CRYOPRESERVATION OF PEREGRINE FALCON SEMEN AND POST-THAW DIALYSIS TO REMOVE GLYCEROL

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ABSTRACT — Peregrine Falcon (*Falco peregrinus*) semen was found to have a mean ejaculate volume, sperm concentration and initial sperm motility of 95 μl, 47 × 10^6 sperm/ml and 70%, respectively. When frozen in a medium containing 0.3, 0.9 or 1.48 M glycerol, post-thaw sperm motility was 29, 47 and 54%. Because of the contraceptive effect of glycerol, a dialysis procedure was developed to remove the cryoprotectant from post-thaw semen. Percent motility during post-thaw incubation was greater in samples from which glycerol had been removed by dialysis than in controls ($P < 0.05$). Of 6 eggs from a single ♀ American Kestrel (*Falco sparverius*) obtained after insemination with frozen-thawed, dialyzed peregrine semen, 2 were fertile and survived to pip. One interspecific hybrid was hatched and raised successfully.

Captive breeding has been used effectively for the conservation of birds of prey for many years. Artificial insemination has been a useful technique in captive breeding programs since the early 1970's (Weaver 1983). However, there are many situations in which the efficiency of a breeding program may be reduced because semen is not available at the time or place where it is needed. The ability to freeze raptor semen would facilitate captive breeding under these and other circumstances. The ability to freeze semen also would permit banking germ plasm from rare or endangered species.

At present, a limiting factor in the use of frozen semen in domestic avian species is the inhibition of fertility by the cryoprotectant in the medium (Brown and Graham 1971; Lake and Stewart 1978; Sexton 1979; Lake et al. 1980; Lake et al. 1981; Graham et al. 1982). In order to overcome this problem, cryoprotectants such as glycerol or dimethylsulfoxide (DMSO) must be removed post-thaw (Lake and Stewart 1978; Lake et al. 1981; Graham et al. 1982) or new, less problematic cryoprotectants must be identified.

The purpose of this study was to develop procedures for processing and freezing raptor semen, using the Peregrine Falcon (*Falco peregrinus*) as a semen source; and to develop a procedure for removing the cryoprotectant glycerol from thawed semen without further loss of sperm viability.

MATERIALS AND METHODS

Semen Collection and Handling. — Semen from 3 adult male Peregrine Falcons was collected up to 2 times/d (Boyd and Schwartz 1983) over a period of approximately 2 m. On each occasion, ejaculates from 1 - 3 ♂♂ were pooled. Semen was diluted 1:3 (v/v) at 20°C in 12 × 55 mm vials containing Lake's freezing diluent (Lake and Stewart 1978), placed directly into an ice water bath (0 - 2°C) and transported to the laboratory (see Table 1). All remaining steps up to freezing and during the thawing process were carried out at 4°C.

Semen Evaluation. — Sperm motility was assessed microscopically by estimating the percentage of sperm moving progressively forward (percent motility). Unfixed smears prepared from samples diluted in freezing diluent were placed on a slide warmer at 37°C for 30 sec immediately prior to evaluation. Percent motility was estimated in several microscopic fields to the nearest 5% using a phase contrast microscope at a total magnification of 400x.

Sperm concentration of the semen diluted 1:3 was determined with a hemacytometer after an additional dilution of 1:1 (v/v) in fixative (4% glutaraldehyde). Duplicate counts of each preparation were averaged for use in calculating sperm concentration of the original ejaculate.

Semen Freezing and Storage. — Aliquots of approximately 50 μl of cooled, diluted semen were placed into 0.25 ml French straws (IMV) for freezing. Diluent was aspirated into the straw ahead of the semen with a small air space separating the 2 liquids. Filling the straws in this way served to seal the polyvinylchloride (PVC) plug at the end of the straw without loss of semen and also prevented straws from floating when placed in liquid nitrogen. Straws were then sealed and loaded into a Planer R204 freezer (2°C). Semen was frozen in nitrogen vapor at 6°C/min to -180°C and then plunged into liquid nitrogen (Brock et al. 1984). One straw from each freezing procedure was then thawed in water (4°C) for evaluation of percent motility. Straws were wiped dry and the semen-containing portion was emptied into precooled tubes (6 × 50 mm). Remaining straws were stored for 1 - 2 months prior to thawing.

Initially, semen was frozen in diluent containing 0.3, 0.9 or 1.48 M glycerol. Percent motility was estimated on aliquots prior to freezing and immediately post-thaw. Based on these initial trials using different levels of glycerol in the freezing medium (Table 2), 1.48 M glycerol was selected for routine use in subsequent experiments.

Method for the Dialysis of Diluent and Diluted Semen. — Dialysis to remove glycerol from the freezing medium and thawed semen was carried out using semi-micro dialysis tubing (2.55 mm diameter, molecular weight cutoff of 12,000 - 14,000, Spectra/Por). Tubing was washed thoroughly in twice distilled water and stored wet at 4°C prior to use. All subsequent steps in the dialysis procedures were carried out at 4°C. Tubing was tied and cut into lengths of 8-10 mm from the tied end, filled with the freezing diluent (1.48 M glycerol) and equilibrated for 10-20 min in the same diluent. Diluent was then completely removed from the tubing and 50 μl aliquots of fresh freezing diluent or of thawed semen were pipetted into the tubing using a fire-polished 200 μl capillary pipet and a capillary suction apparatus (Clay-Adams). The tubing was then closed with a Spectra/Por closure and dialyzed with stirring against 500 volumes of Lake's thawing medium (Lake and Stewart 1978).

Table 1. Characteristics and pre-freezing treatment of Peregrine Falcon semen.

	VOLUME FOR COLLECTION PERIOD[a] (μl)			INITIAL MOTILITY (%)	SPERM CONCENTRATION (× 10^6/ml)	INTERVAL BETWEEN COLLECTION AND COOLING[c] (min)
	Individual Ejaculates	Pooled Ejaculates	All[b] Ejaculates			
$\bar{X}$	95.4	89.0	94.6	70.1	47.4	18.4
S.D.	51.7	38.5	49.7	6.9	16.1	6.8
Range	27-208	50-127	27-208	60-85	29-81	12-45
n	23	3	26	25	15	24

[a] Semen was collected between approximately 0830 - 0930 H and 1630 - 1730 H.
[b] Volumes for pooled ejaculates included 2 samples (partial or whole ejaculates) from 2 individuals and 1 sample from 3 individuals. Volumes are included for individual birds from 3 collection periods in which ejaculates were pooled prior to freezing.
[c] Intervals were timed from collection of the last ejaculate for pooled samples. Freezing was begun within approximately 15 to 30 min after initial dilution and cooling.

Estimation of the Efficiency of Glycerol Removal by Dialysis. — Removal of glycerol from the freezing medium was measured by supplementing the medium with [2-^{3}H]-glycerol (New England Nuclear, 200 μCi/μmol) at a level of 2 × 10^5 dpm/50 μl. Appearance of radioactive glycerol in the dialysate relative to the initial amount placed in the dialysis tubing was used to calculate the rate and extent of glycerol removal.

Preliminary trials (n = 2) indicated that > 99% of the glycerol in the freezing medium was removed after 30 min of dialysis. To establish the time-course relationship of glycerol removal, dialysis of freezing medium containing 1.48 M glycerol was carried out for 2 h against thawing medium containing no glycerol (n = 5). Input samples; 0.2 ml aliquots of the dialysate taken at 0, 0.25, 0.5, 1.0, 2.0, 5.0, 15, 30, 60 and 120 min of dialysis; and residual material in the dialysis tubing were analyzed for ^{3}H content (glycerol) by liquid scintillation spectrometry.

Evaluation of the Effect of Dialysis on Sperm Motility. — Because glycerol was removed so rapidly by the dialysis procedure, damage to sperm due to osmotic effects was considered a potential problem. Therefore, dialysis conditions were established to remove the glycerol more gradually. Material to be dialyzed was transferred at 15 min intervals to thawing solutions containing glycerol decreasing in equimolar increments (1.1, 0.74 and 0.37M and no glycerol). It was assumed that the rate of glycerol equilibration (and thus removal of glycerol from the thawed semen) in these steps was approximately equivalent to that observed in the one-step procedure, and the extent of total glycerol removal was calculated on this basis. An experiment was designed to assess the effect of the step-wise dialysis procedure on falcon sperm motility during post-thaw, post-dialysis incubation. A split-ejaculate technique was used in which all treatments within each experiment were imposed on aliquots of the same ejaculate. At the

Table 2. The effect of glycerol level on pre-freeze and post-thaw motility of falcon sperm. Values are percentages.

	PERCENT MOTILITY					
	PRE-FREEZE			POST-THAW		
Glycerol Level	0.3 M	0.9 M	1.48 M	0.3 M	0.9 M	1.48 M
$\bar{X}$	75	66	70	29	47	54
S.D.	4.1	4.9	7.4	11.0	2.6	5.8
Range	70-80	60-75	60-85	20-45	45-50	45-65
n	4	6	15	4	6	15

Table 3. Post-thaw motility of falcon sperm after dialysis to remove glycerol (n = 4).[a] Values are percentages.

	HOURS OF INCUBATION AFTER DIALYSIS					
DIALYSATE	0	0.5	1.0	1.5	4.0	$\bar{x}$*
1.48 M Glycerol	41	26	24	25	13	26
Four-step Procedure (1.1 M to Glycerol-Free)	43	33	35	31	20	32

[a] Post-thaw, pre-dialysis motility for these samples was 55±7%.
* $P < 0.05$

completion of dialysis, semen was emptied into tubes (6 × 50 mm) at 4°C. Percent sperm motility was determined immediately and after 30, 60, 90 and 240 min of post-dialysis incubation (38°).

Artificial Insemination. — Female peregrines were not available for testing the fertility of post-thaw, dialyzed semen. One unpaired ♀ American Kestrel (*Falco sparverius*) was inseminated with approximately 40 to 50 μl of thawed semen, dialyzed by the step-wise procedure. Six single inseminations were made within 4 h after oviposition and the first egg laid after each insemination was artifically incubated. Thawed samples were maintained at 4°C until the oviduct was everted for insemination. The semen was then transferred to an insemination syringe and deposited into the oviduct (Weaver 1983). Total time between thawing and insemination including dialysis was approximately 90 min.

Statistical Analysis. — Means and standard deviations were calculated for semen characteristics and for motility estimates on semen diluted and frozen in different levels of glycerol. The effects of dialysis on post-thaw motility were analyzed by analysis of variance after arcsin transformation of the percentage data.

RESULTS

Semen characteristics and information related to initial handling of semen are presented in Table 1. Semen was not scored on appearance, but only a low to moderate level of contamination by extraneous cell types and other debris was observed in the ejaculates used for freezing. Initial experiments in which glycerol was the only variable tested (Table 2) indicated that 1.48 M glycerol provided greater protection during freezing than 0.9 M or 0.3 M glycerol based on post-thaw sperm motility. However, glycerol levels were tested on separate semen collections so comparisons were not made on a statistical basis. The response to freezing, when using 0.3 M glycerol, was consistently poor; but the difference in post-thaw motility between 0.9 M and 1.48 M diluents was small, especially when expressed as the difference between pre-freeze and post-thaw motility (13 vs 16%).

Rate and extent of glycerol removal during a single step dialysis of samples are presented graphically in Fig. 1. Approximately 90% of the glycerol was removed by 15 min of dialysis, and after 30 min glycerol had been completely removed. Based on this rate of equilibration, a sample frozen in 1.48 M glycerol and dialyzed by the step-wise procedure was considered to contain less than 30 mM glycerol post-dialysis.

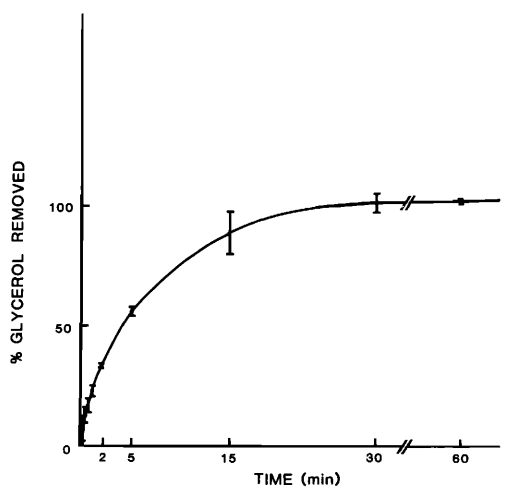

Figure 1. Removal of glycerol from semen diluent by dialysis. [^{3}H]-glycerol was added to diluent containing 1.48 M glycerol and the appearance of radioactivity in the dialysate was measured. Bars represent standard deviation for each time point (n = 5).

A comparison of post-thaw motility for sperm dialyzed by the step-wise procedure or directly against the freezing medium (1.48 M glycerol) is presented in Table 3. It is apparent that sperm survived the dialysis procedure with fair to good motility. During post-dialysis incubation motility declined with both treatments ($P < 0.01$). Although no significant time × treatment interaction was found, percent motility remained sufficiently higher after glycerol removal to demonstrate an advantage over the control treatment ($P < 0.05$). A consistent difference in the motility pattern was also observed between the dialysis treatments. Sperm from which glycerol had been removed exhibited a greater velocity, and motility was more progressive with less amplitude in the flagellar motion than with sperm in glycerol. This difference was not qualified but was readily apparent to other observers. Sperm in suspensions after glycerol removal also seemed more resistant to dessication on slides prepared for microscopic examination than those remaining in high glycerol medium, based on maintenance of motility.

Of 6 American Kestrel eggs potentially fertilized by frozen, thawed and dialyzed Peregrine Falcon semen, 2 eggs were fertile and developed to pip. One of these young died at pip while the other interspecific hybrid hatched and was raised successfully.

DISCUSSION

At present, there is very little detailed information on semen characteristics of raptorial species. This study provides such information on ejaculate volume, sperm concentration and percent motile sperm for semen from the Peregrine Falcon. Values for these characteristics have also been reported for the American Kestrel (Bird and Lague 1977). A comparison of the semen characteristics for these 2 species indicates an 8-fold greater ejaculate volume for the peregrine which approximates the difference in body weight between it and the kestrel. Sperm concentration is over 30% greater for the peregrine. The much greater total sperm/ejaculate for the peregrine may be a necessary adaptation for ensuring adequate sperm numbers at the site of fertilization in this larger species. Percent motile sperm appears to be slightly higher for the peregrine than the kestrel, but this may be due to differences in conditions under which sperm were examined. This type of information on semen parameters is necessary for making the most effective use of artificial insemination in the species of interest, and for effective processing of semen for cryopreservation. A knowledge of semen characteristics may also serve as a basis for comparison when examining the effects of environment or environmental contamination on reproduction (Bird and Lague 1977).

In the present study, we found that peregrine semen freezes well in Lake's diluent. It appears that a broad range in glycerol level might be acceptable, but more definitive work is required to establish the optimum glycerol concentration. Brock et al. (1984) reported excellent post-thaw motility for kestrel semen frozen in Lake's diluent, but fertility of the semen was < 5%. The requirement to remove glycerol and other cryoprotectants from post-thaw semen in order to obtain acceptable fertility has been established in domestic avian species (Brown and Graham 1971; Lake and Stewart *op. cit.*; Lake et al. 1980; Lake et al. 1981; Graham et al. *op. cit.*). This also may be true for falcon semen. Removal of glycerol from post-thaw cock semen by dilution and centrifugation greatly improves fertility (Lake and Stewart, *op. cit.*), but this approach is not practical when working with microliter quantities of falcon semen. Graham et al. (*op.cit.*) reported that the level of cryoprotectant (DMSO and ethylene glycol used in combination) necessary to maintain vigorous post-thaw motility of turkey semen depressed fertility. Use of dialysis to remove the cryoprotectant significantly improved fertility; although dialysis time, dialysate composition and pH, and semen-to-dialysate ratio all influenced the level of fertility observed. Dialysis can be adapted for use with the small semen volumes associated with raptorial species and is a milder approach for removing cryoprotectant.

Lake et al. (1980) demonstrated that in order to minimize its inhibitory effect on fertility in the Domestic Chicken (*Gallus* spp.), glycerol must be reduced to a level below 1% (0.11 M) in diluted semen. It is apparent from the present study that under the appropriate conditions glycerol can be reduced below the level of 1% within 30 min by dialysis. By controlling the sample volume/dialysate ratio or by adjusting the level of glycerol in the dialysate, rate of glycerol removal can be regulated to minimize the post-thaw to insemination interval necessary to remove cryoprotectant while maintaining optimal sperm viability. In this study, man-

ipulations required to transfer and dialyze the microliter volumes of frozen semen were carried out with only a small reduction in motility. Maintenance of post-thaw motility was slightly, but significantly improved with glycerol removal. The relevance of greater velocity in these samples is not readily apparent. However, differences observed in motility of sperm after glycerol removal may translate into enhanced sperm survival in the more favorable environment of the female reproductive tract.

The results of this study are based on a limited number of observations, leaving many questions regarding cryopreservation of falcon semen unanswered. However, several important points can be drawn from these results. Peregrine semen can be frozen using glycerol as a cryoprotectant with good post-thaw sperm motility, and the glycerol can be rapidly removed from post-thaw semen by dialysis without substantial loss of sperm motility. The techniques used in these procedures are simple, relatively inexpensive, and can be adapted for practical application. Finally, the development of 2 kestrel eggs in a clutch of 6 suggests that post-thaw dialysis is potentially useful for successful breeding with frozen falcon semen. Use of homologous species for insemination may provide a more useful measure of fertility. Refinement of these procedures and use of additional females to test fertility will help to establish whether post-thaw glycerol removal will make the use of frozen semen a practical approach to captive breeding of falcons and other birds of prey.

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