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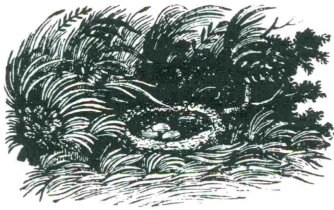

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Male Noble Macaw *(Ara nobilis cumanensis* or *Diopsittaca nobilis cumanenis),* also known as Red Shouldered Macaw distinguished by a horn colored upper mandible
Male Severe or Chestnut Fronted Macaw *(Ara severa)*
Male Yellow-collared Macaw *(Ara auricollis)*
Male Illiger's Macaw *(Ara maracana)*
Female Medium Sulphur-crested Cockatoo *(Eleonora) (Cacatua galerita eleonora)*
Male Brazilian Crested Cardinal *(Papoaria cucullata)*
Female Green-winged Macaw
Female Scarlet Macaw
Female Blue and Gold Macaw ●

Cryogenic Preservation of Budgerigar Semen

by Tim Hargrove
College Park, Maryland

Popularity of pet birds has increased tremendously in recent years, resulting in the importation of large numbers of birds, especially psittacines. During this same time period, there has been an unprecedented increase in the rate of habitat destruction in the tropics. The combination of these two factors has caused a decrease in the number of individuals in many wild psittacine populations.

It would be very beneficial to find ways of increasing the productivity of captive birds and, simultaneously, increase their genetic diversity. Cryogenic semen preservation is one way of achieving these goals. A frozen sperm bank could ensure the maintenance of a large degree of genetic diversity, should individuals be lost, and it is cheaper than maintaining large, captive populations. A sperm bank would also decrease the need to import large numbers of individuals and thus aid in maintaining wild populations. A few individuals could occasionally be captured to supply "new blood" to the captive flock or, if possible, semen samples could be collected from individuals in the wild instead of importing the birds.

Photos by Tim Hargrove

Massage holding position using thumb and forefinger on sides of abdomen.

Collecting budgie semen.

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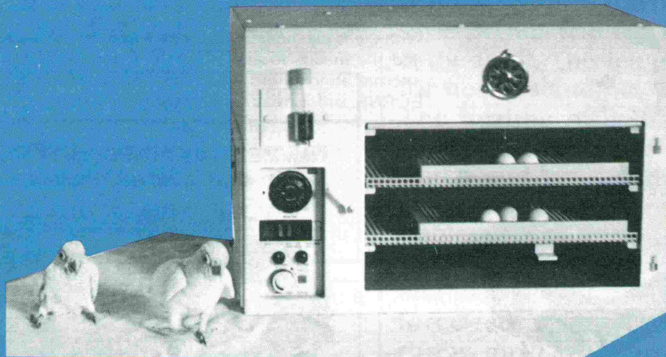

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The freezing of avian semen was first attempted in 1941 using chickens. In the years that followed, the study of cryogenic preservation of avian semen was limited to domesticated species, primarily chickens and turkeys. In 1978, it was reported that Greater Sandhill Cranes *(Grus canadensis tabida)* had been produced using frozen-thawed semen. Semen freezing has also been achieved in the Red-crowned *(G. japonensis),* White-naped *(G. vipio),* Whooping *(G. americana) Cranes,* and Florida and Mississippi Sandhill Cranes *(G. canadensis pratensis* and *G.c. pulla,* respectively), Peregrine Falcon *(Falco peregrinus)* and the American Kestrel *(F. sparverius).*

The procedure for freezing avian semen is basically the same for all species. Once the semen is collected and its volume determined, it is diluted with a diluent or semen extender, which has an osmolality and pH similar to that of avian seminal plasma (the fluid produced by the males in which the sperm cells are suspended). Dilution serves to prolong sperm viability during *in vitro* storage. Diluents should consist of an exogenous energy source, chelating agents to protect against toxic ions, a buffer, and a proper osmotic concentration. Most diluents used for freezing semen do not contain antibiotics, but if semen is stored above 0°C, these should be added. The typical constituents of poultry semen extenders are given in Table 1. Following mixing, and prior to freezing, the diluted sample is held at 5°C for a given length of time which is referred to as the holding time. This allows the spermatozoa and diluent to become thoroughly mixed.

At the end of the holding time a cryoprotectant (CP) is added; in birds, this is usually dimethylsulfoxide (DMSO). The CP aids in protecting the spermatozoa during the freezing and thawing process. The diluted semen with the CP is then held at 5°C for a given length of time to allow the CP to equilibrate the spermatozoa; this is referred to as the equilibration time. The spermatozoa are then frozen. The samples are thawed in a 2° to 3°C ice bath when it comes time to artificially inseminate a female.

There are several steps in the above procedure which are the same for most avian species. However, there are other steps which appear to be species- or genus-specific. The holding and equilibration temperature, the CP, the freezing rate from 5°C to -196°C (the temperature of liquid nitrogen), and the temperature at which the samples are thawed appear to be very similar for most avian species.

However, the diluent, dilution ratio (semen:diluent), final concentration of the CP in the diluted semen, and the holding and equilibration times appear to be species-specific.

In this study, the effects of two different diluents, three dilution ratios, two DMSO concentrations, and four holding and equilibration times on post-thaw motility of budgerigar semen were examined. The techniques used were similar to those used on poultry and crane semen.

The two diluents examined in this study were Beltsville Poultry Semen Extender (BPSE), which was used on Sandhill Cranes, and an acetate diluent, which yielded better fertility in poultry than most other poultry semen extenders.

The three dilution ratios were 1:1, 1:2 and 1:3 and the two DMSO levels were five and ten percent (v/v).

The holding and equilibration times that were tested were 15, 30, 45, and 60 minutes each since times of 15 to 60 minutes had been reported to be optimum for several different avian species.

Male and female budgerigars were housed independently of each other but were within visual and/or vocal contact. Samples were collected every other day from any given male since semen volume decreased if samples were collected on more than two consecutive days or if the samples were not collected for more than one week.

Semen was collected by the massage technique into nonheparinized microhematicrit capillary tubes. Two or more samples from different males were pooled to give a volume of at least 0.006 ml prior to dilution. The samples were then transferred to a 0.250 ml polystyrene microcentrifuge tube and diluted with either BPSE or the acetate diluent, in ratios of 1:1, 1:2 and 1:3. The diluted samples were then transferred to a

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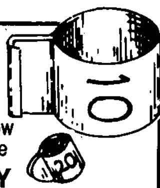

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5 °C refrigerator where they were held for 15, 30, 45 or 60 minutes, after which the CP (DMSO) was added in bulk (5 or 10% v/v). The samples were then allowed to equilibrate for 15, 30, 45, or 60 minutes before being transferred to the cryogenic chamber where they were cooled at a rate of 1 °C/minute from 5 °C to -25 °C, and then plunged into the liquid nitrogen and kept frozen. All samples remained frozen at -196 °C for at least 24 hours before thawing. This freezing rate differs slightly from that used on other avian species.

Preliminary trials indicated that dilution ratios of 1:2 and 1:3 in combination with 10% DMSO were yielding better post-thaw motilities than 1:1 with 10% DMSO or any of the dilution ratios using 5% DMSO. Therefore, all subsequent semen samples were frozen using dilution ratios of 1:2 and 1:3 with 10% DMSO.

A total of 64 different combinations were tested involving the two different diluents (BPSE and acetate), two dilution ratios (1:2 and 1:3), and four different holding and equilibration times. Five of these combinations (treatments) yielded post-thaw motilities greater than or equal to 70% (70% or more of the spermatozoa showed forward movement when thawed; Table 2). All of these five treatments were the result of using a 1:2 dilution ratio and four of the five treatments used the acetate diluent. Holding and equilibration times for these five combinations were

Table 1
Common constituents of poultry semen extenders*

Primary Function	Constituent
Buffer	TES, Phosphates (Na + or K +), TRIS
Energy source	Fructose, Glucose, Inositol, Raffinose
Chelator	Glutamate, Albumen, Milk
Osmotic balance	Magnesium chloride and sodium acetate Potassium citrate and sodium chloride
Antibacterial	Gentamycin, Penicillin, Streptomycin

*Sexton, T.J. 1979. Preservation of poultry semen — a review. In H.W. Hawk, ed. Beltsville Symposium on Agricultural Research 3:159-170.

Table 2
The five treatments which yielded mean motilities equal to or greater than 70%. Motility expressed as mean percent (± 1 SD).
HT = Holding Time; ET = Equilibration Time.

DILUENT	MEAN (%)	HT (min)	ET (min)
BPSE	77 (13)	45	45
Acetate	72 (13)	30	30
	70 (5)	45	45
	75 (10)	60	45
	72 (23)	60	60

between 30 and 60 minutes. These five treatments were determined not to vary significantly from each other.

Of the variables tested, the diluent and dilution ratio appear to have the most significant effect on post-thaw motility, followed by equilibration time. The holding time did not appear to have a significant effect. The four variables (diluent, dilution ratio, and holding and equilibration time), however, have a significant interaction, i.e. one variable will have an effect on one or more of the other variables.

Since this research has been completed, Samor et al. (1988) achieved a fertility rate of 66.6% in budgerigars using frozen-thawed semen. The technique they used was similar to that used for the Peregrine Falcon and the American Kestrel.

These relatively simple techniques could be applied to any avian species. The equipment necessary to freeze semen would cost less than $10,000, which is cheaper than buying several pairs of the more expensive psittacine species. Many of the larger private breeders could afford to do this. If several breeders throughout the country had this equipment and they wanted some "new blood" in their breeding flock, they could ship frozen semen samples which would be cheaper and safer than shipping the individual bird.

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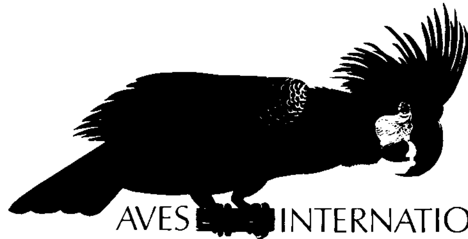

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