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Characteristics of fresh and frozen–thawed red wolf (*Canis rufus*) spermatozoa

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Abstract

Ejaculates of the red wolf (*Canis rufus*) were evaluated immediately after collection and freeze–thawing to initiate a reproductive database for this endangered species. Electroejaculates from 13 adult red wolves collected during the breeding season (February–March; $n = 25$; 1–3 collections/male) had a mean volume of 4.7 ± 0.7 ml, $146.5 \pm 25.7 \times 10^6$ spermatozoa/ml and 71.2% motile spermatozoa. The mean proportion of cells with normal morphology was $73.6 \pm 3.2\%$ (range, 20.3–93.7%), with 64% of ejaculates (16/25) containing 70–90% normal spermatozoa. The four most predominant abnormalities were a coiled flagellum (8.1%), a bent flagellum (4.7%), a bent midpiece with no cytoplasmic droplet (3.3%;), and a detached head defect (6.4%). After cooling in glycerolated extender, semen was frozen using a pelleting method on dry ice before plunging into liquid nitrogen. Pellets were thawed in phosphate buffered saline and examined for % sperm motility, normal morphology, viability and intact acrosomes. There was a decline ($P < 0.05$) in sperm motility ($\sim 40\%$) and percentage of normal sperm (11.9%) after freezing, but no change in the proportion of viable cells. After freezing, there was a marked decline ($P < 0.05$) in the proportion of intact acrosomes from 74.5% to 55.5% which was accompanied by an increased proportion ($P < 0.05$) of partial acrosomes from 11.9% to 35.8%. These data demonstrate that, although red wolf spermatozoa can survive freeze–thawing using a technique common for domestic dog sperm, the finding of significant acrosome damage reveals (1) likely species

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specificity in the *Canis* genus and (2) the need for refining sperm cryopreservation technology for the red wolf.

Keywords: Red wolf; *Canis rufus*; Canine; Spermatozoa; Freezing

1. Introduction

During this century, members of the genus *Canis* have faced unrelenting human persecution and severe reduction of habitat in North America, leading to endangered classification of the grey wolf (*Canis lupus*), red wolf (*C. rufus*) and Mexican wolf (*C. lupus baileyi*) over large expanses of their historical range (Mech, 1970, 1982; Carbyn, 1987; U.S. Fish and Wildlife Service, 1990). In 1980, the red wolf was considered extinct in the wild, following planned removal of remnant individuals from its final range. The red wolf's long-term survival now depends on initiatives taken by conservation biologists from numerous government agencies and nongovernmental organizations (NGOs) through a combined captive breeding and reintroduction program (U.S. Fish and Wildlife Service, 1990).

Due to the small number of founder animals available to the captive breeding programme ($n = 13$), establishment of a genome resource bank (in the form of frozen/thawed semen) and the use of artificial insemination could assist in maximizing genetic diversity while serving as a reservoir of genetic material once an animal has been released into the wild (Ballou, 1992; Wildt, 1992; Johnston and Lacy, 1995). Therefore, efforts to develop assisted reproductive technologies have been recommended by the Red Wolf Species Survival Plan (SSP)© and Recovery Plan, and initiated at the Point Defiance Zoo and Aquarium's Red Wolf Breeding Facility in Graham, WA, USA (U.S. Fish and Wildlife Service, 1990). Because only anecdotal and general information are available on the reproductive biology of this species, the specific objectives were to establish electroejaculate characteristics for the red wolf and to evaluate the effects of established domestic dog (*C. familiaris*) commercial cryopreservation techniques on red wolf spermatozoa.

2. Materials and methods

2.1. Animals

Adult, male red wolves ($n = 13$, five of which were proven breeders) were maintained at the Red Wolf Breeding Facility of the Point Defiance Zoo and Aquarium. Animals were housed in conspecific pairs or with multiple conspecifics in large, outdoor pens (15 m × 31 m–31 m × 31 m) that contained natural substrate, foliage and concrete dens (239 cm × 172 cm × 147 cm) with bedding material. Wolves were fed a commercially-available, dry dog food daily and water ad libitum. Semen collections were attempted from 3 January 1995 through 31 March 1995, ranging from one to three collection attempts/animal, with a total of 25 ejaculates collected.

2.2. Semen collection, freezing and evaluation

Males were fasted 1 day prior to anaesthesia/electroejaculation. On the day of semen collection, each male was captured, transferred to a wire crate (94 cm × 58 cm × 61 cm) for holding and transported to an indoor collection site. Wolves were anaesthetized using 175.0 mg Telazol (tiletamine hydrochloride and zolezapam, Fort Dodge Laboratories, Fort Wayne, IN, USA) administered i.m. by hand syringe. Before semen collection, the length and width of each testis was recorded using measuring calipers, the penis was catheterized with a 5 Fr, 55.8 cm long polypropylene catheter (Sherwood Medical, St. Louis, MO, USA) and the bladder drained of urine. Testis volume was estimated based on the formula for a cylinder with spherical ends ($[\pi \text{width}^2 * (\text{length} - \text{width})/4] + [\pi \text{width}^3/6]$, Oberg et al., 1986).

Electroejaculation was performed using a P.T. Electronic Model 302 ejaculator and no. 4 (1.6 cm diameter) rectal probe (P.T. Electronics, Boring, OR, USA), based on the methods of Platz and Seager (1978). A set of three to five stimulation periods with voltages ranging from three to eight were used to achieve ejaculation, with each period consisting of multiple (~25–30) on–off stimuli and a 5–7 min rest between each period. Semen from each stimulation period was collected into plastic containers (there were no attempts to fractionate the ejaculates) and the total volume recorded. The voltage(s) that elicited ejaculation varied among individuals.

For each electroejaculate, semen from all series was pooled and evaluated for percent sperm motility (using subjective estimates) and concentration (using a haemocytometer method) based on methods described by Wildt et al. (1983) and Hay et al. (1997a,b). To permit morphologic and acrosomal evaluations, two aliquots (~10 μl each) of each ejaculate were placed on separate, clean glass slides (labelled A and B), and a smear of each was made by rolling the end of a Pasteur pipette through the drop and across the slide. After air-drying, slide A was stained with eosin/nigrosin (Society for Theriogenology, Hastings, NE, USA) to determine the % of viable spermatozoa (Christiansen, 1984), whereas slide B was stained with Spermac® (Stain Enterprises, Wellington, South Africa) to allow evaluating spermatozoal morphology and acrosomal integrity (Oettlé, 1986; Oettlé and Soley, 1988; Hay et al., 1997a,b). Evaluations were made by microscopic examination at 1000×. A total of 100 cells was counted from slide A; viable spermatozoa did not take up stain and were clear whereas dead spermatozoa stained purple. Morphological assessment and acrosomal status were both evaluated using Spermac® stained spermatozoa. A total of 300 spermatozoa from each ejaculate was evaluated for morphological characteristics, and the percentage of normal and each abnormality type was calculated; these spermatozoa also were evaluated specifically for acrosomal integrity using previously described characterizations for the domestic dog (Oettlé, 1986; Oettlé and Soley, 1988; Hay et al., 1997a,b). Morphological abnormalities were divided into those involving the head, midpiece or flagellum. Intact acrosomes were identified by a uniform blue color in the distal portion of the sperm head, while the post-acrosomal region was stained pink. Partial or damaged acrosomes were those in which the blue stain in the distal portion of head region was disrupted, uneven or irregular in appearance. Missing acrosomes were identified by blue color in the equatorial region only or pale, even pink color in the acrosomal region.

In preparation for cryopreservation, semen was centrifuged for 5 min at approximately $500 \times g$. After removing the supernatant, the resulting pellet was resuspended to its original volume in a commercial, egg yolk-based glycerolated extender (International Canine Semen Bank, ICSB; Sandy, OR, USA). Diluted semen was held at 5°C for 30 min and then frozen using a pelleting method previously described for the domestic dog (Platz and Seager, 1977) and grey wolf (Seager et al., 1975). Single drops of cooled semen were dispensed from pre-cooled Pasteur pipettes onto small indentations in a block of dry ice. After 3 min, the pellets were released into a bath of liquid nitrogen, transferred into a labeled Nalgene vial (Gibco Laboratories, Grand Island, NY, USA) and stored immersed in a liquid nitrogen tank (−196°C). Samples were thawed by retrieving one to two pellets, placing them in a tube containing ~0.5 ml of phosphate buffered saline (38°C) and agitating the tube and its contents for ~2–5 min in a 38°C waterbath. Evaluation of sperm motility, viability and morphology were as described above for fresh semen.

2.3. Statistical analyses

Values are reported as means ± the standard error of the mean (SEM). Differences in sperm cell characteristics between fresh and frozen–thawed semen were assessed using a paired Student's t test or Chi square analysis.

3. Results

Mean testicular dimensions for the red wolf during the breeding season were 41.0 ± 0.4 mm in length and 23.3 ± 0.4 mm in width with a combined testicular volume of 30.89 ± 1.4 cm^3. Although there was no difference in testis size during the entire breeding season ($P > 0.05$), there was a tendency for testicular volume to decrease at each collection for five animals subjected to semen collection three times in the 2-month period of February and March.

Mean electroejaculate volume was 4.7 ± 0.7 ml (range, 1–13.5 ml), with an average spermatozoal motility and viability of 71.2 and 84.1%, respectively (Fig. 1A) and a mean concentration of $146.5 \pm 25.7 \times 10^6$ spermatozoa/ml. Mean number of morphologically normal spermatozoa was 73.6 ± 3.2% (Fig. 1A; range, 20.3–93.7%), with the greatest proportion of ejaculates (16/25, 64%) containing between 70–90% normal-appearing spermatozoa. Only a single ejaculate contained <50% normally-shaped spermatozoa. From the average of 26.4% abnormal spermatozoa present in an ejaculate, the four predominant abnormalities were: a coiled flagellum, a bent midpiece with no cytoplasmic droplet, a bent flagellum or a detached head defect (Table 1), with each being ≤9% of the total sperm evaluated. There were no differences ($P > 0.05$) in ejaculate volume, sperm concentration, % motility, % of normally-shaped sperm or intact acrosomes between proven and unproven breeder males (data not shown). With the exception of two animals, there were no differences among ejaculates within males. In these two males, values for semen volume (~1 ml), concentration ($< 100 \times 10^6$/ml)

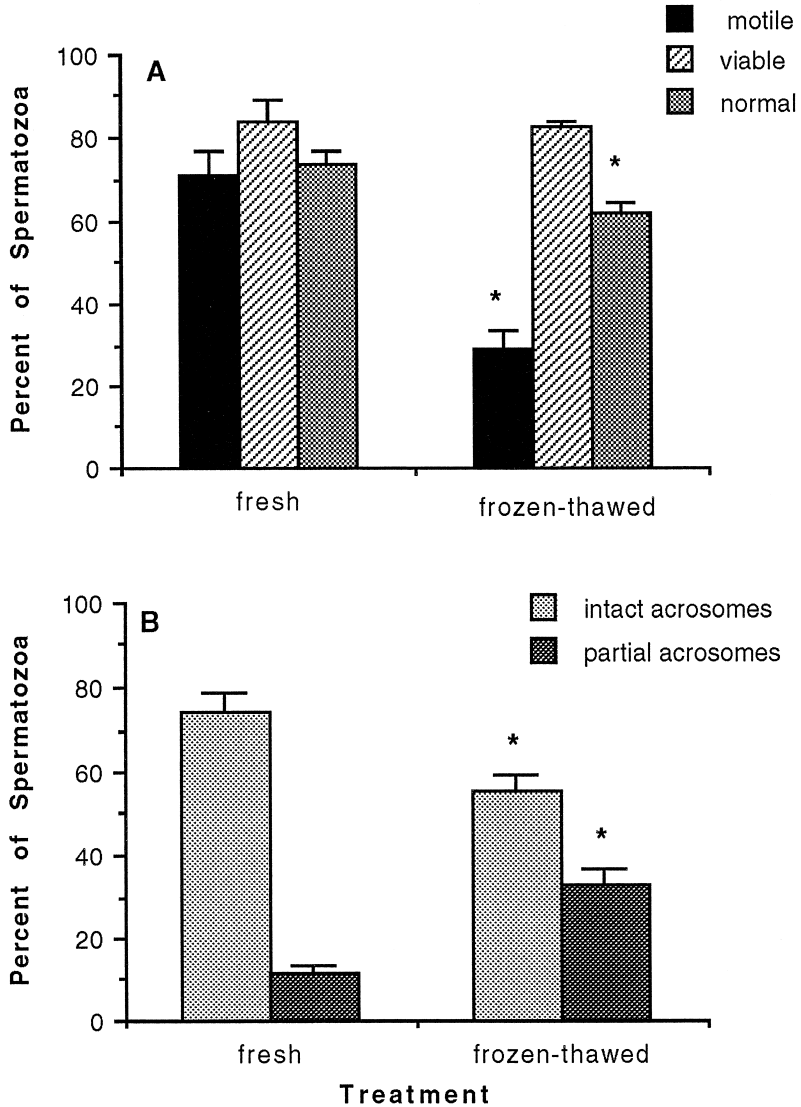

Fig. 1. Mean % sperm motility, viability and normal morphology (A) and mean % intact and partial acrosomes (B) for fresh and frozen–thawed red wolf spermatozoa. * Indicates significant differences between fresh and frozen–thawed spermatozoa ($P < 0.05$).

and motility (10%) of a single ejaculate collected late in the breeding season (31 March) were substantially less than previous ejaculates.

After freezing, there was a decline in sperm motility ($\sim 40\%$, $P < 0.05$), but no change in the proportion of viable spermatozoa (Fig. 1A). The percentage of structurally-normal spermatozoa also decreased after freezing (Fig. 1A; $P < 0.01$), with an increase in the proportion of cells with a bent flagellum (14%) accounting for this

Table 1
Mean sperm cell abnormalities from freshly-collected and frozen–thawed red wolf semen ($n = 25$ ejaculates)

Characteristic	Mean Percent (±SEM)	
	Fresh	Frozen–thawed
Head abnormalities		
Macrocephalic	0.03±0.02	0.02±0.02
Microcephalic	1.4±0.4	1.9±0.7
Bicephalic	0.04±0.03	0.1±0.1
Knobbed acrosome	0.2±0.1	0.1±0.1
Detached head	6.4±1.9	5.3±1.5
Midpiece abnormalities		
Abnormal midpiece	0.2±0.1	0.02±0.02
Bent midpiece without droplet	3.3±0.8	1.4±0.5
Bent midpiece with droplet	0.4±0.2	
Flagellum abnormalities		
Coiled flagellum	8.1±1.6	7.9±1.2
Biflagellate	0.1±0.04	0.04±0.03
Bent flagellum	4.7±0.8	19.4±2.8
Bent flagellum tip	0.04±0.03	0.1±0.1
Proximal droplet	0.3±0.1	0.04±0.03
Distal droplet	0.1±0.1	0.1±0.1
Bent neck	1.1±0.3	1.7±0.5

change (Table 1). Additionally, a significant decline in acrosomal integrity (Fig. 1B) was observed, with the proportion of intact acrosomes decreasing from 74.5 to 55.5%, accompanied by an increase in the proportion of partial acrosomes from 11.9 to 35.8% between the fresh and frozen–thawed groups, respectively ($P < 0.05$). The percentage of missing acrosomes did not differ ($P < 0.05$) between the two groups (13.6 and 8.4% for fresh and frozen–thawed spermatozoa, respectively).

4. Discussion

This is the first report describing ejaculate characteristics from freshly-collected and frozen–thawed red wolf semen. Collection of spermic ejaculates during January, February and March in the American Northwest was consistent with the tightly synchronized breeding season of late February through early March observed in captive and free-ranging conspecific female red wolves (Waddell, 1995). Ejaculate characteristics for red wolves did not differ between proven and non-proven breeders and, with the exception of semen volume and sperm concentration were consistent with domestic dog data. Sperm concentration in red wolf semen collected by electroejaculation was approximately half that collected manually from the domestic dog and is likely the result of increased accessory gland secretion provoked by electroejaculation. For a group of 28 proven-breeder dogs, England and Allen (1989) reported mean sperm motility and

normal sperm morphology as 89 and 88%, respectively, with an average volume of 1.2 ml and a mean concentration of 299.6×10^6 cells/ml in manually collected ejaculates. In 30 ejaculates from four mongrel dogs collected by manual stimulation, we found the mean volume to be 2.3 ml, with an average concentration of 277×10^6 sperm/ml and a mean sperm motility of 83% (Hay et al., 1997b). The percentage of spermatozoa with normal morphology in these ejaculates was 90%, with 95% undamaged acrosomes as evaluated by Spermac® staining. Although other investigators report various semen characteristics for the domestic dog, sperm motility and morphology usually lie in the same range (75% motile and 79% normal, Oettlé, 1993; 80% viable and 69% normal, Morton and Bruce, 1989). Therefore, although there is some variance among individual dogs and among laboratories, there are close similarities in fresh semen characteristics between the red wolf and the domestic dog.

A previous study of red wolves (Koehler et al., 1994) reported a high incidence of spermophagy in the semen of six animals, suggesting that this particular abnormality may be due to inbreeding effects related to the original small number of founders. Inbreeding is known to compromise sperm quality in the domestic dog (Wildt et al., 1982). Although this is a serious consideration for the red wolf, seminal characteristics obtained in the present study for this species were not indicative of a serious sperm quality problem. The sperm donors in our study had low inbreeding coefficients (range, 0–.0782). Nonetheless, semen quality alone is not always a clear index of fertility potential. To complement morphological and sperm motility observations, our laboratory has developed a homologous sperm/oocyte penetration assay for the domestic dog which can be used as a measure of assessing spermatozoal functionality. Results from contemporary studies indicate that traits such as sperm motility, viability and acrosomal status are positively correlated with oocyte penetration in the domestic dog, but that one variable alone cannot serve as an accurate predictor of gamete interaction (Hay et al., 1997b). A logical next step for studying red wolf spermatozoa is to determine their ability to interact with domestic dog oocytes in vitro, as an assay of functional capacity.

Numbers of viable spermatozoa (82%) and intact acrosomes (55%) in red wolf semen after freeze–thawing were greater than observed in the domestic dog (55 and 24%, respectively) using similar evaluative techniques (Hay et al., 1997a,b). In contrast, sperm motility for the red wolf after thawing was poor ($\sim$ 28%) compared to studies in the domestic dog ($\sim$ 70%; Hay et al., 1997a,b). Therefore, although red wolf spermatozoa appeared viable after freezing, poor motility (likely attributable to changes in flagellum morphology negatively affecting sperm motility) may represent a reduction in fertilizing potential of these cells. This hypothesis, along with evaluating function of freshly-collected spermatozoa, could be tested in the red wolf by subjecting fresh and frozen/thawed spermatozoa to the oocyte penetration assay. Preliminary data from ongoing studies suggest that red wolf spermatozoa can penetrate domestic dog oocytes (Hay et al., unpublished data).

The large proportion of bent flagella in thawed red wolf spermatozoa suggested a possible ‘cold-shock’ effect, such as that observed in bull and ram sperm (Watson, 1981), or osmotic shock attributable to glycerol exposure. Whether this flagellum damage occurred during cooling (prior to freezing) or during freezing is unknown, but it likely accounted for the observed loss of motility after thawing. Further studies of

changes during pre-freeze cooling and freezing would help us better understand when damage is occurring. We have found in dog sperm that rapid cooling over a short time period (30 min) or addition of glycerol during cooling reduces oocyte penetration in vitro, even though morphological damage is minimal (Hay et al., 1997a). Oettlé (1986) has demonstrated that dog sperm motility and acrosomal damage occur in both the pre-freeze cooling and freezing phases, but the most marked decline occurs after thawing. Therefore, more in-depth studies examining each stage of cryopreservation are needed to gain a better understanding of when and how morphological and acrosomal damage occurs and how it can be prevented.

Accounts of successful assisted reproduction in non-domestic canids are sparse. Seager et al. (1975) reported the birth of wolf pups following artificial insemination (AI) using frozen–thawed semen and a method for cryopreservation similar to that described here. Additionally, one litter of red wolf pups has been born after AI by surgical deposition of fresh semen into the uterine horns (Waddell and Platz, personal communication) thus demonstrating the biological competence of sperm deposited 'artificially'. In domestic dogs, the factors deemed most important for achieving successful AI are: (1) seminal quality; (2) sperm handling and insemination techniques; and (3) timing of insemination (Linde-Forsberg, 1991; Silva et al., 1996). Although it does not appear that seminal quality will be a deterrent to successful AI in red wolves, data from this study suggested that improvements in cooling and/or cryopreservation techniques may be needed to enhance spermatozoal motility and normal morphology. Additionally, previous attempts with AI for this species have included daily vaginal cytology and blood sample collection for progesterone analysis from manually-restrained females to estimate time of ovulation (Waddell and Behrns, personal communication). Although serum progesterone is useful for detecting ovulation and timing AI in the domestic dog (Linde-Forsberg, 1991), it is possible that taking a blood sample from a stress-susceptible, essentially wild, estrual red wolf bitch could negatively impact on AI success (Liptrap, 1993). Therefore, studies examining a combination of behavioral cues and faecal hormone analysis are now underway to develop a non-invasive method for detecting time of ovulation in the red wolf (Wagener et al., and Walker et al. unpublished data). By using this multidisciplinary approach, we anticipate making more rapid advances towards understanding the reproductive biology of this endangered species and developing assisted reproductive technologies useful for genetic and conservation management.

5. Conclusions

Semen from red wolves can be successfully collected using electroejaculation, with seminal and sperm characteristics similar to the domestic dog. This suggests that inbreeding depression is not having a major effect on reproductive fitness of the male red wolf. However, the marked decrease in sperm motility and increase in flagellum defects after freeze–thawing indicates that further studies are needed to optimize spermatozoal function after cryopreservation.

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