CRYOPRESERVATION OF SEMEN OF THE AMERICAN KESTREL

Falco sparverius

A Thesis

by

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Submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements
for the degree of

Master of Science

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UNIVERSITÉ McGILL

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Suggested short title:

Cryopreservation of kestrel semen.

To my friends at Mac,

the best group of people I've ever known.

ABSTRACT

M.Sc. M. Kelly Brock Renewable Resources

Cryopreservation of Semen of the American Kestrel

Falco sparverius

Artificial insemination in captive propagation programs is playing an increasingly important role in the maintenance of several endangered avian species. Cryogenic preservation of semen from these species could be critical to their conservation. In this respect, semen from the American kestrel, Falco sparverius, was used as model for the study of cryogenic preservation of falcon spermatozoa.

In a preliminary study, current poultry cryotechnology was modified to assess the feasibility of freezing kestrel semen. The effectiveness of known cryopreservative compounds, glycerol, dimethylsulfoxide, and dimethylacetamide (DMA), was evaluated for protecting kestrel spermatozoa throughout the various steps of cryopreservation. Using spermatozoal motility and fertilizing capacity as criteria, a diluent containing glycerol (in a ratio of 1 part semen to 3 parts diluent) was superior in preventing motility loss (20-40% remained motile post-thaw), while semen containing DMA (in a ratio of 1 part semen to 1 part diluent) yielded

higher fertility (28.6%).

In a second set of experiments, further evaluations were made of glycerol and DMA as cryopreservative compounds, in ratios of 1:3 and 1:1 with kestrel semen, respectively. Glycerol was superior to DMA in preventing motility loss (57.4% pre-freeze and 40.7% post-thaw vs. 49.5% pre-freeze and 13.0% post-thaw, respectively).

With subsequent characterization of morphology, the biological effects of cryopreservation on kestrel spermatozoal integrity were evaluated. DMA offered better protection than glycerol to spermatozoal morphology (54.6% of acrosomes remained intact pre-freeze and 49.6% post-thaw vs. 49.2% pre-freeze and 41.1% post-thaw, respectively).

Higher fertility was obtained with semen containing DMA (52.2% pre-freeze and 30.4% post-thaw) than with semen containing glycerol (13.6% pre-freeze and 11.8% post-thaw). Unlike DMA however, glycerol, once inseminated exhibited a contraceptive effect, which confounded the effects of motility and morphological integrity on fertility.

ABREGE

M.Sc. M. Kelly Brock Ressources renouvelables

La cryoprèservation du sperme de la Crècerelle Amèricaine, Falco sparverius.

L'insèmination artificielle, dans un programme de propagation en captivitè, revêt une importance accrue dans le maintient de plusieurs espèces d'oiseaux en voie d'extinction. La prèservation par la congèlation du sperme peut donc jouer un rôle prèpondèrant dans la conservation de ces espèces. Ainsi, le sperme de la crècerelle amèricaine, Falco sparverius, a servi de modèle pour l'étude de mèthodes de prèservations cryogèniques des spermatozoides de faucons.

Dans une ètude prèliminaire, la mèthode actuelle de congèlation du sperme de volaille a ètè modifièe pour èvaluer la possibilitè de congeler le sperme de crècerelle. L'efficacitè de produits cryoprèservatifs, glycèrol, dimethylsulfoxide (DMSO), et dimethylacetamide (DMA), à prèserver les spermatozoides de crècerelles durant les ètapes de la cryoprèservation a ètè èvaluèe. Utilisant le critère de rendement du taux de motilitè des spermatozoides et leur capacitè de fertilisation, un diluant contenant du glycèrol (dans une proportion d'une partie de sperme pour trois de diluant) s'est montrè supèrieur à prèvenir une perte du taux de motilitè

(20 à 40% est demeurè motile après dècongèlation); cependant, le sperme contenant le DMA (dans une proportion d'une partie de sperme pour une de diluant) a produit une fertilitè plus èlevèe (28.6%).

Lors d'une seconde sèries d'expèriences, les effets biologiques de la cryoprèservation sur l'intègritè des spermatozoides de crècerelles ont ètè èvaluès par une caractèrisation de leur morphologie. Le glycèrol ètait supèrieur au DMA dans la prèvention de la perte de motilitè (57.4% prè-congèlation et 40.7% post-dècongèlation, contre 49.5% prè-congèlation et 13.0% post-decongèlation, respectivement). Le DMA offrait une protection supèrieure à celle du glycèrol quant à l'ultrastructure du spermatozoide (54.6% d'acrosomes sont demeurès intacts lors de la prè-congèlation et 49.6% lors de la post-decongèlation, contre 49.2% prè-congèlation et 41.1% post-dècongèlation, respectivement). On a èlagement obtenu une fertilitè plus èlevèe avec le sperme contenant du DMA (52.2% prè-congèlation et 30.4% post-dècongèlation) qu'avec le sperme contenant du glycèrol (13.6% prè-congèlation et 11.8% post-dècongèlation). Toutefois, le glycèrol, (à l'encontre du DMA), une fois inséminé, a prèsentè un effet contraceptif qui brouillait les effets de la motilitè et de l'intègritè de l'ultrastructure sur la fertilitè.

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ACKNOWLEDGEMENTS

I would like to thank Ian Ritchie, Laird Shutt, and fellow Raptor Centre graduate students for their advice and assistance in setting up the facilities necessary for the study, and the Raptor Centre summer interns of 1983, 1984, and 1985: Nancy Shackell, Linda Pacioretty, Sandy Wagner, and especially Jacqueline Peltier, for their invaluable assistance with semen collection and artificial insemination. I am also indebted to the other interns and graduate students who lent a hand when needed. For assistance with statistical analyses, I extend much of my appreciation to Dave Gordon and Peter Alvo. I also would like to thank Louis Blais and Maria Harvey for providing the French translation of the abstract.

My sincerest gratitude goes to my advisors: Dr. George Ansah who guided me with his expert knowledge of cryopreservation, Dr. Roger Buckland who gave me the use of his laboratory and equipment, and especially to Dr. David Bird, a man who truly cares about his students.

For financial assistance, I wish to thank the Department of Renewable Resources, the Canadian National Sportmen's Fund, World Wildlife Fund (Canada), Natural Sciences and Engineering Research Council of Canada, and the government of the state of Maryland, USA.

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PREFACE

Concern for the welfare of declining falcon populations in North America has led to the establishment of captive propagation programs (Porter and Wiemeyer 1970; Fyfe 1975; Weaver and Cade 1983). The advantages and disadvantages of captive propagation have been described (Conway 1978) and the use of aviculture has become a vital conservation measure (Wayre 1967).

Despite some success with natural pairing and mating, overall efficiency at producing progeny has been relatively low for many reasons, including incompatibility, extreme aggressiveness, crippling disabilities, skewed sex ratios (Gee and Temple 1978), problems with sex identification, human imprinting, abnormal reproductive behavior, nervousness (Mendelssohn and Marder 1970), delayed attainment of sexual maturity, and the maintenance of breeding condition (Lake 1978).

The literature discusses several small-scale, yet successful attempts to breed raptors by artificial insemination, i.e. prairie falcons (Falco mexicanus) (Boyd et al. 1977), golden eagles (Aquila chrysaetos) (Grier 1973), goshawks (Accipiter gentilis) (Berry 1972), and red-tailed hawks (Buteo jamaicensis) (Temple 1972). Only one large-scale experiment with American kestrels (Falco sparverius)

has been conducted to demonstrate comparable productivity between artificial insemination and natural mating (Bird et al. 1976). Thus, the advantages of artificial insemination have been summarized (Bird et al. 1976; Boyd 1978; Gee and Temple 1978; Lake 1978) and the procedure has become routine in many breeding programs.

In recent years, as the knowledge of captive management and the need for successful breeding of avian species have increased, avicultural endeavors have become more scientific. Accordingly, a next logical step to improve efficiency of artificial insemination is short- and long-term semen storage.

Methodologies for cryopreservation of avian semen have been described for chickens (Gallus domesticus) (Lake et al. 1981), turkeys (Meleagris gallopavo) (Graham et al. 1982), and cranes (Grus canadensis) (Gee et al. 1985). The overall objective of the present study is to use the American kestrel as a model for investigating cryopreservation of falcon semen.

This thesis is divided into two sections, each written as a manuscript to be submitted for publication. Section I, formatted for submission to "Raptor Research," descriptively discusses preliminary trials during which modifications to poultry cryotechnology were developed for suitability

to the model species Falco sparverius. Section II, formatted for submission to "The Auk," discusses in greater detail the specific effects of cryopreservation on the morphology, motility, and fertilizing capacity of spermatozoa of Falco sparverius. Dr. David M. Bird, as thesis advisor, will appear as co-author on each manuscript. Data collection and analyses were conducted by this author independently.

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SECTION I. Application of Poultry Cryotechnology to Falcon Semen: A Preliminary Study.

ABSTRACT

In the spring of 1983 and 1984, semen collected from unpaired male American kestrels was mixed in ratios of 1 part semen to 1 part diluent (1:1), or 1:3, with the following diluents and cryopreservative compounds: Beltsville Poultry Semen Extender (BPSE) and 4% dimethylsulfoxide (DMSO), Lake's diluent and 13.6% glycerol, or Lake's diluent and 12.3% dimethylacetamide (DMA). Diluted semen samples were frozen in liquid air at a rate of 6°C/min from 5°C down to -196°C.

Using light microscopy, motility of spermatozoa in fresh, diluted, and frozen-thawed semen was compared. Spermatozoa in semen diluted 1:1 with Lake's diluent containing glycerol, retained the highest motility (40 - 60%). However, when frozen-thawed, a greater percentage of cells (20 - 40%) remained viable in semen diluted by a glycerol diluent in a ratio of 1:3 than 1:1 (1 - 20%). Overall, glycerol was superior to DMA (in Lake's diluent) for preventing motility loss of kestrel spermatozoa, while 0% spermatozoal motility was apparent in semen containing BPSE and DMSO.

Retention of fertilizing capacity of frozen-thawed spermatozoa was determined by artificially inseminating female kestrels. When not frozen-thawed, semen with glycerol, or DMA, in a ratio of 1:1 and

1:3 respectively, yielded 0% fertility. Semen frozen-thawed with DMA in a ratio of 1:1 yielded 28.6% fertility, while that frozen-thawed with glycerol in a ratio of 1:3, yielded 12.5% fertility. Comparatively, fertility achieved with fresh semen was 50.0%.

Although DMA was not as effective as glycerol in preventing post-thaw motility loss of kestrel spermatozoa, it yielded a higher fertility rate.

INTRODUCTION

In recent years scientific aviculture has become a vital conservation measure for the propagation of many threatened or endangered species. Artificial insemination, clutch manipulation, artificial incubation, and the hand-rearing of young are playing increasingly important roles in the maintenance of some of these species in captivity.

However, one frequently encountered problem is that associated with gene pools. Original stocks are often small in number, thus limiting genetic diversity. Because one cannot predict how a species will be affected by inbreeding, genetic variability should be maintained at the highest level by maximizing interbreeding (Lovejoy 1978). Thus, to preserve a species is to preserve its gene pool.

One scheme to achieve this goal is to mate unrelated lines. Presently, a breeding loan, for example the transfer of a bird from one institution to another, is the most common method employed, but several disadvantages are attributed to this method. These include the stress of handling and transportation inflicted upon potentially valuable breeders, as well as the time needed for the new arrival to acclimate to its new surroundings and its new mate. Even then, successful breeding still cannot be guaranteed. A more efficient means might be to

exchange semen rather than birds for the purpose of artificial insemination. Semen from a highly productive male could be collected and cryogenically treated for short- or long-term storage, exchange between institutions or groups, for use after the bird is no longer capable of producing semen, and to increase the number of females bred by individual males (Lake 1978).

Unlike mammals where both male and female gametes can be collected for cryopreservation (Graham 1978; Mazur 1980), only the avian male gametes can be cryogenically stored. The techniques involved in the cryogenic preservation of poultry semen have been summarized, and over 90% fertility has been achieved using frozen-thawed semen (Lake et al. 1981). Much advancement in this field has been achieved with domestic species and the general principles can be used as a guideline for the development of techniques for non-domestic species (Lake 1978). Although Sexton and Gee (1978) modified a method developed for poultry semen for use with the sandhill crane (Grus canadensis), little work has been reported for direct comparisons of different freezing techniques, or diluents for freezing the spermatozoa of other non-domestic avian species.

Using the American kestrel (Falco sparverius) as a model raptor species (Bird 1982), the following

objectives were set for the present study:

1. To compare the effectiveness of diluents containing different cryoprotective compounds (in different ratios with semen) in preserving the motility of kestrel spermatozoa before and after freezing and thawing.

2. To obtain fertility with frozen-thawed spermatozoa, and the production of healthy progeny.

MATERIALS AND METHODS

Breeding Stock. Sixty-three pedigreed American kestrels in 1983, and 34 pedigreed American kestrels in 1984, 1 and 2 years of age, were maintained at the Macdonald Raptor Research Center of McGill University, for this study. Descriptions of care, feeding, and breeding are detailed elsewhere (Bird 1982).

Beginning 20 April, 1983, 20 of 30 unpaired male semen donors were tethered by means of falconer's jesses, swivels, and leashes to perches at floor level, for ease in handling during semen collection. They were exposed to artificial light which followed the natural photoperiodic regime. Five of the 10 remaining males were allowed free flight in each of 2 flight rooms (6.10 x 6.10 x 2.29 m) with exposure to natural light. This allowed for comparisons on how handling stress affected the degree of contamination

by urates and feces during semen collection. Thirty-three unpaired females were housed in individual pens ranging from holding boxes (0.80 x 0.43 x 0.56 m) to flight pens (2.44 x 1.22 x 2.44 m), all provided with nest boxes. Most of the females were in auditory and/or visual contact with the males.

Beginning 18 April, 1984, 17 unpaired males were tethered to perches 1.37m above floor level to reduce stress caused by fear as the investigators entered the rooms, but otherwise treated similarly to those in 1983. These males were exposed to artificial light which followed the natural photoperiodic regime. Seven "teaser" females with access to nest boxes, but not to the males, were tethered in alternating positions to stimulate and perhaps prolong semen production. Ten additional unpaired females to receive artificial insemination were housed in individual flight pens (2.44 x 1.22 x 2.44 m), each provided with a nest box. These females were exposed to the same photoperiod as the other birds, and were without auditory or visual contact with the males.

Semen Collection. The technique of semen collection by massage, described by Bird et al. (1976), was modified by drawing the semen up in a dry non-heparinized microhematocrit tube. In 1983 semen was collected every second day, 3 times per week, for

7 weeks from 20 April to 8 June. In 1984 semen was collected every 3 days from 18 April to 8 June. All semen samples contaminated by urates or feces were discarded

The amount of semen donated by each male was calculated by using the formula for the volume (V) of a cylinder, $V = \pi r^2 h$, where r is the radius of the inner diameter of the microhematocrit tube, and h is the length of the tube filled with semen (measured in mm).

Upon collection, individual semen samples were cooled immediately down to approximately 5°C (Shaffner et al. 1941) by placing the tubes on a rack above ice in a plastic tackle box to protect them from environmental factors (sunlight, rain, dirt, dust, etc.) Thirty to 40 minutes elapsed from the first to the last collection. After all the samples were obtained, the semen was brought for processing to a cold room maintained at 5°C.

Semen Evaluation. Prior to treatment, individual semen samples (collected each day) were transferred from the microhematocrit tubes and pooled in a 5ml glass test tube. Total volume of fresh semen was measured using a straw and a glass microliter syringe. The percent motility of spermatozoa in fresh and treated semen (see Freezing Trials below) was subjectively estimated on a scale of 0 to 5

(Table 1) (Bird and Lague 1977) by placing approximately 4 ul of semen on a glass slide and examining at 400X, using light microscopy.

In 1984, spermatozoa concentrations of pooled samples were determined using samples diluted 1 part semen to 3 parts diluent (1:3). The following formula for determining cell concentration by the hemacytometer method was used:

$$\frac{N \times D}{V} = \frac{N \times D}{5\ (0.2 \times 0.2 \times 0.1)} = \frac{N \times D}{.02 mm^3} \times 10^3,$$

where N is the average number of cells counted in each corner and middle secondary square of the hemacytometer, D is the dilution factor, and V is the volume of the hemacytometer chamber. The final concentration value is expressed as $x10^6$/ml.

The pH of fresh semen was determined using Fisher's Alkacid Full Range pH kit.

Freezing Apparatus. The Macdonald freeze-thaw apparatus described by Mitchell et al. (1975) consisted of a variable speed kymograph which was used to lower 1/2 ml plastic straws containing the semen samples into a wide mouth liquid air tank. Inserted into the mouth of the tank was an aluminum tube encased in styrofoam. The insulated tubing and wide mouth tank contained static vapor above the liquid air which generated a temperature gradient of approximately 5°C at the top to -196°C at the bottom.

A thermocouple was inserted in a straw containing a diluent sample and the temperature was recorded on an automatic printer during the freezing process.

Freezing Trials. A series of trials was conducted to measure percent survival of spermatozoa following the addition of a diluent containing a cryoprotective compound, and freezing in liquid air to -196°C at a descent rate of an average of 6°C/min (Mitchell et al. 1977). A single trial consisted of 1 application of a treatment to a single pooled semen sample. Table 2 outlines the protocol followed.

The following formulations for diluents were used.

BPSE (Sexton 1977)

	g/100ml distilled H_2O
Potassium diphosphate . $3H_2O$	0.1270
Sodium glutamate	0.0867
Fructose	0.0500
Sodium acetate.$3H_2O$	0.0430
TES	0.0195
Potassium monophosphate	0.0065
Magnesium chloride.$6H_2O$	0.0034
Potassium citrate	0.0064

pH = 7.5

LAKE'S GLYCEROL DILUENT (Lake and Stewart 1978)

	g/100 ml distilled H_2O
Sodium glutamate monohydrate	1.92
Fructose	0.80
Magnesium acetate (tetrahydrate)	0.08
Potassium acetate (anhydrous)	0.50
Polyvinyl pyrrolidonin (MW 10,000)	0.03
Glycerol	13.64

pH = 6.8

LAKE'S DMA DILUENT (Lake and Ravie 1984)

	g/100 ml distilled H_2O
Sodium glutamate monohydrate.....	1.92
Glucose.....	0.80
Magnesium acetate (tetrahydrate).....	0.08
Potassium acetate (anhydrous).....	0.50
Polyvinyl pyrrolidonin (MW 10,000).....	0.03
N,N-Dimethylacetamide.....	12.36 ml

pH = 6.8

Fresh stock solutions of Lake's diluents were made once a week throughout the testing periods.

In 1983 a total of 15 trials was conducted. Three trials consisted of subjectively estimating and comparing the percent motility of spermatozoa after a) fresh samples were pooled, b) 30 minutes following dilution at a ratio of 1 part semen to 1 part Beltsville Poultry Semen Extender (BPSE), and c) 15 minutes of equilibration after the addition of 4% (v/v) dimethylsulphoxide (DMSO) (Sexton and Gee 1978). Five trials consisted of subjectively estimating spermatozoal motility after a) fresh samples were pooled, b) dilution at a ratio of 1:1 with Lake's glycerol diluent, and c) freezing in liquid air and thawing. Seven additional trials using Lake's glycerol diluent were carried out as above, using a dilution ratio of 1:3. Rapid thaw consisted of transferring frozen semen samples immediately from the liquid air into a 30-50C waterbath. In 4 of the latter 12 trials the glycerol was removed from the frozen-thawed samples by adding

Lake's glycerol-free diluent stepwise and with gentle mixing, centrifugation, and resuspension of cells in the glycerol-free diluent (Lake and Stewart 1978). Motility of spermatozoa in these samples was thereafter estimated again.

In 1984 a total of 8 trials was conducted. Three trials consisted of subjectively estimating percent motility of spermatozoa on the hemacytometer and glass slides after a) fresh samples were pooled, b) dilution at a ratio of 1:1 with Lake's DMA diluent, and c) freezing in liquid air and thawing. Five additional trials were carried out as above, using a dilution ratio of 1:3. As in 1983 the frozen samples were thawed in a 3°-5°C waterbath.

Artifical Insemination. To test the fertilizing capacity of frozen-thawed spermatozoa, unpaired female kestrels in laying condition were artificially inseminated (AI), as described by Bird et al. (1976). From 3-28 May, 1983, a total of 10 female kestrels were artificially inseminated, with a mean volume of 35 ul of semen per insemination. Three females were inseminated, one time each, with frozen-thawed semen containing glycerol, at a dilution rate of 1:1. Seven females were inseminated with frozen-thawed semen containing glycerol, at a dilution rate of 1:3. Two of these 7 females received multiple inseminations and 5 received single inseminations

only.

From 25 April to 6 June, 1984, each of 8 female kestrels were artificially insemination, one time only. Three females received an average of 25.5 ul of fresh semen, 3 females received an average of 22.3 ul of frozen-thawed semen containing DMA, at a dilution rate of 1:1, and 2 females received an average of 53.0 ul of frozen-thawed semen containing DMA, at a dilution rate of 1:3.

Because the duration of fertility in kestrels can extend up to 12 days (Bird and Buckland 1977), each egg laid up to 10 days post-AI was considered as potentially fertile and was collected sequentially for artificial incubation. Fertility was determined by candling the eggs after 8 days of incubation. Eggs candled as infertile were opened and examined macroscopically for the presence of a blastodisc and blood islands (Bird et al. 1984).

RESULTS

The mean $\pm$ S.D. individual semen collection volume was 9.4 $\pm$ 6.5 ul (n=196) in 1983 and 15.2 $\pm$ 11.8 ul (n=192) in 1984. The mean $\pm$ S.D. volume of pooled semen samples was 92.3 $\pm$ 21.5 ul (n=18) in 1983 and 145.5 $\pm$ 67.4 ul (n=20) in 1984. The mean $\pm$ S.D. concentration of spermatozoa per pooled sample in 1984 was 40.6 $\pm$ 17.6 x 10^6/ml. The pH of fresh

semen was between 6.5 and 7.0 (volumes were too low for more accurate measurement). Semen collected from tethered males tended to be less contaminated with urates or feces than that collected from males allowed free flight. Likewise, males tethered above floor level appeared more at ease in the presence of the investigators which further facilitated the collection of clean samples.

The median value for percent motility of spermatozoa, in pooled samples of fresh semen, was 60% (from a mean scale value of 3.3 (40-60%) in 1983 and 4.1 (60-80%) in 1984). Percent motility of spermatozoa in each treatment group is summarized in Table 3. When fresh semen was diluted with BPSE, motility was reduced from 40 - 60% down to 0%, and no viable cells were observed after the addition of, and equilibration with DMSO. A higher percentage of spermatozoa remained viable upon dilution of fresh semen at a ratio of 1:1 than 1:3, with the glycerol-containing diluent. Following freezing at 6°C/min from 5°C down to -196°C, and a rapid thaw in a 5°C waterbath, further reduction of motility occurred for both ratios. However, a higher percentage of cells remained viable in semen diluted at a ratio of 1:3. Furthermore, a reduction of spermatozoal motility to 0% was obtained during the procedure to remove the glycerol from the frozen-

thawed semen (Lake and Stewart 1978). The addition of the DMA-containing diluent to fresh semen suppressed motility in both the 1:1 and 1:3 treatment groups. However, unlike glycerol, a further reduction of motility following freezing and thawing was not seen. In the thawed samples containing DMA, motility was greater overall in a semen-to-diluent ratio of 1:1.

In 1983, all 9 eggs were infertile for the glycerol 1:1 frozen-thaw group, and 2 of 14 eggs yielded 12.5% fertility for the glycerol 1:3 frozen-thaw group (Table 4).

In 1984, a fertility of 50.0% (5/10 eggs) was achieved with fresh semen. In the DMA 1:1 frozen-thaw group, two of 7 eggs were fertile yielding 28.6% fertility, while all 5 eggs were infertile in the DMA 1:3 frozen-thaw group.

DISCUSSION

Factors affecting cell survival during freezing and thawing are two-fold: a) intracellular growth of ice crystals, and b) precipitation of solutes in high concentration due to cellular dehydration (Meryman 1956; Mazur 1970, 1980). Several neutral solutes of low molecular weight have proven to be adequate protectors of living cells against damage due to

freezing and thawing by virtue of their affinity for water. Such compounds include glycerol, dimethlysulphoxide, and dimethylacetamide which penetrate and protect the cells. The mechanism of action of these cryoprotective agents within the cell involves lowering the temperature at which freezing occurs, altering the habit of ice crystallization, and lowering the salt concentration in equilibrium with ice (Nash 1966).

In the present study, small semen collection volumes precluded the division of samples into aliquots to compare 2 dilution ratios in a single trial. Massaging the males for semen every 3 days in 1984 instead of every 2 days perhaps accounted for the improved volume and quality of the semen. Furthermore, the presence of teaser females tethered in alternating positions with nest boxes between males in 1984 may have acted as additional stimuli for the onset and improved quality of semen production.

Research for refinements in the technique for cryopreservation of fowl spermatozoa has included the use of glycerol, as it has proven to be superior to, and less hazardous than other compounds examined (Lake et al. 1980). At a ratio of 1:3 with kestrel semen, a diluent containing glycerol proved to be the most effective cryoprotector of kestrel spermatozoa

as well. This is reflected in the post-thaw motility scores in Table 3. The BPSE and DMSO appeared detrimental, as spermatozoal motility was apparently suppressed to 0%. However, volume restrictions of the kestrel semen limited further applications or modifications of this treatment. Further investigations are necessary to determine if different dilution ratios of semen to BPSE and DMSO reveal an effectiveness of these compounds to protect the motility of kestrel spermatozoa throughout the freeze-thaw process.

The values presented in Table 3 for the motility of spermatozoa in semen at a ratio of 1:1 with diluent, may be deceiving. For example, the post-thaw motility score for DMA 1:1 was higher than that prior to freezing, possibly due to a difference in motilities evaluated on the hemacytometer as compared to glass slides in 1984. Regardless of quantitative error however, DMA qualitatively offers good protection to kestrel spermatozoa through freezing and thawing; and may be as effective as glycerol.

High fertility rates (>90%, Lake et al. 1981) have been obtained in poultry using semen frozen in the presence of glycerol (1.1M final strength, Lake et al. 1981). However, when not reduced in concentration to below 0.163M in the thawed semen, the presence of the glycerol in the cells decreases

fertility (Lake et al. 1980). Although this contraceptive phenomenon in chickens (Gallus domesticus) is based on fertility per female per fertility period by a single insemination, low fertility was also seen in kestrel eggs following multiple inseminations per female per fertility period (Table 4). Any attempt to remove the glycerol from the thawed semen prior to AI by the technique described by Lake and Stewart. (1978) resulted in a loss of all remaining viable cells. Although Parks et al. (1985) have achieved some success with removing glycerol from frozen-thawed semen of peregrine falcons (Falco peregrinus) using a dialysis technique, numbers of donating males and low semen volumes in the present study did not facilitate the employment of either extraction procedure. It was thought that multiple inseminations may compensate for lost cells from previous inseminations for each subsequent egg, however it appears that the contraceptive mechanism prevails regardless of the insemination scheme. Comparatively, as DMA was not as effective as glycerol in protecting the motility of kestrel spermatozoa, it did not severely impair fertility.

The small sample sizes were inadequate for statistical analyses, but certain trends are indicated for future studies of the cryopreservation

of kestrel spermatozoa. Glycerol, in Lake's diluent, was superior to DMSO and DMA in protecting the motility of the spermatozoa through freezing and thawing, more so at a ratio of 1:3 than 1:1, semen to diluent. Due to the fragility of the kestrel spermatozoa, removal of glycerol from the cells is not possible without modification of an extraction technique. Subsequently, the presence of glycerol in the thawed semen at the time of insemination leads to low fertility. As an alternative, DMA in Lake's diluent, at a ratio of 1:1 with kestrel semen, offers sufficient protection to spermatozoa for the retention of fertilizing capacity. Lake and Ravie (1984) investigated alternative cryoprotectants to glycerol to overcome the contraceptive phenomenon in poultry and found the results with DMA encouraging enough to warrant further research. The fertility obtained in chicken eggs using DMA is not as high as that obtained with glycerol, however employing a multiple insemination scheme may prove to be a valuable tool in overcoming the problem.

The results of the present study are encouraging for further investigation into the cryopreservation of falcon spermatozoa, as healthy viable young falcons can and have been produced with frozen-thawed semen. Continued research into the biological effects of freezing falcon semen may prove to have

important implications for captive propagation of endangered raptorial species.

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Table 1. Scores used to represent subjective estimates of percent motility of kestrel spermatozoa (from Bird and Lague 1977).

SCORE	DESCRIPTION
0	No evident motility.
1	1 - 20% spermatozoa slightly oscillatory or progressively motile. Low number of spermatozoa.
2	20 - 40% spermatozoa progressively motile including those that may have been caught by debris but are moderately oscillatory. Spermatozoa numbers may be low.
3	40 - 60% spermatozoa progressively motile; some caught by debris but fairly vigorous. Spermatozoa numbers fair.
4	60 - 80% spermatozoa are progressively motile, few caught by debris but very vigorous. Many spermatozoa.
5	80 - 100% spermatozoa are progressively motile. Many spermatozoa.

Table 2. General scheme for testing effectiveness of cryoprotectants in kestrel semen and for evaluating fertilizing capacity of spermatozoa.

1.	Collect semen and cool to 5°C.
2.	Pool semen samples and maintain at 5°C throughout the remaining procedure.
3.	Estimate motility of spermatozoa in fresh semen according to Bird and Lague (1977).
4.	Dilute semen samples 1:1 or 1:3 with BPSE and 4% (v/v) DMSO (Sexton and Gee 1978), Lake's glycerol-containing diluent (Lake and Stewart 1978), or Lake's DMA-containing diluent (Lake and Ravie 1984).
5.	Estimate motility of spermatozoa in diluted semen samples, and calculate spermatozoa concentration with samples diluted 1:3.
6.	Freeze diluted semen samples at an average of 6°C/min (Mitchell et al. 1977) from 5°C down to -196°C in static liquid air vapor.
7.	Thaw frozen samples in 5°C waterbath.
8.	Estimate spermatozoa motility in the thawed samples.*
9.	Artificially inseminate females with fresh or frozen-thawed semen.
10.	Artificially incubate eggs collected sequentially and determine fertility by candling after 8 days of incubation.

* In some cases glycerol was removed before step 8.

Table 3. Percent motility of kestrel spermatozoa in fresh, pre-freeze, and post-thaw semen samples.

	Pre-freeze		Post-thaw	
	Scale value	%	Scale value	%
Fresh	3.3 ('83) 4.1 ('84)	40-60	-	-
BPSE	0.3	0	-	-
DMSO	0	0	-	-
Glycerol				
1:1	3.0	40-60	1.9	1-20
1:3	2.8	20-40	2.4	20-40
DMA				
1:1	2.3	20-40	2.8	20-40
1:3	1.8	1-20	2.3	20-40

Table 4. Fertility of kestrel eggs following artificial insemination with fresh or frozen-thawed semen. Numbers in parentheses indicate actual number of fertile eggs/total in each treatment group.

Fresh (1984)	38.5%	(5/13)	2 Hatched and Fledged 1 Post-natal Death 1 Late Embryo Death 1 Early Embryo Death
Glycerol (1983)			
1:1	0%	(0/10)	
1:3	8.3%	(2/24)	1 Hatched and Fledged 1 Died Pipping
DMA (1984)			
1:1	28.6%	(2/7)	1 Hatched and Fledged 1 Early Embryo Death
1:3	0%	(0/6)	

CONNECTING STATEMENT

Section I discussed trends associated with the suitability of modifying current poultry cryotechnology for a raptorial species, Falco sparverius. Section II discusses, in greater detail, the biological effects of freezing falcon semen on spermatozoa morphology, motility, and fertilizing capacity.

SECTION II. The Pre-freeze and Post-thaw Effects of Dimethylacetamide and Glycerol on the Morphology, Motility, and Fertilizing Capacity of Spermatozoa of the American Kestrel.

ABSTRACT

In the spring of 1985, the morphology of kestrel spermatozoa was characterized, and the biological effects of dimethylacetamide (DMA) and glycerol on spermatozoa were assessed in the various steps of cryopreservation.

Spermatozoa, fixed in 1% gluteraldehyde and stained with fast green FCF, were examined under the light microscope. The mean ± S.D. of head length was 3.4 ± 0.7 um, acrosome width 2.7 ± 0.5 um, acrosome length 3.3 ± 0.7 um (which covered 75% of the head), tail length was 26.4 ± 5.6 um. Only spermatozoa frozen-thawed with glycerol, which increased in size, showed a significant change.

Morphological damage was evaluated in stained spermatozoa under the light microscope by categorizing acrosomes according to state of integrity (intact, swollen or loose, partially detached or deteriorated, or completely detached). Unlike DMA, dilution with glycerol had injurious effects on acrosome integrity. During freezing neither compound completely prevented some morphological damage, however DMA offered better protection overall than glycerol.

Motility of spermatozoa in fresh semen (63.2%), as evaluated by phase-contrast microscopy, was suppressed by both compounds after dilution (57.4%

remained motile in semen containing glycerol and 49.5% in semen containing DMA). Further reductions in spermatozoal motility occurred after freezing, however a greater percentage of cells remained viable in semen containing glycerol than in semen containing DMA (40.7% and 13.0%, respectively).

By means of artificial insemination of female kestrels, semen containing glycerol, either freshly diluted or frozen-thawed, yielded the lowest fertility (13.6% and 11.8%, respectively), while fresh semen and that diluted with DMA yielded the highest (56.5% and 52.2%, respectively). Semen frozen-thawed with DMA yielded an intermediate fertility rate (30.4%).

In evaluating the pre-freeze and post-thaw effects of DMA and glycerol on kestrel spermatozoa, it was apparent that each compound may have protected a different part of the cell. For semen containing DMA, motility and integrity of spermatozoa could be associated with the resulting fertility rates. However, as documented in other avian species, glycerol once inseminated, exhibited a contraceptive effect which confounded its influence on resulting fertility rates.

INTRODUCTION

Conserving genetic variability for captive propagation by storing semen cryogenically has been investigated in two non-domestic avian species, the sandhill crane (Grus canadensis) (Sexton and Gee 1978) and the American kestrel (Falco sparverius) (Brock et al. 1983). These early studies reported on the modification of poultry cryotechnology to suit each respective species for eventual use in the aviculture of closely related endangered species. In neither study however, were the biological effects of cryopreservation on the spermatozoa assessed. Several studies involving poultry (Westfall and Harris 1975; Marquez and Ogasawara 1977; Bakst and Sexton 1979; and Maeda et al. 1984ab) reported on the ability of the cryopreservative agents to prevent freeze-induced morphological damage of spermatozoa, as well as motility loss. Maintaining the integrity of the spermatozoon's acrosome which contains the essential enzymes for egg penetration, is vital for fertilization to occur (Palmer and Howarth 1973). Loss of either acrosomal integrity or motility due to some other structural damage, will render the spermatozoon incapable of fertilizing an egg. Gee et al. (1985) have advanced their research to assessing post-thaw morphological damage to frozen crane spermatozoa, but such information is still lacking

for the American kestrel.

The objectives of the present study are to 1) characterize the morphology of the spermatozoa of the American kestrel, 2) compare the effectiveness of glycerol and dimethylacetamide (DMA) in preventing morphological damage and motility loss of kestrel spermatozoa before and after freezing from 5°C down to -196°C in liquid air, and 3) compare fertility obtained from fresh semen, and semen treated with glycerol or DMA.

MATERIALS AND METHODS

Breeding stock. A total of 118 pedigreed American kestrels, 1-5 years of age, and maintained at the Macdonald Raptor Research Centre, were used in this study. Descriptions of care, feeding, and breeding are detailed elsewhere (Bird 1982). Sixty-three unpaired male semen donors were divided into 3 groups of 29, 19, and 15 and were tethered by means of falconer's jesses, swivels, and leashes to perches 1.37 m above floor level, in seperate flight room locations (6.1 x 6.1 x 2.3 m). From a total of 60 female kestrels, 55 were housed in individual breeding pens (2.4 x 1.2 x 2.4 m), each provided with a nest box. All 55 females were visually isolated from male kestrels, and most were without auditory contact. The remaining 5 females were housed in

groups of 2,2, and 1, respectively, with each group of males.

On 9 February, 1985, each of the 3 groups of males and each of 3 groups of 5 females were placed in one of the 3 locations used in the study. Beginning 10 February with 10 1/2 L: 13 1/2 D, the kestrels were photoperiodically advanced by adding 1/2 hour/week of artificial light to the natural photoperiodic regime. The kestrels were maintained at 13 L:11 D from 17 March to 6 April when the natural photoperiod reached 13 L: 11 D. Thereafter, natural photoperiod was maintained. In 1 of the 3 locations only artificial light was used whereas in the other 2 locations artificial light was used to enhance the natural light entering the rooms.

From 10 February to 3 March the males were allowed free flight in association with the females. From 3 March, and for the duration of the study, the males were tethered as previously described, and 10 of the 15 females were placed in individual breeding pens. Five "teaser" females (2,2 and 1) remained with each group of males as described above.

Semen collection. Semen was collected in dry non-heparinized microhematocrit tubes by the massage technique as described by Bird et al. (1976). Each of the 3 groups of males was ejaculated for semen once every 3 days, i.e. 1 group per day, from 11

March to 6 June, 1985. From 7-14 June, semen was collected from individuals only when needed for artificial insemination (AI).

The amount of semen donated by each male was calculated by using the formula for the volume (V) of a cylinder, $V = \pi r^2 h$, where r is the radius of the inner diameter of the microhematocrit tube, and h is the length of the tube filled with semen (measured in mm).

Upon collection, each sample was cooled to approximately 5°C by placing the tubes on a rack above ice in a plastic tackle box to protect them from environmental factors. Fifteen to 30 minutes elapsed from the first to the last collection. After all the samples were obtained, the semen was brought for processing to a cold room maintained at 5°C. All samples contaminated by urates or feces were discarded.

Prior to treatment the semen samples were transferred from the microhematocrit tubes and pooled in a 1.5 ml plastic microcentrifuge tube. Total volume was equal to the summation of individual samples collected each day.

Spermatozoa concentrations of pooled samples were determined using samples diluted 1 part semen to 3 parts diluent (1:3). The following formula for determining cell concentration by the hemacytometer

method was used:

$$\frac{N \times D}{V} = \frac{N \times D}{5\,(0.2 \times 0.2 \times 0.1)} = \frac{N \times D}{0.2\text{mm}^3} \times 10^3,$$

where N is the average number of cells counted in each corner and middle secondary square of the hemacytometer, D is the dilution factor, and V is the volume of the hemacytometer chamber. The final concentration value is expressed as $x10^6$/ml.

Freezing. The apparatus for freezing was the Macdonald freeze-thaw apparatus described by Mitchell et al. (1975). It consisted of a variable speed kymograph which was used to lower 1/2 ml plastic straws containing the semen samples into a wide mouth liquid air tank. Inserted in the mouth of the tank was an aluminum tube encased in styrofoam. The insulated tubing and wide mouth tank contained static vapor above liquid air which generated a temperature gradient of approximately 5°C at the top to -196°C at the bottom. A thermocouple was inserted in a straw containing a diluent sample and the temperature was recorded on an automatic printer during the freezing process.

Semen samples were frozen in aliquots of 50-70 ul for 1:1 dilutions and 80-100 ul aliquots for 1:3 dilutions (see protocol below), and at a rate of approximately 6°C/min from 5°C down to -196°C. Upon completion of the freezing process the frozen samples

were transferred in liquid air to a small, portable liquid air storage tank. All samples to be used for AI were frozen for 2-5 weeks prior to thawing. When more than 1 aliquot per treatment was frozen in a single trial (see below), 1 sample from each frozen treatment was thawed immediately for motility evaluation and for fixation (see morphology, acrosome integrity, and motility below). Rapid thawing consisted of transferring the frozen samples immediately from the liquid air into a 3-5°C waterbath.

A total of 37 complete trials (defined as enough semen to freeze 1 or more aliquots of diluted semen, with both cryoprotectants from a single fresh, pooled sample) were conducted with 112 semen samples (aliquots). Incomplete trials were those where most of the semen was used for AI in the fresh state (neat or diluted), leaving too little or none of either cryoprotectant treatment to freeze.

Experimental protocol. Fig. 1 outlines diagrammatically the general scheme of investigation followed in the present study. Based on a preliminary study (Brock and Bird MS) the pooled semen sample for each day of the study period was divided into 5 treatment groups:

1. fresh semen - Control

2. fresh semen diluted in a ratio of 1:1 with Lake's dimethylacetamide (DMA) diluent and unfrozen (Lake and Ravie 1984) - DUF
3. fresh semen diluted in a ratio of 1:3 with Lake's glycerol diluent and unfrozen (Lake and Stewart 1978) - GUF
4. semen diluted with DMA and frozen in liquid air and later thawed - DTh
5. semen diluted with glycerol and frozen in liquid air and later thawed - GTh.

Fresh stocks of diluents were made once a week throughout the test period.

All statistical analyses were conducted by the Statistical Analysis System (SAS) (Ray 1982) at the 0.05 level of significance.

Morphology. Ten ul of semen from each of the 5 treatment groups was fixed in 10 ul of a 1% gluteraldehyde in phosphate buffered saline solution, maintained at 5°C (Hayat 1981). Semen collected from 20 March to 17 April, 1985 was used to work out a consistent, effective staining technique. Upon the addition of the gluteraldehyde fixative, the semen samples were concentrated at room temperature either by centrifugation at 600 rpm for 30 min, or by sedimentation over a period of 2 - 16 h (however most samples sat for only 4-5 h). Fifteen ul of supernatant was removed and 5 ul of concentrated

fixed semen was placed on one end of a warmed slide. Five ul of one of the following stains was mixed thoroughly with the fixed semen:

1. 1 part 1% fast green FCF/ 2 parts 1% eosin B/ 1.7 parts ethanol (Wells and Awa 1970)
2. substitution of eosin B above, with phyloxin B or nigrosin
3. 1 part 1% fast green FCF / 1.7 parts ethanol.

One third of the flat surface of a second warmed slide was placed over the semen/stain mixture allowing it to diffuse between both slides. A quick sliding motion, length-wise, was used to produce a uniform distribution of the smears on each slide, and to separate the two slides (Salisbury et al. 1942). The duplicate smears were dried immediately with a warm air blower.

Each duplicate slide was examined by light microscopy using a magnification of 1000X (oil immersion) and a blue filter to effectively distinguish the heads, acrosomes, and tails of the spermatozoa.

From 18 April to 27 May, the dimensions (length and width) of the acrosome and head, the distance between the top of the acrosome and top of the head, the distance between the bottom of the acrosome and the bottom of the head, and tail length were measured using a disc ocular micrometer in the right

eyepiece, calibrated with a stage micrometer. From as many as 10 randomly located cells examined per duplicate slide, measurements were taken from only those cells which appeared "intact" for all treatments (Control n=179, DUF n=32, DTh n=16, GUF n=6, and GTh n=6). Data obtained from the control group were analyzed by date (n=22 sample days), by single analysis of variance (ANOVA) (Steele and Torrie 1960), and discriminate function analysis, Wilks' lambda, (Legendre and Legendre 1983) for differences among the dimensions of cell structures over time. Means, standard deviations (S.D.), and coefficients of variation (CV), were calculated for each morphological structure.

The effects of the treatments on cell dimensions were analyzed using multiple analysis of variance (MANOVA) (Legendre and Legendre 1983), and discriminant function analysis, Wilks' lambda.

Acrosome integrity. For all treatments, up to as many as 10 randomly located cells per duplicate slide were examined from 16 April to 27 May, 1985 (as described above). Each acrosome examined was subjectively evaluated and placed into 1 of 4 categories of integrity (Fig. 2):

1. intact (I)
2. swollen or loose (S)
3. partially detached or deteriorated (P)

4. completely detached (D).

A G-test (Sokal and Rohlf 1981) was performed on data from the control group to test for an effect of time on the percentage of acrosomes in all categories of integrity (n=24 sample days). G-tests were also performed to test for differences in the percentage of acrosomes in all categories, between the control and each treatment group, and between the 2 cryoprotectants after dilution and freezing (n=6 sample days). Log linear analyses (Sokal and Rohlf 1981) were conducted to test for treatment/date interactions.

Motility. The percent motility of spermatozoa in fresh and treated semen was subjectively estimated using phase-contrast microscopy (400X), on a scale of 0 - 100% by placing 6 ul of semen under a cover slip on a clean glass slide.

Paired comparison T-tests (Steele and Torrie 1960) were performed to test for differences in spermatozoal motility, between treatment groups 1 (Control) and 2 (DUF), 1 and 4 (DTh), 1 and 3 (GUF), 1 and 5 (GTh), 2 and 3, and 4 and 5.

Artificial insemination. Of a total of 55 unpaired females, ~~33~~ in laying condition served as recipients for AI. Each female beginning egg-laying was randomly assigned to 1 of the 5 treatment groups. To keep spermatozoa concentrations constant

the volume of semen to be inseminated was adjusted according to the dilution factor. Eight females received fresh semen (approximately 15 ul/AI), 6 females received semen from the DUF treatment group (approximately 30 ul/AI), 7 females received semen from the DTh treatment group (approximately 30 ul/AI), 6 females received semen from the GUF treatment group (approximately 60 ul/AI), and 6 females received semen from the GTh treatment group (approximately 60 ul/AI).

The AI technique has been described by Bird et al. (1976). Each female was inseminated after the first and every subsequent egg, within 2-6 hours following oviposition. The first 2 eggs were pulled from the nest box. The first, always infertile, was discarded. The second, usually present at an advanced stage of development at the time of the first insemination (as determined by palpation), was kept for artificial incubation, but not included in the fertility data. All subsequent eggs were left in the nest box until a clutch of 5-6 eggs was partially or fully completed. If natural incubation proceeded, the clutch was left with the female for 4 days before being pulled for artificial incubation. Otherwise the clutch was pulled for artificial incubation 4 days after the laying of the last egg. For females which recycled to lay a second

clutch following the removal of the first, the AI protocol and treatment for each bird was repeated.

Fertility was determined by candling the eggs (excluding the first 2 as mentioned above) after 10 days of incubation. Eggs candled as infertile were opened and macroscopically examined for the presence of a blastodisc and blood islands (Bird et al. 1984). A G-test was performed to test for differences among the fertility rates obtained from each treatment group.

Teaser females using nest boxes were allowed to lay full clutches and to incubate for 4 days. Eggs laid by these females outside of nest boxes were removed sequentially to avoid breakage. All eggs were removed to keep the females reproductively active and visible to the males.

RESULTS

Semen collection. The mean ± S.D. individual semen collection volume was 13.7 ±9.8 (n=835) ul. Overall semen production for each of the 3 groups of males is presented in Fig. 3. The mean ± S.D. volume of pooled semen for the birds located in A, B and C were 176.4 ± 78.9 ul (n=29), 116.3 ± 47.8 ul (n=29), and 117.7 ± 64.7 ul (n=26), respectively. Not all males donated semen on any given day throughout the study period. The number of donations for each of

the 3 locations ranged from 3 to N, where N is the total number of males in each group. The overall mean ± S.D. volume of pooled semen samples was 137.5 ± 70.3 ul (n=84). The mean ± S.D. concentration of spermatozoa in pooled samples was 33.0 ± 16.6 x 10^6/ml (n=77).

Morphology. The Wells and Awa stain (1970) was effective in the initial attempt to identify the acrosome and head of a kestrel spermatozoon. However, with either eosin B or phyloxin B, the stains were inconsistent in distinguishing cell structures. Fast green, with or without nigrosin, provided the most effective and consistent results and was used throughout the remainder of the study (Fig. 3, as indicated by arrows.)

The general morphology of the kestrel spermatozoon is illustrated in Fig. 4. There were no significant changes in the dimensions of the cell structures in the control group over the period of data collection (ANOVA, HL $p = 0.3464$, AW $p = 0.0867$, AL $p = 0.4659$, DT $p = 0.5158$, DB $p = 0.3735$, TL $p = 0.0782$, Wilks' lambda $p = 0.4359$), thus the results below were obtained from pooling all cells examined throughout the study period.

The mean ± S.D. of head length (HL) was 3.4 ± 0.7um (n=179, CV=21.1); acrosome width (AW) 2.7 ± 0.5um (n=176, CV=19.7); acrosome length (AL) 3.3 ±

0.7um (n=176, CV=20.9); distance between the top of the acrosome and the top of the head (DT) 0.8 ± 0.3um (n=160, CV=36.0); distance between the bottom of the acrosome and the bottom of the head (DB) 0.9 ± 0.4um (n=159, CV=41.1); and tail length (TL) 26.4 ± 5.6um (n=135, CV=20.3). Among all the treatments however, it was possible to discriminate intact cells from the GTh group from all other cells (Wilks' lambda p = 0.0003). The significant changes had occurred in the dimensions of the head length (4.4 ± 0.86um, n=6, CV=19.7, p = 0.0035), acrosome width (3.3 ± 0.9um, n=6, CV=26.0, p = 0.0005), and the distance between the bottom of the acrosome and bottom of the head (1.7 ± 0.5um, n=3, CV=31.6, p = 0.0011).

Acrosome Integrity. The percentage of acrosomes in all categories of integrity, for all treatments, are presented in Table 1. Significant changes in the frequency of acrosomes in each category of integrity, by date, were found within the control group (p = 0.0003). However, observation points plotted on scattergrams indicate that these differences may be random and are probably artifacts of smear preparations (Fig. 5). Among treatments, no treatment/date interactions were found to be significant (Control vs DUF, p = 0.9619; Control vs Dth, p = 0.8953; Control vs GUF, p = 1.0000; Control vs GTh, p = 0.9988), thus the results below were

obtained by pooling the data in each treatment group.

Treatment effects on acrosome integrity were found to be significant between the control group and a) GUF ($p = 0.0004$), where most acrosomes shifted from the intact category into the swollen or loose category, b) GTh ($p = 0.0001$), where the major shifts were from intact to swollen or loose and partially detached or deteriorated, and c) DTh ($p = 0.0335$), where acrosomes shifted from the intact to the swollen or loose category of integrity.

There was no significant difference between the control and DUF treatment groups ($p = 0.4123$). The percentage of the acrosomes found between the DUF and GUF treatment groups were significantly different ($p = 0.0001$), particularly in the category of swollen or loose acrosomes, as well as between the DTh and GTh treatment groups ($p = 0.0027$), where the greatest differences in the percentage of acrosomes were seen in all non-intact categories of integrity.

Motility. The mean percentage $\pm$ S.D. of spermatozoa motility was 63.2 $\pm$ 13.4% in the control group, 49.5 $\pm$ 13.2% in the DUF group, 13.0 $\pm$ 9.5% in the DTh group, 57.4 $\pm$ 13.5% in the GUF group, and 40.7 $\pm$ 12.6% in the GTh group. All mean motility values were significantly different from each other ($p = 0.0001$ for each pair-wise comparison).

Fertility. Fertility of each individual female, with insemination information, is given in Table 2 along with treatment totals. Fertility among some of the treatment groups were significantly different ($p = 0.0003$). Semen containing glycerol, either unfrozen or frozen-thawed, yielded the lowest fertility, while fresh semen and semen diluted with DMA yielded the highest. Semen frozen and thawed in the presence of DMA was not as effective for fertilization as its unfrozen counterpart or fresh semen, yet neither was it as ineffective as glycerol-containing semen.

DISCUSSION

By advancing the reproductive recrudescence of American kestrels by photoperiod manipulation, semen collection began 6 weeks earlier than in previous years (Brock and Bird MS). Semen production by males in all 3 locations showed a similar pattern (Fig. 3) throughout the study period. The pooled semen volumes differed with the number of donating males, however, no other effects were observed in semen characteristics which might be associated with difference in donor location. Interestingly, considerable decreases in the volume of pooled samples (Fig. 3) corresponded with periods of stormy weather, although Bird and Lague (1977) reported no

significant correlations between weather factors and semen characteristics.

Many factors besides photoperiod, however, may act as stimuli for the onset of breeding condition in birds (Wingfield and Farner 1980). For this reason, teaser females were allowed to freely associate with the tethered males and to have access to several nest boxes, in sight of the males, as an attempt to maximize fresh semen quality. Whether these factors actually acted as additional stimuli is unknown, however in one case, a teaser female laid 2 clutches of fertile eggs, and 2 other females laid 3 clutches of fertile eggs.

Prior to assessing pre-freeze and post-thaw effects on kestrel spermatozoa it was necessary to characterize the morphology of the cell. Unlike the spermatozoa of chickens (Gallus domesticus), turkeys (Meleagris gallopavo), or cranes (Grus sp.) which have elongated heads and small conical acrosomes (Grigg and Hodge 1949; Wakely and Kosin 1951; Russman 1981), the head of the kestrel spermatozoon was described by Bird and Lague (1977) as jellybean-shaped. In their study, Bird and Lague (1977) measured one cell under a scanning electron microscope and determined the head to be roughly 4 um long, 2 um wide, with a tail length of approximately 15-20 um. However, they were unable to identify the

acrosome. In the present study a more detailed and structured investigation was made to identify and obtain accurate measurements of the acrosome, head, and tail.

A differential acrosome stain was developed for several mammalian species by Wells and Awa (1970) for the purpose of assessing acrosomal characterisitics using light microscopy. The fast green FCF component effectively stains the acrosomes of spermatozoa from several domestic mammals a pale translucent green, while the eosin B stains the heads red. After examining several smears prepared with the Wells and Awa stain, the effect on mammalian spermatozoa was seen in only a few kestrel spermatozoa where the acrosomes had become partially detached from the heads. Overall, the differential stain was effective in the initial breakthrough of discovering the acrosome and its general morphology in relation to the head of the cell, but it was inconsistent for the purpose of obtaining structural dimensions. In most cases the entire cell would be stained by eosin B making it difficult to distinguish the acrosome. Phyloxin B, a stain with characteristics similar to eosin B, was also inconsistent. By substituting the head staining material with a background stain, nigrosin (or leaving it out completely), it was possible for the fast green FCF to consistently and

effectively stain the acrosome. In most cases, the uncovered portion of the head was also stained green, but the demarcation between acrosome and head remained well defined.

The dimensions of spermatozoon structures for several species are given in Table 3. The shape of the kestrel cell is elliptical, resembling mammalian spermatozoa rather than those of other avian species studied (Fig. 4). The similarity between the acrosomes of kestrel and bull spermatozoa is noteworthy. Hancock (1952) described the morphology of the living bull spermatozoon as having a closely applied acrosome cap (or plasma membrane) which overlays a tighter, more compact cap, the acrosome, and which, according to Saake and Almquist (1964), covers 55-60% of the anterior portion of the head. In a well- stained kestrel spermatozoon, the acrosome, which is nearly as long as the head, forms a tight fit over approximately 75% of the anterior portion of the head (Fig. 2a). This relationship made it impossible to obtain measurements of head width, as no demarcation could be seen between the borders of the head and the acrosome at the widest point of the cell. Furthermore, whether or not the kestrel acrosome described in this study is the acrosome as such, or is a structure homologous to the plasma membrane of the bull, is unknown.

Among all of the treatments, kestrel spermatozoa frozen and thawed in the presence of glycerol, showed a significant change in size. Harris et al. (1973) described a "ballooning" effect of fowl spermatozoa as a result of rapid freezing and thawing. A glycerolized diluent added to fresh semen was not detrimental to fowl spermatozoa (nor was it for the kestrel). However, the cryopreservative was not effective during freezing and thawing in preventing some morphological damage such as extensive swelling or disrupted cytoplasmic membranes. Furthermore, the heads became more globular in appearance, although the acrosomes tended to remain intact. Similar effects (distended membranes lengthwise along the cell, with some manifestations as bent or crooked necks) were also seen in spermatozoa of turkeys and muscovy drakes (Cairna moschata) (Marquez and Ogasawara 1977; Bakst and Sexton 1979; Maeda et al. 1984a,b). The changes seen in glycerol frozen-thawed kestrel spermatozoa can also be described as ballooning, as the heads had elongated and the acrosomes (which remained intact) widened, probably as a result of enlarging heads. According to Harris et al. (1973), the extent of damage to frozen-thawed spermatozoa depends on the type and concentration of cryopreservative used. They found that contrary to the inability of 8% or 16% glycerol to prevent

morphological damage, 8% dimethylsulphoxide (DMSO) provided some protection. Maeda et al. (1984a), on the other hand, found 1M glycerol to be superior to 3 other cryopreservatives at the same concentration, including DMSO. In this study there were no significant changes in intact kestrel spermatozoa protected with 12.6% DMA, before and after freezing. However, the number of cells per treatment decreased with dilution, the freeze-thaw process, and smear preparation (further dilution), making conclusive analysis difficult. Nevertheless, before it can be determined which cryoprotectant, glycerol or DMA, is better in protecting the morphology of kestrel spermatozoa, a closer examination of freeze-induced injury must be made.

As noted, the general shape of the kestrel spermatozoon is quite different from other avian species studied. Overall, the cell is very small in size, but the relatively large acrosome made it possible to investigate pre-freeze and post-thaw morphological damage associated with cryopreservation by use of light microscopy alone.

The presence and need of a trypsin-like enzyme in the acrosomes of avian spermatozoa for penetration of the vitelline membrane of the hens' ova during fertilization, was demonstrated by Palmer and Howarth (1973) and Langford and Howarth (1974).

Damage to the acrosome resulting in the leakage of this enzyme may become responsible for decreased fertility (Maeda et al. 1984b). This was found to be the case in mammals, as bulls with heritable acrosome abnormalities were those with a history of subfertility (Saake et al. 1968).

By studying freeze-induced injury to muscovy drake spermatozoa, Maeda et al. (1984b) found frequent incidences of abnormal acrosomes. In their studies, Maeda et al. (1984a,b), categorized acrosome integrity into 3 groups: separated, swollen, and other abnormalities, i.e. detaching, rough, and disintegrating. They suggested that swelling occurs first, leading to a partial detachment or disintegration of the acrosome before final separation occurs. Using glycerol as a cryopreservative, Maeda et al. (1984a,b) found for frozen-thawed chicken and muscovy drake spermatozoa, respectively, that 32.1% and 1.1% had suffered swollen acrosome damage, 37.6% and 19.6% suffered other abnormalities of the acrosome, and 30.4% and 2.2% of the acrosomes separated from the cells. In comparing the abilities of several cryoprotectants to prevent these freeze-induced injuries to acrosomes, Maeda et al. (1984a) found that overall, glycerol was superior to DMSO, ethylene glycol, and methylformamide. This finding was also supported by

the work of Westfall and Harris (1975) who compared the efficacies of the same cryoprotectants using the membrane digestion technique.

Acrosome abnormalities similar to those described above were observed in kestrel spermatozoa. The percentage of cells from the control group, which occurred in all categories of integrity, appeared to be significantly different at random throughout the study period. This finding may be an artifact of smear preparation, or may be due to pooling semen donated by individual males at different stages of reproductive condition, as no significant treatment/date interactions were found.

As suggested by Maeda et al. (1984b) for muscovy spermatozoa, the degree of injury to kestrel acrosomes increased from intact to swollen or loose. In the latter cases, the acrosome either appeared "bloated," thus rendering the top of the head invisible, or the acrosome had lost its secured appearance over the head. The next order of injury was a partially detached or deteriorated acrosome, which either swollen or with a disrupted membrane, covered less than 75% of the head. The highest degree of injury was a completely detached acrosome, where the unstained head appeared more spherical than elliptical with a shadow of the once present acrosome visible (Fig. 2).

Unlike glycerol, where many swollen or loose acrosomes were found, DMA did not cause cellular damage after dilution. Whether or not a swollen acrosome renders the spermatozoon incapable of fertilizing an egg is unknown. Although Maeda et al. (1984a) found a high percentage of swollen acrosomes in chicken spermatozoa in semen diluted with glycerol (32.1%), Westfall and Harris (1975) claim no damage to chicken spermatozoa under these conditions, based on the ability of the spermatozoa to digest membranes. Conversely, Hancock (1952), described "dead" bull spermatozoa as having lost the definite outline of the head, and the acrosome as having an irregular outline, loosely covering the head. It may be supposed that partially detached or deteriorated and detached acrosomes do render spermatozoa incapable of starting or completing the fertilization process.

In assessing freeze-induced injury, neither glycerol nor DMA were effective enough to prevent some morphological damage to the kestrel acrosome. Comparatively, more intact acrosomes became swollen or loose, and partially detached or deteriorated when frozen in the presence of glycerol than with DMA. However, when considering the difference in the percentage of detached acrosomes between frozen-thawed treatments, it should be clarified at this

point that in many cases, semen containing glycerol did not dry properly during slide preparation, resulting in "blurred" smears. The number of spermatozoa with detached acrosomes (the most difficult state of integrity to evaluate unless a clear, well-defined specimen is located) were low in the glycerol treatments (Table 1), and this may be reflected as a bias toward the ratio of acrosomes found in other categories of integrity. Nonetheless, DMA showed an overall superiority to glycerol in protecting the morphology of kestrel spermatozoa throughout the various steps of cryopreservation.

Besides having intact acrosomes the spermatozoa must also be motile enough to reach the ova from the intravaginal site of insemination. The initial motility of spermatozoa in fresh semen was suppressed as a result of dilution, with further decreases after freezing and thawing. Differences were also seen in the effectiveness of either of the 2 cryoprotectants in preventing motility loss. A greater percentage of cells remained viable when treated with glycerol than when treated with DMA, at both steps of the cryopreservation process. This finding was supported by the work in preliminary research trials (Brock and Bird MS). Furthermore, although DMA was not tested, Westfall and Harris (1975) found similar results in that glycerol was superior to other cryoprotective

compounds, when the ability of these agents were evaluated for preventing motility loss before and after freezing. Therefore, as suggested by Maeda et al. (1984a), a compound capable of protecting one part of the cell, e.g. the acrosome, does not necessarily protect another, e.g. the midpiece, which generates spermatozoa motility (Marquez and Ogasawara 1977). Although the midpiece was not identifiable there were definite differences in the abilities of either glycerol or DMA to protect motility, with glycerol being superior.

The best criterion for evaluating the effectiveness of glycerol and DMA in preventing pre-freeze and post-thaw damage to kestrel spermatozoa, is the retention of fertilizing capacity. Fertility may be directly correlated with motility and morphological integrity, as without motility the cells cannot traverse the female tract to reach the ova, or once there, cells without intact acrosomes may not be able to penetrate the vitelline membrane for fertilization. Unfortunately, such direct correlations were not possible in the design of the present study. However, fertility achieved by fresh semen, diluted semen containing either glycerol or DMA, and frozen-thawed semen containing either glycerol or DMA can be compared.

Fertility achieved with fresh semen in the

present study (Table 2) was similar to that achieved overall by AI by Bird et al. (1976). Interestingly, a factor which may be attributed to these relatively low fertility rates is perhaps inherent to normal kestrel semen composition. Bird and Lague (1977) described kestrel semen as clear to deep amber in color, with the concentration of "debris" increasing as color intensified. They also noted lowered motility of spermatozoa in deep amber semen. In the present study frequent observations were made of spermatozoa being engulfed by large spherical cells in the semen. These large spherical cells, definitely not spermatozoa, comprise the debris referred to by Bird and Lague (1977), and are probably macrophages. A positive association of abnormal spermatozoa and macrophages was found in yellow-colored semen of turkeys (Marquez and Ogasawara 1975; Thurston et al.1975) and guinea fowl (Numidia meleagris) (Thurston et al. 1982). A decrease in fertility from 92% down to 57% in turkeys was attributed to the presence of abnormal cells and lowered spermatozoa concentration in yellow semen (Marquez and Ogasawara 1975). What actually causes the yellow semen syndrome in turkeys and the presence of a large concentration of macrophages in such semen is unknown. Marquez and Ogasawara (1975) made reference for the aid of macrophages in the disposal

of unejaculated spermatozoa remaining in the reproductive tract. This may apply to the kestrels in this study, for if ejaculation was not forced by massage, the semen would not be used by these otherwise non-breeding males.

Although motility was suppressed to some degree by diluting semen in the presence of DMA, fertility was comparable to that obtained with fresh semen as acrosome integrity remained unaffected. However, motility loss after freezing is most likely responsible for a subsequent decrease in fertility. Even though some significant changes in acrosome integrity were not prevented by DMA during freezing, the effect was not as drastic as the reduction in motility.

Unlike DMA, the absolute fertilities achieved with semen in the presence of glycerol, either unfrozen or frozen-thawed cannot be solely associated with reductions in either motility or acrosome integrity of spermatozoa. Glycerol in semen is known to have a contraceptive effect in the female reproductive tract if not reduced in concentration prior to insemination (Lake et al. 1980). Nevertheless, on a relative basis, the post-thaw decreases in motility and acrosome integrity of spermatozoa may not be of concern, as fertility remained unaffected by the freezing process. It may

be deduced therefore, that fertility was affected primarily by the impaired-fertility phenomenon of glycerol.

To summarize, by evaluating pre-freeze and post-thaw effects of DMA and glycerol on the spermatozoa of the American kestrel, each compound may have acted cryoprotectively on different parts of the cell. DMA was more effective than glycerol in overall protection of spermatozoa morphology, leaving fertility dependent upon the chance of a relatively few viable, motile cells reaching the ova. On the other hand, glycerol was superior to DMA in protecting spermatozoa motility, leaving fertility dependent upon the integrity of the cells reaching the ova. Until modifications can be made in the poultry technique for reducing the glycerol concentration in kestrel semen (Brock and Bird MS), fertility achieved is not worthwhile for practical routine use of glycerol for cryopreservation. However, Lake and Ravie (1984) are encouraged with DMA as a possible alternative to glycerol in protecting fowl spermatozoa during cryopreservation, and the results are encouraging for its use with falcon semen. By taking extreme care in handling kestrel semen with DMA during the various steps of cryopreservation, sufficient motility can be maintained in frozen-thawed semen to improve

fertility.

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Table 1. Frequency (N) and percentage of acrosomes in all categories of integrity for all treatment groups.

	Intact	Swollen/ Loose	Partially detached/ Deteriorated	Detached
Control[a]	58.7	25.6	9.9	5.8
N	71.0	31.0	12.0	7.0
DUF[a]	54.6	26.9	8.4	10.1
N	65.0	32.0	10.0	12.0
DTh[b]	49.6	36.1	5.9	8.4
N	59.0	43.0	7.0	10.0
GUF[c]	49.2	44.3	6.6	0.0
N	30.0	27.0	4.0	0.0
GTh[d]	41.1	41.1	16.4	1.4
N	30.0	30.0	12.0	1.0

Treatment groups with different superscripts are significantly different ($p<.05$).

Table 2. Artificial insemination and fertility results for individual female kestrels in all treatment groups.

Treatment	Female #	% Fertility (#fertile/total)	Mean ± S.D. Conc./AI x 10^8	Mean ± S.D. % Motility/AI
Control	1119	50.0 (1/2)	0.7 ± 0.5	55 ± 7.1
	1300	67.7 (2/3)	0.7 ± 0.1	50 ± 10.0
	1080	0.0 (0/1)	1.0 ± 0.0	75 ± 0.0
	1227	67.7 (2/3)	0.9 ± 0.6	40 ± 26.4
	1204	40.0 (2/5)	0.9 ± 0.3	63 ± 14.0
		50.0 (1/2)*	N.D.	55 ± 7.1
	1208	100.0 (1/1)	1.2 ± 0.0	50 ± 0.0
	1132	0.0 (0/1)	0.7 ± 0.0	50 ± 0.0
	1108	80.0 (4/5)	0.5 ± 0.2	67 ± 15.7
Total		56.5 (13/23)[a]	0.7 ± 0.3	57 ± 15.9
DUF	1230	83.3 (5/6)	0.5 ± 0.2	48 ± 24.9
		50.0 (2/4)*	0.5 ± 0.1	57 ± 5.8
	839	50.0 (1/2)	0.5 ± 0.0	50 ± 0.0
	1097	50.0 (1/2)	0.6 ± 0.0	65 ± 21.2
	1134	0.0 (0/3)	0.6 ± 0.4	53 ± 5.8
	1259	67.7 (2/3)	0.7 ± 0.1	68 ± 7.6
		0.0 (0/2)*	0.6 ± 0.0	65 ± 21.2
	1253	100.0 (1/1)	0.6 ± 0.0	50 ± 0.0
Total		52.2 (12/23)[a]	0.6 ± 0.1	56 ± 15.6
Dth	1071	25.0 (1/4)	0.6 ± 0.3	16 ± 8.5
	1143	33.3 (1/3)	0.7 ± 0.4	13 ± 5.8
		0.0 (0/2)*	0.0 ± 0.0	18 ± 10.6
	1178	50.0 (2/4)	0.7 ± 0.1	16 ± 7.5
	A228	33.3 (1/3)	0.4 ± 0.2	12 ± 7.6
	562	0.0 (0/2)	0.5 ± 0.0	18 ± 17.7
	1133	0.0 (0/3)	1.0 ± 0.3	18 ± 7.6
	1104	100.0 (2/2)	0.6 ± 0.3	10 ± 0.0
Total		30.4 (7/23)[b]	0.7 ± 0.3	16 ± 7.7

* Females had recycled after removal of first clutch.
N.D. Not determined.
[a, b] Totals with different superscripts are significantly different ($p<0.05$).

Table 2. Continued.

Treatment	Female #	% Fertility (#fertile/total)	Mean + S.D. Conc./AI x 10^8	Mean + S.D. % Motility/AI
GUF	1024	16.7 (1/6)	0.8 ± 0.2	53 ± 13.7
		25.0 (1/4)*	0.8 ± 0.2	69 ± 10.3
	1019	0.0 (0/1)	0.2 ± 0.0	50 ± 0.0
	1218	0.0 (0/1)	0.5 ± 0.0	40 ± 0.0
	1005	0.0 (0/2)	0.9 ± 0.0	50 ± 0.0
	1255	0.0 (0/4)	0.6 ± 0.1	75 ± 15.0
	1020	25.0 (1/4)	0.5 ± 0.1	58 ± 5.0
Total		13.6 (3/22)c	0.6 ± 0.2	60 ± 13.9
GTh	1256	0.0 (0/4)	0.2 ± 0.1	28 ± 15.0
		0.0 (0/1)*	0.3 ± 0.0	35 ± 0.0
	1182	0.0 (0/3)	0.5 ± 0.3	47 ± 5.8
	1092	33.3 (2/6)	0.6 ± 0.2	47 ± 5.2
		0.0 (0/2)*	0.9 ± 0.2	53 ± 17.7
	1096	20.0 (1/5)	0.5 ± 0.2	40 ± 0.0
	1072	0.0 (0/5)	0.5 ± 0.0	52 ± 4.5
	982	0.0 (0/4)	0.4 ± 0.1	44 ± 7.5
		25.0 (1/4)*	0.6 ± 0.2	28 ± 9.6
Total		11.8 (4/34)c	0.5 ± 0.2	42 ± 11.7

* Females had recycled after the removal of the first clutch.
c Totals do not differ significantly ($p>0.05$).

Table 3. Morphological measurements in um of spermatozoa from selected mammalian and avian species. A dash indicates no value given in the reference.

Species	Head L	Head W	Acrosome L	Acrosome W	Tail L	Reference
Chicken	14.0[a]	0.5[a]	1.3-1.5[a]	0.1[a]	90.0[b]	[a] Grigg and Hodge 1949 [b] Lake et al. 1968
Turkey	9.1	0.8	1.8	-	61.0	Marquez and Ogasawara 1975
Guinea Fowl	12.8	0.49	1.8	0.47	59.0	Thurston et al. 1982
Muscovy Drake	10.9	0.55	1.8	0.4	71.0	Maeda et al. 1984b
Bull	9.0	4.0	5.0[a]	-	44.0	Sorenson 1979 [a] Calculated according to Saake and Almquist 1964
American Kestrel	3.4	-	3.3	2.7	26.4	This study.

Figure 1. Experimental protocol for investigating the biological effects of DMA and glycerol on kestrel spermatozoa before and after freezing.

$\bar{X}$ ul Fresh Semen

↓

6 ul, Motility evaluation
10 ul, Fixed in 10 ul 1% gluteraldehyde
15 ul, AI

↓

$\bar{X}$ - 16 - 15 ul, Fresh semen[a]

↙ ↘

Fresh semen[a] /2

↓

Diluted 1:1,
Lake's DMA diluent

↓

6 ul,
Motility evaluation;
10 ul semen,
in 10 ul Fixative;
30 ul, AI

↓

Remaining semen frozen,
50-70 ul aliquots

↓

6 ul,
Motility evaluation;
10 ul semen,
in 10 ul Fixative;
30 ul, AI

Fresh semen[a] /2

↓

Diluted 1:3,
Lake's glycerol diluent

↓

6 ul,
Motility evaluation;
10 ul semen,
in 10 ul Fixative;
60 ul, AI;
12 ul,
Concentration
determination

↓

Remaining semen frozen,
80-100 ul aliquots

↓

6 ul,
Motility evaluation;
10 ul semen,
in 10 ul Fixative;
60 ul, AI

Figure 2. Categories of integrity of the acrosomes of kestrel spermatozoa. a. Intact, b. Swollen, c. Partially detached, d. Detached. Magnification is 5848X, (oil immersion).

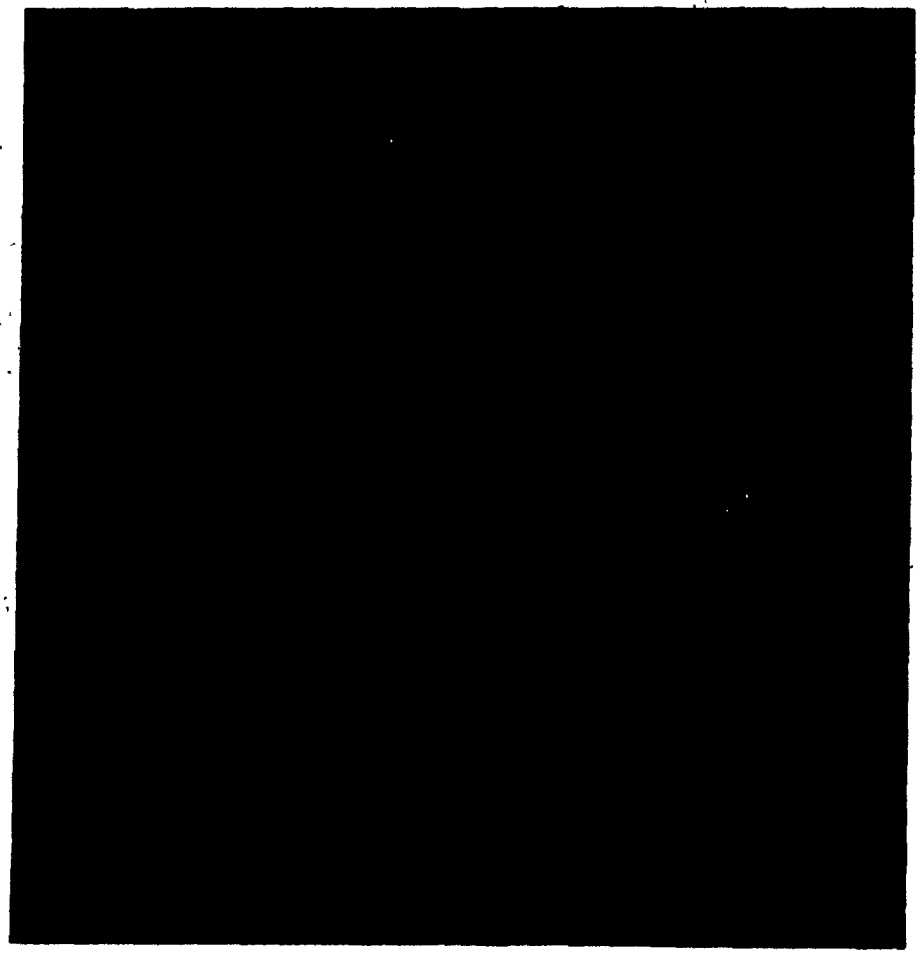


Figure 3. Semen production of kestrels in study locations A, B and C from March to June 1985. Values represent the volume of pooled individual semen samples collected throughout the study period.

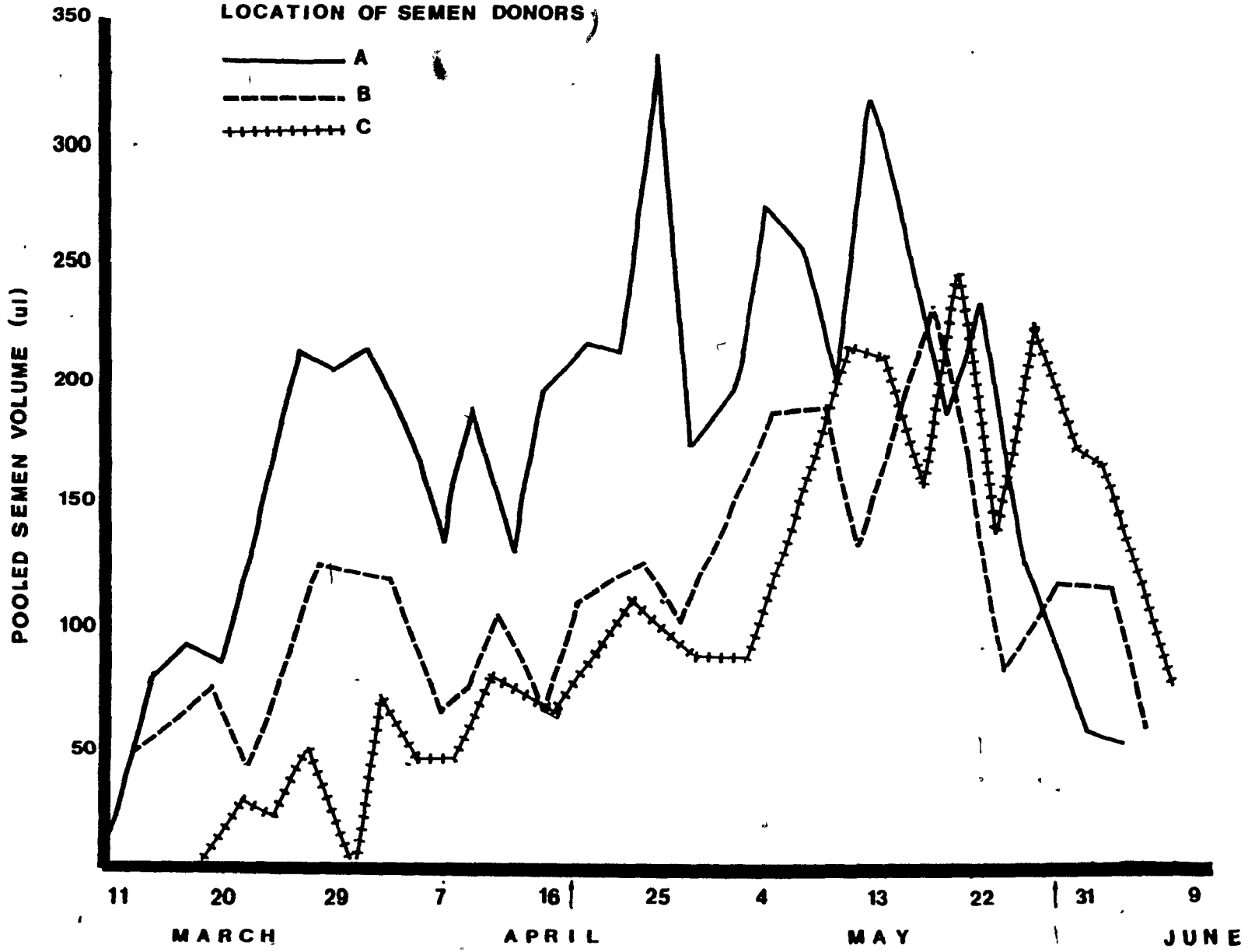
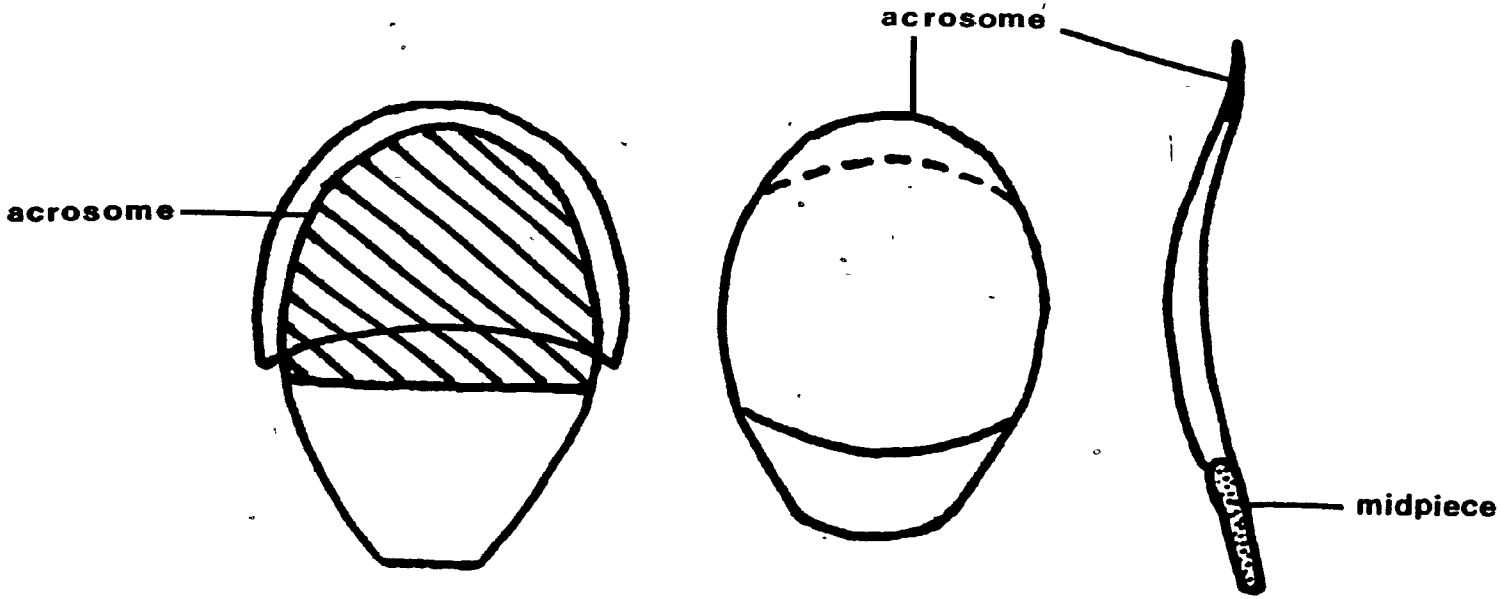

LOCATION OF SEMEN DONORS
A
B
C
POOLED SEMEN VOLUME (ul)
350
300
250
200
150
100
50
11
20
29
7
16
25
4
13
22
31
9
MARCH
APRIL
MAY
JUNE

Figure 4. Diagrammatic illustration of the general morphology of bull, kestrel, and chicken spermatozoa (minus tails). The dimensions of cell structures are located in Table 3.



Bull

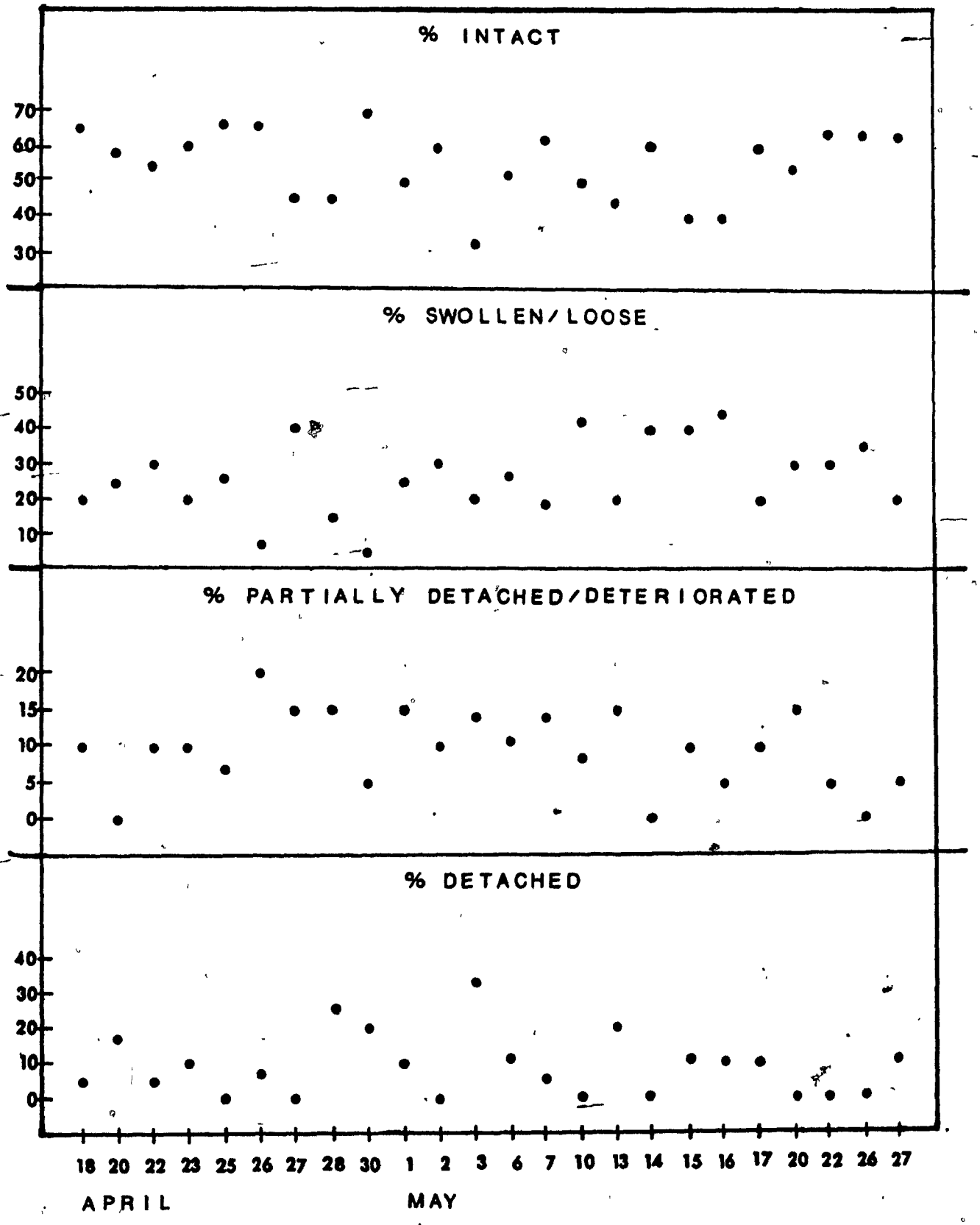
Hancock 1952

Kestrel

Chicken

Grigg & Hodge 1949

Figure 5. Scattergrams of observation points of acrosome integrity within the control group, over time. Values are percent cells found in each category of integrity, on each sampling day.


% INTACT
70
60
50
40
30
% SWOLLEN/LOOSE
50
40
30
20
10
% PARTIALLY DETACHED/DETERIORATED
20
15
10
5
0
% DETACHED
40
30
20
10
0
18 20 22 23 25 26 27 28 30 1 2 3 6 7 10 13 14 15 16 17 20 22 26 27
APRIL
MAY

GENERAL CONCLUSIONS

In a preliminary study, certain trends revealed differences in the effectiveness of several cryoprotective compounds to protect kestrel spermatozoa through the process of freezing and thawing. Glycerol, in Lake's diluent (at a ratio of 1 part semen to 3 parts diluent, 1:3) was superior to DMSO and DMA in protecting the motility of kestrel spermatozoa. However, DMA, in Lake's diluent (1 part semen to 1 part diluent, 1:1) was superior in protecting the fertilizing capacity of frozen-thawed kestrel spermatozoa.

Further evaluation, in a second set of experiments, revealed significant differences ($p<0.05$) in a) the ability of glycerol to protect spermatozoal motility through dilution and freeze-thawing of semen, and b) the effectiveness of DMA to protect the fertilizing capacity of spermatozoa in diluted and frozen-thawed semen. With subsequent characterization of kestrel spermotozoal morphology, DMA was more effective than glycerol in the prevention of morphological damage to spermatozoa in diluted or frozen-thawed semen.

In conclusion, fertility with semen containing DMA may be dependant upon the chance of motile cells reaching the ova, while fertility with semen containing glycerol may be dependant upon the

integrity of cells that reach the ova. However, the presence of glycerol in the semen at the time of insemination leads to low fertility, and due to the fragility of kestrel spermatozoa, removal of glycerol from the cells is not possible without investigations into an extraction procedure. As an alternative, DMA in Lake's diluent offers a degree of protection for the retention of some fertilizing capacity of frozen-thawed kestrel spermatozoa, and its potential for routine use in captive breeding programs deserves further investigation.