chicken semen stored at 5°C. Poultry Science 57:277-284

Skinner, J.L. (1974) Infertility and artificial insemination. In Raising Wild Ducks in Captivity (D.O. Hyde ed.) E.P Dutton and Co., Inc. NJ pp147-152

Steele, R.G.D. and J.H. Torrie (1960) Principles and procedures of statistics. McGraw-Hill, New York, NY

Szell, A. and J.N. Shelton (1987) Osmotic and cryoprotective effects of glycerol-sucrose solutions on day-3 mouse embryos. J. Reproduction and Fertility 80:309-316

Szell, A. and J.N. Shelton (1986) Role of equilibration before rapid freezing of mouse embryos. J. Reproduction and Fertility 78:699-703

Tajima, A., E.F. Graham and D.M. Hawkins (1989) Estimation of the relative fertilizing ability of frozen chicken spermatozoa using a heterospermatic competition method. J. Reproduction and Fertility 85:1-5

Takeda T., R.P. Elsden and G.E. Seidel (1987) Use of sucrose during removal of cryoprotectants after thawing eight-cell mouse embryos. Theriogenology 28:101-108

Tan, N.S.(1980) The frequency of collection and semen production in Muscovy ducks. British Poultry Science 21:265-272

Tai, J.J., C. Tai, Y.M. Su, H.H. Haung, T. Terada and M. Wantanabe (1983) Studies on the cryopreservation of drake semen. Proceedings Fifth World Conference on Animal Production 2:183-184

Tarapovski, V.P. (1977) A comparison of the effectiveness of natural mating and artificial insemination in fowls in individual cages. Poultry Abstracts 3:630 (Abstr.)

Ward, P. and B.D.J. Batt (1973) Propagation of captive waterfowl. Wildlife Management Institute Washington, D.C. 64pp.

Watanabe, M. (1961) Effect of diluents and storage on fertility of duck semen. Japanese J. of Animal Reproduction 7:38-40 cited by Renganathan (1982)

Watanabe, M. (1957) An improved technique of the artificial insemination in ducks. J. Faculty of Fish and Animal Husbandry, Hirohima University 1:363-370

Westfall, F.D. and G.C. Harris (1975) The ability of cryopreservatives to prevent motility loss and freeze-thaw damage to the acrosome of chicken spermatozoa. Cryobiology 12:89-92

Wilcox, F.H. and C.S. Shaffner (1958) The effect of diluent handling methods and added fructose on the fertilizing ability of chicken spermatozoa after storage. Poultry Science 37:1353-1356

Wishart, G.J. (1989) Physiological changes in fowl and turkey spermatozoa during *in vitro* storage. British Poultry Science 30:443-454

Wishart, G.J. (1985) Quantitation of the fertilizing ability of fresh compared with frozen and thawed fowl spermatozoa. British Poultry Science 26:375-380

Zavaleta, D. and F. Ogasawara (1987) A review of the mechanism of the release of spermatozoa from storage tubules in the fowl and turkey oviduct. World's Poultry Science 43:132-139

APPENDIX 1. Distribution of treatment groups for determining drake access and age effects on reproductive performance

Physical		Visual-Auditory		Auditory Only	
♂	♀	♂	♀	♂	♀
3Y	3A	3Y	3A	3Y	3A
3Y	3Y	3Y	3Y	3Y	3Y
3A	3A	3A	3A	3A	3A
3A	3Y	3A	3Y	3A	3Y

Y = Yearlings, A = Adults.

APPENDIX 2. Composition of Beltsville (BPSE) and Lakes (LAKE) Poultry Semen Extenders

Function	Compound	Extender	
		BPSE[a]	LAKE[b]
Buffer	Dipotassium phosphate.$3H_2O$	1.27	-
	Monopotassium phosphate	0.07	-
	BES*	-	3.06
	TES'	0.20	-
Chelator	Sodium glutamate	0.87	1.52
Osmotic balance	Sodium Acetate.$3H_2O$	0.43	-
	1N-Sodium hydroxide	-	5.8 ml
	Potassium citrate	0.06	0.12
	Magnesium acetate.$4H_2O$	-	0.08
	Magnesium chloride.$6H_2O$	0.03	-
Energy source	Glucose	-	0.60
	Fructose	0.50	-
	Distilled H_2O	100 ml	100 ml
	pH	7.5	7.05

Values are in grams unless otherwise stated.
[a] Adapted from Sexton (1977).
[b] Adapted from Lake and Ravie (1979).
*BES = NN-Bis(2-hydroxyethyl)-2-aminoethane sulphonic acid.
'TES= N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

APPENDIX 3. Analysis of variance for clutch size for Mallard pairs of different ages with physical, visual-auditory, or auditory only contact

Dependent Variable: Clutch Size

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	11	1177.56	107.05	2.05	ns
Contact	2	206.72	103.36	1.98	ns
Age	3	118.89	39.62	0.76	ns
Contact*age	6	851.94	142.00	2.72	ns
Error	24	1250.67	52.11		
Total	35	2428.22			

ns = Not significant $P < 0.05$.

APPENDIX 4. Analysis of variance for days to first egg from start of trial for Mallard pairs of different ages with physical, visual-auditory, or auditory only contact

Dependent Variable: Days to First Egg

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	11	317.02	28.82	0.68	ns
Contact	2	96.74	48.37	1.15	ns
Age	3	110.69	36.90	0.88	ns
Contact*age	6	87.78	14.63	0.35	ns
Error	19	800.33	42.12		
Total	30	1117.35			

ns = Not significant $P < 0.05$.

APPENDIX 5. Analysis of variance for down deposited as a percentage of clutch size for Mallard pairs of different ages with physical, visual-auditory, or auditory only contact

Dependent Variable: Clutch Size

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	10	2157.15	215.71	0.41	ns
Contact	2	462.56	231.28	0.44	ns
Age	3	1151.64	383.88	0.73	ns
Contact*age	5	541.59	108.32	0.21	ns
Error	15	7903.52	526.90		
Total	25	10060.66			

ns = Not significant $P < 0.05$.

APPENDIX 6. Analysis of variance for plasma testosterone concentration of yearling and adult Mallard drakes

Dependent Variable: Testosterone Concentration

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	36	65.62	1.82	2.39	**
Age	1	0.32	0.32	0.25	ns
Drake(age)	21	26.93	1.28	1.68	*
Time	7	30.27	4.32	5.67	**
Time*age	7	7.39	1.06	1.38	ns
Error	35	103.00	0.76		
Total	71	168.62			

** = Significant at $P < 0.01$.
* = Significant at $P < 0.05$.
ns = Not significant $P < 0.05$.

APPENDIX 7. Analysis of variance for arc sine transformed percentage of abnormal sperm after storage at 5 °C in Beltsville or Lakes Poultry Semen Extender in comparison to neat semen

Dependent Variable: Arc sine Transformed Percent Abnormal Sperm

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	11	17952.48	1632.04	128.84	**
Treat	2	9577.47	4788.74	378.06	**
Time	3	6105.82	2035.27	160.68	**
Treat*Time	6	4458.32	743.05	58.66	**
Error	30	380.01	12.67		
Total	41	18332.49			

** = Significant at $P < 0.01$.

APPENDIX 8. Analysis of variance for arc sine transformed percentage of dead sperm after storage at 5 ^{0}C in Beltsville or Lakes Poultry Semen Extender in comparison to neat semen

Dependent Variable: Arc sine Transformed Percent Dead Sperm

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	11	431.92	39.27	3.83	**
Treat	2	99.92	49.96	4.87	**
Time	3	266.92	88.97	8.67	**
Treat*Time	6	65.08	10.85	1.06	ns
Error	48	492.68	10.26		
Total	59	924.60			

** = Significant at $P < 0.01$.
ns = Not significant $P < 0.05$.

APPENDIX 9. Analysis of variance for sperm motility score after storage at 5 °C in Beltsville or Lakes Poultry Semen Extender in comparison to neat semen

Dependent Variable: Motility score

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	11	65.25	5.93	12.06	**
Treat	2	24.40	12.20	24.81	**
Time	3	28.58	9.53	19.38	**
Treat*Time	6	12.27	2.04	4.16	**
Error	48	23.60	5.93		
Total	59	88.85			

** = Significant at $P < 0.01$.

APPENDIX 10. Repeated measures analysis of variance for percent fertility of semen (fresh or stored) in Beltsville or Lakes Poultry Semen Extenders

Dependent Variable: Percent Fertility

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model					
Treat	2	0.53	0.26	2.48	ns
Time	1	16.51	16.51	155.78	**
Treat*time	2	1.00	0.50	4.72	*
Error	32	3.39	0.11		
Week	3	0.63	0.21	5.10	**
Week*treat	6	0.97	0.16	3.92	**
Week*store	3	0.17	0.06	1.39	ns
Week*treat*store	6	0.45	0.08	1.82	ns
Error (week)	96	3.96	0.04		

** = Significant at $P < 0.01$.
* = Significant at $P < 0.05$.
ns = Not significant $P < 0.05$.

APPENDIX 11. Analysis of variance for effect of different concentrations of glycerol in BPSE on the fertilizing capacity of unstored chicken spermatozoa

a) Dependent Variable: Percent Fertility

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	7	37672.27	5381.75	8.39	**
Treatment	3	30548.46	10182.82	15.87	**
Replicate	1	3348.35	3348.35	5.22	*
Treat*Repl	3	3775.46	1258.49	1.96	ns
Error	40	25661.13	641.53		
Total	47	63333.40			

N.B. Values used in the above analysis were data obtained on Days 2-8.
** = Significant at $P < 0.01$.
* = Significant at $P < 0.05$.
ns = Not significant $P < 0.05$.

b) Dependent Variable: Percent Fertility

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	7	38337.76	5476.82	9.52	**
Treatment	3	29143.66	9714.55	16.89	**
Replicate	1	7525.36	7525.36	13.08	**
Treat*Repl	3	1091.85	363.95	0.63	ns
Error	39	22435.87	575.28		
Total	46	60773.64			

N.B. Values used in the above analysis were on data obtained on Days 9-15.
** = Significant at $P < 0.01$.
ns = Not significant $P < 0.05$.

c) Dependent Variable: Percent Fertility

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	7	37447.12	5349.59	11.42	**
Treatment	3	30387.80	10129.27	21.61	**
Replicate	1	4834.06	4834.06	10.32	**
Treat*Repl	3	2225.26	741.75	1.58	ns
Error	40	18745.37	468.63		
Total	47	56192.49			

N.B. Values used in the above analysis were on data obtained on Days 2-15.
** = Significant at $P < 0.01$.
ns = Not significant $P < 0.05$.

APPENDIX 12. Analysis of variance for effect of ethylene glycol and propylene glycol concentration on chicken egg fertility

a) Dependent Variable: Arcsin Transformed Fertility

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	9	18133.00	2014.78	2.26	*
Treatment	4	13227.38	3306.84	3.70	**
Replicate	1	148.21	148.21	0.17	ns
Treat*Repl	4	4757.42	1189.35	1.33	ns
Error	50	44670.07	893.40		
Total	47	62803.07			

N.B. Values used in the above analysis were arcsin transformed data obtained on Days 2-8.
** = Significant at $P < 0.01$.
* = Significant at $P < 0.05$.
ns = Not significant $P < 0.05$.

b) Dependent Variable: Arcsin Transformed Fertility

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	9	23102.16	2566.91	5.82	**
Treatment	4	17897.26	4474.31	10.14	**
Replicate	1	413.44	413.44	0.94	ns
Treat*Repl	4	4791.47	1197.87	2.71	*
Error	50	22069.65	441.39		
Total	59	45171.81			

N.B. Values used in the above analysis were on arcsin transformed data obtained on Days 9-15.
** = Significant at $P < 0.01$.
* = Significant at $P < 0.05$.
ns = Not significant $P < 0.05$.

c) Dependent Variable: Arcsin Transformed Fertility

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	9	12888.17	1432.02	2.94	**
Treatment	4	10373.70	2593.42	5.32	**
Replicate	1	586.91	586.91	1.20	ns
Treat*Repl	4	2059.45	514.86	1.06	ns
Error	44	21438.20	487.23		
Total	53	34326.37			

N.B. Values used in the above analysis were on arcsin transformed data obtained on Days 2-15.
** = Significant at $P < 0.01$.
ns = Not significant $P < 0.05$.

APPENDIX 13. Analysis of variance for effect of sucrose concentration on chicken egg fertility

a) Dependent Variable: Arcsin Transformed Fertility

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	11	81638.22	7421.66	15.82	**
Treatment	5	68439.61	13687.92	29.19	**
Replicate	1	8062.38	8062.38	17.19	**
Treat*Repl	5	5136.23	1027.24	2.19	ns
Error	60	28139.71	4689.00		
Total	71	109777.94			

N.B. Values used in the above analysis were arcsin transformed data obtained on Days 2-8.
** = Significant at $P < 0.01$.
ns = Not significant $P < 0.05$.

b) Dependent Variable: Arcsin Transformed Fertility

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	11	43165.76	3924.16	11.88	**
Treatment	5	40550.63	8110.13	24.55	**
Replicate	1	1913.74	1913.74	5.79	*
Treat*Repl	5	701.38	140.28	0.42	ns
Error	60	19824.68	330.41		
Total	71	62990.44			

N.B. Values used in the above analysis were on arcsin transformed data obtained on Days 9-15.
** = Significant at $P < 0.01$.
* = Significant at $P < 0.05$.
ns = Not significant $P < 0.05$.

c) Dependent Variable: Arcsin Transformed Fertility

Source of Variation	df	Sum of Squares	Mean Square	F-Value	Inference
Model	11	51250.85	4659.19	15.28	**
Treatment	5	46154.03	9230.81	30.27	**
Replicate	1	3358.58	3358.58	11.01	**
Treat*Repl	5	1738.24	347.65	1.14	ns
Error	60	18296.79	304.95		
Total	71	69547.65			

N.B. Values used in the above analysis were on arcsin transformed data obtained on Days 2-15.
** = Significant at $P < 0.01$.
ns = Not significant $P < 0.05$.