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# Kinematic activity of gray wolf (*Canis lupus*) sperm in different extenders, added before or after centrifugation

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ABSTRACT

We evaluated two approaches to improving *in vitro* wolf sperm survival. Both approaches aimed to reduce the exposure of sperm to prostatic fluid resulting from electroejaculation: (1) use of extender formulations recently developed for the domestic dog (the most closely related domestic species); and (2) dilution of ejaculate shortly after semen collection. Three commercial extenders were compared with the TRIS-based extender we had previously used. We also compared the effects on motility of adding extender immediately after collection to our previous protocol in which extender was added after centrifugation. Both subjective and objective (computer-assisted semen analysis program) kinematic measurements were made. Relatively minor differences were noted (and not in total or progressive motility) between the centrifugation protocols. Two of the commercial extenders resulted in significant improvement in motility over the TRIS-based extender and one of the other commercial extenders at 8 hours after collection (mean ± SEM; total motility was 68.3 ± 4.0% and 70.0 ± 4.0% compared with 53.3 ± 4.0% and 55.0 ± 4.0%, respectively; progressive motility 58.6 ± 5.4% and 57.1 ± 5.4% compared with 32.8 ± 5.4% and 39.3 ± 5.4%; $P < 0.05$). We inferred that components in two of the commercial dog extenders might provide more protection for wolf sperm, prolonging their motility.


## 1. Introduction

The Mexican gray wolf (*Canis lupus baileyi*), a subspecies of the gray wolf, was listed as endangered by the US Fish and Wildlife Service in 1976. Considered extinct in the wild at the time of listing, reintroductions in Arizona, New Mexico, and the Sonoran region of Mexico have relied entirely on wolves bred in captivity. The current captive population consists of 308 wolves maintained in 53 zoos and related facilities in the United States and Mexico [1]. Because the entire current population of captive and free-ranging Mexican wolves are descended from six or seven founders [2], very careful genetic management is required to maintain population health. Each year, breeding pairs are selected based on kinship values to maintain or increase gene diversity and to avoid inbreeding. To preserve male genes from this limited population for future use, a frozen semen bank was created at the Saint Louis Zoo in 1991 under the auspices of the US Fish and Wildlife Service Mexican wolf recovery program. At present, this semen bank holds samples from 89 individual Mexican wolves.

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The objective of the semen bank is to serve as a genetic resource for eventual use in AI or other types of assisted reproduction. In effect, this extends the reproductive lives of individual wolves and allows them to be included in future genetic management. However, the usefulness of the semen bank is limited by the quantity and quality of the stored semen samples, so it is important to evaluate promising new methods that might enhance their reproductive utility.

During our more than 20 years of experience with Mexican wolf semen, we found that wolf sperm have shorter longevity and lose motility earlier than do dog sperm. This was surprising, because the gray wolf is widely considered to be the direct ancestor of domestic dogs [3], so wolf and dog sperm were expected to be similar. To begin to evaluate possible reasons for the difference, we used gray wolves, as a model for the Mexican wolf subspecies, and determined that one source of variability between dog and wolf sperm long-term motility was the semen collection method [4]. Because wolves cannot be handled without anesthesia, semen must be collected by electroejaculation, which prevents separation of the sperm-rich fraction from the prostate fluid; conversely, the prostatic fractions can be discarded during semen collection in the domestic dog. Considering the results of that study, wolf sperm longevity might be improved by methods that protect sperm from the adverse effects of prostate fluid. We decided to test two possible approaches: (1) evaluate newer semen extenders formulated specifically for domestic dogs; and (2) remove the prostate fluid sooner from the freshly collected sample.

The growing popularity of sperm cryopreservation and AI for the domestic dog has stimulated research and commercial development of improved products that can sometimes also be useful for nondomestic canids, e.g., wolves. Because of restricted access to Mexican gray wolves, we again used generic gray wolves as models for their endangered counterparts in this study. We chose three extenders to test with wolf semen. In experiment I, we compared three semen extenders to the standard TRIS/egg yolk extender we had previously used, and, in experiment II, we tested whether addition of semen extender soon after collection would improve sperm longevity.

## 2. Materials and methods

### 2.1. Animals

Because Mexican wolves are endangered, handling for semen collections must be minimized. Therefore, we typically evaluate new products and protocols with generic gray wolves before using them with Mexican wolf samples. For experiments I and II, we collected one ejaculate from each of six gray wolves at the Wildlife Science Center, Forest Lake, MN, USA, in January 2010. The wolves, ranging in age from 4 to 7 years, and weighing 40 to 50 kg, were maintained in outdoor enclosures with access to den boxes, fed carcasses of white-tailed deer, and given continual access to water. This project was approved by the Animal Care and Use Committee of the St. Louis Zoo.

### 2.2. Semen collection

Semen was collected under general anesthesia (5 mg/kg IM, ketamine hydrochloride [Ketaset; Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO, USA] and xylazine [0.5 mg/kg IM, Rompun; Bayer Corp., Shawnee Mission, KS, USA]) and maintained by supplemental ketamine (1.25 mg/kg IV or IM). Just before stimulation the urinary bladder was flushed with sterile saline to minimize urine contamination of samples. An 8-French polypropylene catheter (Sovereign; Tyco Kendall Healthcare, Mansfield, MA, USA) was coated with sterile lubrication (K-Y; Johnson & Johnson) and passed through the urethra into the bladder. After urine was aspirated, saline was repeatedly infused and aspirated until the effluent was clear.

Semen was collected by electroejaculation (Model 12 Electroejaculator; G & S Instruments, Midlothian, TX, USA) using a 3-cm diameter probe with three, 7.5-cm long, linear electrodes (PT Electronics, Boring, OR, USA) placed ventrally in the rectum, above the prostate and pudendal nerves. Stimulation was increased slowly until the hind-limbs extended, returned to zero, and repeated rhythmically at gradually increasing voltage, with a cycle of approximately 5 seconds, until ejaculation. Stimulation was not standardized by voltage but rather, to effect; that is, the amount of stimulation delivered was determined by the extent of response, which can be affected by depth of anesthesia. Maximum current did not exceed 300 mAmp (the amount of current reaching the tissues). Stimulation was repeated as long as the sample appeared cloudy, an indicator of sperm presence. The same operator performed electroejaculation on all wolves.

### 2.3. Experiment I: Comparison of four extenders

#### 2.3.1. Semen sample preparation

Immediately after collection, an aliquot was taken from each of the six ejaculates to calculate concentration (Makler Counting Chamber; Sefi-Medical Instruments, Haifa, Israel). Each sample was then split into four equal volumes and each was centrifuged at 700× *g* for 5 minutes. After centrifugation, the supernatant was discarded and the resultant pellet from each of the samples was gently resuspended in one of the four test extenders (CaniPro Chill 5 [Minitube of America, Verona, WI, USA], Fresh Express [Synbiotics, Kansas City, MO, USA], Kenney Formula [Reproduction Resources, Walworth, WI, USA], and a TRIS/egg yolk extender [containing TRIS, glucose, anhydrous citric acid, penicillin, streptomycin, milli-Q water, and 20% egg yolk]) as previously described [5] to a concentration of $50 \times 10^6$ cells per mL.

#### 2.3.2. Motility analysis

In experiment I, assessment of total and progressive sperm motility was conducted subjectively; the same observer assessed all samples starting immediately after centrifugation and resuspension with extender (Time 0) and at 1, 2, 4, 8, and 24 hours. Samples were kept at room temperature (26 °C) and just before motility assessment, a 0.5-mL subsample was warmed to 37 °C. To determine total motility, defined as the percentage of cells moving in any manner, the observer counted the first 20 cells lying along or crossing an imaginary line down the center of the field of view. This was repeated five times in random fields