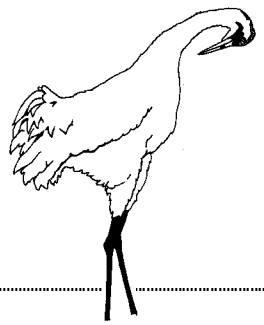
Special Techniques, Part B: Cryopreservation



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Chicken semen was first frozen in 1941 (Shaffner et al. 1941). Since that time, frozen semen has produced fertile eggs from various species of raptors (Brock 1986; Parks et al. 1986; Gee et al. 1993), cranes (Gee et al. 1985; Gale 1987; Hargrove et al. 1993 unpubl.), geese (Gee and Sexton 1990), and psittacines (Hargrove 1986; Samour et al. 1988).

Maximizing fertility and geneticdiversity are important forbreedingendangeredspecies incaptivity, andcryogenic preservationof semen canaccomplish both. If gamete productionis asynchronous,the female may beartificially inseminatedwith frozen-thawedsemen samples(Fig. 11B.1). Furthermore, frozen semen bankscan protect the foundergenepool forgenerations.

Fig. 11B.1 *Bruce Williams holding the first crane chick, a Greater Sandhill Crane, ever produced from frozen semen, 1978.*
Photo George F. Gee

Collection and Dilution

Avian semen was first collected by the massage technique in chickens (Burrows and Quinn 1939). This same technique has been applied to semen collection in other avian species including cranes (Archibald 1974; Gee and Temple 1978; Chapter 11A). Some research projects may require collecting semen by narrow-mouthed devices (e.g., a 0.25-mL caraway or a 0.35-mL natelson capillary tube), but normally, samples are collected in wide-mouthed devices such as a close-ended funnel (35-mm diameter cup with 50-mm stem; Fig. 11A.2) or some other similar glass container. Any surface which comes into contact with the semen should be clean, dry, sterile, and never contain soap residue. Immediately after collection, draw a tiny (ca 0.01 mL) sample into a capillary tube (as described in Chapter 11A). This tube is later examined for sperm concentration, motility, and for live-dead counts. Few cells in the tube die in the hour or less needed to return to the laboratory if the weather is cool or if the tube is stored in a cool container (ice bath or thermos). Transfer the ejaculate to a Pasteur pipet sealed at the small end. Determine the volume with an open-ended tom cat catheter (14 cm) attached to a 1-cc syringe. Dilute the ejaculate with one part crane semen extender (1:1), which is a modified version of the Beltsville Poultry Semen Extender (BPSE; Sexton 1977; Gee et al. 1985). Label the Pasteur pipet for identification and cover it with parafilm to prevent evaporation and contamination. These procedures are performed at ambient temperature.

Place the Pasteur pipet inside a larger test tube and transfer the two into an insulated ice bath (0-4° C). The water level in the ice bath should be sufficiently high to cover at least the lower portion of the tube containing the diluted ejaculate. The tube-within-a-tube arrangement slows the rate at which the ejaculate cools, thereby promoting survival of the spermatozoa. The top of the insulated container is closed between samples to maintain the desired temperature and to decrease the effects of airborne contamination and sunlight. Uncontaminated semen may be held in this manner up to 3 hours prior to freezing (Gee 1991).

Sample Preparation

Upon arrival at the lab, the 0.01-mL sample in the capillary tube is examined for motility, concentration, and urate contamination. Samples showing good concentration with little or no urate contamination,

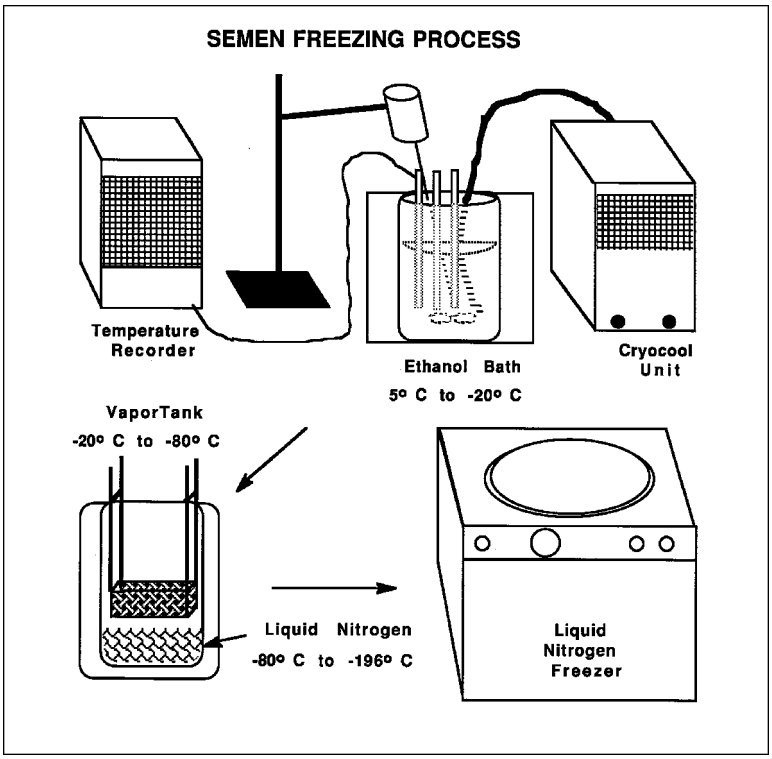


FIG. 11B.2 *Semen freezing process.*

irrespective of the degree of motility, are prepared for freezing.

As mentioned earlier, semen is diluted when collected with an equal volume of crane extender. Samples that will be frozen are diluted by one-half the volume of the diluted ejaculate with 24% dimethylsulfoxide (DMSO) in crane extender to get a final 8% DMSO concentration (one part semen, one part extender, and one part DMSO extender). When adding the DMSO, the 24% DMSO in crane extender and diluted ejaculate should be at the same temperature (0-4° C). DMSO helps protect the sperm from damage during freezing and thawing. For the maximum cryoprotectant effect with the least amount of cell toxicity, the ejaculate should equilibrate with DMSO for 15 minutes in the ice bath (Gee 1991).

While the semen sample is equilibrating, the ethanol bath (in which the straws containing the semen are placed for freezing) is cooled to 5° C. For the ethanol bath, we use a Neslab Agitanor (an insulated multipurpose bath with stirrer; see Appendix) which is cooled by a Neslab Cryocool Immersion Cooler CC-100 (a refrigeration compressor immersion probe; see Appendix). The stirrer in the ethanol bath maintains a constant temperature around each cane. (Canes are 29.3 cm long, semicircular with a flat top and bottom and are constructed of aluminum). A temperature probe is placed in the ethanol bath on a semen cane. The probe consists of a Type T bimetal thermocouple in a semen straw containing 8% DMSO in crane extender and is sealed on top with vinyl plastic putty (e.g., Critoseal, see Appendix). The thermocouple is attached to a temperature-recording unit (Honeywell Electronik III, Type T; see Appendix), a single pen strip chart recorder with a temperature range of 5° to –200° C.

Near the end of the equilibration period, heat-seal the straws on one end. These straws are either 0.2- or 0.5-mL and are labeled by male ID and colony. First, reserve a small residual semen sample (0.01 mL) for density and live-dead counts (these counts are performed after the samples are frozen). Then, transfer the remaining semen sample to the straws (using a tom cat catheter and a 1-cc syringe) and heat-seal the other end. The sealed straws are transferred to a consecutively labeled cane (e.g., cane 88-64 is the 64th sample frozen in 1988) and placed in the ethanol bath.

The first step of the freezing process (Fig. 11B.2) is cooling the samples in the ethanol bath from +5° to –20° C at a rate of –1° C/min. Second, cool the samples from –20° to –80° C at a rate of –50° C/min by placing them in liquid nitrogen vapor. This may be accomplished by holding the samples in the neck of the storage tank or by placing them in a vapor tank filled with 5 cm of liquid nitrogen. Third, plunge the samples into liquid nitrogen (–196° C; a freezing rate of –160° C/min; Gee et al. 1985). If a vapor tank is used in steps two and three, the samples should remain in the vapor tank for 5 min before transferring to the liquid nitrogen storage tank.

Determine sperm density using a hemacytometer. First, draw up a 0.01-mL subsample of ejaculate you prepared for freezing. Then dilute this 50:1 by adding 0.5 mL neutral formalin. You can purchase neutral formalin or prepare your own by adding 5 g sodium bicarbonate and 1 mL commercial formalin to 100 mL distilled water. Thoroughly mix the semen and formalin and allow to sit for 5 min. Mix the sample and place a small portion of this solution on a hemacytometer (Fig. 8.7). Wait 10-15 min for the sperm cells to settle into one focal plane (if more time is allowed

the sample may dehydrate). At 100x magnification, count the sperm cells within 5 of the 25 small squares in the l-mm square. Then calculate density with the following formula:

(No. Sperm Cells × Dilution × 4000)/No. Squares Counted = No. Sperm Cells/0.001 mL Ejaculate.

The number of smallest squares counted is 80 (5 × 16) if only one grid of the hemacytometer is counted and 160 if both sides are counted. To get the number of sperm cells/mL, multiply the calculated value by 1000.

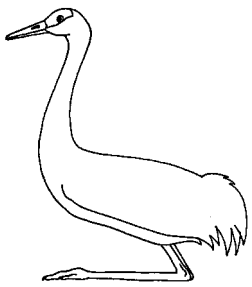
For example, if both sides of the hemacytometer add up to 145 sperm cells and the dilution is 200x, then the results are as follows:

(145 × 200 × 4000)/160 = 725,000 sperm/0.001 mL = 725 million/mL.

Knowing the original undiluted frozen volume, the percent live, and the density, one can calculate the number of live sperm cells in the freshly frozen sample. For example, if

0.05mL = original ejaculate volume,
725 million/mL = density, and
90% of the sperm cells were alive,
then 0.05 × 725,000,000 × .900 = 32,625,000 live sperm cells in the original sample.

Live-dead counts are made with 5% eosin and 10% nigrosin stain (Burrows and Quinn 1939; Hackett and Macpherson 1965). Place one drop of semen on a clean dry glass slide, one drop of 5% eosin next to the drop of semen, and 3 drops of 10% nigrosin next to the eosin. Mix the eosin and sperm and let sit for 5-10 seconds. Then mix with the 3 drops of 10% nigrosin, spread the sample like a blood smear, and air dry it quickly. Do *not* dry over a flame, but a flow of warm air (e.g., from a hair dryer) may be used. Glue a 24 × 50 mm coverslip to the slide with Permount (see Appendix), balsam, or other mounting medium. The slide can be examined now or later for live and dead sperm. Live sperm appear white against the blue (nigrosin) background; dead sperm appear red (eosin) or pink against the blue background. Determine percentage live by counting 300 sperm on each of three slides or 500 on each of two slides.



Use of Frozen Semen

To thaw frozen samples for insemination, transfer the canes from the storage tank to an ice bath (crushed ice saturated with water, 0.5° C) for 3-5 min. Remove the straw containing the thawed semen, dry the surface, and cut off one end. Transfer the thawed semen with a tom cat catheter attached to a 1-cc syringe from the straw to a closed end Pasteur pipet in an ice bath or keep in the syringe for insemination. Examine the samples for motility and live cells as described earlier.

Use the equivalent of two to four ejaculates for each insemination to compensate for the loss of cells during freezing (50-60%) and on the sides of tubes, straws, and transfer devices. To achieve good fertility, use 15-20 million live cells per insemination (Gee et al. 1985). Samples should be inseminated immediately after thawing and in the same manner as fresh semen.

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