Aus der Klinik für kleine Haustiere der Tierärztlichen Hochschule Hannover
und dem Research Department des Saint Louis Zoo, St. Louis, Missouri, USA

Cryopreservation of Mexican gray wolf (*Canis lupus baileyi*) semen -

evaluation of different times and rates of pre-freeze cooling and Equex pasta® supplementation - in comparison with semen of the domestic dog and generic gray wolf (*Canis lupus*)

INAUGURAL-DISSERTATION
Zur Erlangung des Grades einer
DOKTORIN DER VETERINÄRMEDIZIN
(Dr. med. vet.)
durch die Tierärztliche Hochschule Hannover

Vorgelegt von
Claudia Zindl
aus Singen/Hohentwiel

Hannover 2006

Bibliographische Information der Deutschen Bibliothek

Die Deutsche Bibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliographie; Detaillierte bibliographische Daten sind im Internet über http://dnb.ddb.de abrufbar.

1. Auflage 2006

ISBN 3-938026-89-8

Verlag: DVG Service GmbH
Frankfurter Straße 89
35392 Gießen
0641/24466
geschaeftsstelle@dvg.net
www.dvg.net

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Tag der mündlichen Prüfung: 29. Mai 2006

Die Dissertation wurde gefördert durch ein Kurzstipendium des Deutschen Akademischen Austauschdienstes

Meinen Eltern

Contents

Abbreviations

ALH	lateral head displacement
ATP	adenosine triphophate
ANOVA	analysis of variance
AZA	American Zoo and Aquarium Association
BCF	beat cross frequency
BEEM	ballistic electron emission microscopy
BHT	butylated hydroxytoluene
BSA	bovine serum albumine
Ca^{2+}	calcium ions
Canid TAG	Taxon Advisory Group of canid species for AZA conservation programs
cAMP	circular adenosin triphophate
CASA	computer assisted semen analysis
chap	chapter
6-CFDA	6-carboxyfluoresceindiacetate
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CO_2	carbon dioxide
ext	extender
EZA	European Zoo Association
e.g.	exempli gratia
h	hour

HEPES	N-(2-hydroxyethyl)piperazinN-2-ethane
HOST	hypoosmotic swelling test
HT	Hamilton Thorn IVOS-10
HSP90	90 kDa heat-shock protein
i.e.	id est
IMP	intramembraneous particle
IUCN	The World Conservation Union
kg	kilogram
LIN	linearity
LN_2	liquid nitrogen
max	maximum
mg	milligram
min	minimum
mins	minutes
µg	microgram
µl	microliter
mOsm	milliosmol
n	number of dogs/generic gray wolves/Mexican wolves
OEP	Orvus Es paste
p	probability of error
pH	negative decadic logarithm of the hydrogen ion concentration
PI	propidium iodide
PUFA	polyunsaturated fatty acids
RT	room temperature

SDS	sodium dodecyl (lauryl) sulphate
SEM	statistic error of the mean
SMI	sperm motility index
SQA	sperm quality analysis
SSP®	Species Survival Plan
STR	straightness
TRIS	Tris(hydroxymethyl)-aminomethane
USFWS	United States Fish and Wildlife Service
VAP	average path velocity
VCL	curvilinear velocity
VSL	straightline velocity
WAZA	World Association of Zoos and Aquariums
WCSRC	Wildlife Canid Survival and Research Center
wb	water bath

1 Introduction

Recovery of the Mexican gray wolf* (*Canis lupus baileyi*), a subspecies of the generic gray wolf (*Canis lupus*), depends on careful genetic management of the captive population. Because of their monogamous mating system, transfer of gametes using cryopreservation and artificial insemination is preferable to breaking pair-bonds and transfer of animals. As there are only few Mexican wolves available for evaluating the requisite technology, such as sperm cryopreservation, it has been necessary to use the generic gray wolf as a model and to base the techniques on those developed for the domestic dog, the wolf's closest relative.

As cooling is one of the critical steps in semen freezing, the effect of various cooling rates on semen quality was compared in both generic gray and Mexican wolves. Spermatozoa of several species require a rest of several hours during cooling, before freezing, to develop maximal resistance to the effects of freezing. The first studies on cooling and equilibration of canine spermatozoa stated that none of the temperatures or time factors significantly affected the survival rate (MARTIN 1963a, b). A number of studies have dealt with the effects of slow and rapid cooling of canine semen (e.g. OLAR et al. 1989; HAY et al. 1997b). Some investigations (OSINOWO and SALAMON 1976; OETTLÉ 1986a) showed that the duration of the cooling process is an important factor. However, most studies have not fully evaluated cooling rates or equilibration time before freezing and have used arbitrary values (ENGLAND 1993).

Equex STM paste (Nova Chemical Sales, Scituate, USA) has shown beneficial effects on frozen-thawed dog semen with regard to post-thaw motility, thermoresistance, increased longevity and plasma membrane integrity (ROTA et al. 1997; PEÑA and LINDE-FORSBERG 2000b). For Equex pasta (Minitüb, Tübingen, Germany) these effects were missing (PEÑA et al. 2003a, b).

Computer-assisted semen analysis has been widely used in evaluation of dog semen since more than 10 years (GÜNZEL-APEL et al. 1993; SMITH and ENGLAND 2001; RIGAU et al. 2001; ROTA et al. 2001; IGUER-OUADA and VERSTEGEN 2001a, b; RJISSELAERE et al. 2003). It has been shown to be a useful tool in investigating motility of fresh and frozen-thawed semen.

* The name 'Mexican gray wolf' will be abbreviated with Mexican wolf in this study

Electron microscopic semen evaluation has been performed in several species including the canine. Sperm damage by cooling and freezing-thawing processes (OETTLÉ and SOLEY 1988; BURGESS et al. 2001), different cryopreservation methods (STRÖM HOLST et al. 1998) and the influence of different diluting media and temperatures (SIRIVAIDYAPONG et al. 2000) were investigated.

The aim of the present study was to investigate the effects of various cooling times, cooling rates and extender components on semen quality in domestic dogs as well as in generic gray and Mexican wolves using conventional spermatological methods and additional tools of semen evaluation such as computer-assisted and electron microscopic analysis.

2 Literature Review

2.1 Carnivore Conservation

The decline of large predator populations is a global problem (WEBER and RABINOWITZ 1996). There are approximately 34 wild species in the family Canidae, 22 of which are considered threatened or endangered under the CITES or the United States Fish and Wildlife Service (USFWS) Endangered Species Act. Most carnivores come into conflict with people because of their predatory habits: red foxes (*Vulpes vulpes*) kill chicken and wolves (*Canis lupus)* kill sheep. For these reasons the majority of carnivore species have been persecuted for hundreds, or even thousands of years (BOITANI 1995). *Canis lupus* management follows a classic pattern of controlling large carnivores. Only the red wolf (*Canis lupus rufus*), the Mexican wolf (*Canis lupus baileyi*), and the maned wolf (*Chrosycaon brachyurus*), the African wild dog (*Lycaon pictus*), and the bush dog (*Speotos venaticus*) are included in captive breeding and/or re-introduction programs. The Canid Taxon Advisory Groups of the American Zoo and Aquarium Association has designated the Ethiopian, red, maned, and Mexican wolf, and the African wild dog as priority species for conservation efforts. The goal of conservation is to preserve and reintroduce species and preserve genetic diversity with planned breeding. If this cannot be fulfilled artificial reproduction with frozen semen has to be applied (HOLT and MOORE 1988).

2.1.1 The story of the Mexican wolf

The Mexican wolf is an endangered subspecies of the generic gray wolf that once inhabited the southwestern United States and Mexico, extending as far south as the Isthmus of Tehuantepec and is the most highly differentiated North American gray wolf population, genetically distinct, and historically isolated from other gray wolves (NOWAK 1979). Encroachment by European-style farming and animal husbandry, together with predator-control programs, led to the decline of this once-numerous subspecies of the gray wolf. The last wolf recorded in Texas, Arizona and New Mexico was in 1970, 1975 and 1976, respectively. In 1996, apart from occasional unconfirmed reports of wolf sightings within the Mexican Sierra Madre Occidental, none appears to have survived in the wild (GARCÍA-

MORENO et al.1996). The Mexican wolf is now protected within the U.S. under the Endangered Species Act, being the world's strongest national legal protection for endangered species (MCBRIDE 1980; ABBITT and SCOTT 2001). The U.S. Fish and Wildlife Service declared the Mexican wolf to be an endangered species in 1976. In 1977 five wild Mexican wolves (four males and a pregnant female) were caught in Durango and Chihuahua, known to derive from a wild population of Mexican wolves (GARCÍA-MORENO et al. 1996) for use in a captive-breeding program. In 1982 the USFWS approved the Mexican Wolf Recovery Plan that recognizes the need of recovery of this species by re-establishment in suitable habitats within their historic range using captive-raised wolves in planned reproduction programs. The specific recovery objective for the Mexican wolf is "to conserve and ensure the survival of *Canis lupus baileyi* by maintaining a captive breeding program and re-establishing a viable, self-sustaining population of at least 100 Mexican wolves in the middle to high elevations of a 5.00 square mile area within the Mexican wolf's historic range" (USFWS 1982). In 1993, the Captive Management committee reorganized as a Species Survival Plan (SSP®,) became the first bi-national SSP® programme. The mission of the Mexican Wolf programme has been to support the re-establishment of the Mexican wolf in the wild through captive breeding programmes, public education, and research (SIMINSKI 2002). Three certified lineages, McBride, Aragon and the Ghost Ranch lineage were established and by analysis of hypervariable microsatellite loci it was found that the three captive populations were established with different founders at different times and are genetically more similar to each other than to any other population of dog or wolf-like canid, and they shared alleles that were rare in other canids. All three lineages were included in the breeding program in 1995 (GARCÍA-MORENO et al. 1996; HEDRICK et al. 1997). Management of the breeding program became the responsibility of the holding institutions in 1995, with the formation of the Mexican Wolf Captive Management committee (LINDSEY and SIMINSKI 2003) working in collaboration with the governmental agencies in both countries. The existing population descends from seven founders from those three lineages (GARCÍA-MORENO et al. 1996; SIMINSKI 1998). In 2005 the captive population consisted of 309 individuals in 47 institutions (USA and Mexico). The first reintroduction of Mexican wolves in the USA occurred in 1998 at the Apache National Forest in Arizona and by the end of November 2004 there were 51 to 56 free-ranging wolves in eastern Arizona. The overall goal set by the 1994

AZA SSP® Master Plan is to preserve 75% of the gene diversity in captivity for 50 years. Although 81% of the genetic diversity of the population has been retained (SIMINSKI 1998), it could be increased by more effective management of the captive population. One management practice is to increase the generation time so that the loss of genetic diversity is slower. Breeding recommendations for the captive population focus on genetically under-represented animals of pairing to produce offspring with low inbreeding coefficients (MUSSON 2001). Like captive Swedish wolves, Mexican wolves show signs of inbreeding depression (KALINOWSKI et al. 1999; FREDRICKSON and HEDRICK 2002) that can be a problem for breeding success.

2.1.2 Role of biotechnology in carnivore conservation – semen banking

Because reproduction is the essence to species survival, the reproductive biologist plays an essential role in single species conservation (WILDT 1992). The potential is tremendous, particularly if genetic material is periodically collected and introduced into the captive population. Long-term storage of semen by cryopreservation, with high recovery rates on thawing, is essential for the establishment of genetic resource banks of endangered species. For an increasing number of species, captive breeding represents their best chance to avoid extinction. The key to actual practical implementation of assisted reproductive technology for wildlife conservation is the systematic preservation and use of frozen spermatozoa and embryos, the primary constituents of genetic resource banks (WILDT 1992). BALLOU (1992) has recently discussed the benefits of gamete cryopreservation (sperm, ova, embryos) for captive management programs. Artificial insemination and semen preservation have been identified as powerful tools in breeding programs, because this would allow the storage of semen from genetically valuable animals. Frozen biological material could be used to infuse new vigor into genetically stagnant captive populations while eliminating the need to remove more animals from the wild and also may be used for restoring genetic vigor into free-living populations (WILDT 1992). The disadvantages of those reproductive technologies are that, if not managed well, inbreeding and variability of response are pre-programmed (NICHOLAS 1996). Inbreeding depression is a major concern in the management of small populations (LACY et al. 1995). Among the most important threats faced by captive populations apart

from inbreeding is the loss of genetic variability. Reproductive technology can best assist captive breeding programs in this task by developing strategies that effectively increase the genetic contribution of new wild founders to a population. Because of the small founder populations these factors imply serious risks (BALLOU 1984). Detailed records kept at the European Zoo Association (EZA) since the founder populations were first established, have allowed the calculation of inbreeding coefficients for all individuals. It is shown that semen collection and preservation, with future intent of artificial insemination, can make significant contributions to the maintenance of genetic diversity if careful consideration is given to the selection of donor males. Semen collected of individuals which have not yet contributed to the population can be used later, through artificial insemination, to produce offspring which is genetically equivalent to acquiring new wild-caught stock. Since a primary role of reproductive technology is the maintenance of genetic diversity, semen should be collected from all male founders of the population (BALLOU 1984). The World Conservation Union (IUCN) has recognized the potential role that captive breeding may play in conservation efforts and has recommended that vertebrate taxa numbering less than 1000 individuals in the wild should be considered for captive breeding (IUCN 1987). The goal should be to preserve over the next 200 years at least 90 % of the genetic variability, that existed in the source population. It is prudent to have as many founders as possible to increase the genetic variability because otherwise rare alleles will not survive and the sooner the group will reach the target population size (SOULÉ et al. 1986).

To successfully manipulate the reproduction of a species it is essential to understand its reproductive physiology (BYERS et al. 1990). Without understanding the fundamentals of reproductive processes, assisted breeding can never become consistently successful. In practice current methods of assisted reproduction applied to threatened or endangered species are rather inefficient, undoubtedly because of insufficient knowledge of basic reproduction of non-domestic species (LEIBO and SONGSASEN 2002). Assisted breeding techniques that are effective in humans and cows are not necessarily applicable to wolves. Additionally reproductive mechanisms among animal groups are highly varied (WILDT et al. 1992). WILDT and BUSH (1984) considered it as necessary to standardize electroejaculation protocols for better comparability of results, to establish individual characteristics of electroejaculats, and examine various methods and diluents for successful semen freezing

with regard to cellular integrity and fertilizing capacity. Regarding the Mexican wolf semen, traits have been characterised, extensive development of semen cryopreservation techniques has been conducted (ASA 2001; MUSSON 2001) and semen from genetically valuable individuals is maintained in semen banks.

2.1.3. Seasonality of canid reproduction

Wild canid reproductive biology includes features that are unusual among mammals, including monogamy, seasonal monoestrus, exceptionally long proestrous and diestrous phases, a copulatory lock or tie, incorporation of adult young into the social group, behavioural suppression of mating in these subordinate young, obligate pseudopregnancy in subordinate females, alloparental care (ASA et al.1998), and elevations in progesterone prior to ovulation that are also observed in domestic canids (GUDERMUTH et al. 1998; HAY et al. 2000). Also, ovulated canid oocytes have a germinal vesicle and the meiotic division is not complete at ovulation (YAMADA et al. 1992, dog). Reproduction is seasonal for most wild canid species (HELLE and KAUHALA 1995), which breed from January to March in species native to the Northern hemisphere (FARSTAD 2000a, b). Although much has been published on the life history of gray wolves, relatively little information is available for Mexican subspecies. According to GARCÍA-MORENO et al. (1996), BERNAL and PACKARD (1997) and HEDRICK et al. (1997) the Mexican wolf breeds only once a year. Coinciding with female oestrous periods, a seasonal peak in testicular size and sperm production has been shown in male individuals of the generic gray wolf (SEAGER et al.1975), of coyotes (*Canis latrans*; GREEN et al. 1984) and the red wolf (GOODROWE et al. 1998). A seasonal increase in testicular weight from January to March is correlated with a peak in plasma testosterone levels in blue foxes (*Alopex lagopus;* SMITH et al. 1985) and red foxes (*Vulpes vulpes;* LEFEBRE et al. 1999); however, no cyclicity in plasma testosterone levels has been observed in maned wolves (*Chrysocyon brachyurus;* VELLOSOS et al. 1998). Male domestic dogs (*Canis familiaris)* have significantly higher testosterone levels in August and September (FALVO et al. 1980), however, they do not appear to have any seasonal cyclicity and are showing spermatogenesis and breeding throughout the year (TAHA et al. 1981).

Puberty in both female and male gray wolves (defined as first spermatozoa production and first ovulation) usually occurs at 22 months of age (ASA and VALDESPINO 1998). Puberty in male domestic dogs starts between 8 to 8.5 months of age (depending on breed), with a rapid increase in testicular growth observed at 5.5 and 9 months of age (MAILOT et al. 1985). Semen quality improves as sexual maturity is completed.

2.2 Semen collection by electroejaculation

The first account of a canine ejaculate obtained by electrical stimulation was supplied by TINET (1939) (cited in BARTLETT 1962). The electric stimulation mimics the neural and muscular responses of the various compounds involved in the natural ejaculatory processes - physiological reflexes mediated by parasympathetic, sympathetic and somatic nerve fibers as shown in cat as an example in SEMANS and LANGWORTHY (1938) and ROOT and BARD (1947). By stimulation of parasympathetic fibers (S1-S3) or pelvic nerves, sympathetic fibers (L1-L2) or hypogastric nerves and pudendal nerves (parasympathetic, sympathetic, somatic fibers) erection, spermatozoa and seminal plasma emission and sperm ejaculation do occur, respectively. Electroejaculation in dogs has been recognized as unsuitable because of small ejaculate volume, urine contamination and psychological damage of the animals (GEHRING 1971; BIEDERMANN 1951). In exotic animals though it is the only practicable technique to collect semen. Electroejaculation has been used to collect semen from wild canid species, such as the generic gray wolf, fox (FARSTAD 1996; FARSTAD et al. 1992a, b), red wolf (GOODROWE et al. 1998, 2001), and from other wild species (HOWARD et al. 1986 African elephant; SWANSON et al. 1996, ocelot; HOWARD et al. 1992, cheetah; CASSINELLO et al. 1998, gazelles). In domestic cats electroejaculation is the favoured method to collect semen (HERRON et al. 1986), while in the dog semen collection by manual manipulation is prefered (LINDE-FORSBERG 1991). Results of semen collections by electroejaculation can be poor or variable, depending on the species. In humans viability of electroejaculated spermatozoa is decreased with poor survival after incubation and a moderately impaired cervical mucus penetration (DENIL et al. 1992). Results do not necessarily represent the quality and quantity of semen produced by natural ejaculation and

seminal fluid characteristics are altered by this method of collection. HIRSCH et al. (1991) explain this by neurological injury of men and subsequent seminal dysfunction with unbalanced and unphysiological gland secretion. AXNÉR et al. (1998) suppose that the process of electroejaculation may favour removal of aged spermatozoa with pre-existing tail defects. Electrical stimulation causes a greater volume of seminal plasma to be released probably due to overstimulation of the accessory glands (PLATZ and SEAGER 1978, cat). Voltage of stimulation may affect the osmolarity of semen and semen collected by artificial vagina has a lower pH than semen deriving from electroejaculation (DOOLEY and PINEDA 1986). Contamination of semen with urine is one of the disadvantages of electroejaculation particularly in canid, ursid and felid species (CHRISTENSEN and DOUGHERTY 1955; HOWARD 1993). The animals have to be anaesthetized or deeply sedated which has an effect on the animal and the collection. The type and depth of anaesthesia may interact with the success of electroejaculation. Furthermore, advantages and disadvantages of light and deep anesthesia are controversely discussed and there are no detailed physiological studies in this field. It is suggested that computer-assisted electrical stimulation may improve the results (HOLT 2001). Electroejaculation at present is the only safe method for repeated semen collections from non domesticated species. OLAR et al. (1983) and WOODALL and JOHNSTONE (1988a) found total scrotal width being strongly correlated with testicular weight to be a useful predictor of dog's ability to produce and ejaculate spermatozoa and being a convenient index of testicular size and potential sperm production. Mass of epididymis is variable not only with body weight but also with mating system. A positive allometry of epididymal mass and sperm numbers is related to promiscuous mating and sperm competition in stray dogs (WOODALL and JOHNSTONE 1988b).

2.3 Temperature decrease < 37°C as part of the cryopreservation process

The cryopreservation process includes different procedures to prepare a suspension of cells for long-term storage at temperatures below –80 °C, followed by rewarming to body temperature at thawing. Incubation (38 °C, 4 h), cooling (to 15 °C or 5 °C) and subsequent freezing reduced the proportion of live boar spermatozoa compared with fresh semen (MAXWELL and JOHNSON 1997a, b). All steps should be optimized to provide survival of

sufficient cells to achieve impregnation (AMANN 1999). Cryopreservation of semen can include a cooling period followed by equilibration.

Cooling is defined by the period of lowering the temperature of semen from room temperature to 5 °C, or 0 °C (HAY et al. 1997a, b; BATEMAN 2001). Cooling is the first temperature change known to alter the physical properties of all cell membranes (HAMMERSTEDT et al. 1990) and thus is one of the variables potentially affecting the success of artificial insemination with frozen-thawed semen. Spermatozoa of most species need to be cooled (to approximately 5 °C) before freezing to develop maximal resistance to freezing stress (ENGLAND 1993). The effects of cooling on sperm function are influenced by the length of incubation at falling temperatures, the final temperature attained and by the cooling rate, defined as speed of temperature decrease. The increased resistance acquired by spermatozoa during cooling appears to be beneficial during the equilibration period and for freezing (POLGE 1980). Apart from the beneficial effects of a cooling period of diluted semen, negative effects on spermatozoal function have also been identified.

Equilibration is a short time storage at 5 °C following cooling to help adapt semen to this specific temperature and extender before freezing. In the very early experiments on bull semen POLGE and ROWSON (1952) noted that sperm motility was increased in samples that had been diluted and stored at 2 °C for 18 h or 8 h before freezing as compared with samples frozen soon after collection and dilution. An advantage of equilibration has also been realized in semen samples frozen rapidly in straws and in this case an interaction between equilibration time and thawing rate was found (GILBERT and ALMQUIST 1978). In porcine semen equilibration significantly reduced motility loss during freezing and storage (POLGE 1980). A 12 h equilibration period at 5 °C resulted in better post-thaw progressive motility of equine spermatozoa (CROCKETT et al. 2001). Cryopreservation results of bull semen improved by adding at least 2 h of equilibration at 5 °C to the period of slow cooling (DHAMI and SAHNI 1993; DHAMI et al. 1996). Equilibration can also describe short storage of semen at temperatures between 37 °C and 5 °C, like 30 °C, acquiring cold shock resistance during 2.5 h and 4.5 h incubation in boar semen (PURSEL et al. 1972). The beneficial effect of equilibration may be associated with changes in the sperm membrane increasing its resistance to cold shock. Equilibration of boar spermatozoa at room temperature for 1 h was required to obtain the maximum protective effect of phosphatidylserine,

accelerating the attainment of the maximum resistance of plasma membrane (BUTLER and ROBERTS 1975). If boar semen is incubated for several hours at 25 °C, the detrimental effects of subsequent cooling can be almost completely abolished (ROBERTSON et al. 1988a). Boar semen held at 15 °C for 3.5 h before cooling to 5 °C, was less affected by freezing and thawing. Spermatozoa not held during cooling exhibited loss of viability and acrosome integrity after freezing and thawing (BUTLER and ROBERTS 1975). On the contrary, ALMLID and JOHNSON (1988) stated that holding boar semen prior to freezing is no advantage. Storage at 15 °C may protect sperm plasma membrane from cooling damage especially for 'poor cooler' stallions compared to storage at 4 °C (BATELLIER et al. 2001). Chilling is defined as storage of diluted semen at 5 °C for more than a couple of hours intended for sperm transport that can take several days.

Supercooling is the temperature decrease from 0 °C to –6 °C, when extracellular ice nucleation takes place, followed by freezing to –196 °C, which is characterized by a complete 'quietness' of metabolic activity of the spermatozoa. The stress caused by freezing and thawing is different stress caused by cooling, as during freezing spermatozoa are stressed by ice crystalization and solution effects depending on freezing rate in addition to temperature effects (POLGE 1980).

2.4 Molecular basis of cooling cells

The occurrence of cold induced morphological and biochemical alterations strongly suggests that the plasma membrane is one of the primary sites for sperm injury sustained during cooling (DE LEEUW et al. 1990a). Although the mechanisms of plasma membrane damage are not completely understood, there is increasing evidence that membranes are compromised due to reordering of membrane lipids during cooling and rewarming, thus disturbing the lipid-lipid and lipid-protein associations required for normal membrane function.

2.4.1 Membrane structure and thermotropic behaviour

The plasma membrane is the direct contact area between the sperm and its environment during transport and storage in the male and female reproductive tracts. Biochemically it is a lipid bilayer with integrated proteins. The structural integrity lies in the physical properties of phospholipids. Mixtures of phospholipids induce different physical stages of the membrane and the membrane rigidity depends on the homo- or heterogeneity and saturation of the fatty acids. Cholesterol is an important lipid component that contributes to a more condensed structure and decreased fluidity of the sperm membrane. Part of intrinsic or integral membrane proteins, which are released only if the lipid bilayer is dissolved by detergents, are transport proteins which enable ions or metabolites to pass down a concentration gradient or by membrane potential. The sodium pump maintains a high internal K^+- and a low Na^+-concentration; the Ca^{2+}-pump maintains a low cytosolic Ca^{2+}-concentration. The plasma membrane may be considered as a two-dimensional fluid surrounded by an environment with which it is not miscible. The interior is highly disordered fluidity (bulk fluidity), the region close to the polar head groups is relatively rigid (membrane viscosity). It is a fundamental property of phospholipid bilayers to exhibit thermotropism, und thus to undergo in aqueous suspension an abrupt change from a disordered fluid (liquid-crystalline state) to a highly ordered hexagonal lattice of fatty acyl chains (crystalline-array or gel state) over a specific temperature range. The temperature at the midpoint of this phase change is the transition temperature, and the change in state has been called the lipid phase transition, gel-liquid transition or order-disorder transition. The transition temperature depends on the structure of the hydrocarbonate chains (QUINN 1985) and each kind of lipid has a phase transition temperature, arranging fatty acid chains in a parallel and rigid form (MORRIS and CLARKE 1987). The plasma membrane is divided into five specialized regions (acrosome, equatorial region, post-acrosomal region, midpiece and principal piece). Each region has a distinct lipid composition, as required for the association of specialized functions (LADHA 1998). Within a membrane compartment, lipids are generally miscible and exhibit flexibility for lateral movement as long as the temperature is above a critical point (BUHR et al. 1989; LADHA 1998). If the membrane temperature is reduced below the phase transition temperature of an individual kind of lipid, all molecules of that lipid aggregate in microdomains of lipid gel

(AMANN 1999; Fig. 1) and reorganization of the bilayer is possible, but restricted to particular domains of the cell surface and not always entirely reversible (LADHA 1998). This aggregation together with the crystallization of hydrocarbonate chains results in trapped proteins (QUINN 1985) and thus may be contributing to cryoinjury during cooling with loss of sperm viability (ORTMAN and RODRIGUEZ-MARTINEZ 1994).

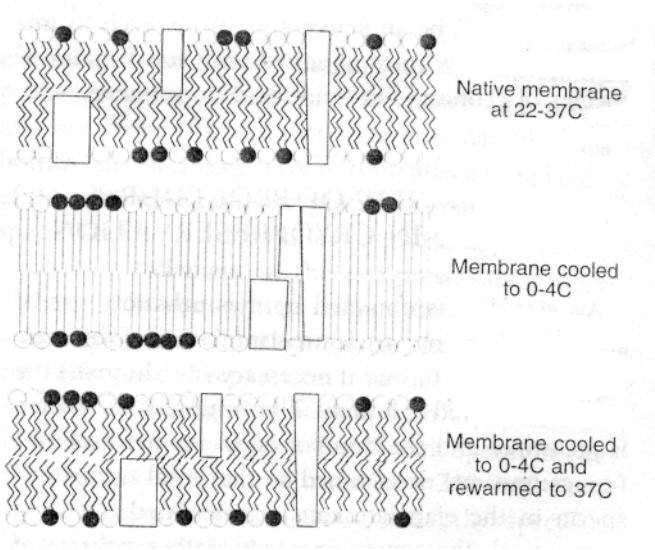


Fig. 1 Schematic representation of a sperm plasma membrane and changes induced therein by cooling and warming (AMANN 1999)

2.4.2 Thermotropic behavior and membrane function

Plasma membrane fluidity is to a certain degree physiological (BUHR et al. 1989). Changes of membrane lipid association is thought to provide the reason for the fluidity for membrane mediated events leading to fertilization (PARKS and GRAHAM 1992). The drastic rearrangement of membrane components which occurs upon cooling below the phase transition temperature alters the biological and biophysical properties of the membranes (QUINN 1981). Increase in membrane viscosity followed by phase separation will both have direct effects on membrane function. Some cells may recover, but if the cells lack the potential of compensating changes in membrane phase behaviour at the reduced temperature, they will be trapped in a state of permanent phase change or transition (MORRIS and

CLARKE 1987). The end result are clusters of certain kind of lipids, redistribution of proteins at other locations and aggregation in fewer sites, a rigid membrane, gaps in membrane integrity and resultant leaks as well as hampered function of enzymes or receptors (AMANN 1999).

2.4.2.1 Sperm damage caused by low temperature *per se*

Passing through phase transition temperatures during the cooling period, the plasma membrane is described by a stable balance of gel- and liquid-phases with increasing membrane permeability (MORRIS and CLARKE 1987). As proteins are stable only within a limited temperature range (WATSON and MORRIS 1987), during cooling protein molecules are excluded by the ordered arrays of membrane lipids, and become concentrated within regions of remaining membrane fluidity (HOLT and NORTH 1984), the so-called intramembranous particle (IMP) redistribution (DE LEEUW 1990a, b). Thus cooling-induced segregation of proteins may alter lipid-protein interactions within the membrane in case that nonbilayer lipids cannot re-establish their original protein associations. This leads to altered protein function (PARKS and GRAHAM 1992; AMANN 1999), resulting in biochemical changes. Gaps or leaky and unstable borders are formed between microdomains of lipid gel and fluid portions of the membrane, facilitating membrane rupture or fusion with other membranes. Fluidity of head membranes decreased at 25 °C but the rate of decrease is significantly lower for membranes of cooled sperm cells. Head plasma membranes of fresh or fresh-extended spermatozoa maintain a normal rate of fluidity decline until below 10 °C and undergo molecular reorganization already at 25 °C. Cooling to 5 °C reduces the rate of fluidity change in the plasma membrane of boar spermatozoa (BUHR et al. 1989) and ram spermatozoa experience drastic changes through the temperature range 15 to 10 °C, showing markedly increased proportion of immunoreactive cells (HOLT and NORTH 1991), and when the temperature is lowered to 5-0 °C (ORTMAN and RODRIGUEZ-MARTINEZ 1994). Sperm membrane changes are reduced if semen is kept at 15 °C, but are increasing by cooling to 5 °C, which is thought to be the primary cause of damage associated with cryopreservation in boar (MAXWELL and JOHNSON 1997a, b). Exposing porcine sperm cells to cold temperatures the percentage of propidium iodide stained cells increased (SCHIFFER 1996).

Changes in membrane fluidity are influencing ion permeability and transport. Cooling induces calcium uptake, especially in bull and ram spermatozoa, and less dramatic in human, dog, rabbit, or fowl spermatozoa (QUINN and WHITE 1966). Membrane potential and calcium concentration have a clear effect on the survival rate of the spermatozoon interfering with the normal membrane events at fertilization (BUHR et al. 1994). The Ca^{2+}-ATPase function is highly dependent on the physiochemical properties of the lipid environment. Phospholipid hydrolysis has been implicated in cold-induced membrane damage resulting in a major thermal phase transition of lipids in the direct vicinity of the calcium-stimulated ATPase molecule with a change in the activation energy of the enzyme reaction. This if followed by irregularities in ion permeability of ram spermatozoa at 22 to 24 °C (HOLT and NORTH 1985) and below 17 °C (HOLT and NORTH 1986) or 16 °C respectively (ROBERTSON and WATSON 1986) accompanied by a progressive accumulation of intracellular calcium, that is also seen in boar (SCHIFFER 1996) after exposure to cold temperatures. Cooling and storage of diluted ram semen at 5 °C over 96 h resulted in a significantly higher amount of intracellular calcium than in fresh semen, followed by a progressive decrease in viability and accompanied by a steady increase in acrosomal detachment (ROBERTSON and WATSON 1987). The ability of bovine spermatozoa to control Ca^{2+} transport is clearly compromised after the commercial cryopreservation procedures of dilution, cooling, freezing and thawing (BAILEY and BUHR 1994) and implies that cryopreservation may strongly affect all aspects of spermatozoal Ca^{2+}-control (LEOPOLD 1994).

As secondary reactions to loss of selective permeability of the plasma membrane, cooling precipitated flagellar injury causing a marked decline in the proportion of progressively motile spermatozoa, especially once the temperature fell below 10 °C (SIMPSON and WHITE 1986, ram), below 16 °C (HOLT et al. 1988, ram) or to 8 °C and 0 °C, respectively in boar (GILMORE et al. 1996). Decreasing number of motile cells was correlated with lower content of docosahexaenoic acid (NISSEN and KREYSEL 1983, human), decrease of HSP90 (90 kDa heat-shock protein) during the first hour of cooling to 5 °C (HUANG et al. 1999, boar) and increasing production of lipid peroxides (AMANN 1999). In contrast, cooling did not induce change in motility characteristics in domestic cat spermatozoa (PUKAZHENTHI et al. 1999).

A high percentage of spermatozoa have damaged acrosomal membranes (WATSON 1975b). According to JONES and STEWART (1979) dilution and cooling of bull semen to 5 °C caused acrosomal swelling in about 50 % of the spermatozoa. Within the temperature range of 16 to 8 °C spermatozoa underwent a 'folding process' or acquired a localized swelling of the flagellum. Temperature decline through the range of 10 °C caused a sudden asymmetric bending of the flagellum in the midpiece region of diluted spermatozoa (GILMORE et al. 1996, boar) and in domestic cat spermatozoa cooling induced massive acrosomal membrane damage (PUKAZHENTHI et al. 1999). In contrast to these results, in rabbit sperm low fertility and low numbers of normal embryos are related to membrane damage caused by freezing-thawing process but not by cooling (MOCÉ and VICENTE, 2002).
Results suggest a close relationship between cold susceptibility, lipid phase transition and lipid profile in animal gametes. Lipid phase transition and membrane fluidity occurring at the same temperature when plasma membrane damage is evident (ARAV et al. 2000).

2.4.2.2 Sperm damage caused by cold shock

Cold shock stands for cellular injury following rapid cooling and has long been recognized in mammalian spermatozoa (reviewed by WATSON 1981). Cold shock damages sperm of many mammalian species, but cell injury is not due to exposure to a critical temperature; it is the rapidity of temperature change from 20 °C to just above 0 °C (WATSON and MORRIS 1987), disrupting sperm on three levels; membrane molecular organization, intracellular Ca^{2+} regulation, and gross morphology and motility (ROBERTSON et al. 1990). Cooling from 20 to 0 – 5 °C over 2 or 3 hours eliminates this damage (HOLT and NORTH 1991) and a cooling rate of 30 °C/min in contrast resultes in significantly higher numbers of propidium iodide-positive cells to fresh semen (MEDRANO et la. 2002).
Spermatozoal membranes have insufficient time for lateral diffusion and redistribution of components within the plane of the membrane to occur with subsequent setting of proteins into solid phase lipids, thus inhibiting activity of proteins with following loss of enzymes and phospholipids. As the rate of cooling is reduced the probability of undercooling will decrease with more time for gel-phase growth (WATSON 1981, WATSON and MORRIS 1987). As

described for plasma membrane damage by temperature *per se*, the same mechanisms take place but the process will markedly depend on the composition of the membranes. Several studies have indicated that susceptibility of the cells to cold shock is related to the composition of the sperm membranes, particularly the lipid constituents. Showing large interspecific differences with respect to the sensitivity of spermatozoa, cold shock has been observed in sperm of several domestic species (PURSEL et al. 1972, boar; BUTLER and ROBERTS 1975; ROBERTSON et al. 1988a, boar; BAMBA and CRAN 1992; QUINN and WHITE 1966, bull; QUINN et al. 1969, 1980, ram; GLOVER and WATSON 1985, cat). Those species in which cold shock is most profound display the most pronounced phase events, as e.g. porcine (DROBNIS et al.1993). Differences in susceptibility to cold shock are considered to be determined by asymmetry of the plasma membrane and lipid composition. A high cholesterol to phospholipid ratio is beneficial as cholesterol cause fatty acids to condense and thus form a reasonably impermeable and cohesive membrane structure (WHITE 1993). It is more likely that cold shock has to be attributed to differences in the fatty acid composition of the membrane (DE LEEUW et al. 1990a, b) linking the high ratio of unsaturated to saturated phospholipid fatty acids to a high susceptibility of spermatozoa to cold-shock (DARIN-BENNETT et al. 1973). A relatively small amount of lipid peroxidation of unsaturated fatty acids can lead to extensive loss of lipids and enzyme activity (NISSEN and KREYSEL 1983). The variability of polyunsaturated fatty acid (PUFA) concentration within one species was found to be due to genetic diversity and differences in nutrition and climate (ARAV et al. 2000).

The plasma membrane was considered to be the primary site of lesion associated with early cold-shock damage with a change in pattern of sperm motion and membrane permeability (QUINN et al. 1969; QUINN et al. 1980, both ram) being affected by rearrangements of the ultrastructure during cooling. Under slow cooling protein molecules are excluded from the growing phospholipid lattice and are concentrated into regions of fluid lipid, thus membrane enzymes are concentrated into regions of lipids which are still fluid. This frequently resulted in reduced enzyme activity. Proteins are potentially vulnerable to rapid cooling when they dissociate into biologically inactive species which may or may not reassemble upon rewarming (WATSON and MORRIS 1987). Together with plasma membrane changes, loss of membrane selective permeability properties occur as an early cold shock injury. An

important role of ions, especially of Ca^{2+} was stated by QUINN and WHITE (1966), who clearly established Na^{+} and Ca^{2+} accumulation and K^{+} and Mg^{2+} loss in cold-shocked ram and bull spermatozoa by a permanent change in membrane permeability. The inability of the cells to restore Ca^{2+} levels after cold shock suggests that the membrane changes were irreversible, implying structural alteration rather than simply low temperature inhibition of metabolic processes (QUINN and WHITE 1966).

A substantial increase in boar sperm Ca^{2+} levels was observed after slow cooling (3 h in water bath at initially 25 °C) to <10 °C (ROBERTSON et al. 1988a). NISHIMURA (1993, boar) showed that slow cooling (0.3 °C/min to 15 °C) induced an influx of Ca^{2+}-ions decreasing motility. Sperm motility is affected by diminished anaerobic and respiratory activity in ram and bull spermatozoa following rapid cooling and rewarming. Levels of ATP are reduced and are not resynthesized (WATSON and MORRIS 1987). Sudden cooling deprives sperm of the ability to generate flagellar beats because of ATP depletion which occurs during the cooling phase (HOLT et al. 1992). In human semen the optimal cooling rate is published to be 0.5 – 1°C/min; when semen is cooled faster, viability decreases (MAHADEVAN and TROUNSON 1984). In equine cryopreservation protocols, cooling rates ≤ 0.05 °C/min (from 20 °C to 5 °C in 320 min) were considered proper (KAYSER et al. 1992) as shown by a tendentious better sperm motion characteristics directly after cooling compared to faster cooling. In contrast to these results are findings by VIDAMENT et al. (2000) where centrifugation and glycerol addition at 22 °C followed by cooling from 22 °C to 4 °C at a moderate rate (0.3 °C/min, in 60 min) resulted in an improvement of post-thaw motility, spermatozoa recovery rate and per cycle fertility. Ejaculated and diluted sperm from the impala (*Aepyceros melamopus*), wart hog (*Phacochoerus aethiopicus*) and elephant (*Loxodonta africana*) tolerated slow cooling (~0.15 °C/min) better than abrupt cooling (~50 °C/min) as shown by sperm motility (GILMORE et al. 1998). In cat spermatozoa rapid cooling (4 °C/min to 5 °C) was not more detrimental to motility than slow cooling (0.5 °C/min to 0 °C) (PUKAZHENTHI et al. 1999). A 2 h cooling time at 10 °C or 28 °C with subsequent cooling to 5 °C is essential for cattle semen, however buffalo semen can be frozen successfully after 30 min of cooling at 10 °C and subsequent cooling to 5 °C regarding post-thaw motility showing a significant influence of species-by-cooling time interaction (DHAMI et al. 1992). Sperm viability parameters of slow cooled (0.1 °C/min) bull spermatozoa were insignificantly higher than for faster cooled

(4.2 °C/min) batches, suggesting that a slow cooling procedure is not more beneficial than a fast cooling procedure for post-thawing results. However, the results indicate that the incidence of capacitation-like changes in spermatozoa post-thawing do not seem to depend on the cooling rates used (JANUSKAUSKAS et al. 1999).

Acrosomal swelling is one of the effects of cold shock (QUINN et al. 1969). In cat sperm rapid cooling (4 °C/min to 5 °C) is more detrimental to acrosome integrity than slow cooling (0.5 °C/min to 0 °C) inducing significant acrosomal damage (PUKAZHENTHI et al. 1999). The extent of temperature-induced damage was significantly mitigated by slow cooling (0.05 °C/min), revealing a partial or complete loss of acrosomal integrity, changes resulting from serious structural damage to sperm membranes rather than from capacitation events.

2.5 Cooling, equilibration and chilling in canine species

2.5.1 Cooling period

Dog spermatozoa were found to be more resistant to temperature shock at temperatures below 15 °C than sperm of bull or ram and even faster cooling than with semen of these species might be tried (WALES and WHITE 1959), which may be due to the low polyunsaturated : saturated fatty acid ratio of the sperm phospholipids (DARIN-BENNETT et al. 1974). Temperature related alterations in the organization of structural components of the plasma membrane have profound effects upon its properties, associated with changes in permeability and ability to undergo fusion (HOLT and NORTH 1986).

The cooling phase of canine semen cryopreservation protocols has not yet been standardized since the beginning of successful cryopreservation in this species. Within extensive amount of papers dealing with canine semen cryopreservation, the cooling period is described by cooling time (ranging from 30 min to 24 h with or without an additional equilibration period) or cooling rate (°C/min), initial and final temperature (38 °C and 22 °C respectively; 8 - 0 °C) (table 1).

Table 1: Literature survey presenting 41 years of canine semen cryopreservation and their cooling protocols (acr. = acrosome, cond.=conditions, dil. = dilution with extender, equil. = equilibration time, grad.=gradually, integr.=integrity, membr.=membrane, morph.=morphology, ns = not specified, pen. = penetration, pl.=plasma, prog. = progressive, RT = room temperature, surround.=surrounding, temp.=temperature) [final temperature coincides with equilibration temperature if not specified otherwise]

	initial temp (time)	**cooling time / cooling rate**	**surround. cond.**	**frozen in**	**final temp**	**equil. time**	**investigated parameters**
FOOTE 1964a	ns	grad.	ampoules (2 ml)	no	5 °C	1, 4, 8, 12 d	motility (no freezing)
FOOTE 1964b	ns	ns	ampoules (2 ml)	ampoules (2 ml)	5 °C	5 h and 6 h	motility
SEAGER et al. 1975	22-23°C	90 min	cold room (5°C)	pellets	5 °C	no	conception rates
PLATZ and SEAGER 1977	22-23°C	90 min	cold room (5°C)	pellets	5 °C	no	motility, morph.
ZALEWSKI and ANDERSEN BERG 1983	32°C	45 min	ns	straws (0.5 ml)	5 °C	2.5 h	acr. integr.
PROVINCE et al. 1984	37°C	45 min +15 min	vial in 50 ml tube (+25ml wb at 37°C) in cold room (5°C) in cold room	vials (3ml)	5 °C	5760 min	motility, longevity
BATTISTA et al. 1988	23-35°C	60–120 min	ns	pellets (100 µl) straws (0.5 ml)	5 °C	1 – 2 h	motility
FARSTAD et al. 1992a (fox)	37°C	120 min	tube at 5 °C	straws (0.5 ml)	5 °C	no	motility morph.
FARSTAD et al. 1992b (fox)	ns	120 min	at 5°C	straws (0.5 ml)	5 °C	no	AI- results
THOMAS et al. 1993	22°C	60 min	ns	pellets (100 µl) straws (0.5 ml)	4 °C	no	motility
GÜNZEL-APEL 1994	RT 10min	180 min	fridge	straw (0.5 ml)	5 °C	no	Ns

Table 1 (continued)

	initial temp (time)	**cooling time / cooling rate**	**surround. cond.**	**frozen in**	**final temp**	**equil. time**	**investigated parameters**
KUMI-DIAKA and BADTRAM 1994	ns	ns	ns	ns	5 °C	24 h	acr. reaction HOST, motility morph.
SILVA and VERSTEGEN 1995; PIRLOT et al. 1988	ns	120 min	ns	straws (0.5ml)	4°C	2 h	motility, viability, morph.
NÖTHLING et al. 1995	30°C	300 min	15ml tube in beaker (7x9 cm) + water (30°C) in fridge	straws (0.25ml)	5°C	no	mean litter size
ROTA et al. 1995	ns	ns	ns	ns	4°C	4 d	motility, morph., acr. integr.
ENGLAND and PONZIO 1996	ns	9 min	tube	straws (0.5ml)	5°C	10 d	motility, longevity morph. HOST
HAY et al. 1997a	38°C	120 min	vial (6ml) in 15ml tube (+wb at 38°C) in crushed ice	straws (0.5ml)	0°C	30min	motility, morph., acr. integr.
KOUTSAROVA et al. 1997	ns	180 min	ns	pellets (200µl)	4°C	no	motility
IVANOVA-KICHEVA et al. 1997	ns	180 min	ns	pellets and tubes (5 cm^3)	4°C	no	motility morph., acr. integr.
ROTA et al. 1997	RT	45 min	vial in cooler (4°C)	straws (0.5ml)	4°C	30 min	motility, plasma membr. and acr. integr.
ROTA et al. 1998	RT	60 min	vial in cooler (4°C)	straws (0.5ml)	4°C	no	motility longevity viability
PEÑA et al. 1998a,b	RT	120 min	ns	straws (0.5ml)	4°C	no	motility longevity acr. integr.
IVANOVA et al. 1999	ns	180 min	ns	pellets (200µl)	4°C	no	motility, HZA acr. integr. morph.

Table 1 (continued)

	initial temp	**cooling time / cooling rate**	**surround. cond.**	**frozen in**	**final temp**	**equil. time**	**investigated parameters**
ROTA et al. 1999a	ns	70–140 min	ns	straws (0.5ml)	ns	no	# pregnant bitches, # fetuses
ROTA et al. 1999b	ns	24 h	fridge	ns	4°C	15 min at RT	CASA, plasma membr. integr., capacitation
SZASZ 2000	ns	120 min	tube (10ml)	straws (0.5ml)	5°C	no	motility acr. integr.
YILDIZ et al. 2000	32°C	45 min	ns	straws (0.25ml)	5°C	2 h	motility viability acr. integr.
PEÑA and LINDE-FORSBERG 2000a,b	ns	60 mi	tube (10ml) in cooler	straws (0.5ml)	4°C	no	motility longevity plasma membr. integr.
BURGESS et al. 2001	ns	9min	tubes	straws (0.5ml)	5°C	3.5 h	plasma membr. integr.
ROTA et al. 2001	ns	60 min	ns	straws (0.5ml)	4°C	no	motility viability acr. integr.
SIRIVAIDYAPONG et al. 2001	ns	360 min	ns	ns	4°C	no	motility viability acr. integr.
MUSSON 2001	16°C	60 min	15ml tube in 500ml beaker (+ 250ml water at 16°C) in fridge	pellets (50µl)	4°C	no	motility, viability, morph.
HEWITT et al. 2001	ns	9min	5ml tube (+ tissue paper) in 20ml tube in fridge (4°C)	straws (0.5 and 0.25ml)	4°C	no	motility, viability, acr. integr. oocyte pen.
KONG et al. 2001	ns	120 min	cold room	straws (0.5ml)	5°C		viability

Table 1 (continued)

	initial temp (time)	**cooling time / cooling rate**	**surround. cond.**	**frozen in**	**final temp**	**equil. time**	**investigated parameters**
YU et al. 2002	RT 20min	10 min	straws (0.25ml) in fridge (4°C)	straws (0.25ml)	4°C	no	motility, plasma membr. integr.
THIRUMALA et al. 2003	RT	5°C/min	ns	sample pan (10 µl)	extracel ice nucleat.	no	permeability parameters

Still some authors did carry out experiments studying different cooling rates and their influence on canine semen (table 2).

Table 2: Literature survey presenting experiments in cooling time (*), cooling rate (**) and equilibration (***) in canine semen cryopreservation (acr. = acrosome, cond.=conditions, dil. = dilution with extender, equil. = equilibration time, grad.=gradually, integr.=integrity, membr.=membrane, morph.=morphology, ns = not specified, pen. = penetration, pl.=plasma, prog. = progressive, RT = room temperature, surround.=surrounding, temp.=temperature) [final temperature coincides with equilibration temperature if not specified otherwise]

	initial temp (time)	**cooling time /cooling rate**	**surround. cond.**	**frozen in**	**final temp**	**equil. time**	**investigated parameters**
MARTIN 1963a*/***	30°C	60 min 120 min		ampules (1ml) stored at –79°C	5°C 5°C	0 min 60min	motility
MARTIN 1963b***	30°C	120min		ampules (1ml) stored at –79 °C	5°C	0 min 240min 300min	progr. motility, viability
YUBI et al. 1987***	37°C	60 min (at 45, 37, 30, 24, 7°C) +15min	tubes in wb or cold room (7°C) in cold room	straws (0.5 ml)	7°C	180min	motility. viability morphology.

Table 2 (continued)

	initial temp time)	cooling time / cooling rate	surround. cond.	frozen in	final temp	equil. time	investigated parameters
OLAR et al. 1989 */***	37°C	120 min	16x150 mm vial in 250ml flask (+200ml 37°C wb)	straws (0.5 ml)	5°C	no	motility
	37°C	60 min (45 to 8°C 15 on rack to 5°C)	16x150 mm vial in 50 tube (+40ml 37°C wb)	straws (0.5 ml)	5°C	60min or 120min	motility
	37°C	120 min (105 to 8°C 15 on rack to 5°C)	16x150 mm vial in 250ml flask (+200ml 37°C wb)	straws (0.5 ml)	5°C	60 min or 120min	motility
	37°C	180 min (165 to 8°C 15 on rack to 5°C)	16x150 mm vial in 600ml beaker (+400ml 37°C wb)	straws (0.5 ml)	5°C	60min or 120min	motility
	37°C	60 min	16x150 mm vial in 50 tube (+40ml 37°C wb)	straws (0.5 ml)	5°C	60min	motility
BOUCHARD et al. 1990 **	22°C	1°C/min 0.3°C/min 0.1°C/min	ns	ns	4°C	120 h	progr. motility velocity

Table 2 (continued)

	initial temp (time)	**cooling time / cooling rate**	**surround. cond.**	**frozen in**	**final temp**	**equil. time**	**investigated parameters**
STRÖM et al. 1997 ** PEÑA et al. 1999 **	RT	120min 60min	ns	straws (0.5 ml)	4°C 4°C	no	motility, pl. membr.and acr. integr., morphology,
HAY et al. 1997b**	38°C	30 min 180 min	2ml in tube in 15 ml vial (+wb at 38°C) in crushed ice 2ml in tube in 50 ml vial (+wb at 38°C) in crushed ice	straws (0.5 ml) straws (0.5 ml)	0°C 0°C	30min 30min	motility viability morphology oocyte penetration
GOODROWE et al. 2001 ** (red wolf)	ns	30 min 120 min	15 ml tube in fridge (5°C) 15 ml tube in 250 ml beaker (+ wb at 37°C) in ice bath (0°C)	pellets (50 µl) pellets (50 µl)	5°C 0°C	no no	motility, morphology, acr. integr.

Table 2 (continued)

	initial temp time)	cooling time / cooling rate	surround. cond.	frozen in	final temp	equil. time	investigated parameters
	Ns	(0.6 °C/min) thereupon >>>> (0.25 C°/min)	fridge >>in ice bath to 0 °C	15ml tube	5 °C 0 °C	no no	
BATEMAN 2001 **		(0.14 C°/min) thereupon >>>> (0.09 °C/min)	fridge >>in ice bath to 0 °C	15ml tube in 50ml tube (+30 ml wb at 37 °C) in 250ml beaker (+160 ml wb at 37 °C)	5 °C 0 °C	no no	motility, PSA-acr. integr. morphology, ZPA

OLAR et al. (1989) tested different cooling rates (see table 2) and found no effect of cooling time to 5 °C on canine spermatozoal motility prior to freezing, but a cooling time by equilibration time interaction. Canine spermatozoa withstood a range of cooling and equilibration times with no detrimental effect on spermatozoal motility prior to freezing. However, there were differences in spermatozoal motility immediately after thawing. In a review by CONCANNON and BATTISTA (1989) best results have been usually obtained by initiation of cooling as soon as possible after semen collection and a slow cooling rate to reduce 'cold-shock' damage (i.e., cooling at 0.1 to 0.2 °C/min over 2-3 h rather than at 0.3 to 0.5 °C/min over 0.5-1.0 h). However in another study, medium (0.3 °C/min) and fast (1.0 °C/min) cooling rates resulted in a higher percentage of total and progressive motility and higher sperm velocities both after cooling than in slowly (0.1 °C/min) cooled semen (BOUCHARD et al. 1990). Cooling spermatozoa to 0 °C was shown to have no detrimental effect if cells were cooled slowly (ENGLAND 1993). Marked decreases in intact acrosomes, post-thaw motility and normal morphology of spermatozoa after treatment were detected, however, no differences were observed between cooling treatments with regard to either cooling or freeze-thaw of red wolf spermatozoa (GOODROWE et al. 2001). Two authors

cooled dog semen do 0 °C, which already is part of the supercooling period with starting ice nucleation process. HAY (1996) observed that after fast cooling to 0 °C (~40 min) with >2 h equilibration there was a decrease in cooled spermatozoa with regard to percentage intact and undamaged acrosomes. Fast cooling procedure (in 0.5 h to 0 °C) resulted in poorer acrosomal integrity, motility and oocyte penetration than slow cooling (in 3 h to 0 °C) (HAY et al. 1997b). Pre-freeze cooling of semen to 5 °C compared to 0 °C resulted in better sperm motility and acrosome integrity after cooling, but did not influence post-thaw sperm motility, acrosome integrity, morphology or ZPA (BATEMAN 2001).

2.5.2 Equilibration period

MARTIN (1963a) found that ageing of canine semen might modify the rate of penetration of glycerol and affect the need for an equilibration period. A period of about 5 h equilibration prior to freezing has depressed the percentage of motile cells after thawing (MARTIN 1963b). But equilibration at 5 °C for longer than 1 h is beneficial for dog semen cryopreservation (CONCANNON and BATTISTA 1989). It may allow the spermatozoa to a minor extent to recover from some stressing associated with dilution proven by best post-thaw motility and highest percentage of live spermatozoa when cooled 4 h at 5 °C compared to 2 and 6 h equilibration time (ENGLAND 1992). In contrast, equilibration time at 5 °C had no effect on prefreeze canine spermatozoal motility (OLAR et al. 1989) compared with initial sperm motility. These results were confirmed by ROTA et al. (1997) who showed that the equilibration time (30 min at 5 °C) had no significant effect on motility and plasma membrane status of compared fresh and equilibrated samples. STRÖM et al. (1997) compared two cryopreservation methods and found that the integrity of the plasma membrane was not affected by equilibration with either method.

2.5.3 Chilling period

Storage of diluted canine semen at a temperature of 5 °C for 24 h did not significantly impair the physical and functional characteristics of the canine spermatozoa (KUMI-DIAKA and BADTRAM 1994); the percentages of progressively motile spermatozoa, positively responding spermatozoa of the hypoosmotic swelling test (HOST), acrosome-reacted spermatozoa, acrosomal defects and live spermatozoa did not significantly differ between fresh and chilled samples. However, storage at 4 °C advanced the occurrence of hyperactive spermatozoa and acrosome reaction. Storing semen at 5 °C probably initiates/triggers the acrosome reaction therefore taking place earlier in chilled semen compared with fresh semen, when both were placed in canine capacitating medium. Preservation of diluted dog semen at 4 °C for 4 days using egg-yolk TRIS extender seemed to be more successful than using other extenders (ROTA et al. 1999b). Although cooled semen has a limited lifespan, it has been proven that using conventional methodology dilution and cooling is the most suitable technique, provided that the samples is used within approximately 5 days of collection. Short-term storage of canine semen should be preferably performed by cooling and rewarming (ENGLAND and PONZIO 1996) as the freeze-thaw process causes significant sperm damage with the morphology deteriorating most quickly. Nevertheless, capacitation-like changes in dog semen seem to be both initiated and accelerated by the preservation procedures (ROTA et al. 1999b). Furthermore canine sperm binding capacity decreases from day 1 to day 4 of chilling (STRÖM HOLST et al. 2000). At 4 °C, egg yolk extender best maintained sperm motility, as found by computer-assisted semen analysis. The results indicated that fresh dog semen of good quality can be preserved for up to 10 days (IGUER-OUADA and VERSTEGEN 2001b). Storage for 6 h at 4 °C did not affect sperm motility, viability or acrosome integrity, irrespective of the dilution treatment. However, sperm motility and viability decreased significantly after freezing and thawing, while the acrosome morphology of viable spermatozoa was not affected by chilling or freezing and thawing (SIRIVAIDYAPONG et al. 2001).

2.6 Extender additives

A good extender is expected to have:

- nutrients as an energy source
- to buffer against harmful changes in pH
- to provide a physiologic osmotic pressure and concentration of electrolytes
- to prevent growth of bacteria
- to protect cells from cold-shock during the cooling procedure and
- to have cryoprotectants that reduce sperm cell damage during freezing and subsequent thawing.

Extenders used for semen freezing are based on those initially developed for semen preservation at 4 °C. They have incorporated egg yolk for reduction of cold shock and glycerol for protection against freezing damage (CONCANNON and BATTISTA 1989). Different extender additives have been studied in order to improve the protecting and preserving effects on spermatozoa.

2.6.2 Extender additives containing sodium dodecyl sulphate

Sodium dodecyl sulphate $[CH_3(CH_2)_{10}CH_2OSO_3]^- Na^+$- is a water-soluble anionic detergent and wetting agent, used to solubilize proteins (HELENIUS et al. 1979). Because of its surface-active properties it is widely used in cleaners, pharmaceuticals, personal care products and in biochemical research involving electrophoresis (SINGER and TJEERDEMA 1993). Sodium dodecyl sulphate (SDS) is the most common name, but sodium lauryl sulphate (SLS) is used synonymously (SINGER and TJEERDEMA 1993).

SDS is the main component of commercially available extender additives like Orvus Es Paste (OEP, Procter and Gamble, Co., Cincinatti, OH, USA) (KATO et al. 1990; THOMAS et al. 1992), Orvus Es Paste (OEP, Nova Chemical Sales, Scituate, Inc., MA, USA) (ROTA et al. 1997; TSUTSUI 2000a, b, c), Equex STM paste (Nova Chemical Sales, Scituate, Inc., MA, USA) (NÖTHLING and VOLKMANN 1993; ROTA et al. 1997, 1999a; PEÑA and LINDE-FORSBERG 2000b; PEÑA et al. 2003a, b) and Equex pasta (Minitüb, Tiefenbach, Germany;

PEÑA et al. 2003a, b). SDS is also available as single component (SDS, Schwarz/Mann Biotech, Division of ICN Biomedicals. Inc., Cleveland, OH, USA) (PEÑA et al. 1998c).

SDS has been included in extenders for several species and is one of the best studied additives so far. In the early 80s, Orvus ES paste (OEP) was one of the first additives to be used in boar extender. Orvus ES paste had a beneficial effect on sperm motility independent of the cooling rate. The effects on the acrosomes however was more pronounced after 2 h cooling than after 6 or 12 h cooling (PURSEL et al. 1978). In frozen-thawed (pellets) boar sperm SLS (2.0-2.8 mg/ml sperm-extender suspension) improved post-thaw percentages of normal acrosomes and progressively motile spermatozoa after a 3 h post-thaw incubation at 37 °C (KATO et al. 1990). The addition of different amounts of Equex STM paste or Orvus ES paste, to extenders for the freezing of semen has been found to be beneficial in the boar (*Sus scrofa;* PURSEL eta l. 1978), stallion (*Equus caballus* MARTIN et al. 1979), mouse (PENFOLD and MOORE 1993), and domestic dog (THOMAS et al. 1992; ROTA et al. 1997).

Among several hypothesis on the mode of action, one is that SDS/SLS may alter egg-yolk contained in the extender, by solubilizing active molecules, especially protective lipids in the egg-yolk (ARRIOLA and FOOTE 1987; PENFOLD and MOORE 1993), and thus possibly increasing its protective effects during cryopreservation (PURSEL et al. 1978). SDS elicits both physical and biochemical effects on cells, with the membrane as primary target structure, indicating an indirect positive effect of SDS on the sperm membranes (SINGER and TJEERDEMA 1993). A protective effect on the acrosome was detected by KATO et al. (1990) suggesting that SDS increases the content of protein and SH groups in semen, thereby reducing the harmful effects of hydrogen peroxide on sperm. TSUTSUI et al. (2000b) confirmed earlier findings that acrosomes are protected by addition of SDS (OEP). PEÑA and LINDE-FORSBERG (2000b) presumed a direct protective effect of SDS on sperm membranes, by reducing lipid phase transitions and/or protecting the functioning of membrane ionic pumps, which resulted in lower intracellular calcium concentrations, reduced hyperactivity and prolonged post-thaw thermoresistance of spermatozoa. Sperm subpopulations showing prolonged post-thaw survival and longevity on one hand and high intracellular Ca^{2+} concentrations on the other hand were detected. SDS may protect sperm membranes during freezing and thawing by a molecular reorganization of the membranes that allow the Ca^{2+}-pumps to continue working even under stress. Although the Ca^{2+}-pumps might

work with less efficiency than before cryopreservation with subsequent increase of intracellular calcium concentration, they are still able to keep the intracellular Ca^{2+} concentration under the critical threshold (PEÑA et al. 2003a, b).

Post-thaw, canine semen frozen with 0.5% Equex STM paste tended to have higher percentage of totally and progressively motile spermatozoa, longer *in vitro* survival (ROTA et al. 1999a), also in the thermoresistance test (STRÖM HOLST et al. 2000) and better motility characteristics (PEÑA and LINDE-FORSBERG 2000b) than semen frozen without Equex. However, inclusion of Equex STM paste did not affect pregnancy rates after AI with frozen-semen (ROTA et al. 1999a) although it showed a higher capacity bind to the zona pellucida *in vitro* (STRÖM HOLST et al. 2000). The protective effect of SDS on dog spermatozoa was found to be more pronounced if it was added only immediately before freezing, suggesting that prolonged exposure to SDS or to SDS-treated egg-yolk lipoproteins may exert a direct negative effect on sperm membranes and that the beneficial effect of SDS may depend on concentration and time of exposure (PEÑA and LINDE-FORSBERG 2000b).

2.7 Hypoosmotic swelling test

The plasma membrane plays an essential role in the communication between the sperm cell and the external medium (GARBERS and KOPF 1980; CALVETE et al. 1996). The HOS-test is used to determine the functional integrity of the sperm's plasma membrane. It is based on the fact that fluid is transported through an intact cell membrane under hypoosmotic conditions until a balance between ions is reached between the inside and outside of the cell. Fluid influx leads to cell expansion, causing a bulging of the plasma membrane. The fibres of the sperm tail (axonemal complex) are normally closely surrounded by the plasma membrane. If the plasma membrane balloons under hypoosmotic conditions, the tail fibres are curling and bending within the plasma membrane. These tail changes can readily be observed by a phase contrast microscope. Since it is not possible to clearly identify the plasma membrane of an unstained spermatozoon by light microscopy, the entire tail will appear curled or bent and the phenomenon is often referred to as 'tail curling'. Spermatozoa with a chemically and physically intact membrane will show 'tail curling' or 'swelling' under hypoosmotic

conditions, whereas spermatozoa with an disintegrated membrane will not. The biochemical integrity of the plasma membrane is essential for sperm function, not only for the metabolic processes involved in sperm motility but also for the events occurring during fertilization, e.g. capacitation, acrosome reaction, and binding of the spermatozoa to the egg-surface (JEYENDRAN et al. 1992). A nonfunctional membrane as determined by lack of swelling is assessed to be a serious impairment of fertility (STARK and BERNT 1989; JEYENDRAN et al. 1992;). The HOS-test is used in the evaluation of human semen quality and provides a statement about physiological, functional and structural integrity, as the tail fibres coil several times within the swollen membrane in case of a positive reaction of the spermatozoa (SCHRADER et al. 1986). The HOS-test is classified as a vital stain with the advantage that plasma membrane integrity is tested without using chemical components which may stress the spermatozoa (PETZOLD and ENGEL 1994). The plasma membrane of mammalian spermatozoa has domains of different composition and ultrastructure (FRIEND 1982; HOLT 1984) with clear demarcation between them (BEARER and FRIEND 1990). These domains (acrosomes, equatorial segment, post-acrosomal region, midpiece and tail) have different functions, each contributing to sperm status. Accordingly, acrosomal status doesn't provide information about tail status. For obtaining a complete picture of sperm quality the functional and structural integrity of the sperm plasma membrane of the tail must be taken into account. In domestic animals the first studies were done by LINDAHL and DREVIUS (1964) who incubated bull spermatozoa for 15 min in Ringer solution and studied plasma membrane changes by electron microscopy. CORREA and ZAVOS (1994) observed in bull semen a positive correlation between motility and intact plasma membrane on one hand and a good volume regulation capacity on the other hand. The HOS-test is assessed as reliable, sensitive and reproducible assay also for boar semen (100-150 mOsm) and useful for detecting subpopulations of subviable cells (VAZQUEZ et al. 1997). Neither motility nor acrosome morphology were able to reflect the ion shift, and the HOS-test has been shown to indicate cold shock induced damage more sensitively than motility and acrosome evaluation (PÉREZ-LLANO et al. 2001). Because of its predictive value in selecting individual ejaculates from stored canine semen samples for insemination, the HOS-test may be a useful addition to standard semen analysis (KUMI-DIAKA 1993). KUMI-DIAKA and BADTRAM (1994) and RODRÍGUEZ-GIL et al. (1994) demonstrated a significant association between sperm

motility and acrosome integrity to the volume regulation capacity indicating the value for assessing sperm viability and the potential of the HOS-test to have predictive value in screening out subfertile dogs with apparently normal sperm quality.

2.8 Computer-assisted semen analysis

Sperm motility is an important parameter in evaluating quality of fresh semen and the effect of different treatments during the cryopreservation period. In connection with the fertilization process characteristic motility patterns are required for sperm moving through the oviduct towards the oocyte for oocyte penetration. Subjective optical microscopic evaluation has been shown to result in variations of 30-60 %, estimating motility parameters in the same ejaculates (DEIBEL et al. 1976; CHONG et al. 1983; JEQUIER et al. 1983; DUNPHY et al. 1989). A more objective evaluation of semen motility is a track semen analysis of individual spermatozoa, that allows an accurate calculation of different semen parameters such as concentration and motility, including the percentage of progressively motile spermatozoa, linearity and velocity (MATHUR et al. 1986; NEUWINGER et al. 1990). Computer-aided semen analysis (CASA) was first proposed by DOTT and FOSTER (1979), and is now commonly used in human andrologic centres. AUGER and DADOUNE (1988) investigated the effect of freezing on motility parameters in human sperm and MAHONY et al. (1988) correlated velocity and swimming pattern with fertility. The average path velocity (VAP) was shown to be of prognostic value in prediction of the oocyte fertilization rate in humans (MAK et al. 1994). There are different systems of detecting spermatozoa and evaluating motility parameters. CHAN et al. (1989) used a system for computerized analysis of sperm movement characteristics and differential sperm tail swelling patterns in predicting *in vitro* fertilizing capacity of human sperm. The sperm quality analyser (SQA), used by ZAVOS et al. (1996) in bull semen detects variations in the optical density of motile spermatozoa, providing a sperm motility index (SMI) that is based on various sperm parameters including the concentration, morphology and acrosomal status of motile spermatozoa, and is found to be suitable for rapid and reliable screening of sperm samples. Apart from motility analysis GRAVANCE et al.

(1997) evaluated gross head morphology of equine sperm by sperm head morphometry analysis.

Characterizing motility during maturation of rat spermatozoa, YEUNG et al. (1992, HT HTM 2030) showed that caput spermatozoa had low straight-line (VSL) and averaged-path (VAP) velocities and low path straightness, whereas mature cells displayed high VSL, VAP and STR. The magnitude of the velocity parameters 2 h after incubation in capacitating medium being correlated to successful inseminations of sows were identified as the most consistent indicators of fertility (HOLT et al. 1997, Hobson Sperm Tracker). Even with considerable variation in semen quality within species (human, rabbit and bull), CASA instruments are capable of high speed objective analysis of many sperm cell characteristics (FARRELL et al. 1995, HT-IVOS). *In vitro* fertilizing capacity of rat spermatozoa is shown to be correlated with the decline in straight-line velocity (VSL) and lateral head displacement (ALH) only during continuous semen analysis for several hours (MOORE and AKHONDI 1996, Hobson Sperm Tracker). Semen motility parameters were evaluated successfully in Tori and Estonian breed stallions (KAVAK et al. 2003, CMA) and dromedary bulls (AL-QARAWI et al. 2002, CMA). Sperm subpopulation patterns were studied in equine (QUINTERO-MORENO et al. 2003) describing that separate subpopulations fo spermatozoa with different motility characteristics coexist in stallion ejaculates and that ejaculates with confirmed fertilizing capacity included subpopulation with high progressive motility and low linearity.

GÜNZEL-APEL et al. (1993) described CASA in dog semen using different systems. These authors and WJICHMAN et al. (1995) worked with the Cellsoft computer videomicrography system (Strömberg-Mika Cell Motion Analyser) based on sperm tail detection. While other research groups referred to different CASA systems evaluating dog semen (SMITH and ENGLAND 2001: Hobson Sperm Tracker; RIGAU et al. 2001: Sperm Class Analyser; ROTA et al. 2001, IGUER-OUADA and VERSTEGEN 2001a, b: Hamilton Thorn IVOS–System; RJISSELAERE et al. 2003: Hamilton Thorn Ceros-System). The HT determines sperm characteristics by detecting and analysing the sperm head positions in sequential video images (DAVIS and KATZ 1993; MORRIS et al. 1996). GÜNZEL-APEL et al. (1993) found a significant correlation between the parameters microscopically estimated and computer analyzed sperm motility. The information provided by CASA is not purely objective but are influenced by the gate and parameter settings chosen by the operator. Therefore careful

attention is required with regard to the internal image settings, the analysis time and the minimum length of sperm track (SMITH and ENGLAND 2001). A variety of parameters and settings between human studies and veterinary studies is presented by VERSTEGEN et al. (2002). Frame rate significantly influenced most of the motility characteristics (RJISSELAERE et al. 2003). Chamber depth of the device where spermatozoa are evaluated is important as well because it may influence the distribution of non-progressive and progressive hyperactivated sperm (LE LANNOU et al. 1992); while the Makler chamber has an additional disadvantage of a possible lack of reproducibility (AGARWAL et al. 1992). Regarding sperm concentration, motility may be underestimated if it is lower than 25 x 10^6 spermatozoa/ml (RJISSELAERE et al. 2003), however AMANN (1989) prefers 10-12 x 10^6 bull spermatozoa/ml to minimize collisions or 'confusion' of the computer.

The influence of sodium dodecyl sulfate on gazelle (*Gazella dama mhorr*) semen was studied by computer assisted semen analysis by HOLT et al. (1996) and protective effect on sperm motility parameters was detected (product name and final concentration of equex were not specified). Addition of 0.5 % equex (product not specified) resulted in a subtle protective effect of gazelle (*Gazella dama mhorr*) spermatozoa motility with suppression of erratic swimming behaviour and significantly higher sperm velocity parameters (VCL, VAP, VSL) and ALH, LIN (ABAIGAR et al. 1999).

2.9 Electron microscpopy

Major acrosome defects like cystic changes of the apex or distinct lipping of the acrosomal rim detected by COUBROUGH et al. (1987) in cheetah semen, confirmed the electron microscopy as a provider of invaluable complimentary information regarding semen ultrastructure. The integrity of the plasma membrane and acrosome of frozen human sperm, as evaluated by transmission electron microscopy (TEM), has been shown to be positively correlated with fertility using artificial insemination (MAHADEVAN and TROUNSON 1984). Seminal parameters of fresh red wolf (*Canis rufus*) semen was evaluated by electron microscopy frequently observing abnormalities associated with head structures and acrosomal defects (KOEHLER et al. 1998).

In slowly cooled (0.25 °C/min) ram and black buck semen samples particle clustering over the post-acrosomal region at the head and the tail was discovered as an irreversible change of plasma membrane that may have contributed to loss of motility and fertility (HOLT and NORTH 1984). In ram semen acrosomes and midpieces were affected by cooling to 5 °C and storage for up to 72 h at this temperature (JONES and MARTIN 1973; PARKS and HAMMERSTEDT 1985). The acrosome changes involved swelling and vacuolation or swelling and vesiculation of the outer acrosomal membrane with discontinuities in the plasma membrane; total loss of the anterior outer membrane was uncommon. Midpieces showed condensation of mitochondria with occasional loss of electron-density and internal structures (WATSON and MORRIS 1987). They showed a localized swelling of the flagellum, particularly in the region of the endpiece (HOLT et al. 1988). Cold shock causes profound changes in the appearance of the corrugated and terminal parts of the acrosome in the majority of ram spermatozoa (QUINN et al. 1969) and in case of severe stress total loss of acrosomal matrix; both the proportion of damaged cells and the extent of membrane destruction is related to the degree of cold shock (WATSON and MORRIS 1987). WOOLEY and RICHARDSON (1978) described ultrastructural changes in human spermatozoa occurring either during or after the thawing procedure. The outer acrosomal membrane often appeared lightly ruffled and the acrosomal content was less homogeneous after dilution. After thawing most acrosomes were more severely swollen and distorted and appeared to have lost much of their content. Mitochondria only were affected by freezing-thawing being more closely packed and angular in profile. After cooling bull and boar semen to 0 °C IMP distribution in the acrosomal region of the sperm head was no longer homogeneous and particle-free areas had formed, with rearrangement of intramembranous particles in the principal piece of the tail, resulting of lateral phase separations (DE LEEUW et al. 1990b). ABRAHAM-PESKIR et al. (2000, bull, horse), however, stated that distortion and separation of the plasma membrane from the acrosomal membrane also reported as freeze-thaw damage (BURGESS et al. 2001, dog) could represent an artefact caused by TEM. Swelling, rarefaction and loss of electron-dense acrosome material due to freeze-thawing in dog spermatozoa has been described by RODRIGUEZ-MARTINEZ et al. (1993) and STRÖM HOLST et al. (1998). A significant amount of damage to the acrosome occurred during dilution, cooling and equilibration of dog spermatozoa (OETTLÉ 1986a). BURGESS et al. (2001) detected undulation and vesiculation

of the plasma membrane and acrosome after a 3.5 h storage at 5 °C. Describing the same results for cooled dog semen, STRÖM HOLST et al. (1998) also found acrosomal swelling with ballooned plasma membrane and plica formations, vesiculation of the outer acrosomal membrane and acrosome detachement. Loss of electron-dense acrosomal material between the outer and inner acrosomal membrane was associated by OETTLÉ and SOLEY (1988) with cold-shock and not with the freeze-thaw process. In cryopreserved blue fox spermatozoa (HOFMO and ANDERSEN BERG 1989), post-thaw changes were characterized by extensive acrosomal changes showing various degrees of disruption and vesiculation. Extensive damage of acrosomal membranes occured after cooling, especially in spermatozoa from teratospermic ejaculates, showing higher susceptibility to cold-induced acrosomal damage compared with normospermia (PUKAZHENTHI et al. 1999).

3 Materials and Methods

3.1 Preliminary cooling and temperature trial

15 ml plastic vials (Fisher Scientific Company, Houston, Texas, USA) filled with different volumes (1 to 8 ml) of semen extender at room temperature were placed into the water baths (one vial per water bath) and held by a wire construction so that the vial was fixed in place in the middle of the water bath. Glass beakers of varying volumes (250 ml, 500 ml, 1000 ml; Fisher Scientific Company, Houston, Texas, USA) were filled with water (250 ml, 500 ml, 1000 ml) at room temperature. The vial holding beakers were placed in the middle shelf of a completely emptied refrigerator, which had a cooling cycle between 2 °C and 6 °C. One vial was placed in a glass beaker filled with 500 ml water at room temperature and placed in an ice water bath in a cooler. This set up was included into the study to investigate a simple cooling system which would be of advantage in the field when no electric current for a refrigerator is available. The temperature changes in water bath and extender were measured by temperature sensors (HOBO H-8 4-channel external, USA) placed in the liquids (water and extender) and in the refrigerator (refrigerator cooling cycle). The data were stored in the temperature sensor and were transferred to the desk computer by means of a specific software (Box Car, USA).

3.2 Semen donors

Domestic dogs (*Canis familiaris*)

Four healthy male adult dogs (two Nova Scotia Duck Tolling Retriever, one Golden Retriever and one Yellow Labrador Retriever) of 1.5 to 5 years were included in the study. They belonged to private owners and were fed a balanced commercially available diet and had fresh water *ad libitum*. The dogs had valid vaccination status and had never reproduced before. In the 5 year old yellow Labrador Retriever a semen collection had been performed before by a veterinarian.

Mexican wolves (*Canis lupus baileyi*) (Fig. 2 and 3)

Six healthy male Mexican wolves belonging to the U. S. Fish and Wildlife Service were designated by the Mexican Wolf Species Survival Plan to participate in the research project. They were housed at the Wild Canid Survival and Research Center in Eureka, Missouri, USA. #566 (‚Tika'), #567 (‚Lakota') and #568 (‚Cheveya') were 3 years old and brothers of the same litter and were housed together in a pen of 313.3 m^2 (17.7 x 17.7 m).

Fig. 2 Mexican wolf in its summer coat

Fig. 3 Mexican wolf in its winter coat

#545 (‚Nacona'; ~3 years old) was housed together with #606 (~4 years old) in a pen of 215.9 m^2 (12.2 x 17.7 m). #78 (‚Taz', 11 years old) was housed separately in a small isolation enclosure of 73.2 m^2 (6.0 x 12.2 m). With one exception (wolf #78, which was a proven breeder and had produced three litters between 1992 and 1994), semen quality and fertility of the wolves was unknown. The animals were fed a commercially available dry chow diet (Mazuri Exotic Canine Diet, PMI Nutrition International, St. Louis; two pounds per wolf per day). Fresh water was provided *ad libitum*. Bones and frozen mice were additionally offered once a week. They had valid vaccination status for distemper, leptospirosis, parvovirosis and rabies and the testing for heart worm and brucella was negative. #606 was diagnosed to be kryptorchid.

Generic gray wolves (*Canis lupus*)

Seven generic gray wolves (#404 and #413; 6 years of age each, #417; 5 years of age, #424, #425, #426 and #427; 3 years of age each) belonged to the Wildlife Science Center, Forest Lake, Minnesota, USA. The animals were held in large outdoor pens either in family packs or in brotherhood groups of three to eight animals. They were fed exclusively deer carcasses that were provided by road kills (one deer per group and week). Fresh water was provided *ad libitum*. They all had valid vaccination status and were treated for heart worm.

3.3 Determination of testicular size

On the anaesthetized animal the testicular size (width, length and depth) of both testicles was measured with calipers (Seager Orchometer™, Booth Medical Equipment Co., Alexander, AR, USA).

Equation for calculation of testicle volume (MUSSON 2001)

$$V_{total} = [(\pi D_{AV}^2) / 2] * [(L_t / 2) - (D_{AV} / 2) + (D_{AV} / 3)]$$

D_{AV} = average of width and depth measurements
L_t = longitudinal measurement

3.4 Semen collection

Domestic dog (*Canis lupus familiaris*)

The semen collections were performed at the St. Louis Zoo Veterinary Hospital, St. Louis, Missouri, USA, without a teaser bitch by manual stimulation of the penis (LINDE-FORSBERG 1991). The collected sperm rich fraction was evaluated immediately under a phase contrast microscope for motility.

Mexican wolf (*Canis lupus baileyi*) and generic gray wolf (*Canis lupus*)

Anaesthesia

In the Mexican wolves semen was collected once per week during seven weeks with 6-7 day intervals from end of January to beginning of March. For sedation the animals were caught in a smaller pen adjacent to their enclosure, and fixed by a neck pole. The sedative drugs [Ketamin, Ketased® (1 mg/kg body weight) Fort Dodge Animal Health, Fort Dodge, Iowa 50501, USA and Xylazine, Rompun® (0.5 mg/kg body weight) Ben Venue Laboratories, Bedforf, Ohio 44146, USA; MUSSON 2001] were administered intramuscularly into the hind limb muscles at a proportion Ketamine : Xylazine = 1:1. Mexican wolves were transported anaesthetized into a trailer, placed on a surgery table, intubated and kept under isofluorane (1-3 %; Isoflo®, Abbott Laboratories, Chicago, Illinois 60064, USA) anaesthesia. In #78 semen collections were limited to one due to delayed recovery from anesthesia.

In the generic gray wolf semen collections were performed twice at the end of February at a two day interval. The anaesthetic drugs (as described above) were injected intramuscularly by a syringe-construction on a stick. The wolves were anaesthetized in their enclosure and then transported into the semen collection location.

Electroejaculation

Using a urinary catheter (Sherwood Medical, St. Louis, Missouri, USA) the urinary bladder was emptied and flushed several times with sterile saline. After measuring both testicles in length, width and depth using calipers (The Seager Orchometer ™, Booth Medical Equipment Co., Alexander, AR, USA), the fur around the ostium praeputiale was cut with scissors and the abdominal area around the penis was cleaned with sterile NaCl-solution and gauze. The surface of the penis tip was washed with sterile saline solution, a sterile drape was placed over the abdominal region and the penis was exposed through a hole in the drape. The electroejaculation probe #4 of the electroejaculator (model 303, 110V, 50/60 Hz; P-T Electronics, 11241 S.E. 362nd, Boring, Oregon 97009, USA) was covered with sterile lube (K-Y lubricating jelly, Johnson & Johnson Medical Inc. Arlington, Texas 76004, USA) and introduced into the rectum (CHRISTENSEN and DOUGHERTY 1955).

The power of stimulation was adjusted to the feedback coming from the animal. One application consisted of several administrations of increasing electric flow until ejaculation.

first application (2 min);

- 10 Amp: increase from 0 Amp to 10 Amp, stay at 10 Amp (1 sec), decrease to 0 Amp
- 20 Amp: increase from 0 Amp to 20 Amp, stay at 20 Amp (1 sec), decrease to 0 Amp
- the application of increasing stimulation in steps of 10 Amp are continued until ejaculation
- when ejaculation occurred at a specific Amp level, stimulations were repeated with this level.

resting period of 5 to 10 min

second application (2 min): same procedure than during the first application

The ejaculated semen was collected in a commercially available plastic cup. The samples were checked for contamination with dirt or urine and, if clean, were evaluated under a phase contrast microscope for motility and were pooled.

3.5 Experimental Design

Dog semen was used prior to experimentation with wolf semen for selective testing of five different cooling times (0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h).

With semen of the Mexican wolves and the generic gray wolves semen a short cooling time (0.5 h) and a long cooling time (2.5 h) were compared with an intermediate cooling time of 1.0 h, that had been used in recent years as part of the Mexican wolf semen freezing protocol. For the cooling time of 0.5 h the semen samples were placed in a 15 ml vial in a refrigerator (5 °C) without surrounding water bath. For the cooling time of 2.5 h the samples in a 15 ml vial stayed in a glass beaker (diameter: 6.5 cm) with 250 ml water. For the control cooling time of 1.0 h the samples in a 15 ml vial were placed in a 400 ml plastic beaker (diameter: 9 cm) with 250 ml water.

Besides the cooling time, the effect of the addition of 1 % Equex pasta (Minitüb, Tübingen, Deutschland) to the semen extender on frozen-thawed semen quality was tested.

As a general guideline a minimum of 1ml semen-extender is needed to get enough pellets (at least 30 pellets of 30 µl) for post-thaw testing and artificial insemination. The volume of 1 ml also was necessary to guarantee the complete immersion of the temperature sensors into the semen-extender during the cooling process.

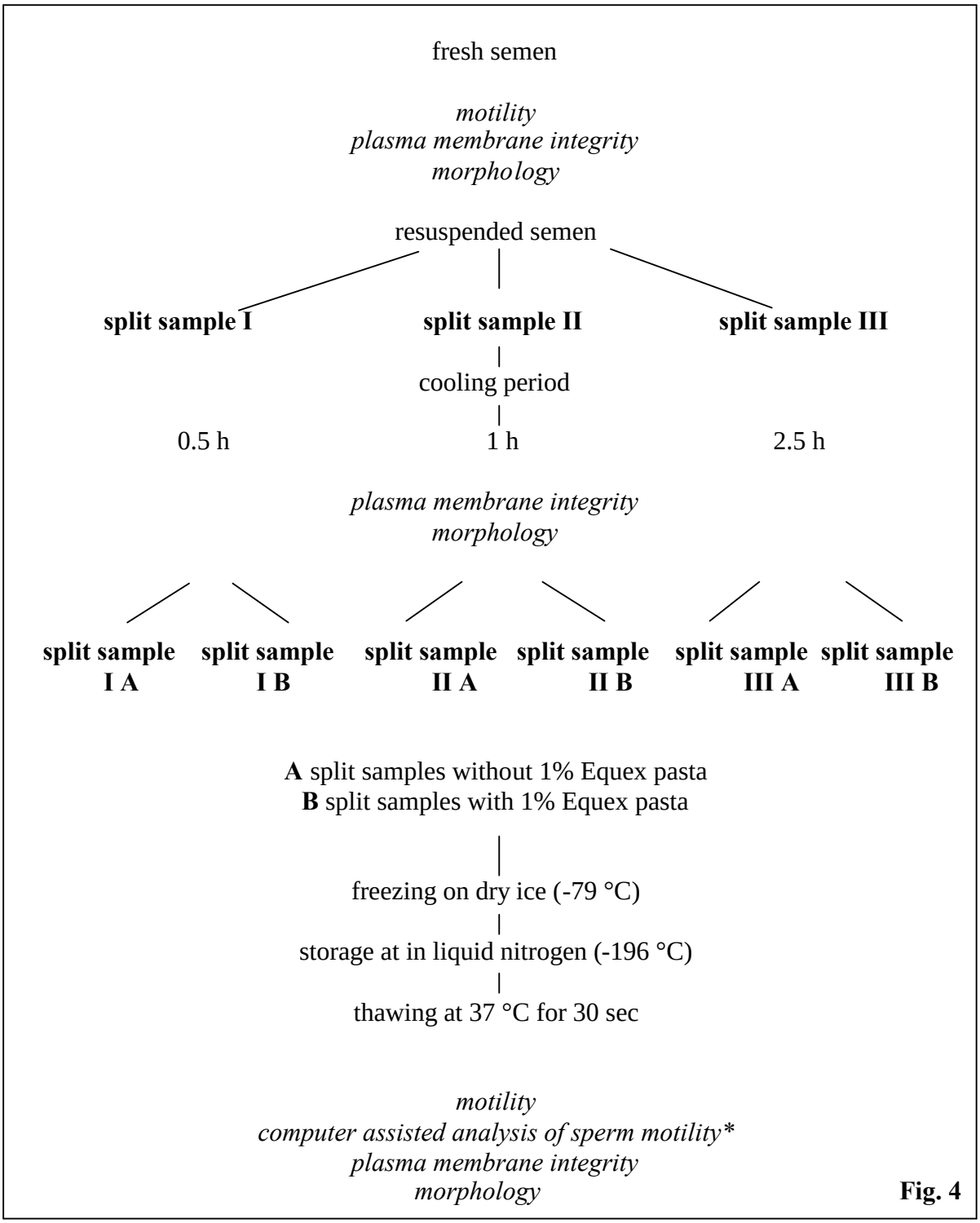


Fig. 4: Experimental design, semen handling and timing of semen evaluation procedures *(parameters in italic typing were evaluated statistically)*; * only semen of Mexican wolf

3.6 Semen handling

Before cooling

The pooled samples were recorded for total volume. For determination of the sperm concentration a drop of semen sample was diluted 1:20, using the Unopette white blood cell/platelet dilutor kit (Becton-Dickinson, Rutherford, NJ, USA) and a Makler cell counter (Sefi Medical Instruments, TS Scientific, Perkasie, PA, USA). Semen was centrifuged (centrifuge: model 228, bench top centrifuge, Fisher) at 3300 RPM (380 x g) for 5 min. All but 1 ml of the supernatant (seminal plasma) was removed and saved in a microcentrifuge tube (Fisher Scientific, Pittsburgh, PA 15219, USA; cat # 05-664-53) for later measurement of pH (Beckman Instruments, Inc. Fullerton, CA 92634-3100, USA) and osmolarity (vapor pressure osmometer, model 550, Wescor Inc., 459So. Main Street, Logan, Utah 84321, USA). Based on the total calculated motile sperm number the amount of extender (9.2.1) to be added was determined.

volume of extender to add
= conc [x 10^6 sperm/ml] * vol I [ml] * mot [%] - vol II [ml] / 40x10^6 sperm/ml

conc = concentration
mot = forward motility
vol I = volume of semen collected
vol II = volume of resuspended semen after centrifugation (here 1 ml)

The extender was added to the semen drop-by-drop holding the vial almost horizontally in an angle and adding the extender not directly into the semen but onto the wall of the vial.

Cooling

All water baths were prepared at the beginning of the day with regular tap water to let the water equilibrate to room temperature in the laboratory or trailer. The ice water bath for the dog semen trial was prepared with 2l and 24 ice cubes right before the semen collection. Each of the vials filled with semen-suspension was put into a water bath and the temperature sensors were immersed into the water baths with an additional sensor immersed in a ‚fake‘

sample of plain extender of the same volume. After the respective cooling time measured by a timer (traceable, 100 h four-channel timer) the sample was removed in its water bath from the Refrigerator (Fig. 5).

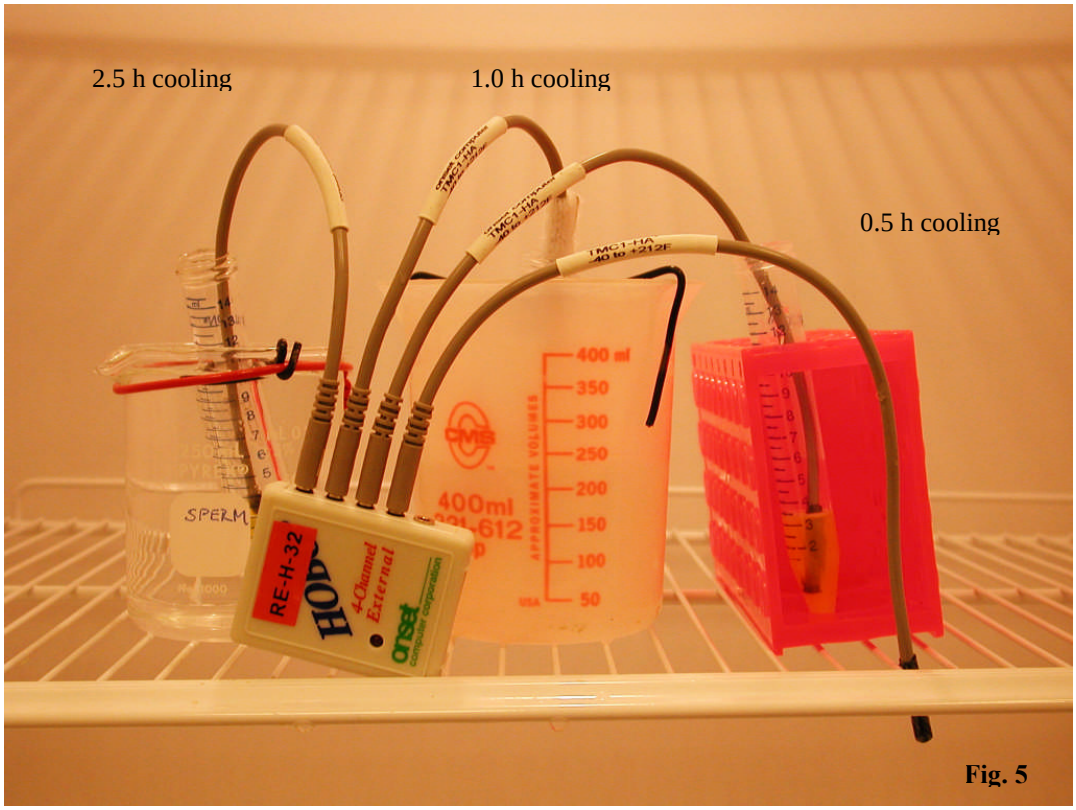


Fig. 5: Refrigerator set up of vials with semen-extender suspension and water baths

transported in its water bath to another room [dogs (1 min), generic gray wolves (15 sec)] or another trailer [Mexican wolves (1-2 min)], respectively, for the addition of Equex pasta and the freezing process.

Addition of Equex pasta

The diluted cooled semen samples were split in two aliquots. To one of those aliquots (B), see fig. 3) 1 % Equex pasta, hand-pre-warmed, was added with a Pasteur pipette at room temperature to the cooled samples.

Freezing

For semen freezing in pellets on dry ice (-79 °C), a nail-board holding 81 1 cm aluminium pegs was pressed on a block of dry-ice (30 x 15 x 5 cm). Drops of 30 µl of the diluted semen were placed with a pipette into hemispherical indentations on the dry ice blocks and were left on the ice for about 1min for complete freezing. Then the pellets were transferred with forceps into labeled capped 3.6 ml cryovials filled with liquid nitrogen. The batch documented the collection date, studbook number of the semen donor, cooling treatment and Equex status of the semen. The cryovials were submersed in the liquid nitrogen tank for storage.

Thawing

For testing of post-thawing semen quality single pellets were thawed in 1 ml of clear TRIS buffer (appendix 9.1.1) warmed up to 37 °C in a 3.6 ml cryovial and shaken vigorously for 30 sec. This procedure took place in the laboratory after the semen has been frozen for at least 6 weeks.

3.7 Semen evaluation

The semen quality was evaluated in freshly collected semen samples as well as after cooling to 5 °C and after the freezing-thawing process as shown in Fig. 4.

Sperm motility by microscopic examination

Motility of the spermatozoa were evaluated subjectively under a phase contrast microscope at room temperature. For this evaluation a drop (~5 µl) of semen sample was placed on a microscope slide (Fisher Scientific Company, Houston, Texas, USA) using a Pasteur pipette and covered with a 22 x 22 mm cover glass. Motility was assessed as percentage of all moving sperm cells, percentage of progressively motile sperm cells, percentage of sperm cells with local motility and twitching tails but no progression and percentage of cells showing no movement. For additional characterization of motility the vigor of sperm cell movement and speed of progressive motility (velocity) were assessed using a scale from

0 = no moving cells

1 = slight side-to-side movement, no forward movement

2 = rapid side-to side movement, no forward movement

3 = rapid side-to side movement, forward movement in spurts

4 = slow, steady forward movement to

5 = vigorous tail movements with good steady forward movement.

On one microscope slide at least four fields at different locations of the slide were evaluated.

Plasma membrane integrity

Sperm plasma membrane integrity in fresh, cooled and frozen-thawed semen was evaluated by means of the Theriogenology-(Eosin-Nigrosin-)stain, the hypoosmotic swelling test (appendix 9.1.2) and a dual fluorescent stain (appendix 9.1.3) procedure.

Evaluating the samples with the Theriogenology-(Eosin-Nigrosin-)stain (Society of Theriogenology, Hastings, Nebraska, USA) one drop (~5 µl) of semen was placed on a microscope slide and mixed with one drop of equal volume of Eosin-Nigrosin stain by use of a pipette tip. With a second microscope slide a smear was made. After a drying period of several minutes the smear was evaluated in a phase-contrast microscope (x 400) and cover slipped with a 50 x 22 mm cover glass and glue some hours later in the laboratory due to logistic reasons. On each slide 100 spermatozoa were counted in a meander-like way, evaluating stained (dead cells) or unstained heads (live cells). The numbers were expressed in per cent of the counted sperm cells.

The hypoosmotic swelling test was performed according to KUMI-DIAKA (1993) and KUMI-DIAKA and BADTRAM (1994) by first mixing 1.0 ml HOST-solution in a 1.5 ml Eppendorf tube with 0.1 ml of sample and incubating the mixture for 60 min at 37 °C in a water bath (Falcon test tube holder and heater, 110/120 V, Cook Veterinary Products, 1100 West Morgan Street, Spencer, Indiana 47460, USA). After incubation, the sample was gently mixed to get a good overall distribution of the sperm cells and one drop (~ 5 µl) of the treated mixture was examined, in thin slide-coverslip preparation, under a phase contrast microscope at 200x and 400x magnification. Two hundred spermatozoa per slide were counted, and the percentage of spermatozoa exhibiting tail curling was determined. In case of the wolf trial 100

µl of a 18.5% formalin solution was added to the samples to fix the sperm cells until evaluation in the microscope was possible.

For the dual fluorescent staining the different solutions were prepared in the lab. Before electroejaculation, 1.0 ml saline medium, 20 µl formalin solution, 20 µl propidium iodide and 20 µl 6-carboxyfluoresceindiacetate were taken out of the freezer to thaw and to be mixed, kept in the dark at room temperature and used within one hour. 300 µl of staining medium were mixed with 100 µl of the semen sample and incubated in a water bath of 30-35 °C for 15 min. After incubation and following repeated gentle mixing a 10 µl drop was placed on a microscope slide, covered with a 50 x 20 mm coverslip and evaluated immediately under the fluorescence microscope (frozen-thawed samples). For staining of fresh and cooled semen samples stained in the field, a drop (30 µm) of Vectashield R Mounting Medium (Vector Laboratories, Burlingame, CA, USA) was added, covered by a 50 x 22 mm coverslip, sealing the edges with clear nailpolish and stored in horizontal position in dark slide boxes in the refrigerator. Evaluation was performed in the laboratory after transport in a cooler. Assessment of generic gray wolf semen samples could be done only 2 - 4 days after semen collection due to the unavailability of a fluorescence microscope in the Wildlife Science Center. For evaluation of the sperm plasma membrane integrity 100 spermatozoa were counted with a magnification of 400x. For the red excitation range (6-CFDA) a combination (B-2A, Nikon) of a dichroic mirror of 510, an excitation filter of 450-490 and a barrier filter of 520 was used and for the green excitation range (PI) a combination (G-A, Nikon) of a dichroic mirror of 580, an excitation filter of 510-560 and a barrier filter of 590 was used. The spermatozoa were classified in the following categories:

- entirely intact plasma membrane: plasma membrane stained entirely (head and tail) green with 6-CFDA and unstained with PI,
- damaged plasma membrane with intact acrosomal membrane: acrosome stained green with 6-CFDA (green ‚cap'), post-acrosomal region stained red with PI
- damaged plasma membrane and damaged acrosomal membran: unstained with 6-CFDA but head is stained red with PI.

Morphology

Sperm morphology was determined with the Spermac®-stain (Sage Biopharma Inc., Bedminster, NJ, USA) (OETTLÉ 1986b, OETTLÉ and SOLEY 1988). Using a pipette a 10 µl drop of sperm samples was placed on a microscope slide. The drop was smeared over the whole slide by rolling the end of a Pasteur pipette through the drop and across the slide and the smears were allowed to dry. For staining the dried samples the steps on the proceeding sheet were followed. After the last washing procedure the slides were air dried and stored in a covered container in the dark until examination. 100 cells were evaluated under 1000x magnification using oil immersion. In the first step morphological abnormalities were distinguished with regard to the sperm head, midpiece and tail. Head abnormalities included acrosome abnormalities (lipped acrosome, acrosome with cyst and abnormal distribution of the acrosome), abnormal head forms (macrocephalic, microcephalic, pyrifrom, round, doubled head, narrow, bizzare from) and abnormal neck forms (detached head and kinked neck). Midpiece abnormalities were counted as coiled, ruptured, thick and kinked midpieces, abaxial insertion of the midpiece in the head region, proximal and distal cytoplasmatic droplets. Tail defects were classified as kinked, coiled or ruptured tails. All abnormalities were taken into account not distinguishing between minor or major defects. In the second step the acrosome was evaluated and varying degrees of acrosomal damage could be demonstrated (OETTLÉ 1986a, b; OETTLÉ and SOLEY 1988).

· intact acrosome: those which possessed a deep green acrosome, midpiece and tail, a distinct pale green equatorial zone and a red post acrosomal region of the head

· swollen acrosome: the ballooned membrane is clearly visible as a pale halo around the head. There is almost no green staining in the acrosome, indicating that most of the acrosomal contents have been lost

· reacted acrosome: fuzzy acrosomes, with decreased green staining affinity and irregularity of staining of the acrosome caused by vesiculation

· lost acrosome: pale pink staining of the area which used to be covered by the acrosome and of the equatorial region

Electron microscopy

An electron microscopical study was done in fresh and cooled semen of Mexican wolf #568. Each of 100 µl fresh and cooled (0.5 h, 1.0 h, 2.5 h) semen samples were put in a separate 1.5 ml Eppendorf microtube, fixed in a glutaraldehyde fixative solution (appendix 9.2.4). After centrifugation for 5 min in a micro centrifuge, the semen pellets were fixed overnight in glutaraldehyde fixative solution at 4 °C and washed three times for 15 min in a washing solution (appendix 9.1.4) next day. Post-fixation occurred in a post-fixation buffer (9.1.4) overnight at 4 °C. This was followed by washing twice for 15 min in distilled water, 1 h of incubation in 2% aqueous uranyl acetate in the dark and dehydration through graded ethanols to 100 %. After washing twice for 15 min in propylene oxide the samples were incubated overnight in a 1:1 mixture of Polybed resin and propylene oxide. The tissue pellets were then incubated in fresh Polybed resin for 6 h, embedded in Polybed resin in BEEM capsules and polymerized overnight at 70 °C. The tissue blocks were trimmed with a razor and sectioned on a Reichert Ultracut E ultramicrotome. Thick plastic sections were cut with glass knives, collected on glass slides, stained with toluidine blue and examined in a light microscope for fixation quality. Thin sections were cut with a diamond knife, collected on 200 mesh copper grids, stained with uranyl acetate and lead citrate and viewed and photographed with a JEOL 100CX electron microscope; NM was 10K and PM was 30K (RYERSE*)

*personal communication RYERSE 29th February 2001, St. Louis, MO, USA

Longevity test

Microscopic evaluation of motility

The frozen-thawed semen was additionally tested for longevity over a period of 7 h. Semen was incubated at room temperature and was put on a warming plate of 37 °C for 5 sec before evaluation. Sperm motility was assessed under a phase contrast microscope at hourly intervals.

Computer assisted sperm motion analysis

Mexican wolf semen was evaluated by computer assisted sperm motion analysis (CASA) [Hamilton Thorn (HT) IVOS-10, MA, USA] using standardized analysis chambers (Microcell slides, chamber depth 10 µm; Spectrum Technologies, 1532 Chablis Road Suite 101, Healdsburg, CA 95448, USA). For 11 h VAP (average path velocity), VSL (straight-line velocity), VCL (curvilinear velocity), ALH (average head displacement), BCF (beat cross frequency), STR (straightness = VSL/VAP) and LIN (linearity = VSL/VCL) were assessed. Elongation (head width / head length), size (in µm and pixels) and intensity of sperm heads were also monitored. The defined set up of the computerized semen analysis by Hamilton Thorn IVOS-10 is presented in the appendix (9.2). Only those spermatozoa were included in the statistics that showed 30 track points (Fig. 6).

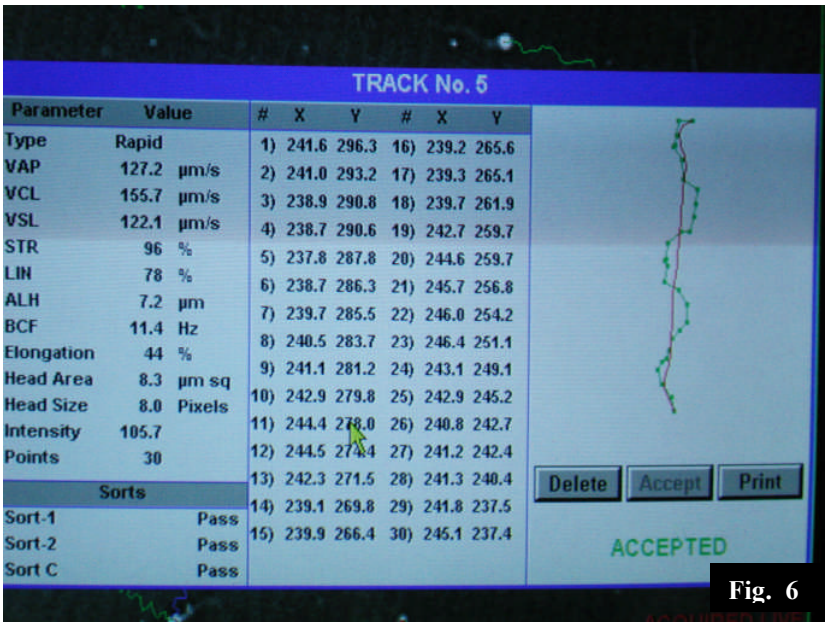


Fig. 6: Example of one sperm track of the computerized evaluation (Hamilton Thorn)

Particles identified as motile spermatozoa ranged in size from 4 to 241.9 pixels. The percent motility was not evaluated because of interference in detection of debris and cells that are non-motile spermatozoa. A 5 µl drop of semen was placed on a cover-slipped prewarmed evaluation slide with calibrated depth of 10 µm, which was then put onto the Hamilton-Thorne-stage. Each semen analysis was based on an evaluation of 20 fields per sample. Five subsequent fields were evaluated one by one. Before the next five fields were added to the analysis the focus was reset manually. As soon as the 20^{th} field was analyzed, the gates were adapted using the playback facility, so that every moving cell was included in the analysis. Samples were kept at room temperature and mounted on a prewarmed slide for each evaluation. Each sample was analyzed in the same manner at time point 0 (right after thawing) as well as after 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 h, respectively, depending on the longevity of the sample. The analysis was stopped if no progressively moving sperm cells were detected on the screen. Sperm concentration and progressive motility were not evaluated because the HT was not able to differentiate egg-yolk particles of sperm head size from non-motile sperm cells (RIJSSELAERE et al. 2003).

3.7 Statistical Analysis

The data were tested for normal distribution using the Shapiro Wilk W test. As data were not normally distributed, highly skewed and with a Kurtosis too flat, they were transformed by ARCSIN transformation prior to significance analysis. The mean, the standard error of the mean (±SEM) and the range (min – max) were calculated for testes volume, ejaculate volume, sperm concentration, total sperm count, pH and osmolarity of seminal fluid as well as for sperm motility, plasma membrane integrity, morphology and acrosome integrity in fresh, cooled, and thawed semen. Due to the small number of animals (domestic dogs: n = 4; Mexican wolves: n = 4; generic gray wolves: n = 7), and data that were not equal, not normally distributed and not independent, a full factorial ANOVA was used to analyse the data set and determine significant differences between semen processing procedures within a species and within the three different stages of semen cryopreservation (fresh, cooled, post-thaw). Differences in motility and motility patterns between semen processing procedures during a longevity test were assessed over 7 (microscope) and 11 (Mexican wolves) h using ANOVA. If significance ($P < 0.05$) or high significance ($P < 0.01$) was indicated, multiple comparison procedure (Tukey-Kramer- and Kruskal-Wallis Z-test) was used to compare semen processing procedures.

4 Results

4.1 Preliminary temperature trial

Extender and water bath had the same temperature at the beginning of the measurements. Mean time required for cooling to 5 °C was significantly different ($P < 0.05$) for extender cooled in the 250 ml water bath and in the 500 ml water bath in ice water ($P < 0.05$). In the 250 ml water bath the extender needed 14.8 min, in the 500 ml water bath 12.4 min, in the 1000 ml water bath 10.1 min and in the 500 ml water bath in ice water 26.0 min longer than the surrounding water.

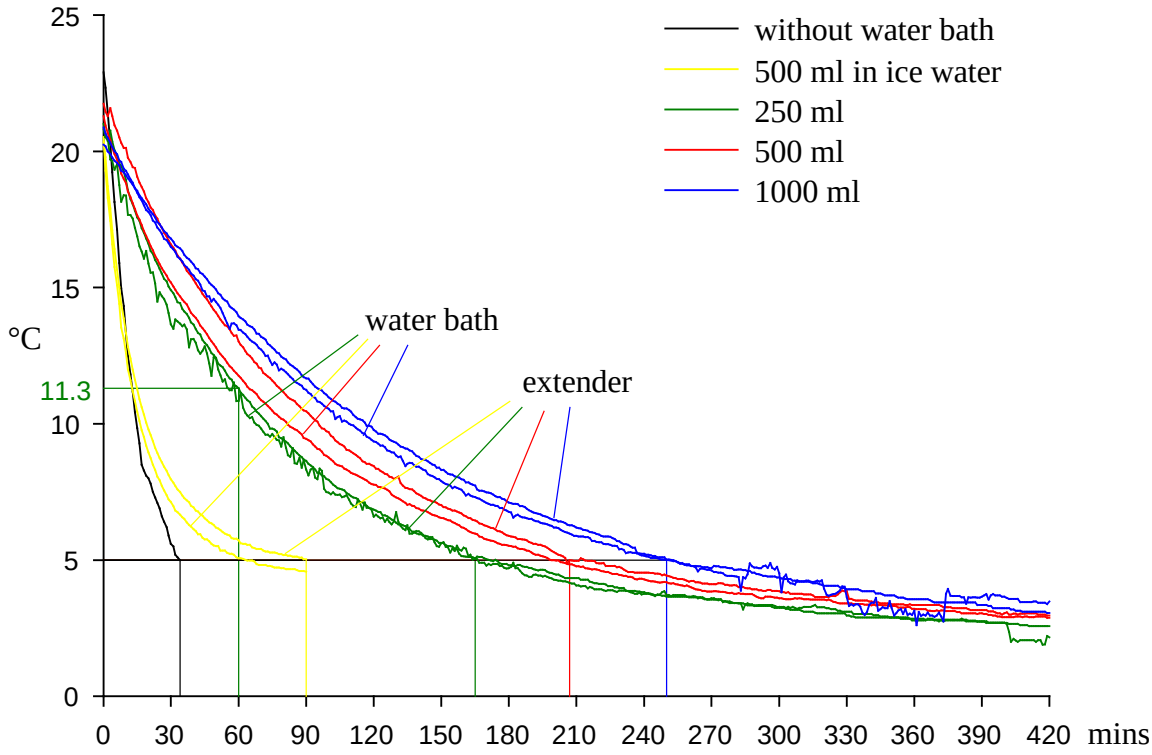


Fig. 7: Cooling curves of different volumes of water (250 ml, 500 ml, 1000 ml, 500 ml in ice water) and of extender, kept in these water baths, or without surrounding water. Drop lines indicate the time (min) used for cooling to 5 °C. At 60 min a drop line indicates the temperature reached by the control water bath in the wolf trial.

Without a water bath the extender was cooled to 5 °C in a mean time of 33.5 ± 3.1 min and in the 500 ml water bath in ice water of 90.2 ± 8.5 min while in the 250, 500 and 1000 ml water baths 165.2 ± 4.2 min, 207.3 ± 4.0 min and 250.3 ± 5.7 min, respectively, were required (Fig. 7). Temperature and time data for all cooling times are also presented in table 9.3.1 (appendix) and Fig. 4.

4.2 Cooling patterns of diluted semen

Results for cooling times of extender suspensions of dog semen in different water baths are presented in table 3. Semen-extender suspensions with an initial room temperature (20.0 – 22.3 °C) reached refirgerator temperature (5.0 – 6.3 °C) during the tested cooling times.

Table 3: Initial temperature (°C), temperature difference (°C) and final temperature (°C) of 1ml extended domestic dog semen cooled without water bath (0.5 h) and water baths of 500 ml in ice water (1.5 h), 250 ml (2.5 h), 500 ml (3.5 h) and 1000 ml (4.5 h) (n = 4 each)

wb volume (ml)	initial temperature (°C)				temperature difference (°C)				final temperature (°C)			
	mean	SEM	min	max	mean	SEM	min	max	mean	SEM	min	max
no wb (m=8)	22.3	0.6	21.0	22.9	16.2	0.7	11.5	17.9	6.1	0.8	4.6	11.0
500 ice (m=5)	20.3	0.6	18.1	21.2	15.8	0.6	14.7	17.3	5.0	1.0	3.2	8.5
250 (m=6)	20.5	0.4	19.0	21.3	14.2	0.6	12.4	15.6	6.3	0.3	5.4	7.0
500 (m=9)	20.3	0.3	18.7	21.3	14.6	0.4	12.8	16.7	5.7	0.2	4.9	6.6
1000 (m=6)	20.0	0.4	18.7	21.3	14.5	0.6	12.3	16.8	5.5	0.3	4.6	6.9

Temperature logger results in the Mexican wolf trial representative for both wolf trials are presented in table 4. Initial temperature of extender and water (19.0 – 19.8 °C) were lower than in the domestic dog trial. Generic gray wolf semen samples cooled for 0.5 h at 0.5 °C/min and 2.5 h at 0.1 °C/min reached temperatures of 5.5 °C and 5.2 °C, respectively. While Mexican wolf semen with the same cooling times resulted in final temperatures of 3.7 °C and 3.3 °C, respectively (table 4). After cooling for 1.0 h at 0.2 °C/min the semen samples had reached 9.8 °C (generic gray wolf) and 7.5 °C (Mexican wolf), respectively (table 4).

Table 4: Initial temperature (°C), temperature difference (°C) and final temperature (°C) of extender cooled without water bath (0.5 h) and water baths of 250 ml cooled for 1.0 h (control) and 2.5 h (n = 4 each) in the Mexican wolf trial

wb volume (ml)	initial temperature (°C)				temperature difference (°C)				final temperature (°C)			
	mean	SEM	min	max	mean	SEM	min	max	mean	SEM	min	max
no wb (0.5 h) (m=22)	19.8	0.5	16.8	26.0	16.2[a]	0.5	11.0	21.4	3.7[b]	0.5	-0.2	7.4
250 (1 h) (m=21)	19.2	0.6	16.0	26.0	11.6[a]	0.6	6.5	19.7	7.5[b]	0.5	3.7	15.2
250 (2.5 h) (m=21)	19.0	0.4	16.8	26.1	15.8[a]	0.4	14.3	22.6	3.2[b]	0.2	2.1	5.4

Significant differences

Single-factor ANOVA:

temperature difference	0.5 h / 1.0 h, 2.5 h	a: $P < 0.01$
final temperature	0.1 h / 0.5 h, 2.5 h	b: $P < 0.01$

4.3 Morphology of testes

Testicular size and volume of generic and Mexican wolves measured during the semen collection period are presented in table 5. Measurements don't reveal a species specific difference. The total testicle volume for generic gray wolf was 41.9 ± 2.1 cm^3 (30.0 – 54.0 cm^3) and for Mexican wolf 43.3 – 1.3 cm^3 (31.7 – 57.0 cm^3).

Table 5: Testicular length, width, and depth (mm) and calculated volume (cm^3) for generic gray (n = 7; m = 13) and Mexican wolves (n = 4; m = 28)

measurement (cm and *cm^3*)		left testicle				right testicle			
		mean	SEM	min	max	mean	SEM	min	max
generic gray wolf	length	41.7	1.3	35.5	50.0	38.5	1.7	23.5	46.4
	width	29.1	1.0	21.8	33.4	28.3	1.7	14.0	39.2
	depth	30.4	1.0	24.0	35.8	29.6	1.4	17.6	37.5
	volume	*22.2*	*1.3*	*14.0*	*30.7*	*19.7*	*1.9*	*6.1*	*28.3*
Mexican wolf	length	44.3	0.7	33.4	52.0	43.7	0.6	36.7	49.0
	width	29.4	0.5	24.0	34.0	29.1	0.4	25.0	33.0
	depth	27.8	0.4	24.0	32.0	26.5	0.5	22.0	31.0
	volume	*22.3*	*0.7*	*16.3*	*30.8*	*21.0*	*0.7*	*14.1*	*29.6*

4.4 Characteristics of dog and wolf ejaculates

Semen characteristics of the three species are presented in table 9.3.2 (appendix). Mean ejaculate volume (7.0 ± 1.0 ml), total sperm count and motility showed highest values in generic gray wolf. Total sperm count was twice as high as in domestic dog and Mexican wolf semen 1590.4 ± 390.0 x 10^6 spermatozoa versus 720.0 ± 204.0 x 10^6 spermatozoa and 756.2 ± 153.9 x 10^6 spermatozoa, respectively. Semen of generic gray wolf also showed highest values in percetange of progressively motile spermatozoa (88.5 ± 0.8 % versus 76.4 ± 3.8 % in domestic dog semen and 77.6 ± 2.0 % in Mexican wolf semen); pH and osmolarity were in physiological ranges. All ejaculate parameters were characterized by large variation.

4.5 Cooled semen

4.5.1 Influence of different cooling times and rates on sperm plasma membrane integrity

Tested cooling times and rates had no effects on sperm plasma membrane integrity with Eosin/Nigrosin stain in either species. The same was true regarding plasma membrane integrity tested with HOST in the domestic dog. There were significant differences ($P < 0.05$) in Eosin-Nigrosin stained spermatozoa between domestic dog and generic gray wolf semen (table 9.3.3, appendix). In generic gray and Mexican wolf semen, cooling for 1.0 h at 0.2 °C/min resulted in slightly better plasma membrane integrity (HOST) than the other two cooling procedures (table 9.3.3; appendix).

4.5.2 Influence of different cooling times and rates on sperm morphology and acrosome integrity

Sperm morphology was not markedly affected by cooling time. The best results were achieved in the domestic dog with 4.5 h cooling ($P > 0.05$), in the generic gray wolf with 0.5 h cooling ($P > 0.05$) and in the Mexican wolf with 1.0 h cooling ($P > 0.05$) (table 6).
In semen of the Mexican wolf cooled for 1.0 h, a significantly higher ($P < 0.05$) percentage of intact acrosomes was found than in samples cooled for 2.5 h, while in the domestic dog and the generic gray wolf no significant differences were observed (table 6).

Table 6: Spermatozoa with normal morphology (%) and intact acrosomes (%) in fresh and cooled semen of domestic dog, generic gray and Mexican wolf after exposure to different cooling times (0.5 h, 0.5 °C/min; 1.0 h, 0.2 °C/min; 2.5 h, 0.1 °C/min) compared with fresh semen (before cooling)

species	cooling time (h)	normal morphology (%)		intact acrosomes (%)	
		mean	SEM	mean	SEM
domestic dog (n=4)	before cooling	59.9	9.4	75.8	7.1
	0.5 (m=9)	68.9	4.3	78.1	4.2
	1.5 (m=8)	70.3	7.9	76.1	6.7
	2.5 (m=8)	63.7	8.5	76.6	6.2
	3.5 (m=8)	71.0	7.7	72.0	7.1
	4.5 (m=8)	67.5	8.3	68.8	7.1
generic gray wolf (n=7)	before cooling	77.0	3.4	87.8	2.8
	0.5 (m=7)	72.3	7.3	82.7	5.6
	1.0 (m=6)	69.0	10.5	78.5	7.7
	2.5 (m=6)	70.7	8.8	78.2	5.4
Mexican wolf (n=4)	before cooling	73.9	4.7	90.6	1.7
	0.5 (m=24)	66.1	4.4	77.9	2.4
	1.0 (m=25)	72.2	4.0	83.6[a]	2.6
	2.5 (m=25)	69.5	4.5	69.0[a]	5.3

Significant differences

Single-factor ANOVA:

Mexican wolf, intact acrosomes (%): 1.0 h / 2.5 h a: $P < 0.05$

4.5.3 Electron microscopic evaluation of sperm morphology

Electron microscopic pictures (m = number of pictures) were taken from fresh semen (m=9) and cooled semen [0.5 h (m=5), 1.0 h (m=10), 2.5 h (m=4)], collected from Mexican wolf # 568 (Fig. 8). With regard to plasma membrane no differences between fresh or cooled samples were observed. These findings can, however, not be underlined by statistical evaluation. The morphological abnormalities were equally distributed among the samples cooled for different cooling times and no specific abnormality could be related to a certain cooling regimen.

A: Fresh sperm with swelling of the acrosomal plasma membrane (pm) detached from the nuclear envelope and with ruffling of the outer acrosomal membrane (oam). Transverse and longitudinal sections of midpieces (mp)

B: Fresh sperm with an intact plasma membrane (pm) and tightly arranged mitochondria (mi); the visible changes are the swollen plasma membrane (pm) and in the same area the mitochondria appear rounded. The cristae are irregular, the intercristal matrix is no longer homogeneous and contains electrotranslucent spaces. Transverse sections of sperm tails are visible

C: Sperm cooled for 0.5 h with swollen and disrupted acrosomes (acr) with protruding acrosomal content (ac) of the sperm head in the upper part of the picture. The second sperm head shows an intact acrosome (acr) but with a swolllen plasma membrane (pm)

D: The transverse sections of a spermatozoal midpiece show a perfect structure with ovoid mitochondria (mi) building the mitochondrial sheath, lying against the tear-drop shaped outer dense fibers (odf) surrounding the axonemal complex with its microtubules (mt) (0.5 h)

E: Sperm cooled for 1.0 h with sperm heads showing intact plasma membranes (pm) in close apposition to the acrosome (acr) in the transverse sections. In the longitudinal section the plasma membrane (pm) is swollen with an underlying disintegrating acrosome showing vesiculation (ves) of the acrosomal content

F: Longitudinal section of a spermatozoa cooled for 1.0 h with mitochondria (mi) of heterogeneous density with electrodense and electrotranslucent spaces. Transverse sections of sperm midpiece (mp) and sperm tail are visible

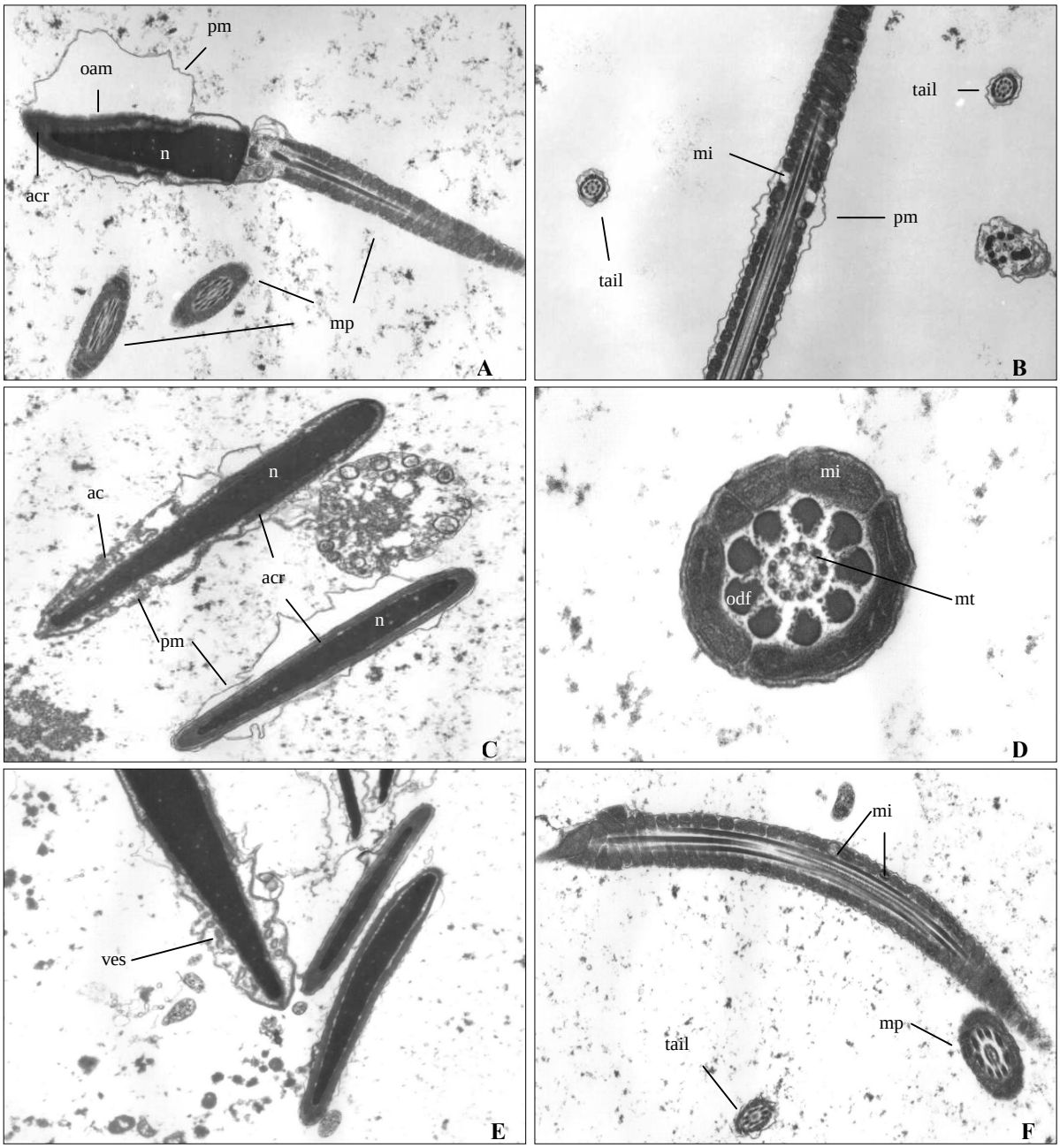


Fig. 8: Electron microscopic pictures of fresh and cooled (0.5 h, 1.0 h, 2.5 h) Mexican wolf semen (collected 28th February 2001 of #568) [ac=acrosomal content, acr=acrosome, mi=mitochondria, mp=midpiece, mt=microtubules, n=nucleus, oam=outer acrosomal membrane, odf=outer dense fibers, pm=plasma membrane, ves=vesiculation] (x 10 000)

4.6 Frozen-thawed semen

Semen of the Mexican wolf survived the freezing-thawing process better than generic gray wolf semen regarding all evaluated semen parameters. Semen of generic gray wolf showed the worst results in all parameters evaluated.

4.6.1 Differences of collected fresh, cooled and frozen-thawed semen

The quality of domestic dog, generic and Mexican wolf semen samples declined significantly during the freezing-thawing process. Semen of all species showed significant differences of frozen-thawed semen to fresh and cooled semen in percentages of functionally intact membranes (HOST, Eosin-Nigrosin; $P < 0.01$) and in domestic dog morphologically normal cells were significantly higher in cooled than in frozen-thawed samples ($P < 0.01$). In both wolf species fresh samples show significantly higher percentages of intact plasma membrane (HOST; $P < 0.01$) than cooled and frozen-thawed samples (table 9.3.4; appendix). In Mexican wolf cooled semen and in domestic dog fresh and cooled semen had significantly higher percentages of intact plasma membrane (HOST, $P < 0.01$) than frozen-thawed samples (table 9.3.4, appendix).

In cooled semen domestic dog had significantly higher percentages of intact plasma membrane (HOST, $P < 0.01$) than wolves and vice versa in Eosin-Nigrosin ($P < 0.01$). In frozen-thawed samples domestic dog had significantly higher percentages of intact plasma membrane (HOST, $P < 0.01$) than Mexican wolf which had significantly higher percentages (HOST, $P < 0.01$) than generic gray wolf. Domestic dog and Mexican wolf showed significantly higher percentages of intact plasma membranes than generic gray wolf (6-CFDA/PI, Eosin-Nigrosin, $P < 0.01$). In Mexican wolf morphologically normal cells were significantly higher than in domestic dog and generic gray wolf ($P < 0.01$) (table 9.3.4, appendix).

4.6.2 Influence of different cooling times and Equex pasta on post-thaw motility

4.6.4.1 Microscopic evaluation

The percentage of progressively motile spermatozoa (%) was significantly ($P < 0.01$) lower in frozen-thawed semen samples than in fresh samples in either species (Fig. 9, table 9.3.5, 9.3.6, appendix). Mexican wolf shows markedly better post-thaw motility results than domestic dog and generic gray wolf Mexican wolf semen cooled in different cooling times showed significantly higher motility than generic gray wolf semen evaluated in any cooling time diluted without or with Equex pasta (Fig. 9, table 9.3.5, appendix).

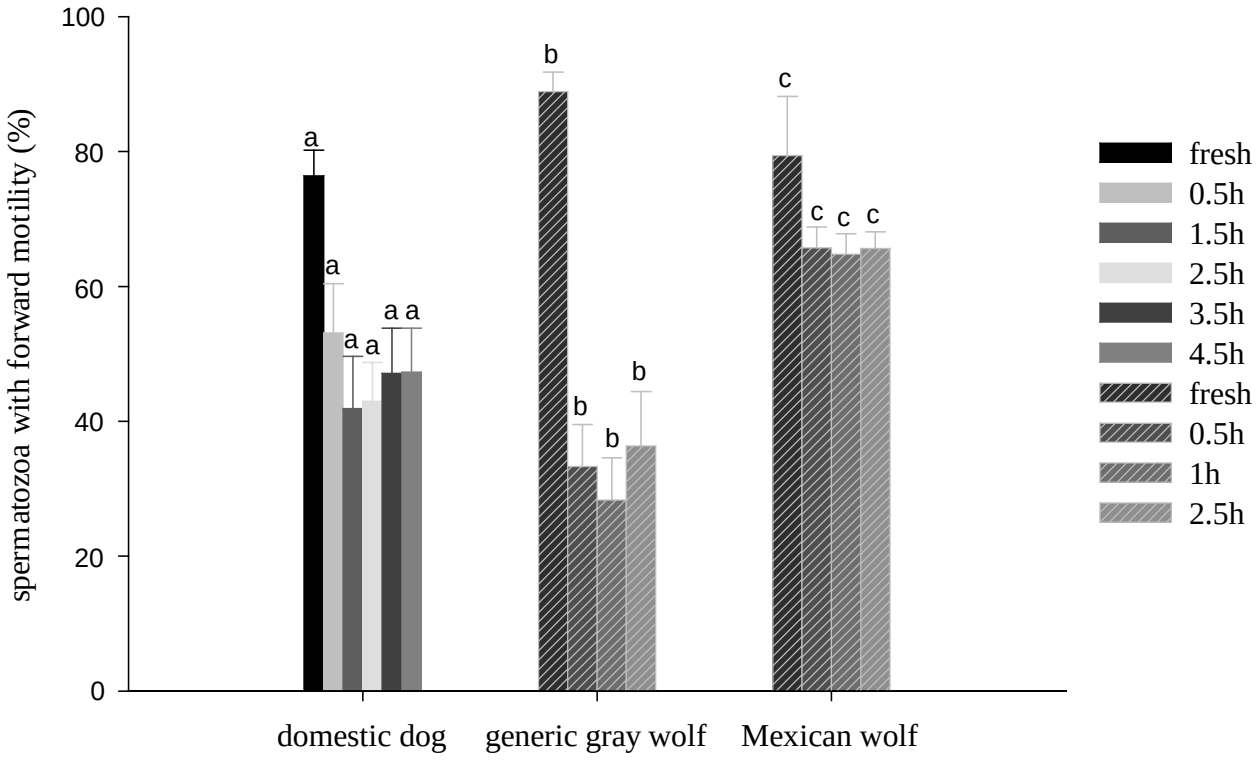


Fig. 9: Percentage of sperm forward motility in fresh and frozen-thawed semen of dog, generic gray and Mexican wolf cooled for different times (dog: 0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h; wolves: 0.5 h, 1 h, 2.5 h) [significant difference, single-factor ANOVA; domestic dog: a (fresh / 0.5h, 1.5 h, 2.5 h, 3.5 h and 4.5 h); generic gray wolf and Mexican wolf: b, c (fresh / 0.5 h, 1.0 h and 2.5 h), $P < 0.01$]

No significant differences were detected during the post-thaw 7 h period in semen of the domestic dog (Fig. 10; table 9.3.6, appendix).

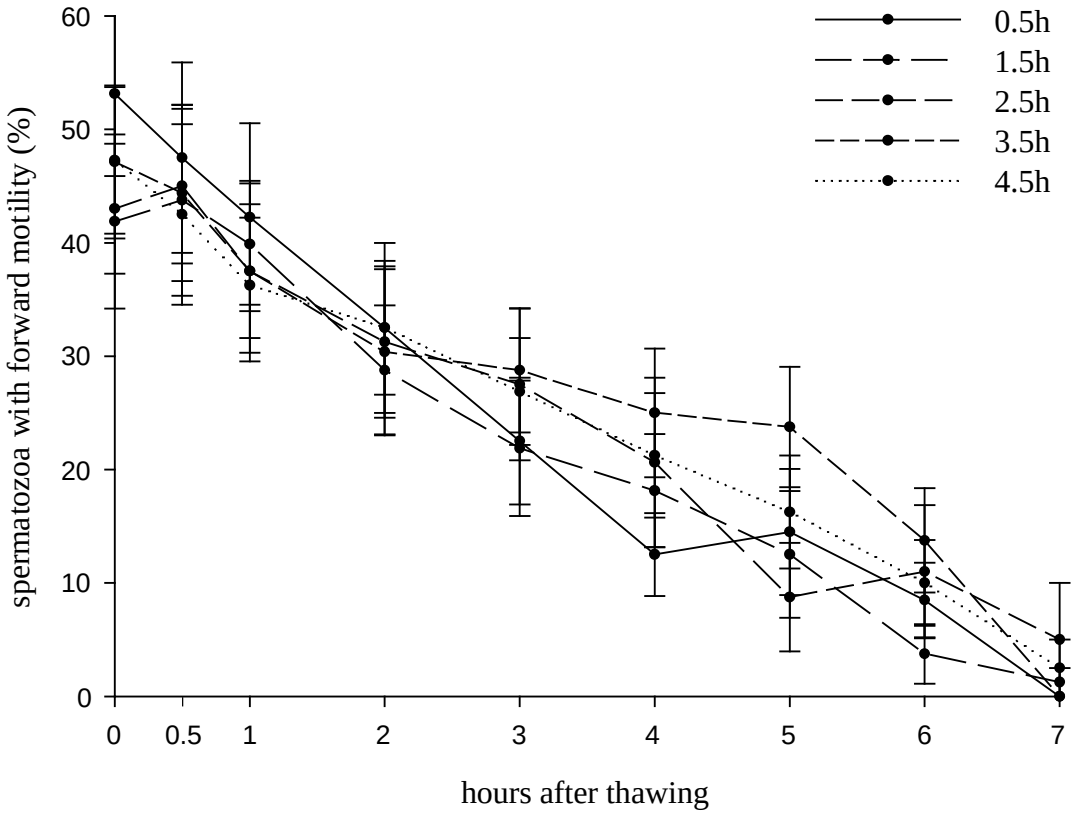


Fig. 10: Percentage of forward motility in frozen-thawed domestic dog semen cooled for different times (0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h) during a 7 h post-thaw longevity test [no significant differences, $P > 0.05$]

In the generic gray wolf, motility decreased fast during the first hours of the post-thaw period. The percentage of forward motility was higher in samples cooled for 2.5 h than in samples cooled for 0.5 h and 1.0 h. The difference was significant ($P < 0.05$) at 4 h and 1 to 4 h after thawing, respectively (Fig. 11; appendix table 9.3.7). In Mexican wolf semen samples cooling time had no effect on post-thaw progressive motility (table 9.3.8, appendix). Motility values of Mexican wolf semen were markedly higher than values of generic gray wolf semen during the entire post-thaw period (Fig. 11; appendix table 9.3.8).

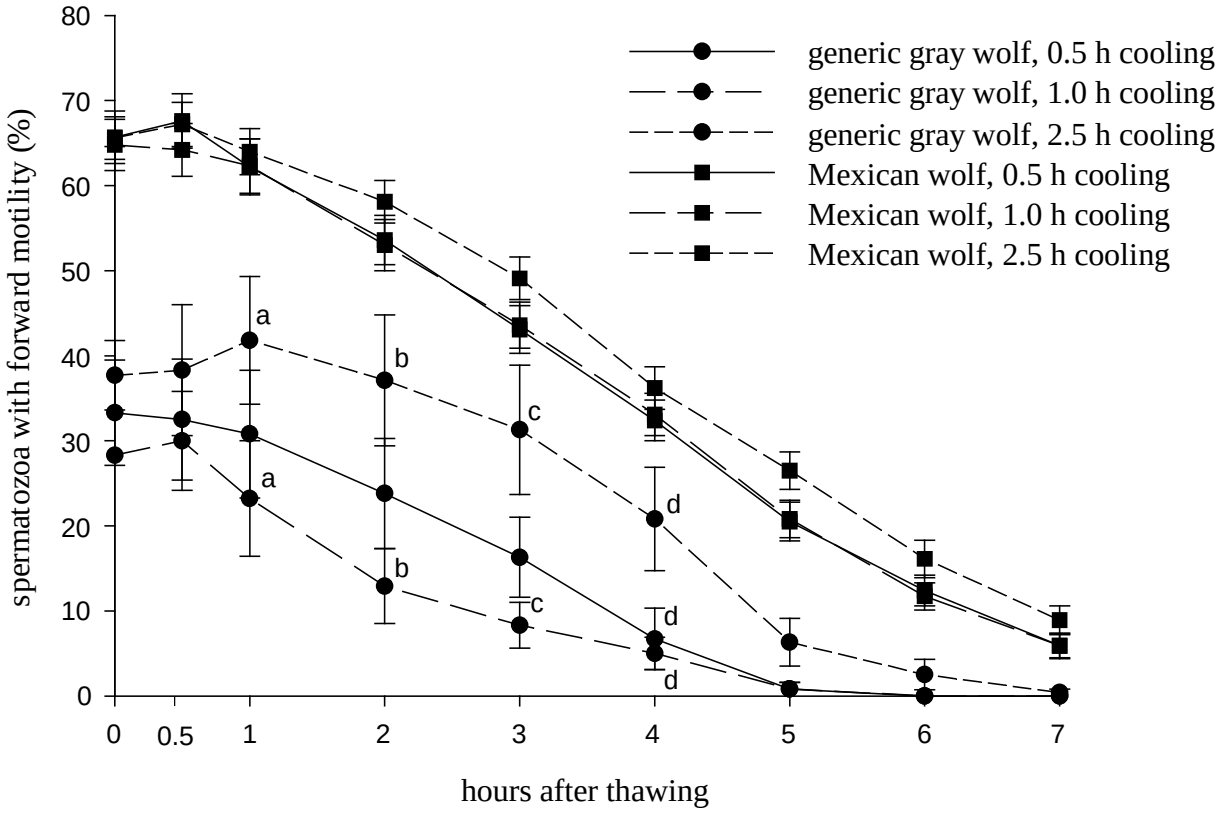


Fig. 11: Percentage of forward motility in frozen-thawed generic gray and Mexican wolf semen cooled for different times (0.5 h, 1.0 h, 2.5 h) during a 7 h post-thaw longevity test [significant differences of values with identical letters: single-factor ANOVA, a, b, c (0.5 h / 2.5 h) $P < 0.05$, d (0.5 h/1.0 h and 2.5 h) $P < 0.05$)]

In both wolf species addition of Equex pasta to the extender did not affect post-thaw sperm motility (Fig. 12; tables 9.3.9 and 9.3.10, appendix), without considering cooling time. Again it was shown that Mexican wolf had markedly better post-thaw sperm motility than generic gray wolf semen. During the first two hours Mexican wolf semen diluted with Equex pasta showed better progressive motility than samples not treated with Equex pasta. Generic gray wolf semen diluted without Equex pasta had best motility during the entire longevity test until semen reached almost 0 % progressive motility.

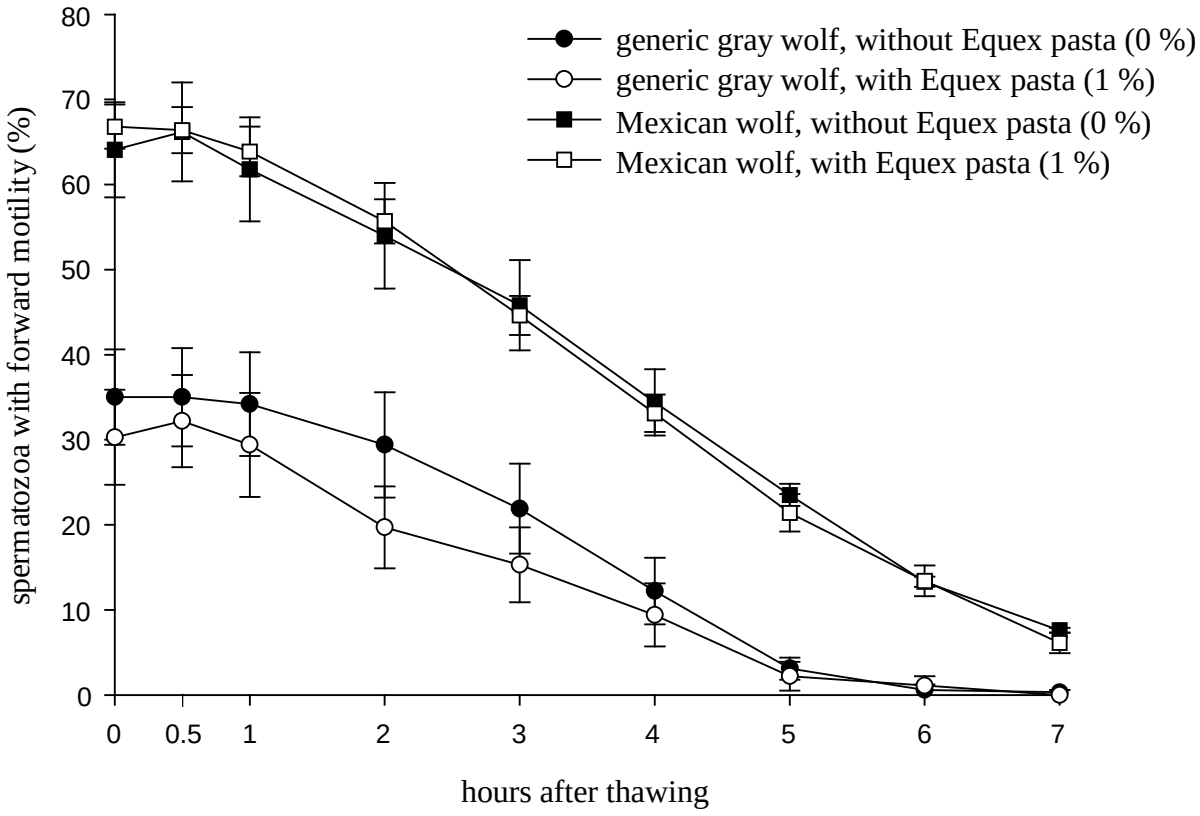


Fig. 12: Percentage of forward motility in frozen-thawed Mexican and generic gray wolf semen without (0 %) or with (1 %) Equex pasta during a 7 h post-thaw longevity test [no significant differences, $P > 0.05$]

4.6.4.2 Computer assisted semen analysis of Mexican wolf semen

Number of spermatozoa evaluated in total = 150 305 (table 7)

Table 7: Number of spermatozoa evaluated per hour post-thaw

hours after thawing	0 %			1 %		
	0.5 h	1.0 h	2.5 h	0.5 h	1.0 h	2.5 h
0	1584	1782	1450	1341	1352	1031
1	3319	2904	2688	2730	2884	1857
2	2546	3243	2659	1961	2379	1792
3	2427	2357	3018	2015	2448	1732
4	2639	2607	2539	2018	1872	2272
5	2852	2862	2893	2235	1878	3067
6	2533	2637	2677	2012	1965	2277
7	2305	1957	3159	2009	1590	1898
8	2358	2164	3397	1686	2398	2162
9	3396	1940	2647	1751	1614	2019
10	1418	1340	1962	1238	1465	1233
11	683	294	756	628	699	785

Measurements of sperm head parameters are presented in table 8.

Table 8: Sperm head parameters measured by Hamilton Thorne IVOS 10 in semen of four Mexican wolves; 150 305 sperm heads were evaluated

parameters	mean	SEM	min	max
elongation (µm)	52.5	0.3	50.2	53.7
size (µm)	7.6	0.1	7.2	8.5
size (pixels)	8.8	0.1	8.3	9.7
intensity	113.3	1.1	104.9	117.0

The computer assisted analysis of sperm motility revealed mean values for average path velocity (VAP of 85.5 ± 0.3 μm/s; 8.2 – 200.3 μm/s), for straightline velocity (VSL of 59.5 ± 0.2 μm/s; 0.1 – 191.4 μm/s), for curvilinear velocity (VCL of 190.5 ± 0.6 μm/s; 10.0 – 493.8 μm/s), for lateral head displacement (ALH of 8.8 ± 0.1 μm; 0.5 – 28.4 μm), for beat cross frequency (BCF of 28.1 ± 0.1 Hz; 0.0 – 60.0 Hz), for straightness (STR of 66.4 ± 0.2 %; 0.0 – 100.0 %) and for linearity (LIN of 32.0 ± 0.1 %; 0.0 – 98.0 %); see also tables 9.3.11 – 9.3.17, appendix).

In semen samples diluted without Equex pasta cooled for 0.5 h the spermatozoa showed significantly higher VAP ($P < 0.05$; Fig. 10, table 9.3.11, appendix), VSL ($P < 0.01$; Fig. 13, table 9.3.12, appendix) right after thawing.

In semen samples treated with Equex and cooled for 1 h significantly higher VAP and VSL ($P < 0.01$) right after thawing at 0 h (Fig. 13, table 9.3.11, 9.3.12, appendix) were found.

Right after thawing samples cooled for 2.5 h had the worst measurements for velocities VAP and VSL.

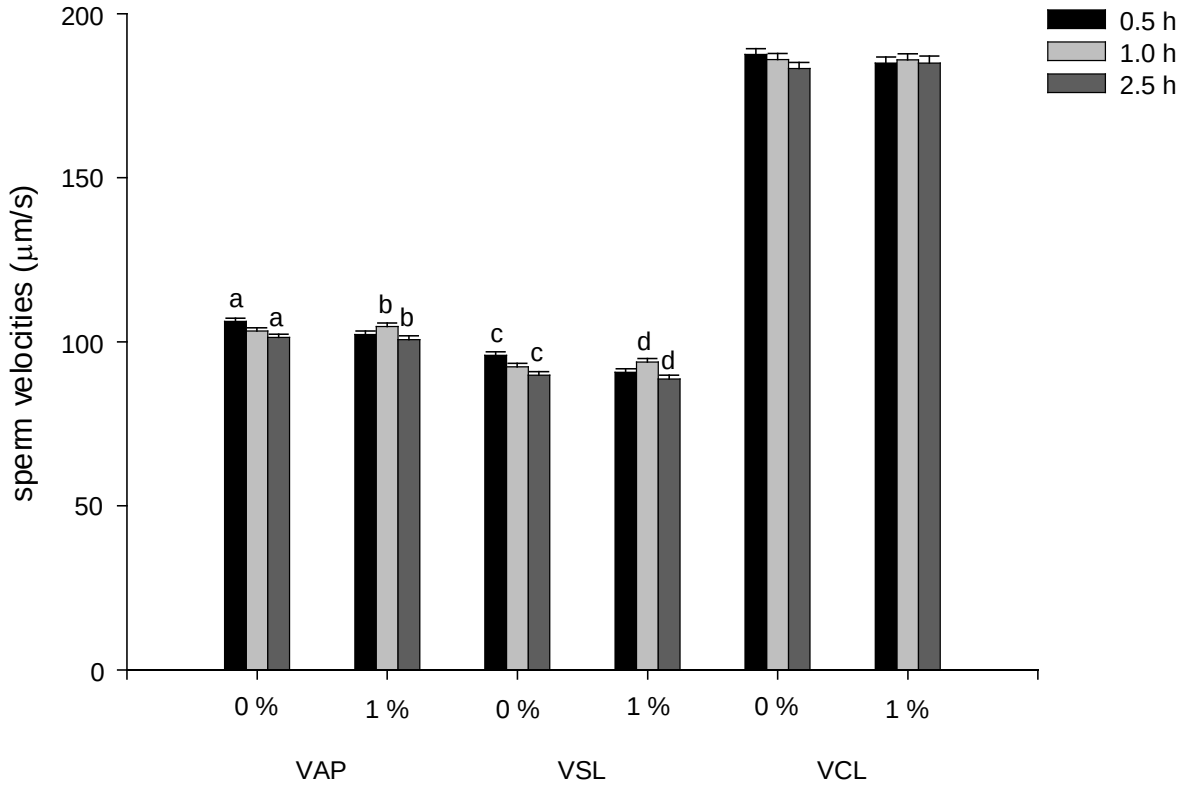


Fig. 13: Sperm speed (µm/s; average path velocity, straight line velocity, curvilinear velocity) of frozen-thawed Mexican wolf spermatozoa cooled for different times (0.5 h, 1.0 h, 2.5 h) and diluted without (0 %) or with (1 %) Equex pasta at thawing H0 [significant differences: influence of cooling time (single-factor ANOVA, a (0.5 h / 2.5 h), c (0.5 h

/ 1.0 h, 2.5 h), d (1.0 h / 2.5 h); P < 0.01); b (1.0 h / 2.5 h) P < 0.05]

The 0.5 h cooled samples also showed significantly higher STR (P < 0.05; Fig. 14, table 9.3.14, appendix) and LIN (P < 0.01; Fig. 14, table 9.3.15, appendix) right after thawing at 0 h than samples cooled for 1.0 h or 2.5 h, when not diluted with Equex pasta. And also showed significantly higher values in STR (P < 0.01; fig. 14, table 9.3.14, appendix) and LIN (P < 0.01; Fig. 14, table 9.3.15, appendix) right after thawing at 0 h than in samples cooled for 0.5 h or 2.5 h. Samples cooled in 2.5 h show significantly lower values in STR and LIN.

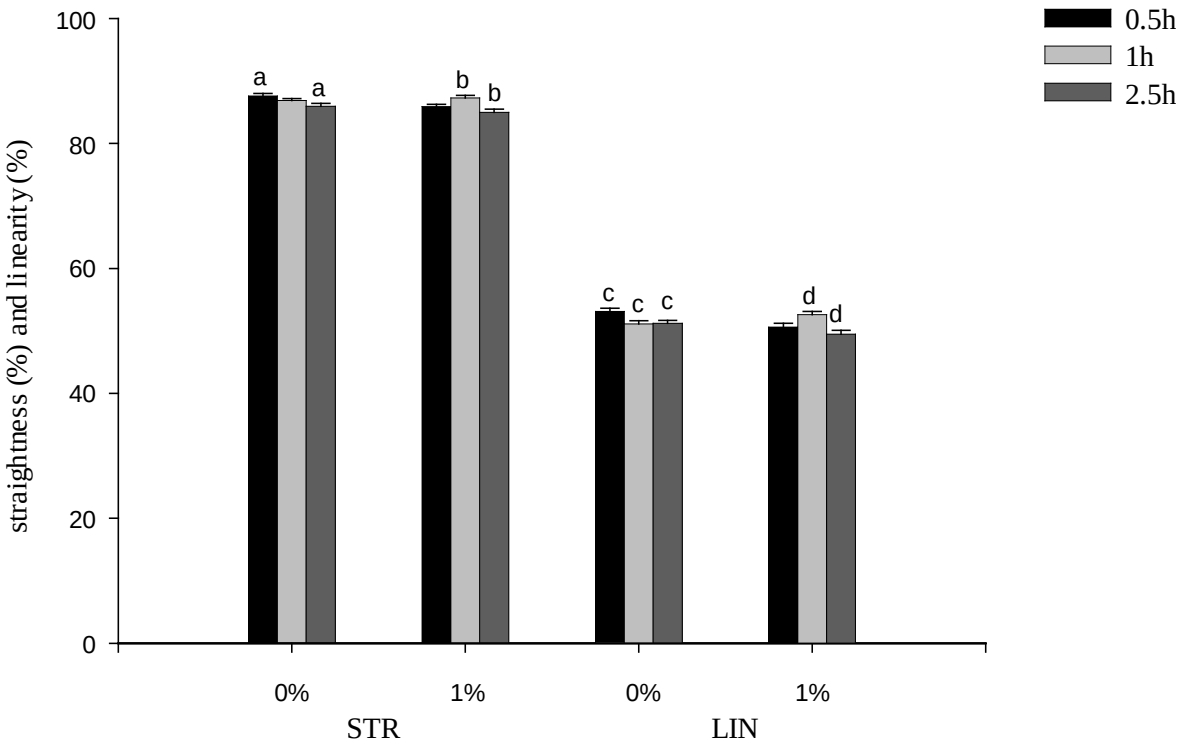


Fig. 14: Straightness (STR, %) and linearity (LIN, %) of frozen-thawed Mexican wolf spermatozoa cooled for different times (0.5 h, 1.0 h, 2.5 h) diluted without (0 %) and with (1 %) Equex pasta at 0 h [significant differences: influence of cooling time; single-factor ANOVA, a (0.5 h / 2.5 h), P < 0.05; b (1.0 h / 0.5 h, 2.5 h), c (0.5 h / 1.0 h, 2.5 h), d (1.0 h / 0.5 h, 2.5 h), P < 0.01)]

Samples cooled for 0.5 h with Equex showed significantly higher values for BCF ($P < 0.01$, Fig. 15, table 9.3.17, appendix). Results for ALH are presented in Fig. 15 and table 9.3.16 (appendix).

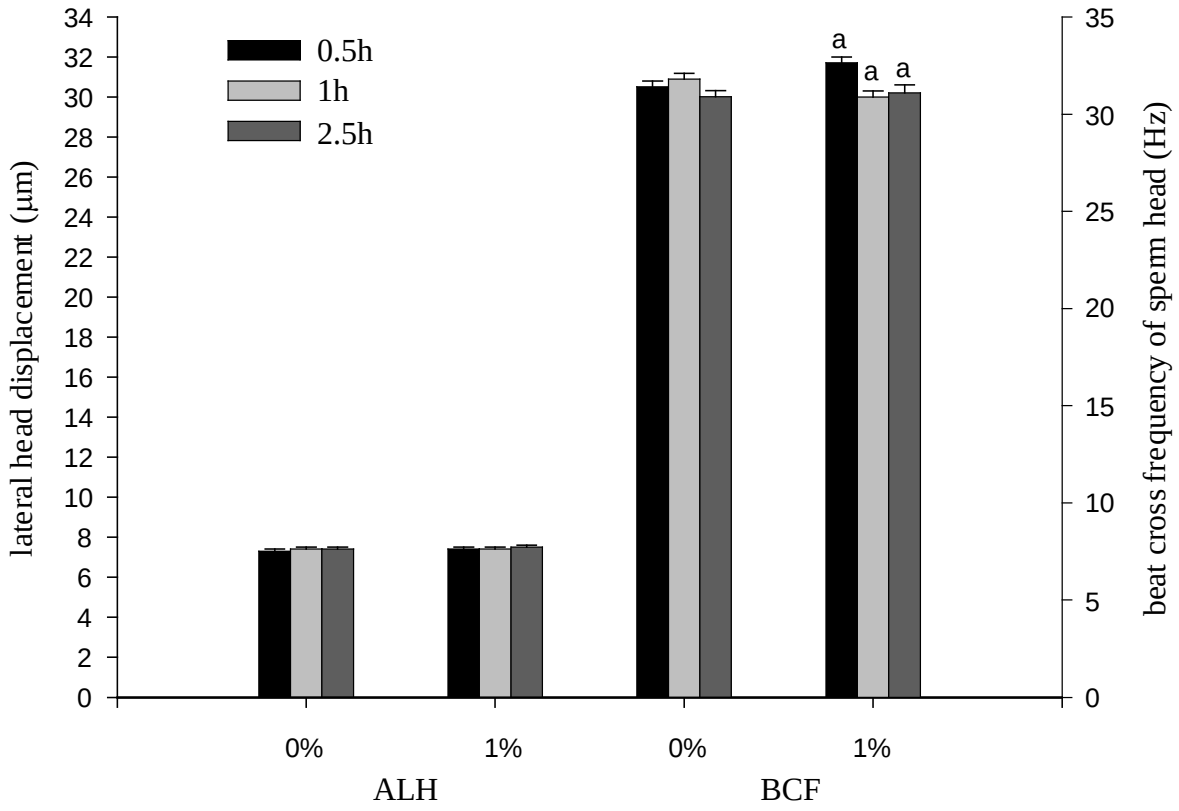


Fig. 15: Lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz) of the sperm head without (0 %) and with Equex pasta (1 %) of frozen-thawed Mexican wolf semen cooled for different times (0.5 h, 1.0 h, 2.5 h) [significant differences: single-factor ANOVA, a (0.5 h / 1.0 h, 2.5 h), $P < 0.01$)]

During the 11 hours of the longevity test samples diluted without Equex pasta showed better results for the first hours of the longevity test compared with the samples containing Equex pasta, but at 3 h (VCL; Fig. 17, table 9.3.18, appendix), 4 h (VAP; Fig. 16, table 9.3.18, appendix) and 6 h (VSL; Fig. 16, table 9.3.18, appendix) of incubation, respectively, significantly better conditions were found in samples diluted with Equex pasta.

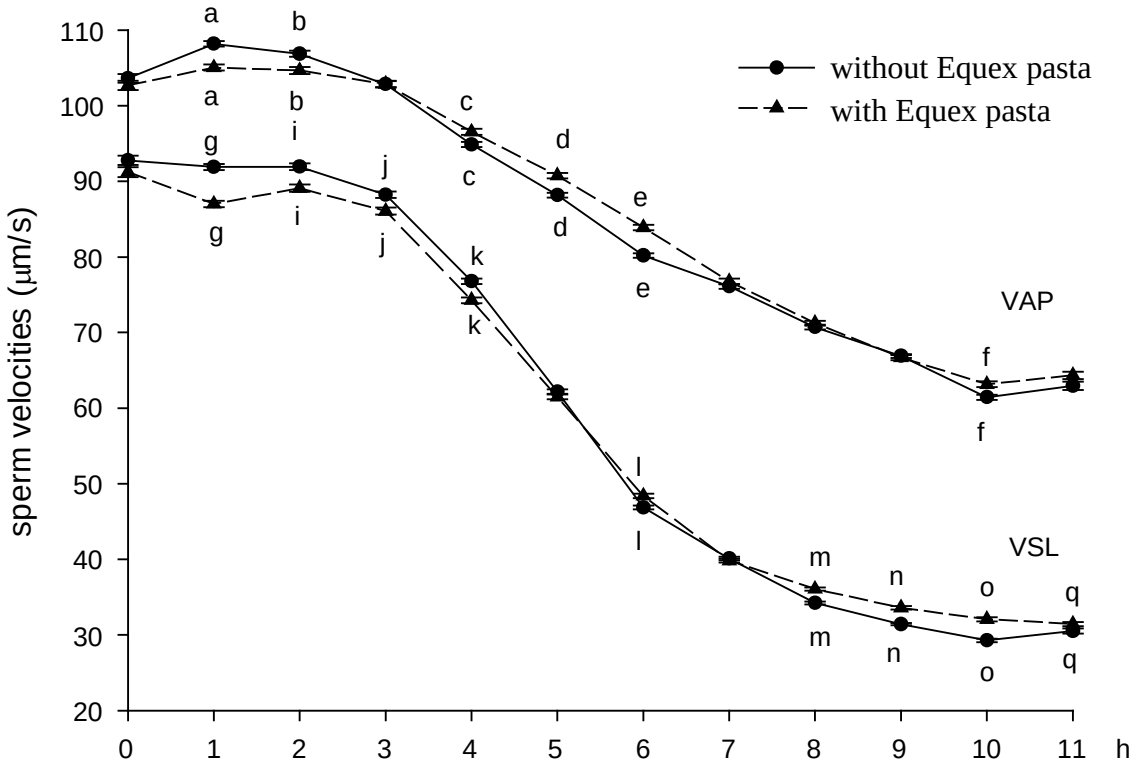


Fig. 16: Sperm speed (VAP, average path velocity, µm/s; VSL, straight line velocity, µm/s) of frozen-thawed Mexican wolf spermatozoa diluted without (0 %) or with (1 %) Equex pasta during a 11h longevity test evaluated by CASA; influence of Equex supplementation (single-factor ANOVA; a, b, c, d, e, f, g, i, j, k, l, m, n, o (0 % / 1 %) $P < 0.01$); q (0 % / 1 %) $P < 0.05$]

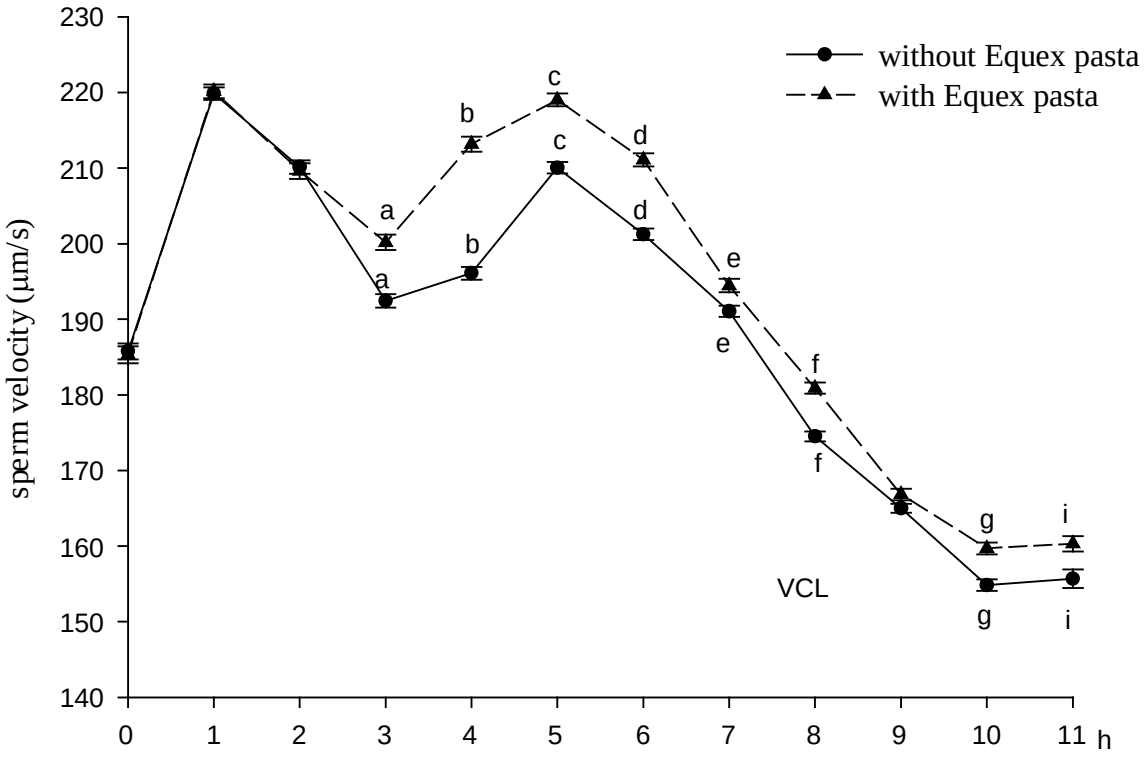


Fig. 17: Sperm speed (VCL, curvilinear velocity, µm/s) of frozen-thawed Mexican wolf spermatozoa diluted without (0 %) or with (1 %) Equex pasta during a 11h longevity test evaluated by CASA; influence of Equex supplementation (single-factor ANOVA; a, b, c, d, e, f, g, i (0 % / 1 %) $P < 0.01$]

The straightness (STR; Fig. 18, table 9.3.19, appendix) and linearity (LIN; Fig. 18, table 9.3.19, appendix) were significantly better in samples diluted without Equex pasta until 8 hours after thawing, when samples diluted with Equex pasta showed better results.

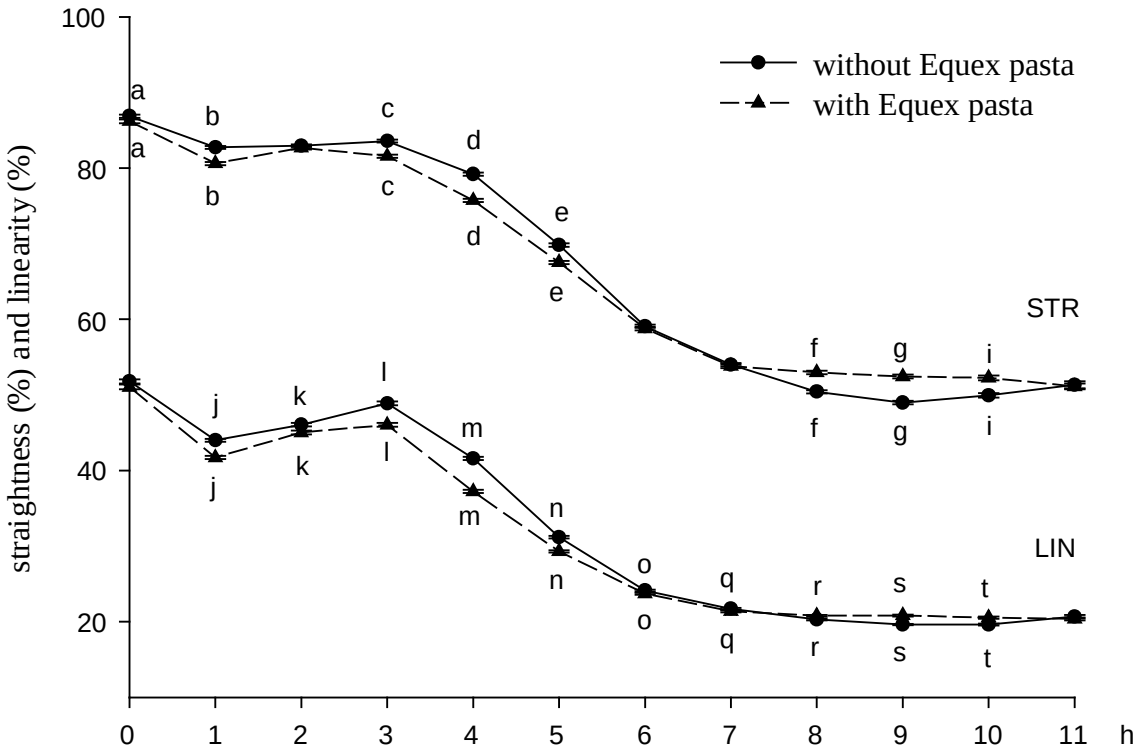


Fig. 18: Straightness (STR, %) and linearity (LIN, %) of frozen-thawed Mexican wolf Spermatozoa diluted without (0 %) and with (1 %) Equex pasta during a 11h longevity test evaluated by CASA; influence of Equex supplementation (single-factor ANOVA; a, o (0 % / 1 %) $P < 0.05$; b, c, d, e, f, g, i, j, k, l, m, n, q, r, s, t (0 % / 1 %) $P < 0.01$]

Furthermore samples diluted with Equex pasta had significantly higher ALH (Fig. 19, table 9.3.20, appendix) at 1 h, 3 – 6 h, 10 & 11 h after thawing and higher BCF (Fig. 19, table 9.3.20, appendix) from 3 to 7 h of incubation.

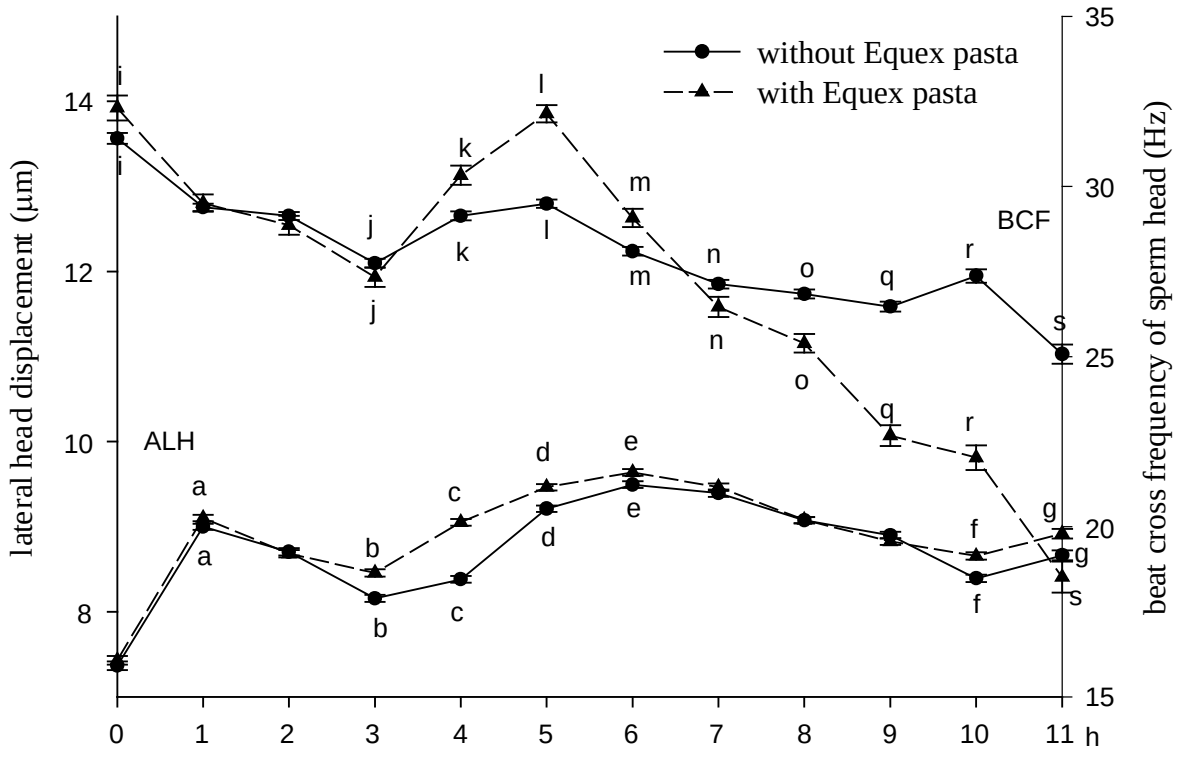


Fig. 19: Lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz) of frozen-thawed Mexican wolf sperm diluted without (0 %) and with Equex pasta (1 %) during a 11h longevity test evaluated by CASA; influence of Equex supplementation [significant differences: single-factor ANOVA; a, j (0 % / 1 %), $P < 0.05$); b, c, d, e, f, g, i, k, l, m, n, o, q, r, s (0 % / 1 %) $P < 0.01$]

4.6.3 Influence of different cooling times and Equex pasta on post-thaw plasma membrane integrity

In samples stained with the **Eosin/Nigrosin** semen of the domestic dog, cooling time had no effect on post-thaw sperm plasma membrane integrity. In both wolf species samples cooled for 2.5 h with or without Equex pasta showed best results ($P > 0.05$). Generic gray wolf showed markedly lower percentages of live spermatozoa in all cooling times tested compared to results in domestic dog and Mexican wolf (Fig. 20, table 9.3.21, appendix).

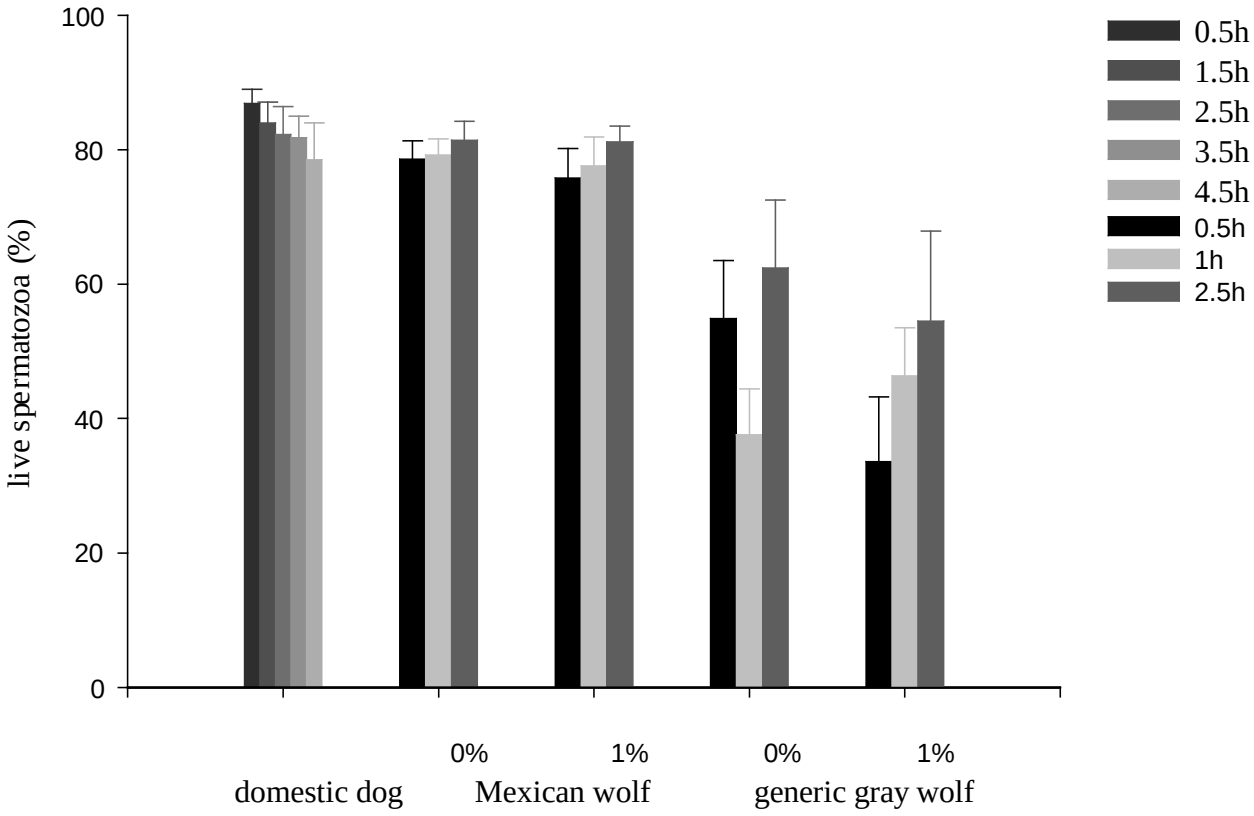


Fig. 20: Plasma membrane integrity (Eosin-Nigrosin, %) in frozen-thawed samples cooled for different times of domestic dog (0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h) and of generic gray and Mexican wolf (0.5 h, 1.0 h, 2.5 h) diluted without (0 %) and with (1 %) Equex pasta

Dog semen samples cooled for 1.5 h showed the highest percentage of spermatozoa with intact plasma membrane (**6-CFDA/PI**) after thawing. Differences between cooling times were, however, not significant (table 9).

Table 9: Spermatozoa with intact plasma membranes (%, 6-CFDA/PI and HOST) in frozen-thawed semen of the domestic dog cooled for different times (0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h)

cooling time (h)	6-CFDA / PI		HOST	
	mean	SEM	mean	SEM
0.5 (m=8)	39.2	6.8	69.2	4.2
1.5 (m=8)	55.6	7.7	67.4	4.1
2.5 (m=8)	52.6	6.2	69.3	4.0
3.5 (m=8)	50.1	6.6	67.6	4.0
4.5 (m=8)	47.1	8.3	63.8	3.1

In generic gray wolf semen samples cooling for 2.5 h have best results ($P > 0.05$). Generic gray wolf semen samples treated with Equex pasta show a significantly higher ($P < 0.05$) percentage of partly damaged plasma membranes (6-CFDA/PI) than samples not treated with Equex pasta (Fig. 21 and table 10).

Table 10: Spermatozoa with intact (%), partly damaged (%) and completely damaged (%) plasma membranes (6-Carboxyfluoresceindiacetate) of frozen-thawed generic gray wolf (n = 7) semen cooled in different cooling times (0.5 h, 1.0 h, 2.5 h) diluted without (0 %) or with (1 %) Equex pasta

Equex pasta	cooling time (h)	intact		partly damaged		completely damaged	
		mean	SEM	mean	SEM	mean	SEM
0 %	all times (m=21)	29.9	5.0	9.6[a]	1.2	60.5	5.0
	0.5 (m=7)	32.2	7.3	9.3	1.5	58.5	7.5
	1.0 (m=7)	20.2	5.3	6.3	1.5	73.5	6.1
	2.5 (m=7)	37.2	11.8	13.2	2.7	49.5	10.5
1 %	all times (m=21)	21.4	3.3	17.2[a]	2.4	61.4	5.1
	0.5 (m=7)	16.1	6.0	17.1	4.2	66.8	9.9
	1.0 (m=8)	21.0	5.0	15.0	3.3	64.0	6.2
	2.5 (m=6)	28.2	6.6	20.3	5.9	51.6	11.5

Significant differences

Single-factor ANOVA:

partly damaged plasma membrane: 0% / 1 % a: $P < 0.01$

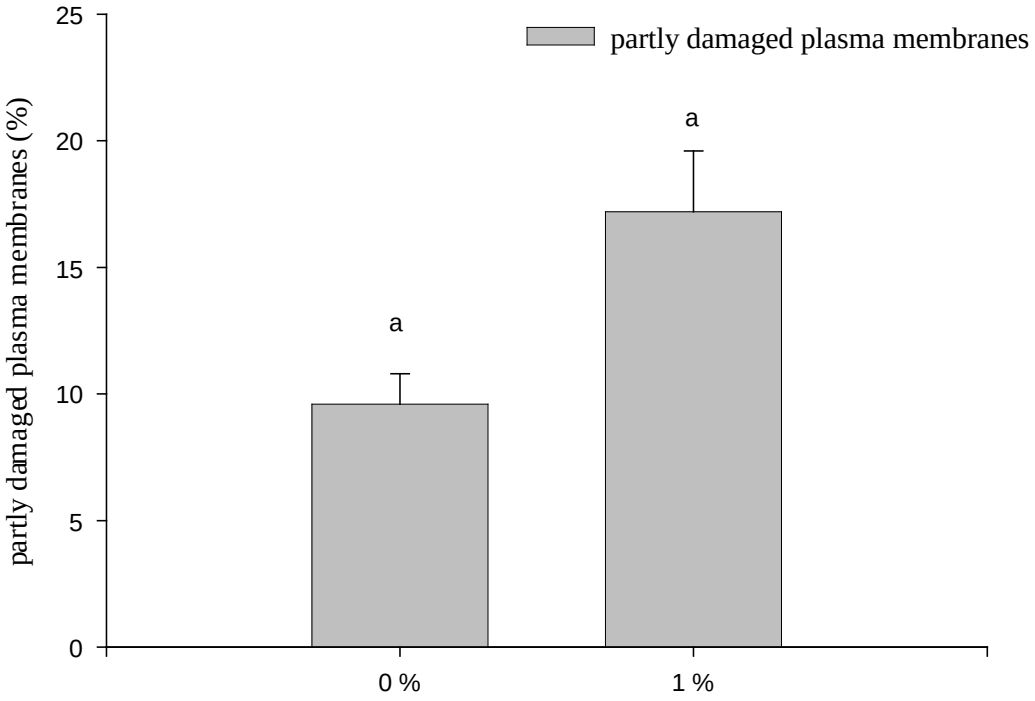


Fig. 21: Spermatozoa with partly damaged plasma membranes (%) in frozen-thawed semen of the generic gray wolf without (0 %) or with (1 %) Equex pasta [significant differences: Equex: single-factorial ANOVA, a: $P < 0.01$)]

Mexican wolf semen samples cooled for 2.5 h showed a significantly higher percentage ($P < 0.05$) of spermatozoa with intact plasma membranes (6-CFDA/PI) than samples cooled for 0.5 h (tables 11 and 12; Fig. 22).

Table 11: Spermatozoa with intact (%), partly damaged (%) and completely damaged plasma membrane (6-CFDA/PI) in generic gray and Mexican wolf semen cooled for different times (0.5 h, 1.0 h, 2.5 h)

species	cooling time (h)	intact		partly damaged		completely damaged	
		mean	SEM	mean	SEM	mean	SEM
generic gray wolf (n=7)	0.5 (m=14)	24.1	5.1	13.2	2.4	62.6	6.1
	1.0 (m=15)	20.6	3.5	10.9	2.2	68.4	4.4
	2.5 (m=13)	33.0	6.9	16.5	3.1	50.5	7.4
Mexican wolf (n=4)	0.5 (m=49)	49.3[a]	2.7	7.7	0.6	43.0	2.8
	1.0(m=49)	54.2	2.5	6.8	0.7	39.0	2.5
	2.5(m=45)	58.0[a]	2.2	9.8	0.7	34.2	2.4

Significant differences

Single-factor ANOVA:

Mexican wolf: 2.5 h / 0.5 h a: $P < 0.05$

In semen samples diluted without Equex pasta integrity of plasma membrane (6-CFDA) was significantly better ($P < 0.01$) than in samples treated with Equex pasta (table 12).

Table 12: Spermatozoa with intact (%), partly damaged (%) and completely damaged (%) plasma membranes (6-Carboxyfluoresceindiacetate) of frozen-thawed Mexican wolf semen cooled in different cooling times (0.5 h, 1.0 h, 2.5 h) diluted without (0 %) or with (1 %) Equex pasta

species	Equex pasta	cooling time (h)	plasma membrane integrity					
			intact		partly damaged		completely damaged	
			mean	SEM	mean	SEM	mean	SEM
Mexican wolf (n=4)	0 %	all times (m=76)	58.9[b]	1.8	6.5	0.4	34.6	1.8
		0.5 (m=26)	55.2[a]	3.5	6.4	0.7	49.0	2.4
		1.0 (m=26)	58.2	3.2	5.5	0.6	50.0	2.4
		2.5 (m=24)	63.7[a]	2.2	7.7	0.5	50.7	2.8
	1 %	all times (m=67)	47.8[b]	2.1	9.9	0.6	43.7	2.4
		0.5 (m=23)	42.7	3.7	9.2	0.9	48.2	4.2
		1.0 (m=23)	49.6	3.9	8.3	1.2	43.0	3.5
		2.5 (m=21)	51.4	3.4	12.3	1.1	41.2	2.4

Significant differences:

Single-factor ANOVA:

intact plasma membranes	0.5 h / 2.5 h	a: $P < 0.05$
intact plasma membranes	0 % / 1 %	b: $P < 0.01$

In Mexican wolf semen, the lowest percentage of spermatozoa with intact plasma membranes (6-CFDA/PI) were seen in samples treated with Equex pasta and cooled for 0.5 h ($P > 0.05$), while the highest percentage was found in samples not treated with Equex pasta and cooled in 2.5 h ($P > 0.05$) (table 12; Fig. 22).

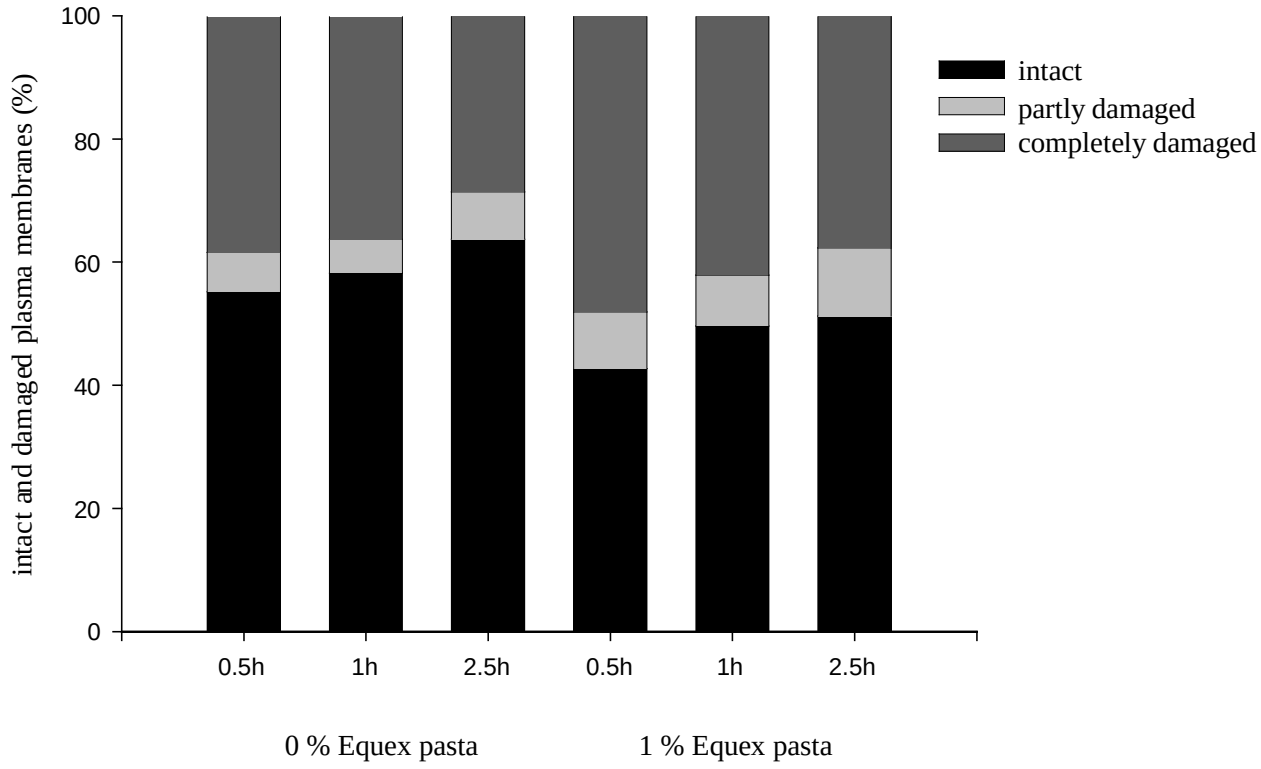


Fig. 22: Intact (%), partly damaged (%) and completely damaged (%) plasma membrane in frozen-thawed Mexican wolf semen cooled for different times (0.5 h, 1.0 h, 2.5 h) without (0 %) or with (1 %) Equex pasta [no dignificant differences, $P > 0.05$]

The **hypoosmotic swelling test (HOST)** clearly showed that wolf semen samples frozen without Equex pasta had higher percentages of spermatozoa with intact plasma membranes than those treated with Equex pasta [generic gray wolf: $P < 0.05$, Mexican wolf: $P < 0.01$] (table 13; table 9.3.22, appendix).

Table 13: Spermatozoa with intact plasma membrane (%, HOST) in frozen-thawed semen of generic gray and Mexican wolf semen diluted without (0 %) or with (1 %) Equex pasta

Equex pasta	0 %		1 %	
	mean	SEM	mean	SEM
Generic gray wolf (n=7)	50.1[a] (m=21)	3.2	39.1[a] (m=21)	4.0
Mexican wolf (n=4)	62.2[b] (m=76)	1.5	51.8[b] (m=67)	1.9

Significant differences

Single-factor ANOVA:

generic gray wolf: 0 % / 1 % a: $P < 0.01$

Mexican gray wolf: 0 % / 1 % b: $P < 0.01$

4.6.4 Influence of different cooling times and Equex pasta on post-thaw sperm morphology and acrosome integrity

In domestic dog, generic gray and Mexican wolf no significant differences were detected in the percentage of morphologically normal spermatozoa in frozen-thawed semen of samples cooled for different cooling times and diluted without or with Equex pasta in ($P > 0.05$, table 9.3.23, appendix). Mexican wolf semen samples treated with Equex pasta had a significantly ($P < 0.05$) lower percentage of morphologically intact acrosomes than samples not treated with Equex pasta (table 14).

Table 14: Spermatozoa with normal morphology (%) in frozen-thawed generic gray and Mexican wolf semen diluted without (0 %) or with (1 %) Equex pasta

Equex pasta	0 %		1 %	
	mean	SEM	mean	SEM
Generic gray wolf (n=7)	61.2 (m=21)	5.7	60.8 (m=21)	6.4
Mexican gray (n=4) wolf	73.8[a] (m=76)	1.3	69.6[a] (m=67)	1.4

Significant differences

Single-factor ANOVA:

Mexican gray wolf: 0 % / 1 % a: $P < 0.05$

Frozen-thawed domestic dog semen samples cooled for 4.5 h showed a significantly lower percentage of spermatozoa with intact acrosome ($P < 0.05$) than samples cooled in 0.5 h (table 15). Percentages of intact acrosomes in dog semen improved with longer cooling times and slower cooling rates. In semen of both wolf species percentage of intact acrosomes are markedly lower (Mexican wolf: 11.7 ± 0.7 %) than in semen of the domestic dog (24.8 ± 1.9); especially in frozen-thawed semen of generic gray wolf (2.4 ± 0.4 %) the percentage of intact acrosomes is extremely low (table 15).

Table 15: Spermatozoa with intact acrosome (%) of frozen-thawed domestic dog, generic gray and Mexican wolf semen cooled for different times (dog: 0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h; wolves: 0.5 h, 1.0 h, 2.5 h) without (0 %) or with (1 %) Equex pasta

species	cooling time (h)	0 %		1 %	
		mean	SEM	mean	SEM
domestic dog (n=4)	0.5 (m=9)	31.9[a]	4.5	-----	-----
	1.5 (m=8)	30.5	3.7	-----	-----
	2.5 (m=8)	23.1	5.0	-----	-----
	3.5 (m=8)	22.6	3.7	-----	-----
	4.5 (m=8)	15.8[a]	2.4	-----	-----
generic gray wolf (n=7)	0.5 (m=7)	3.3 (m=7)	1.0	2.7	1.4
	1.0 (m=8)	2.4 (m=7)	0.4	1.0	0.4
	2.5 (m=6)	2.1 (m=7)	0.7	3.0	1.3
Mexican wolf (n=4)	0.5 (m=23)	11.1 (m=26)	1.7	9.6	1.7
	1.0 (m=23)	13.7 (m=26)	1.5	11.3	2.2
	2.5 (m=21)	13.1 (m=24)	2.1	10.9	1.6

Significant differences

Single-factor ANOVA:

domestic dog 4.5 h / 0.5 h a: $P < 0.05$

Fig. 23: Sperm plasma membrane evaluation with different evaluation methods. Fresh, cooled and frozen-thawed semen of domestic dog and Mexican gray wolf are presented on the following page showing different manners to show reaction to the stain and test.

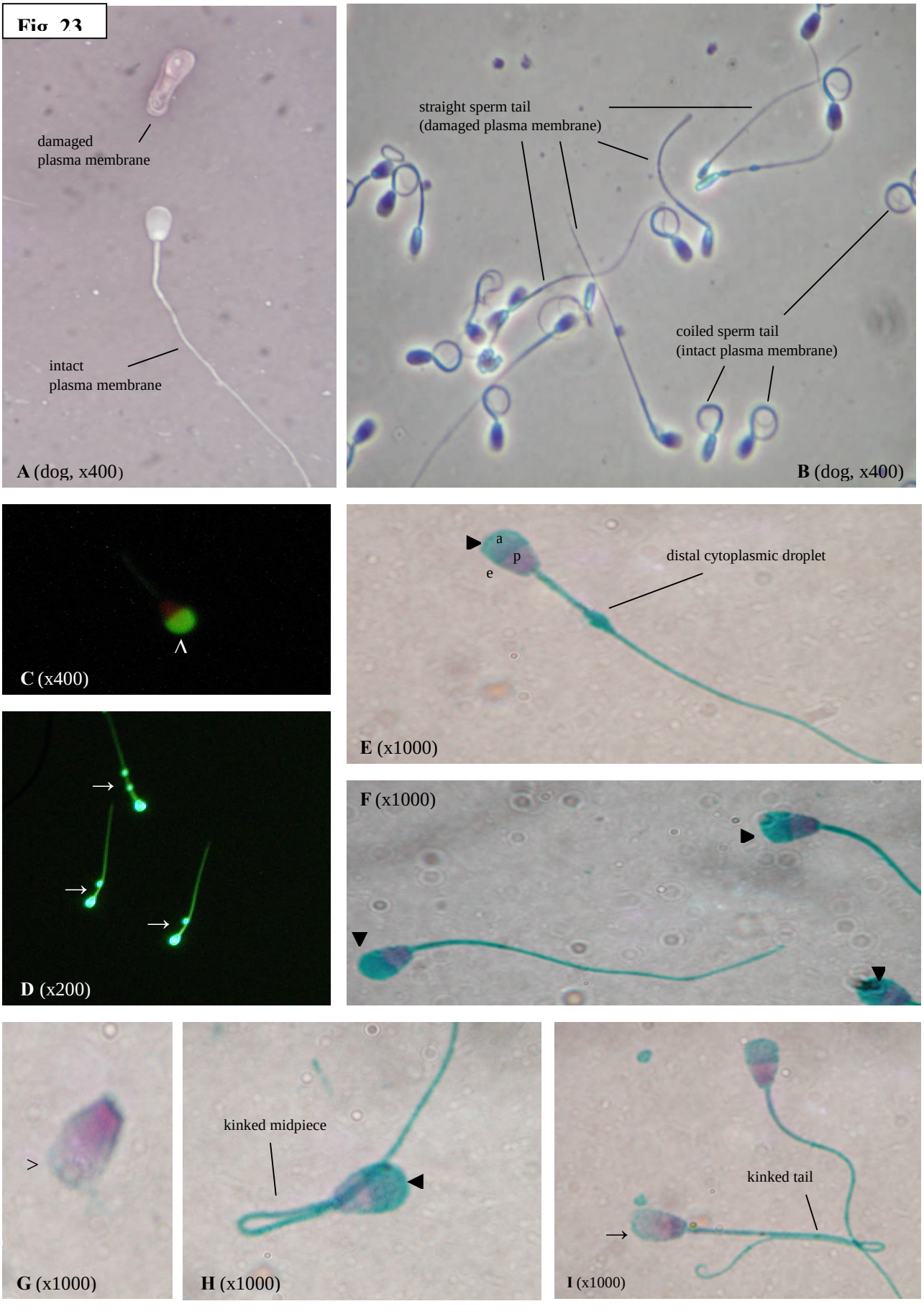
A: Eosin-nigrosin staining showing spermatozoa with intact and damaged plasma membrane (fresh)

B: Hypo-osmotic swelling test showing spermatozoa with functionally intact (coiled sperm tail) and damaged (straight sperm tail) plasma membrane (fresh)

C and D: Frozen-thawed Mexican wolf spermatozoa stained with fluorescent stain (6-CFDA/PI); spermatozoa with damaged plasma membrane and intact acrosomal membrane (acrosome stained green with 6-CFDA/PI (▲), post-acrosomal region stained red with PI; Fig. C). Spermatozoa with entirely intact plasma membrane (stained entirely green with 6-CFDA/PI and unstained with PI; Fig. D) and distal cytoplasmic droplets (→).

E to I: Spermatozoa morphology, stained with Spermac®. Mexican wolf spermatozoa with intact acrosomes (▲); sperm in process of losing its acrosome with reacted acrosome (→; Fig. I); head of spermatozoa without acrosome (>) with the rest of the head stained red (Fig. G). a=acrosome, e=equatorial region, p=post-acrosomal region of the head (F=fresh, E, G-I=cooled and frozen-thawed).

Fig. 23


damaged
plasma membrane
intact
plasma membrane
A (dog, x400)
straight sperm tail
(damaged plasma membrane)
coiled sperm tail
(intact plasma membrane)
B (dog, x400)
C (x400)
D (x200)
a
p
e
distal cytoplasmic droplet
E (x1000)
F (x1000)
G (x1000)
kinked midpiece
H (x1000)
kinked tail
I (x1000)

5 Discussion

In the present study the effects of different cooling times and rates from room temperature to 5 °C on the quality of domestic dog, generic gray and Mexican wolf semen were investigated. An additional split sample served to determine possible effects of sodium dodecyl sulphate (Equex pasta®) on semen quality. Specific attention was directed to differences between the canid species, domestic dog and wolf. The evaluation of the semen was ensued by basal and advanced spermatologic evaluation methods. In the Mexican wolf semen samples post-thaw motility parameters were additionally evaluated by computer assisted semen analysis.

5.1 Species differences of ejaculate and fresh semen characteristics

Sperm concentration of generic gray and Mexican wolf were lower than that of domestic dog indicating that by electroejaculation the accessory sex glands are overstimulated (PLATZ and SEAGER 1978). The low total sperm count of domestic dog may have been caused by insufficient stimulation in absence of an estrous bitch. A lower pH in electroejaculated semen compared with manually collected semen as stated in DOOLEY and PINEDA (1986) could not be confirmed.

All three species had good fresh semen characteristics. The only significant difference between species was seen in the higher percentage of Eosin-Nigrosin-stained cells in generic gray wolf in comparison to domestic dog, that by itself is difficult to interpret. Mexican wolf showed excellent fresh semen characteristics (89.0 ± 1.3 % cells with intact plasma membrane; 73.9 ± 4.7 % morphologically normal cells and 90.6 ± 1.7 % cells with intact acrosome) thinking of the danger of a high inbreeding coefficient with subsequent low sperm quality compared to values obtained for fresh red wolf (*Canis rufus*) semen (KOEHLER et al. 1998).

5.2 Testes measurements

Testicular length and width of both generic gray (50-60 kg) and Mexican wolf (26-36 kg) were similar to those measured in these species by MUSSON (2001), but testicular

measurements for generic gray wolf in this study was slightly higher. Mexican wolf having a lower body weight has a greater total testicle volume than generic gray wolf in the present study; here the small number of evaluated animals has to be taken into account. Comparing the results of this study with measurements published for dogs by OLAR et al. (1983) and by GÜNZEL-APEL et al. (1994) it is indicated that the anatomical and functional conditions of dog and wolf testes are very similar.

5.3 Influence of the cooling process on semen

5.3.1 General observations

Going through cryopreservation, sperm has been shown to be vulnerable to cooling injury. Cooling injury has been studied extensively in ram sperm (WATSON 1981) and cold shock especially is considered to be a severe treatment for sperm (DE LEEUW et al. 1990a, b). In all three species the quality of semen deteriorated during cooling. Plasma membrane integrity was significantly affected by the cooling period. The fluorescent stain did not work out in fresh and cooled samples in all species, probably due to technical reasons of the conditions in the field. Therefore these results cannot be taken into consideration for the discussion. The percentages of spermatozoa with intact plasma membrane (hypoosmotic swelling test) in generic gray and Mexican wolf semen decreased significantly in comparison to dog spermatozoa indicating a higher susceptibility of wolf spermatozoa to cold temperatures. In both wolf species fresh samples show significantly higher percentages of intact plasma membrane (hypoosmotic swelling test) than cooled samples. These results confirm ROBERTSON et al. (1988a) who already found in human sperm that plasma membrane integrity decreased as cold stress increased. The observation by HOLT and NORTH (1994) that plasma membrane integrity of ram spermatozoa was maintained throughout the cooling process cannot be confirmed for the canid species. After the cooling period the percentage of acrosome-reacted spermatozoa had markedly increased and confirms findings of BURGESS et al. (2001) who found an immediate increase of acrosomal damage during cooling. The same was found in GOODROWE et al. (2001) confirming decreasing percentage of

spermatozoa with intact acrosome. However, acrosome integrity did not deteriorate as significantly during cooling as during the freeze-thaw process as shown by ZALEWSKI and BERG (1983) in fox semen. Cooling may be specific in its injurious effects on plasma membrane (QUINN et al. 1980). By inducing phase separations between membrane lipid domains (HOLT et al. 1988) being associated with the thermotropic lipid phase transition (ZERON et al. 2002) plasma membrane damage occurs. Additionally there are other reactions of the membranes to the phase transition temperature (DAVIS and BYRNE 1980; HOLT and NORTH 1985; DE LEEUW et al. 1990a, b; PARKS and LYNCH 1992) with profound effects upon membrane properties. Increased intracellular calcium has been observed after sperm cooling and may be a result of cooling-induced lipid phase alteration of calcium ion channel proteins. Since an increase in intracellular calcium is associated with initiation of capacitation, this calcium increase after cooling could contribute to the capacitation-like characteristics observed in cooled sperm (BAILEY and BUHR 1994; BAILEY et al. 2000; WATSON 2000). The resulting altered protein function (PARKS and GRAHAM 1992; AMANN 1999), lead to biochemical changes as indicated by disturbed functional activity of plasma membrane shown by a decreased percentage of spermatozoa in the hypoosmotic swelling test in all species included in the present study. These mechanisms seemed to occur in this present study represented by decreased plasma membrane and acrosome integrity compared to fresh semen, as a sign of capacitation-like changes found in ROTA (1998) and ROTA et al. (1999b) initiated at preservation.

5.3.2 Specific influence of cooling rate, cooling time and final temperature attained

The cooling procedures studied, differed in their cooling rates, in the duration of exposure to cold temperature and the final temperature attained which all influenced semen quality characteristics after the cooling period. Cooled samples unfortunately have not been evaluated for motility and can therefore not be discussed. No significance was detected in influence on plasma membrane integrity in either species. The cooling time of 1.0 h used in semen of generic gray and Mexican wolf was insufficient to cool the diluted semen to the required 5 °C before entering the freezing period, but finished at 7 °C (Mexican gray wolf) and 9 °C (generic gray wolf). Significant differences in Mexican wolf semen only were seen in quality

of acrosome. Mexican wolf semen cooled for 1.0 h showed significantly higher percentages of intact acrosomes than samples cooled for 2.5 h. MARTIN (1963a) found 1.0 h cooling time satisfactory, but other than in MARTIN's study (1963a), Mexican wolf semen, cooled for 1.0 h did reach 7 °C. The other cooling times of 0.5 h with a cooling rate of 0.5 °C/min and 2.5 h with a cooling rate of 0.1 °C/min did reach 5 °C causing more damaged acrosomes than cooling to 7 °C irrespective of faster cooling rate or longer cooling time. This may have been due to the prevented exposure to a critical temperature below 7 °C which would cause the plasma membrane to undergo certain changes, resembling capacitation-like changes with subsequent acrosome reaction as found in other species than canid (FULLER and WHITTINGHAM 1997, mouse; MAXWELL and JOHNSON 1997b, pig; JONES and STEWART 1979, bull). In Mexican wolf spermatozoa, an additional lipid phase transition might take place at a temperature lower than 7.0 °C. With this hypothetic lipid phase transition the plasma membrane would undergo an additional reordering of lipids with subsequent membrane damage. As different lipid domains exist, multiple phase transitions may be possible (LADHA 1998). For ram spermatozoa the phase transition temperatures are 28-35 °C, 24-26 °C and 15-17 °C (HOLT and NORTH 1986) and in boar spermatozoa at 32 °C and 6 °C (CANVIN and BUHR 1989). The occurrence of phase transitions may be bound to specific temperatures in canid species, and species differences in membrane lipid composition may be responsible for the varying stabilities during cooling, reported for the bull, ram and boar (HOLT and NORTH 1984; DE LEEUW et al. 1990b). Also PARKS and LYNCH (1992) confirmed major species differences in the molecular features of sperm membrane lipids. Semen of generic gray wolf may even have different phase transition temperatures than Mexican wolf because generic gray wolf semen deteriorates more when held longer in cold temperatures, even when only cooled to above 9 °C, than cooled fast to 5 °C. There was no significant difference between cooling rates or time and earlier studies evaluating dog semen cooling protocols didn't get uniform results concerning cooling rates and times. In BATEMAN (2001) the longer time dog semen was cooled, the further decrease in percent intact acrosomes was detected. In contrast HAY et al. (1997b) detected rapid cooling (0.5 h) to be more damaging than slow cooling (3 h) not only for acrosome integrity but also resulting in lower percentage of progressive motility. However, in this study final temperature attained after cooling was 0 °C, having a more detrimental effect on spermatozoa

compared to cooling to 5 °C (BATEMAN 2001) and this might shadow the worse results when cooled faster. Cooling of 0.6 °C/min to 5°C was found to be adequate for cooling of domestic dog semen (BATEMAN 2001) in contrast to GOODROWE et al. (2001) who did not find a difference in cooling rate. PUKAZHENTHI et al. (1999) found significant acrosomal damage in all tested cooling rates, but the extent of temperature induced damage was significantly mitigated by slow cooling (0.5 °C/min) to 0 °C, similar to the results in Mexican wolf semen taking also into consideration the final temperature attained.

5.3.3 Electron microscopic findings after the cooling period

Although the electron microscopic evaluation in the present study is not representative in Mexican wolf spermatozoa, the results may give an idea about the ultrastructure of damaged spermatozoa by cooling to 5 °C. No striking differences were found in fresh and cooled samples or samples cooled for different times. The most detrimental effects occur at the plasma membrane, the acrosome and the mitochondria. As well in fresh and in cooled semen, swelling of the plasma membrane and disintegrating acrosomes are apparent. In fresh semen acrosomal detachment of the nuclear envelope with ruffling of the outer acrosomal membrane was detected similar to the results obtained in red wolf semen by KOEHLER et al. (1998) in the head region. Cooled semen, in either cooling time have mitochondria with heterogeneous density. The discovered particle-clustering as an irreversible change of plasma membrane in slowly cooled (0.25 °C) ram spermatozoa (HOLT and NORTH 1984) could not be confirmed with the present study. Distortion and separation of the plasma membrane from the acrosomal membrane found in all samples could represent an artefact caused by TEM (ABRAHAM-PESKIR et al. 2000, bull, horse). The possibility that spermatozoa may nevertheless have suffered subtle changes during cooling cannot be disregarded. Plasma membrane undulation also occured during cooling (BURGESS et al. 2001; STRÖM HOLST et al. 1998). Further acrosomal damage was evident with swelling and vacuolation or swelling of the outer acrosomal membrane including acrosome detachement in cooled ram (JONES and MARTIN 1973; PARKS and HAMMERSTEDT 1985) and dog spermatozoa (OETTLÉ et al. 1986a; STRÖM HOLST et al. 1998). These changes with ruffling of the outer acrosomal membrane, also evident in the present study were also seen in extended and at room temperature

equilibrated human semen or could indicate a specific sensitivity towards environmental influences or methodological inadequacies (WOOLEY and RICHARDSON 1978). Vesiculation of acrosomal content was evident in cooled spermatozoa (BURGESS et al. 2001) confirmed by the results in the present study. Extensive damage of acrosomal membranes with loss of acrosomal material, not found in the Mexican wolf semen, only was described in spermatozoa being damaged by cold-shock (QUINN et al. 1969; WATSON and MORRIS 1987; OETTLÉ and SOLEY 1988) or by cooling of teratospermic spermatozoa (PUKAZHENTHI et al. 1999). In cooled semen no changes were detected at the mitochondria and only were affected by freezing-thawing (WOOLEY and RICHARDSON 1978). The damage of mitochondria seen in the electron microsopic Mexican wolf sperm pictures correspond with results of a study by WATSON and MORRIS (1987), mid-pieces showing condensation of mitochondria with occasional loss of electron-density and internal structures. As only single mitochondria were detected in the Mexican wolf semen samples this result is not predicative to conclude damage just by cooling. Even though several changes detected by electron microscopy might be related to other procedures during cryopreservation than cooling. DE LEEUW et al. (1990a, b) showed that at reduced temperatures intramembranous components aggregate and redistribute in the head and main piece of the sperm, giving rise to packing faults of membrane lipids, followed by detrimental leakage of sperm cell contents. Additional physical and/or chemical alteration of lipid bilayers are resulting in changing distributions in both head and tail regions of intramembranous particles (IMP). Those intrinsic proteins lying within lipid bilayers are almost evenly distributed in the plasma membrane over the acrosome of uncapacitated spermatozoa, but capacitated spermatozoa has many small patches that are free of IMPs, also occurring during phase transitions at certain temperatures (DE LEEUW 1990a, b).

5.4 Influence of the freezing-thawing process on semen

5.4.1 General observations

Freezing and thawing of semen resulted in a significant deterioration of all parameters in the dog and in both wolf species compared to fresh and cooled semen. Semen of generic gray wolf showing worse results than domestic dog and Mexican wolf. All species showed an increase in spermatozoa with damaged plasma membrane after thawing as stated in a study of boar spermatozoa by ORTMAN and RODRIGUEZ-MARTINEZ (1994). Similar results were obtained in previous studies in dogs (OETTLÉ 1986a, BURGESS et al. 2001) and in ram, where freezing-thawing caused capacitation-like plasma membrane changes (GILLAN et al. 1997). The same was reported for dog (LINDE-FORSBERG 1991, HAY et al. 1997b) and fox semen (FARSTAD 1996). Also a decline in progressive motility as seen in the present study was reported in earlier studies (HAY et al. 1997b, dog; GOODROWE et al. 2001, red wolf). Post-thaw motility was maintained at a low level, except for Mexican wolf, during the post-thaw period similar to conditions described for dog semen (OLAR et al. 1989, ENGLAND 1992, STRÖM HOLST 1999). An extensive acrosomal damage, however, was only seen in Mexican wolf semen and especially in generic gray wolf semen whereas in this species also an extreme loss of post-thaw progressive motility in comparison to fresh semen was apparent. As GOODROWE et al. (1998, 2001) detected a large proportion of partial and missing acrosomes in frozen-thawed red wolf semen with extremely poor post-thaw motility together with the extreme damage seen in the present study might account for a specific vulnerability of wild canid spermatozoa. The markedly better post-thaw results in Mexican wolf semen may be explained by individual variances, mentioned for dog semen by YUBI et al. (1987). It cannot be differentiated whether the acrosomal damage observed at that status resulted indirectly from undetected injury during cooling or directly from freezing and thawing because both cooling and freezing of dog spermatozoa may have both immediate and delayed effects on the ultrastructure of spermatozoa. The immediate effects of cooling and freezing may either kill spermatozoa or render them incapable of fertilization by damaging the acrosome, whereas delayed effects may reduce sperm longevity by altering plasma membrane structure (BURGESS et al. 2001).

5.4.2 Influence of applied cooling rates and times on frozen-thawed semen

Domestic dog semen showed the best results post-thaw when cooled for 0.5 h, generic gray and Mexican wolf semen samples showed the best results post-thaw when cooled for 2.5 h. Post-thaw motility right after thawing was significantly better for domestic dog semen cooled for 0.5 h than for 4.5 h; but not significantly different in either wolf species. However, generic gray wolf semen showed significantly higher percentages of progressive motility in semen cooled for 2.5 h after 1, 2, 3, 4, and 5 h during the post-thaw period. Mexican wolf semen plasma membrane integrity evaluated by fluorescence microscopy cooled in 2.5 h was significantly ($P < 0.05$) better compared with semen cooled for 0.5 h. On the other hand hypoosmotic stress caused less membrane damage in frozen-thawed semen of generic gray wolf semen, when cooled for 0.5 h ($P > 0.05$). This might have been due to the fact that the fluoresecsence stain and the hypoosmotic swelling test are characterizing the status of different plasma membrane components that may not have been equally affected. Acrosome integrity of domestic dog semen cooled for 0.5 h is significantly higher than of semen cooled for 4.5 h. This together with the post-thaw motility, mirrors the results obtained right after thawing and suggests that direct injury from the cooling process prepares for freezing-thawing injury. Results for dog semen confirm findings by MARTIN (1963b) that 5 h cooling leads to a drop in post-thaw motility. This confirms the bad results for dog semen cooled for 4.5 h in the present study preceded by a markedly loss of intact acrosomes during the cooling period. The decrease of spermatozoa with intact acrosome after the pre-freeze cooling period might prepare post-thaw damage. The prolonged cooling time might provide an environment in which damaging membrane redistribution could occur, resulting in the observed increase in the sperm population with reacted acrosomes. In contrast, YUBI et al. (1987) and CONCANNON and BATTISTA (1989) recommended a cooling time of 2-3 h. These differences might be explained by a different freezing rate, having a more pronounced effect on semen frozen after being cooled for a long period of time, as in the present study semen was frozen in pellets. OLAR et al. (1989) stated that dog semen can be cooled over 1, 2 or 3 h and then equilibrated for 1 or 2 h without any consistent effect on post-thaw results, concluding that there is no effect of cooling time or equilibration time at 5 °C in post-thaw motility. The same is true for BATEMAN (2001) who found no difference in motility

between semen cooled at a slow (0.14 °C/min) and fast (0.6 °C/min) cooling rate and only stated that cooling to 0 °C is more detrimental than cooling to 5 °C. Whereas ENGLAND (1993) stated that cooling spermatozoa to 0 °C was shown to have no detrimental effect if cells were cooled slowly. In contrast to the studies which didn't find a difference in cooling rate, the present study found significant differences between a 0.5 °C/min (fast) and a 0.06 °C/min (very slow) cooling rate in domestic dog. The studied cooling rate of 0.14 °C/min (slow) by BATEMAN (2001) might not have been slow enough to detect significant changes. HAY et al. (1997b) did not detect significances either, but theses results cannot directly be compared because the cooling period ended at 0 °C, compared to 5 °C in the present study. GOODROWE et al. (2001) tested a fast and a slow cooling rate (0.5 h to 5 °C and 2 h to 0 °C) with red wolf semen which is comparable with tested cooling times and semen of wild canid species in the present study. That GOODROWE et al. (2001) did not find significant differences in either parameters might be explained by the presumption that fast cooling of red wolf semen tzo 5 °C could have similar damaging effects than cooling slowly but to 0 °C. In contrast to this presumption stands the finding that cation movements caused by freezing ram spermatozoa from 0 °C to –79 °C are more marked than those resulting from rapid cooling from room temperature to 0 °C, so that substantially similar results occur on deep-freezing spermatozoa, whether or not precautions have been taken to cool the semen slowly (QUINN and WHITE 1966). Other than in bull semen where capacitation-like changes do not seem to depend on the cooling rates used (JANUSKAUSKAS et al. 1999) and cat semen (PUKAZHENTHI et al. 1999), the different percentage in progressive motility at different cooling times might be related to the different effect pre-freeze cooling had on plasma membrane integrity, because as plasma membrane damage directly affects ion permeability, motility mechanisms may be hampered. As lipid phase transition is taking place (QUINN 1985), gross membrane damage to spermatozoa can occur (MAXWELL and JOHNSON 1997a, b), lipid molecules aggregate (AMANN 1999), the bilayer is reorganized and might trap proteins (LADHA 1998), disrupting intracellular Ca^{2+} regulation and thus decrease motility (ROBERTSON et al. 1990). Less damage during the cooling period can pave the way for less damage during freezing-thawing. As with slow cooling the plasma membrane has enough time to undergo phase separation, it eliminates possible cold shock damage during cooling (HOLT and NORTH 1991) and less damage might occur during freezing-hawing.

Phase separation is an essential part of capacitation, described by a series of positive destabilizing events that eventually lead to cell death. The high amount of reacted acrosomes in frozen-thawed semen might lead to the conclusion that those spermatozoa with reacted acrosomes might have been capacitated before during the cooling process because it is very common to take the acrosome reaction as an indicator of the completion of capacitation (YANAGIMACHI 1988). Cooling decreased the proportion of viable, uncapacitated spermatozoa at rewarming and accelerated the time course of in vitro capacitation, indicating that capacitation-like changes are initiated by preservation (ROTA 1998). The events occurring in spermatozoa during capacitation with true acrosome reaction forming pointy fusions of the outer acrosomal membrane and overlying plasma membrane are similar to those happening during the cryopreservation process. The relatively unstable outer acrosomal membrane and the overlying plasma membrane may either be destroyed or become detached through the influence of temperature effects during the cryopreservation process. This is called premature or false acrosome reaction of moribund or dead spermatozoa autodigesting the acrosome with following intracellular ions leaking out and external ions penetrating freely into the cells. Especially cooling rate can make spermatozoa moribund with following false acrosome reaction (YANAGIMACHI 1988). Slow cooling may prevent the occurrence of a high number of moribund spermatozoa providing a higher percentage of intact plasma membrane and thus preventing premature acrosome reaction to occur in a higher rate as seen in the wolf species.

The poor post-thaw quality of wolf semen samples cooled for 1.0 h may be due to the relatively high temperature of 7-9 °C (instead of the conventional 5 °C) with which the semen entered the freezing period followed by a fast drop of temperature with a damaging effect on the plasma membrane, similar to the study by YUBI et al. (1987). Dog semen was cooled to 7 °C and frozen immediately on dry ice, resulting in a 50 % drop in motility and 45 % dead sperm, similar to those results found in generic gray wolf.

Reviewing the results for domestic dog semen it can be stated that domestic dog semen of this study had cold shock resistance showing best results when cooled for 0.5 h to 5 °C, confirming WALES and WHITE (1959), DARIN-BENNETT et al. (1974) and HAY et al. (1997b) stating that dog and cat sperm show a higher cold-shock tolerance compared to spermatozoa of other mammalian species, suggesting a specific trait of the carnivores. Semen

of generic gray and Mexican wolf show different behavior when confronted with cold temperature compared to domestic dog semen. Wolf semen cooled for 0.5 h resulted in significantly lower plasma membrane and acrosome integrity post-thaw, without having shown obvious significant differences directly after the cooling period. It can be suggested that no direct cold shock damage was detected after cooling, but indirect damage caused extreme and irreversible membrane change implying structural alteration during freezing rather than simply low temperature inhibition of metabolic processes (QUINN and WHITE 1966).

For post-thaw use generic gray and Mexican wolf semen have to be cooled slowly. These results suggest similar plasma membrane structure of both wolf species, but different behavior of domestic dog membranes, presuming that there is a difference in plasma membrane structure between wolf and dog. Together with the fatty acid profile, cholesterol and the cholesterol : phospholipid ratio are among the major factors which influence membrane fluidity (QUINN 1985; HOLT and NORTH 1986) and transition temperature. Related data of phase transition temperature of canid semen and lipid composition for semen of wolf species has not yet been studied but the different 'cooling behavior' of semen collected from canid species may be explained better when those facts are known. As 0.5 °C/min was the fastest cooling rate used in diluted dog and wolf semen a comparison to a faster cooling rate that might reveal worse effects of cold shock on spermatozoa was not possible. Although the changes in acrosome configuration may not be considered 'damage' at the cooling stage, the changes appear to render these spermatozoa susceptible to extensive damage during freezing and thawing.

5.5 Influence of Equex pasta on frozen-thawed generic gray and Mexican wolf semen

In the present studies semen samples of generic gray and Mexican wolf, diluted without Equex pasta showed significantly better post-thaw results than samples supplemented with 1 % Equex pasta. In both species semen diluted without Equex pasta showed better plasma membrane integrity when tested with the hypoosmotic swelling test; generic gray wolf semen had significantly less spermatozoa with partly damaged plasma membrane and Mexican wolf semen had significantly higher percentage of intact plasma membrane. Motility in generic

gray wolf was slightly higher than in samples diluted with Equex pasta. In Mexican wolf, samples cooled for 2.5h have the best results.

The effects of commercial detergent, sodium dodecyl sulphate (SDS, Equex STM paste) on canine semen frozen in straws have been investigated by a number of authors (THOMAS et al. 1992; ROTA et al. 1997; PEÑA et al. 1998c; ROTA et al. 1999a; PEÑA and LINDE-FORSBERG 2000b; STRÖM HOLST et al. 2000; STRÖM HOLST et al. 2001). The addition of SDS was found to significantly improve the longevity of post-thaw canine sperm motility at 37 °C compared with samples frozen in the same extender without Equex paste (Nova Chemical Sales, Scituate, Ma, USA; NÖTHLING et al. 1995, 0.5 ml / 100.5 ml extender; ROTA et al. 1997, 0.5 %). Beneficial results included a significantly increased proportion of spermatozoa with intact plasma membrane, increased sperm longevity, prolonged maintenance of motility and plasma membrane integrity (ROTA et al. 1997), higher percentage of motile spermatozoa (ROTA et al. 1999a), improved post-thaw survival and thermoresistance of spermatozoa (PEÑA and LINDE-FORSBERG 2000b) and increased binding capacity to the zona-pellucida. These results cannot be confirmed by the present study where Equex pasta was used. The acrosome protective effect by Orvus Es paste (TSUTSUI et al. 2000a, b, c) cannot be confirmed either. BATEMAN (2001) was the first to use supplementation of 1 % Equex STM paste in canine semen frozen in pellets, and found neither an obvious benefit immediately post-thaw nor any detrimental effects on sperm motility, morphology, acrosomal status and ZBA after 3 h incubation at 37 °C.

The beneficial effect of SDS in Equex STM paste is not fully understood. First the active compound of Equex STM paste, sodium dodecyl sulfate, was thought to act through alteration of the egg yolk by solubilization of protecting egg yolk-lipids increasing the egg yolk's protection potential against cold shock and freezing injury (PURSEL et al. 1978, ARRIOLA and FOOTE 1987; PENFOLD and MOORE 1993). Sodium dodecyl sulphate, seems to modify membrane fluidity and thus minimizes the occurrence of capacitation-like changes initiated during cryopreservation in ram semen (WATSON 1995). The protecting effect against lipid phase transitions was confirmed for dog spermatozoa (PEÑA and LINDE-FORSBERG 2000b) including partial protection of functionality of the plasma membrane Ca^{2+}-pumps. The pumps are damaged but still can hold the Ca^{2+}-concentration under a certain threshold, which usually triggers the false acrosome reaction and cell death.

The reason for not detecting the same beneficial effect described by the authors mentioned above, who used 0.5% Equex STM paste (Nova Chemical Sales, Scituate Inc., MA, USA) as a source of sodium dodecyl sulphate might be that the effect of Equex STM paste and Equex pasta in the end is different. PEÑA et al. (2003a, b) showed significantly better post-thaw sperm survival, longevity and quality of sperm movement evaluated by CASA when semen was supplemented with Equex STM paste. The percentage of live spermatozoa with intact acrosomes was not significantly different between Equex pasta and control samples (PEÑA et al. 2003b) confirming in a sense the non-beneficial effect shown in the present study. This may have been due to several reasons. One might be that Equex pasta was not added to the extender in the same concentration, and not at the same stage during the preservation process than in previous studies. Concentration of Equex pasta recommended by the manufacturer for supplementing equine semen is 0.5-1 % at final concentration. The final concentration of 0.5 % Equex pasta was found not to be beneficial (PEÑA et al. 2003a, b) and the final concentration of 1% as tested in the present study did not have any positive effect. Additionally, the possibility of different compositions of both Equex preparations (i.e. the presence of other substances in addition to SDS) cannot be disregarded, as the recipes of the commercially available products are not known. Another explanation may be that wolf sperm plasma membranes react differently to sodium dodecyl sulphate than the membrane of dog sperm. A prolonged exposure of spermatozoa to SDS or to SDS-treated egg yolk lipoprotein (1.0 h equilibration to 4 °C, PEÑA and LINDE-FORSBERG 2000b) may also exert a direct negative effect on the sperm membranes by conferring on them an excessive fluidity. That is why the protective effect of SDS was found to be more pronounced if spermatozoa were exposed to the detergent immediately before freezing than after exposure to SDS throughout the equilibration period (PURSEL et al. 1978, boar; PEÑA and LINDE-FORSBERG 2000b, dog). In the present study Equex pasta was added immediately before freezing, however not as a dilution in extender but added directly into the cooled semen suspension which could cause immediate damage of the cells because of the osmotic capability being a water-soluble anionic detergent. Samples cooled for 2.5 h had the best results when supplemented with Equex in contrast to PURSEL et al. (1978) where the beneficial effect on sperm motility was independent of the cooling rate.

5.6 Influence of cooling times and rates, and Equex pasta on sperm motility in frozen-thawed Mexican wolf semen evaluated by CASA

This is the first study to measure motility parameters in Mexican wolf semen by computer assisted analysis, investigating the effect of different cooling rates and times and the effect of Equex pasta supplementation. Semen samples cooled for 2.5 h showed significantly lower velocity values, straightness, linearity and beat cross frequency right after thawing but increasing the values 3-4 h after thawing. Semen samples not diluted with Equex pasta showed better results during the first hours after thawing until semen samples diluted with Equex pasta show better results after 4 h (VAP), 6 h (VSL), 3 h (VCL), 8 h (LIN) and 3 h (BCF). Independent of the setting, results obtained for frozen-thawed Mexican wolf semen using Hamilton Thorne semen analyser for sperm velocities, were very similar to those found in dog semen by either of the computer systems (Mika Strömberg, GÜNZEL-APEL et al. 1993; Hamilton Thorne, ROTA et al. 2001). The obtained results in the present study cannot be compared to the results obtained by IGUER-OUADA and VERSTEGEN (2001a, Sperm Quality Analyzer) because they used a system of sperm motility index (SMI) to evaluate semen. RIGAU et al. (2001, Sperm Class Analyzer) used different parameters (dance, mean dance, harmonic oscillation) compared to the ones in the present study and results cannot be compared either. ELLINGTON et al. (1993, HT); IGUER-OUADA and VERSTEGEN (2001b, HT); and RJISSELAERE et al. (2003, HT) having similar settings of the CASA, evaluated fresh semen only and found higher velocities, straightness, linearity and lower lateral head displacement and beat cross frequency compared to the present study. Parameters of frozen-thawed dog semen showed higher VAP and LIN (GÜNZEL-APEL et al. 1993) than evaluated Mexican wolf semen. Compared to results by ROTA et al. (2001, HTM) Mexican wolf parameters showed the same range with higher maximum of VAP and VSL, higher ranges of VCL, ALH and BCF, the same range with lower minimum of STR and a lower range of LIN. The high sperm velocities VAP and VCL indicate very active but not necessarily progressively motile spermatozoa, while high BCF values indicate a very strong flagellar action. There might be a species specific difference with wolf spermatozoa having a high velocity but with a less linear path and with head movements presented by BCF more sweeping than in dog (ROTA et al. 2001), BCF being one of the more sensitive parameters in

motility evaluation of spermatozoa (DAVIS and KATZ 1993). Linearity was lower as compared with ROTA et al. (2001), indicating that post-thawed wolf spermatozoa are not moving in a straight line but with deviations. IGUER-OUADA and VERSTEGEN (2001b) mentioned VAP and STR as indicative parameters for progressive motility and ALH and BCF as indicative for the ability to penetrate the zona pellucida at fertilization. With the present results Mexican wolf semen might be able to fulfil those requirements as active, fast cells with extense flagellar movement. An interesting movement for the interpretation of semen motility is hyperactivated spermatozoa. Hyperactivation is a very vigorous movement spermatozoa of many species exhibit as they progress through the female oviduct, before they initiate the acrosome reaction and is associated with zona-free egg penetration and the penetration of zona-intact eggs in several species (BOATMAN and BAVISTER 1984). Capacitation might bring about some changes in the sperm plasma membrane such that energy becomes more accessible to the sperm's motor apparatus. The hyperactivated motility obviously provides the spermatozoa with strong thrusting power. The tail plasma membrane of hyperactivated mouse spermatozoa has a higher 'fluidity' than that of pre-hyperactivated spermatozoa. This indicates that there are alterations of the physical and chemical characteristics of the tail membrane lipids during capacitation of spermatozoa (YANAGIMACHI 1988). ELLINGTON et al. (1993) suggested that as the ability to capacitate and undergo the hyperactive motion pattern is decreased in infertile human males, the identification of hyperactive sperm cells by CASA could serve as an investigative technique for identifying subfertile males. In contrast, MAK et al. (1994) described hyperactivation as a complex phenomenon, which cannot easily be used in prediction of fertilization. The hyperactivated spermatozoa can be visualized as a large amplitude of lateral head movement and a non-progressive trajectory (ZHU et al. 1994) or in subgroups exhibiting circling, thrashing, starspin and helical motility patterns (BURKMAN 1991) that are divided in progressive and non-progressive spermatozoa and depth-dependant (LE LANNOU et al. 1992). Non-progressive ('dancing') movement is described as whiplash-like beatings of the tail, with the sperm head tracing an erratic figure 8, intermitted by a brief linear ('dashing') movement (YANAGIMACHI 1988). The distinguishing characteristics of hyperactivated spermatozoa are VCL > 70 µm/s, ALH > 7 µm, LIN < 30 % and VSL < 30 µm/s (VERSTEGEN et al. 2002). Comparing the results right after thawing of Mexican gray wolf

semen, the spermatozoa belong to the progressive circular pattern. Especially a decrease in VSL, LIN (QUINTERO-MORENO et al. 2003) and STR, indicating a less progressive, less directional path and an increase in ALH and BCF (PEEDICAYIL et al. 1997) is shown. ALH is also considered to be an important parameter affecting the outcome of IVF as it indicates the vigor of flagellar beating together with the frequency of cell rotation, which are important for the progression of spermatozoa into the cervical mucus (VERSTEGEN et al. 2002). In the presented study the evaluated spermatozoa do not show hyperactivation, defined as a combination of the mentioned parameters, evaluated right after thawing. VCL always is > 70 µm/s and ALH always is > 7 µm. Nevertheless, LIN starts to decrease to a value < 30% at 6 h post-thaw and VSL < 30µm/s at 10h post-thaw. Samples cooled faster (0.5h) or those which entered the freezing period with a temperature at 7-9 °C (1.0 h) show signs of hyperactivation earlier, indicating an effect of a possible plasma membrane damage by fast pre-freeze cooling with consecutive hyperactivation and loss of progressive motility post-thaw. This indicates that those samples might have more severe plasma membrane damages and are transferred into the capacitation status earlier than samples cooled in 2.5 h, assuming that motility is a direct consequence of the functional status of spermatozoa (FORD and REES 1990).

In samples supplemented without Equex, parameters showed better values during the first hours of the post-thaw period. In contrast, all velocity parameters were significantly higher when 0.5 % Equex (not specified product, [sodium triethanolamine lauryl sulfate]) was present indicating that Equex had a beneficial effect in maintaining the physiological integrity of the flagellum (ABAIGAR et al. 1999, gazelle) what cannot be confirmed for the first hours post-thaw in the present study. After several hours during the post-thaw longevity test, however, samples diluted with Equex pasta showed better values of evaluated parameters. Despite the bad post-thaw results for motility, plasma membrane and acrosome integrity, Equex pasta might have a certain beneficial effect that only gets apparent during a longer post-thaw period. This should be confirmed with a thermoresistance test in conjunction with evaluation of plasma membrane and acrosome integrity during the post-thaw period.

6 Summary

Claudia Zindl:

Cryopreservation of Mexican gray wolf (Canis lupus baileyi) semen - evaluation of different times and rates of pre-freeze cooling and Equex pasta® supplementation – in comparison with semen of the domestic dog and generic gray wolf (Canis lupus)

Recovery of the Mexican gray wolf (*Canis lupus baileyi*), a subspecies of the gray wolf (*Canis lupus*), depends on careful genetic management of the captive population. Because of their monogamous mating system, transfer of gametes using cryopreservation and artificial insemination is preferable to breaking pair-bonds and transfer of animals. As there are only few Mexican wolves available for evaluating the requisite technology, such as sperm cryopreservation, it has been necessary to use the generic gray wolf as a model and to base the techniques on those developed for the domestic dog, the wolf's closest relative.

The biggest loss of viable spermatozoa is reported to occur during the actual freezing and thawing, with minor changes during cooling and spermatozoa of several species require a rest of several hours during cooling, before freezing, to develop maximal resistance to the effects of freezing.

The aim of the present study was to investigate the effects of various cooling rates and times in domestic dog (0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h) and generic and Mexican gray wolf (0.5 h, 1.0 h, 2.5 h) semen, including investigation of the effect of 1% Equex pasta on wolf semen quality. Semen collected by manual stimulation (dog) or electroejaculation (wolf) was cooled in different times and rates from room temperature to 5°C, in a refrigerator measuring the temperature decline with temperature sensors. 1% Equex pasta was added to the wolf semen before the freezing process, pelleted on dry ice and stored in liquid nitrogen. The influence of treatements was investigated by evaluation of motility, plasma membrane integrity and morphology by conventional spermatological methods in fresh, cooled and frozen-thawed semen and additional tools of semen evaluation such as computer-assisted analysis and electronmicroscopic analysis.

In this study it is confirmed that pre-freeze cooling of dog and wolf semen cause both, loss of plasma membrane integrity and acrosomal damage, with further deterioration after freezing-

thawing. In case of dog semen it is suggested to use a short cooling time (0.5 h) with a fast cooling rate (0.5 °C/min); for cryopreservation of generic and Mexican gray wolf semen, data from the present study support using a longer cooling time and a slow cooling rate of 0.08-0.1°C/min to reach refrigerator temperature (i.e. 5 °C). The addition of 1 % Equex pasta is not recommended. It would be of interest to investigate the effect of 0.5 % Equex STM paste or a different final concentration of Equex pasta and of different equilibration periods of the cooled semen samples to improve the wolf semen cryopreservation protocol

7 Zusammenfassung

Claudia Zindl:

Tiefgefrierkonservierung des Spermas des mexikanischen Wolfes (Canis lupus baileyi)- Untersuchung unterschiedlicher Kühlzeiten und –raten vor dem Einfrieren und Equex pasta® Zugabe – im Vergleich zu Sperma des Hundes und des nordamerikanischen Wolfes (Canis lupus)

Der Schutz vor dem Aussterben des mexikanischen Wolfes (*Canis lupus baileyi*), einer Unterart des nordamerikanischen Wolfes (*Canis lupus*) hängt von sorgfältigem genetischen Management der Population in Gefangenschaft ab. Augrund ihres monogamen Paarungssystems, ist die Übertragung von Keimzellen mittels Tiefgefrierkonservierung und künstlicher Besamung der Trennung von Wolfspaaren und dem Tiertransport vorzuziehen. Da nur noch wenige mexikanische Wölfe der erforderlichen Technologie, wie z.B. Spermatiefgefrierkonservierung, zur Verfügung stehen, wurde es nötig, den nordamerikanischen Wolf als Modell zu nutzen und sich auf diejenigen Techniken zu stützen, die für den Hund, den nächsten Verwandten des Wolfes, entwickelt wurden.

Der größte Verlust an lebensfähigen Spermien entsteht während des eigentlichen Tiefgefrier- und Auftauprozesses, mit minimalen Veränderungen während der Kühlung. Einige Spezies benötigen eine Ruheperiode von mehreren Stunden während der Kühlung, vor dem Tiefgefrierprozess, um eine maximale Widerstandskraft gegen die Auswirkungen der Tiefgefrierkonservierung auszubilden.

Das Ziel der vorliegenden Studie ist es, die Auswirkungen unterschiedlicher Kühlzeiten und –raten auf Spermien des Hundes (0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h) und der nordamerikanischen und mexikanischen Wölfe (0.5h, 1h, 2.5h) zu untersuchen, einschließlich der Auswirkung von 1% Equex pasta auf die Qualität des Wolfspermas. Durch manuelle Stimulation (Hund) und Elektroejakulation (Wolf) gewonnenes Sperma wurde in einem Kühlschrank mit unterschiedlichen Kühlzeiten und -raten von Raumtemperatur auf 5 °C gekühlt, währenddessen der Temperaturabfall mit Sensoren gemessen wurde. Vor dem Tiefgefrierprozess wurde der gekühlten Sperma-Verdünner-Suspension der Wölfe 1 % Equex pasta zugegeben, auf Trockeneis pelletiert und in flüssigem Stickstoff gelagert. Der Einfluss

der Behandlungen wurde mit Hilfe konventioneller spermatologischer Methoden und zusätzlichen Instrumenten wie computergestützter Analyse und Elektronenmikroskopie in frischem, gekühltem und aufgetautem Sperma auf Motilitat, Plasmamembranintegrität und Morphologie untersucht.

In dieser Studie wurde bestätigt, dass das Kühlen von Hunde- und Wolfsspermien vor dem Tiefgefrierprozess sowohl Verlust der Plasmamembranintegrität als auch Schaden der Akrosomenintegrität verursacht, mit einer weiteren Verschlechterung durch de Tiefgefrier- und Auftauprozess. Für die Tiefgefrierkonservierung von pelletierten Hundespermien empfiehlt sich eine kurze Kühlzeit (0.5 h) mit einer schnellen Kühlrate (0.5 °C/min); für die Tiefgefrierkonservierung von Wolfsspermien, unterstützen die Daten dieser Studie die Anwendung einer längeren Kühlzeit (2.5 h) und eine langsame Kühlrate von 0.08-0.1°C/min, bis zum Erreichen der Kühlschranktemperatur (z.B. 5 °C) vor dem Tiefgefrieren. Der Zusatz von 1 % Equex pasta wird nicht empfohlen. Es wäre interessant, die Auswirkung des Zusatzes von 0.5 % Equex STM paste oder von Equex pasta in einer anderen Endkonzentration zu untersuchen, auch unterschiedlich lange Äquilibrierungsperioden der gekühlten Spermaprobe sollten getestet werden, zur Verbesserung des Tiefgefrierprotokolls für Wolfssperma.

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9 Appendix

9.1 Recipes for media

9.1.1 TRIS-egg yolk extender (OLAR et al. (1989) modified)

TRIS buffer:

9.008 g dextrose (glucose)

24.228 g TRIS (Tris[hydroxymethyl]aminomethane)

11.478 g citric acid (anhydrous)

ad 760 ml milli-Q-water

pH 7.45; osmolarity: 329 mOsM

TRIS-egg yolk extender (4% glycerol):

760 ml TRIS base

100 ml Penstrep (10 000 IU/ml Penicillin and 10 000 µg/ml Streptomycine) and

40 ml glycerol

200 ml egg-yolk (commercially available chicken eggs) were added

The extender was prepared in advance, filled in 15 ml vials and kept frozen at –79 °C until use. Equal volumes of the resuspended semen were distributed into three different 15 ml plastic vials.

9.1.2 Hypoosmotic swelling test solution (KEEL and WEBSTER (1990) modified and KUMI-DIAKA (1993), KUMI-DIAKA and BADTRAM (1994))

2.7 g fructose

ad 100 ml milli-Q-water

osmolarity: 60 mOsM

aliquots of 1 ml were pipetted into capped transparent 1.5 ml Eppendorf microtubes and stored at –20°C

9.1.3 Fluorescent stain

[HARRISON and VICKERS (1990); LINDE-FORSBERG*]

Saline medium

4.081 g sodium chloride

0.901 g glucose ($C_6H_{12}O_6$)

0.070 g potassium hydroxide

0.250 g polyvinylpyrrolidon

2.383 g HEPES

ad 500 ml milli-Q-water

pH 7.4 (30°C)

aliquots of 1 ml were pipetted into capped transparent 1.5 ml Eppendorf microtubes and kept frozen at –20°C

Formalin solution

68 ml formaldehyde (37%)

ad 100ml distilled water

aliquots of 1 ml were pipetted into capped transparent 1.5 ml Eppendorf microtubes and stored at –20°C

Propidium iodide solution

0.5 mg propidium iodide

ad 1 ml isotonic sodium chloride

aliquots of 20 µl were pipetted into capped amber 1.5 ml Eppendorf microtubes and stored at –20°C

*personal communication by e-mail with C. Linde-Forsberg, Uppsala, 6th December 2000

6-Carboxyfluoresceindiacetate solution

0.46 mg 6-carboxyfluoresceindiacetate

ad 1 ml isotonic sodium chloride

aliquots of 20 µl were pipetted into capped amber 1.5 ml Eppendorf microtubes and stored at –20°C

9.1.4 Solutions for electron microscopy (*)

Glutaraldehyde fixative solution

2.5 % glutaraldehyde

ad 0.1 M sodium cacodylate buffer (2% sucrose, 2 mM calcium chloride)

pH 7.25

Washing buffer

0.1 M sodium cacodylate buffer (5% sucrose)

pH 7.25

Post-fixation buffer

1 % osmium tetroxide

ad 0.1M sodium cacodylate buffer (2 % sucrose)

pH 7.25

All chemicals used, were purchased at Sigma Chemical Company, St. Louis, Missouri, USA. Penstrep was purchased form Life Technologies, Rockville, Maryland, USA.

* personal communication with Dr. Ryerse in St. Louis, Missouri, USA, 29th February 2001

9.2 Defined set up of the Hamilton Thorn IVOS-10

	value	**unit**
frames acquired	30	
frame rate	60	Hz
min contrast	75	
min cell size	4	pixels
min static contrast	15	
STR threshold	75	%
slow cells	motile	
low VAP cutoff	9.9	µm/s
medium VAP cutoff	50.0	µm/s
low VSL cutoff	15.0	µm/s
head size, non-motile	5	pixels
head intensity, non-motile	70	
min size gate	0.44	
max size gate	4.98	
min size intensity	49	
max size intensity	168	
min size elongation	17	
max size elongation	96	
temperature set	37.0	°C

9.3 Tables

Cooling times and temperatures

Table 9.3.1: Temperature (°C) and differences between the temperature before cooling and 4.99 °C of water baths (250 ml, 500 ml, 1000 ml) in glass beakers and extender (1 – 8 ml) immersed in those water baths or without water bath (no wb) before cooling and the time difference needed to cool to 4.99 °C

wb volume (ml)	initial temperature (°C)				temperature difference (°C)				time difference (min)			
	water bath		extender		water bath		extender		water bath		extender	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
500 ice (1.5 h)	20.5 (m=5)	0.6	20.3 (m=5)	0.6	15.9 (m=5)	0.6	15.3 (m=5)	0.6	66.4[b] (m=5)	5.3	90.2[b] (m=5)	8.5
250 (2.5 h)	20.2 (m=11)	0.1	20.6 (m=11)	0.2	15.2 (m=11)	0.1	15.6 (m=11)	0.2	160.0 (m=11)	4.2	165.2 (m=11)	4.2
500 (3.5 h)	21.0 (m=22)	0.2	21.5 (m=16)	0.2	16.0 (m=22)	0.2	16.6 (m=16)	0.2	194.9[a] (m=22)	3.8	207.3[a] (m=22)	4.0
1000 (4.5 h)	20.3 (m=14)	0.6	20.0 (m=15)	0.5	15.3 (m=14)	0.6	15.0 (m=15)	0.5	240.2 (m=14)	5.7	250.3 (m=14)	5.7
no wb (0.5 h)			22.9 (m=41)	0.3			18.1 (m=41)	0.3			33.5 (m=41)	3.1

Significant differences

Single-factor ANOVA:

Time difference (min),

extender in 250 ml water bath / 250 ml water bath — a: $P < 0.05$

extender in 500 ml water bath in ice water / 500 ml water bath in ice water — b: $P < 0.05$

Ejaculate characteristics

Table 9.3.2: Ejaculate characteristics of domestic dog, generic and Mexican gray wolf (n = number of individuals, m = number of semen samples [dog: sperm rich fraction]

	domestic dog (n = 4; m = 9)		generic gray wolf (n = 7; m = 13)		Mexican wolf (n = 4; m = 32)	
	mean	SEM	mean	SEM	mean	SEM
	range		range		range	
volume (ml)	2.1[a]	0.5	7.0[a]	1.0	4.2[a]	0.5
	0.2 – 5.0		2.0 – 13.0		0.5 – 11.6	
sperm conc (10^6 / ml)	402.2[b]	110.4	271.1[b]	59.4	181.4[b]	26.1
	92.0 – 1188.0		34.0 – 660.0		26.0 – 680.0	
total sperm number (10^6)	720.0	204.0	1597.4	390.0	756.2	153.9
	97.6 – 1782.0		442.0 – 5280.0		65.0 – 4590.0	

pH	6.6	0.2	6.6	0.1	6.8	0.1
	6.0 – 7.3		6.2 – 7.0		6.3 – 7.2	
Osmolarity (mOsM)	363.2	2.5	342.9	9.9	326.3	2.8
	294.5 – 315.0		312.0 – 437.0		302.0 – 365.5	

Motile spermatozoa (%)	76.4[c]	3.8	88.5[c]	0.8	77.6[c]	2.0
	60.0 – 90.0		80.0 – 90.0		45.0 – 90.0	

Significant differences

Single-factor ANOVA:

volume	generic gray wolf / Mexican wolf / domestic dog	a: $P < 0.01$
sperm concentration	generic gray wolf / Mexican wolf / domestic dog	b: $P < 0.05$
motile spermatozoa	generic gray wolf / Mexican wolf / domestic dog	c: $P < 0.01$

Table 9.3.3: Intact plasma membranes (%, Eosin-Nigrosin, HOST) of spermatozoa in cooled semen of domestic dog, generic gray and Mexican wolf after exposure to different cooling times (0.5 h, 0.5°C/min; 1.0 h, 0.2°C/min; 2.5 h, 0.1°C/min) compared with fresh semen (before cooling)

species	cooling time (h)	Eosin-Nigrosin (%)		HOST (%)	
		mean	SEM	mean	SEM
domestic dog (n=4)	before cooling	94.8[a]	2.5	91.8	1.2
	0.5 (m=9)	90.8	2.7	70.3	9.2
	1.5 (m=8)	91.5	2.5	73.5	11.3
	2.5 (m=8)	91.8	3.0	71.8	10.7
	3.5 (m=8)	91.5	3.1	69.9	10.4
	4.5 (m=8)	91.5	3.1	72.9	10.7
generic gray wolf (n=7)	before cooling	98.8[a]	0.4	87.0	1.3
	0.5 (m=7)	99.9	0.1	62.6	6.2
	1.0 (m=7)	99.9	0.1	68.6	2.6
	2.5 (m=7)	100.0	0.0	57.3	6.2
Mexican wolf (n=4)	before cooling	96.0	0.9	89.1	1.3
	0.5 (m=24)	97.8	0.7	67.2	3.1
	1.0 (m=25)	97.8	0.7	73.0	3.2
	2.5 (m=25)	97.9	0.6	65.1	3.2

Significant differences

Single-factor ANOVA:

Eosin-Nigrosin generic gray wolf /domestic dog a: $P < 0.05$

Semen parameters in fresh, cooled and frozen-thawed semen

Table 9.3.4: Spermatozoa with intact plasma membrane (Eosin/Nigrosin, HOST, 6-CFDA/PI; %) and normal morphology (%) in fresh, cooled and frozen-thawed (fr-thaw) semen of domestic dog, generic gray and Mexican wolf semen

		intact plasma membrane (%)						normal morphology (%)	
		6-CFDA/PI		HOST		Eosin-Nigrosin			
		mean	SEM	mean	SEM	mean	SEM	mean	SEM
domestic dog (n=4)	fresh (m=9)	0.4	0.2	91.8^{k}	1.2	94.8^{a,m}	2.5	59.9	9.4
	cooled (m=41)	0.4	0.1	71.7 ^{c,d,l}	4.4	91.4^{e,n}	1.2	68.4^{o}	3.1
	fr-thaw (m=40)	48.9^{f}	3.2	67.4^{g,k,l}	1.7	82.7^{i,m,n}	1.7	59.7^{j,o}	3.3
generic gray wolf (n=7)	fresh (m=12)	0.2	0.1	87.0^{q,r}	1.3	98.8^{a,t}	0.4	77.0	3.4
	cooled (m=21)	0.1^{b}	0.1	62.3^{c,q,s}	1.8	99.8^{e,u}	0.1	72.9	2.4
	fr-thaw (m=42)	27.9^{f}	2.1	40.7^{g,h,r,s}	2.0	51.5^{i,t,u}	2.3	67.1^{j}	2.2
Mexican wolf (n=4)	fresh (m=27)	0.39	0.1	89.1^{v,w}	1.3	96.0^{x}	0.9	73.9	4.7
	cooled (m=77)	1.0^{b}	0.2	68.5^{d,v}	1.8	97.8^{e,y}	0.4	69.5	2.5
	fr-thaw (m=143)	53.7^{f}	1.5	57.3^{g,h,w}	1.3	79.0^{i,x,y}	1.3	71.9^{j}	0.9

Significant differences

Single-factor ANOVA:

Species comparison

Fresh semen samples

Eosin-Nigrosin	generic gray wolf / domestic dog	a: P < 0.05

Cooled semen samples

6-CFDA/PI	Mexican wolf / generic gray wolf	b: P < 0.05
HOST	domestic dog / generic gray wolf	c: P < 0.01
HOST	domestic dog / Mexican wolf	d: P < 0.01

Eosin-Nigrosin	generic gray wolf & Mexican wolf / domestic dog	e: $P < 0.01$

Frozen-thawed semen samples

6-CFDA/PI	Mexican wolf & domestic dog / generic gray wolf	f: $P < 0.01$
HOST	domestic dog / Mexican wolf & generic gray wolf	g: $P < 0.01$
HOST	Mexican wolf / generic gray wolf	h: $P < 0.01$
Eosin-Nigrosin	domestic dog & Mexican wolf / generic gray wolf	i: $P < 0.01$
normal morphology	Mexican wolf / generic gray wolf & domestic dog	j: $P < 0.01$

Cryopreservation period comparison

Domestic dog

HOST	fresh / frozen-thawed	k: $P < 0.01$
	cooled / frozen-thawed	l: $P < 0.01$
Eosin-Nigrosin	fresh / frozen-thawed	m: $P < 0.01$
	cooled / frozen-thawed	n: $P < 0.01$
normal morphology	cooled / frozen-thawed	o: $P < 0.01$

Generic gray wolf

HOST	fresh / cooled	q: $P < 0.01$
	fresh / frozen-thawed	r: $P < 0.01$
	cooled / frozen-thawed	s: $P < 0.01$
Eosin-Nigrosin	fresh / frozen-thawed	t: $P < 0.01$
	cooled / frozen-thawed	u: $P < 0.01$

Mexican wolf

HOST	fresh / cooled	v: $P < 0.01$
	fresh / frozen-thawed	w: $P < 0.01$
Eosin-Nigrosin	fresh / frozen-thawed	x: $P < 0.01$
	cooled / frozen-thawed	y: $P < 0.01$

Table 9.3.5: Percentage of forward motility in fresh and frozen-thawed (immediately after thawing) domestic dog , generic gray and Mexican wolf semen cooled for different times (dog: 0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h; wolves: 0.5 h, 1.0 h, 2.5 h) without (0 %) and with (1 %) Equex pasta

species	cooling time (h)	0 % Equex pasta		1 % Equex pasta	
		mean	SEM	mean	SEM
domestic dog (n=4)	fresh	76.4	3.8	--	--
generic gray wolf (n=7)	fresh	88.9	2.9	--	--
	0.5 (m=12)	38.3[a](m=6)	7.0	28.3[d]	10.5
	1.0 (m=12)	31.7[b](m=6)	9.5	25.0[e]	8.9
	2.5 (m=12)	35.0[c] (m=6)	13.1	37.5[f]	10.6
Mexican wolf (n=4)	fresh	79.4	8.8	--	--
	0.5 (m=49)	64.4[abc] (m=26)	3.9	67.0 [def] (m=23)	5.7
	1.0 (m=49)	64.9 [abc] (m=27)	3.7	64.8 [def] (m=22)	5.0
	2.5 (m=45)	62.8 [abc] (m=24)	3.6	68.8 [def] (m=21)	3.3

Significant differences

Single-factor ANOVA:

Samples diluted without Equex pasta (0 %)

forward motility: generic gray wolf 0.5 h / Mexican wolf 0.5 h, 1.0 h and 2.5 h a: $P < 0.01$

forward motility: generic gray wolf 1.0 h / Mexican wolf 0.5 h, 1.0 h and 2.5 h b: $P < 0.01$

forward motility: generic gray wolf 2.5 h / Mexican wolf 0.5 h, 1.0 h and 2.5 h c: $P < 0.01$

Samples diluted with Equex pasta (1 %)

forward motility: generic gray wolf 0.5 h / Mexican wolf 0.5 h, 1.0 h and 2.5 h d: $P < 0.01$

forward motility: generic gray wolf 1.0 h / Mexican wolf 0.5 h, 1.0 h and 2.5 h e: $P < 0.01$

forward motility: generic gray wolf 2.5 h / Mexican wolf 0.5 h, 1.0 h and 2.5 h f: $P < 0.01$

Table 9.3.6: Percentage of forward motility in frozen-thawed domestic dog semen cooled for different times (0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h) evaluated for 7 hours at 37 °C (forward motility of fresh semen = 76.4 ± 3.8 %) (m= 8)

cooling time(h)	0.5		1.5		2.5		3.5		4.5	
hours after thawing	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
0	53.1	7.3	41.9	7.7	43.0	5.7	47.1	6.7	47.3	6.5
0.5	47.5	8.4	43.8	8.4	45.0	6.8	44.4	7.8	42.5	8.0
1	42.3	8.3	39.9	5.3	37.5	5.9	37.5	8.0	36.3	6.0
2	32.5	7.5	28.8	5.7	31.3	6.7	30.4	7.3	32.5	5.9
3	22.5	5.6	21.9	6.0	27.5	6.7	28.8	5.5	26.9	4.7
4	12.5	3.7	18.1	5.0	20.6	7.5	25.0	5.7	21.3	5.5
5	14.5	5.6	12.5	5.6	8.8	4.8	23.8	5.3	16.3	5.0
6	8.5	3.3	3.8	2.6	11.0	5.9	13.8	4.6	10.0	3.8
7	0.0	0.0	1.3	1.3	5.0	5.0	0.0	0.0	2.5	2.5

Table 9.3.7: Percentage of forward motility in frozen-thawed generic gray wolf semen cooled for different times (0.5 h, 1.0 h, 2.5h) evaluated for 7 hours after thawing (forward motility of fresh semen = 88.9 ± 2.9 %) (m= 12)

cooling time (h)	0.5		1.0		2.5	
hours after thawing	mean	SEM	mean	SEM	mean	SEM
0	33.3	6.2	28.3	6.3	36.3	8.1
0.5	32.5	7.1	30.0	5.8	38.3	7.7
1	30.8	7.5	23.2[a]	6.8	41.8[a]	7.5
2	30.8	7.5	12.9[b]	4.4	37.1[b]	7.7
3	16.3	4.7	8.3[c]	2.7	31.3[c]	7.6
4	6.7[d]	3.6	5.0[d]	1.9	20.8[d]	6.1
5	0.8	0.8	0.8	0.8	6.3	2.8
6	0	0	0	0	2.5	1.8
7	0	0	0	0	0.4	0.4

Significant differences

Single-factor ANOVA:

forward motility, 1 hours after thawing	2.5 h / 1.0 h	a: $P < 0.05$
forward motility, 2 hours after thawing	2.5 h / 1.0 h	b: $P < 0.05$
forward motility, 3 hours after thawing	2.5 h / 1.0 und 2.5 h / 0.5 h	c: $P < 0.05$
forward motility, 4 hours after thawing	2.5 h / 1.0 und 2.5 h / 0.5 h	d: $P < 0.05$

Table 9.3.8: Percentage of forward motility in frozen-thawed Mexican wolf semen cooled for different times (0.5 h, 1.0 h, 2.5 h) evaluated for 9 hours after thawing (forward motility of fresh semen = 77.6 ± 2.0 %)

cooling time (h)	0.5 (m=49)		1.0 (m=49)		2.5 (m=45)	
hours after thawing	mean	SEM	mean	SEM	mean	SEM
0	65.7	3.1	64.8	3.0	65.6	2.5
0.5	67.6	3.2	64.2	3.1	67.2	2.6
1	62.2	3.3	62.3	3.2	64.0	2.7
2	53.6	2.9	53.0	3.0	58.1	2.5
3	43.1	2.8	43.6	2.7	49.1	2.5
4	32.4	2.4	33.1	2.5	36.2	2.5
5	20.5	2.3	20.8	2.2	26.5	2.2
6	12.4	1.8	11.7	1.6	16.1	2.2
7	5.9	1.4	5.9	1.5	8.9	1.7
8	2.2	0.9	2.0	0.9	2.8	1.0
9	0.6	0.7	0.4	0.3	0.3	0.2

Table 9.3.9: Percentage of forward motility in frozen-thawed generic gray wolf semen diluted without (0 %) or with (1 %) Equex pasta evaluated for 9 hours after thawing (forward motility of fresh semen = 88.9 ± 2.9) (m=18)

hours after thawing	0 % Equex pasta (m=18)		1 % Equex pasta (m=18)	
	mean	SEM	mean	SEM
0	35.0	5.6	30.3	5.6
0.5	35.0	5.8	32.2	5.4
1	34.2	6.1	29.4	6.1
2	29.4[a]	6.2	19.7[a]	4.8
3	21.9[b]	5.3	15.3[b]	4.4
4	12.2[c]	3.9	21.9[c]	5.3
5	3.1	1.3	9.4	3.7
6	0.6	0.6	12.2	3.9
7	0.3	0.3	2.2	3.1
8	0	0	1.1	1.1
9	0	0	0	0

Significant differences

Single-factor ANOVA:

Equex pasta

Forward motility, 2 hours after thawing	0 % / 1 %	a: $P < 0.05$
Forward motility, 3 hours after thawing	0 % / 1 %	b: $P < 0.05$
Forward motility, 4 hours after thawing	0 % / 1 %	c: $P < 0.05$

Table 9.3.10: Percentage of forward motility in frozen-thawed Mexican wolf semen without (0 %) or with (1 %) Equex pasta evaluated for 9 hours after thawing (forward motility of fresh semen = 79.4 ± 8.8%)

hours after thawing	0 % Equex pasta (m=76)		1 % Equex pasta (m=67)	
	mean	SEM	mean	SEM
0	64.1	2.1	66.8	2.6
0.5	66.2	2.3	66.4	2.7
1	61.8	2.2	63.9	2.9
2	54.0	2.1	55.7	2.6
3	45.8	2.0	44.6	2.3
4	34.4	1.9	33.1	2.2
5	23.5	1.6	21.4	2.2
6	13.3	1.3	13.4	1.8
7	7.6	1.3	6.1	1.2
8	2.2	0.7	2.5	0.8
9	0.2	0.2	0.8	0.5

Computer assisted analysis of Mexican wolf semen

Table 9.3.11: Average path velocity (VAP, µm/s) of frozen-thawed Mexican gray wolf spermatozoa cooled for different times (0.5 h, 1.0 h, 2.5 h) without (0 %) or with (1 %) Equex pasta during a longevity test evaluated for 11 hours after thawing

hours after thawing	0 % Equex pasta						1 % Equex pasta					
	0.5		1.0		2.5		0.5		1.0		2.5	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
0	106.2[a]	1.0	103.3	1.0	101.3	1.0	102.2	1.1	104.7[j]	1.0	100.7	1.1
1	108.1	0.6	110.4[b]	0.7	105.9	0.7	105.5	0.6	106.2	0.6	102.6[k]	0.8
2	105.0[c]	0.8	107.6	0.6	107.8	0.7	103.0	0.9	105.1	0.7	105.9l	0.8
3	103.2	0.8	101.4	0.8	103.7	0.6	102.0	0.8	103.6	0.7	102.6	0.8
4	95.2	0.6	97.0	0.6	92.4[d]	0.7	96.4	0.7	95.4	0.7	97.6	0.6
5	88.8	0.6	86.9[e]	0.6	88.7	0.5	89.2	0.6	89.1	0.7	92.9[m]	0.5
6	80.2	0.6	80.9	0.5	79.6	0.5	84.6	0.6	82.4[n]	0.7	84.6	0.6
7	77.3	0.6	76.9	0.6	74.7[f]	0.5	80.1[o]	0.7	72.3	0.7	74.4	0.7
8	71.9	0.5	72.7	0.6	68.6[g]	0.4	71.1	0.6	71.0	0.5	71.5	0.6
9	68.6	0.4	66.6	0.5	64.9[h]	0.5	65.3	0.6	70.7[q]	0.7	64.7	0.6
10	61.6	0.6	60.9	0.6	61.7	0.5	62.9	0.6	65.1[r]	0.7	61.1	0.6
11	62.2	0.9	66.9[i]	1.2	62.1	0.8	60.1[s]	0.9	65.7	0.8	66.4	0.8

Significant differences

Single-factor ANOVA:

a) without Equex pasta (0 %)			b) with Equex pasta (1 %)	
0 hours after thawing	0.5 h / 2.5 h	a: P < 0.01	1.0 h / 2.5 h	j: P < 0.05
1 hour after thawing	1.0 h / 0.5 h, 2.5 h	b: P < 0.01	2.5 h / 0.5 h, 1.0 h	K: P < 0.01
	0.5 h / 2.5 h	t: P < 0.01		
2 hours after thawing	1.0 h / 0.5 h, 2.5 h	c: P < 0.01	2.5 h / 0.5 h	l: P < 0.05
4 hours after thawing	2.5 h / 0.5 h, 1.0 h	d: P < 0.01		
5 hours after thawing	1.0 h / 0.5 h, 2.5 h	e: P < 0.05	2.5 h / 0.5 h, 1.0 h	m: P < 0.01
6 hours after thawing			1.0 h / 0.5 h, 2.5 h	n: P < 0.01
7 hours after thawing	2.5 h / 0.5 h, 1.0 h	f: P < 0.01	0.5 h / 1.0 h, 2.5 h	o: P < 0.01
8 hours after thawing	2.5 h / 0.5 h, 1.0 h	g: P < 0.01		
9 hours after thawing	2.5 h / 0.5 h, 1.0 h	h: P < 0.01	1.0 h / 0.5 h, 2.5 h	q: P < 0.01
10 hours after thawing			1.0 h / 0.5 h	r: P < 0.01
11 hours after thawing	1.0 / 0.5 h , 2.5 h	i: P < 0.01	0.5 h / 1.0 h, 2.5 h	s: P < 0.01

Table 9.3.12: Straight-line velocity (VSL; µm/s) of frozen-thawed Mexican wolf semen cooled for different times (0.5 h, 1.0 h, 2.5 h) without (0 %) or with (1 %) Equex pasta during a longevity test evaluated for 11 hours after thawing

hours after thawing	0 % Equex pasta						1 % Equex pasta					
	0.5		1.0		2.5		0.5		1.0		2.5	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
0	95.9[a]	1.0	92.4	1.0	89.8	1.1	90.7	1.1	93.8[i]	1.1	88.6	1.2
1	91.0	0.6	94.5[b]	0.7	90.2	0.7	87.9	0.7	86.6	0.7	86.2	0.9
2	89.6[c]	0.8	92.1	0.7	94.0	0.7	87.9	0.9	87.8	0.8	92.1[j]	0.9
3	89.4	0.8	87.8	0.8	87.6	0.6	86.1	0.8	85.9	0.7	86.3	0.8
4	77.3	0.6	78.7	0.6	74.3[d]	0.7	73.9	0.7	74.3	0.7	74.5	0.6
5	62.1	0.5	61.6	0.5	62.8	0.5	60.4	0.6	59.7	0.6	63.3[k]	0.5
6	46.1	0.4	46.8	0.5	47.7[e]	0.4	46.6[l]	0.5	48.9	0.5	49.6	0.5
7	39.3	0.4	41.1[f]	0.5	40.1	0.3	41.5[m]	0.4	39.0	0.5	38.8	0.4
8	33.7	0.3	34.4	0.4	34.5	0.3	35.0	0.4	35.9	0.3	37.1[n]	0.3
9	31.4	0.3	31.6	0.4	31.3	0.3	32.2	0.3	36.4[o]	0.6	32.5	0.3
10	27.6[g]	0.4	29.7	0.4	30.2	0.3	31.4	0.4	33.4[q]	0.6	31.2	0.4
11	29.3	0.5	33.3[h]	0.7	30.5	0.5	29.7[r]	0.5	32.2	0.5	32.1	0.5

Significant differences

Single-factor ANOVA:

a) without Equex pasta (0 %)			b) with Equex pasta (1 %)	
0 hours after thawing	0.5 h / 1.0 h, 2.5 h	a: P < 0.01	1.0 h / 2.5 h	i: P < 0.01
1 hour after thawing	1.0 h / 0.5 h, 2.5 h	b: P < 0.01		
2 hours after thawing	0.5 h / 1.0 h, 2.5 h	c: P < 0.01	2.5 h / 0.5 h, 1.0 h	j: P < 0.01
	2.5 h / 0.5 h	s: P < 0.01		
4 hours after thawing	2.5 h / 0.5 h, 1.0 h	d: P < 0.01		
5 hours after thaiwng			2.5 h / 0.5 h, 1.0 h	k: P < 0.01
6 hours after thawing	2.5 h / 0.5 h	e: P < 0.05	0.5 h / 1.0 h, 2.5 h	l: P < 0.01
7 hours after thawing	1.0 h / 0.5 h	f: P < 0.05	0.5 h / 1.0 h, 2.5 h	m: P < 0.01
8 hours after thawing			2.5 h / 0.5 h, 2.5 h	n: P < 0.01
9 hours after thawing			1.0 h / 0.5 h, 2.5 h	o: P < 0.01
10 hours after thawing	0.5 h / 1.0 h, 2.5 h	g: P < 0.01	1.0 h / 0.5 h, 2.5 h	q: P < 0.01
11 hours after thawing	1.0 h / 0.5 h, 2.5 h	h: P < 0.01	0.5 h / 1.0 h, 2.5 h	r: P < 0.01

Table 9.3.13: Curvilinear velocity (VCL; µm/s) of frozen-thawed Mexican wolf semen cooled for different times (0.5 h, 1.0 h, 2.5 h) without (0 %) or with (1 %) Equex pasta during a longevity test evaluated for 11 hours after thawing

hours after thawing	0 % Equex pasta						1 % Equex pasta					
	0.5		1.0		2.5		0.5		1.0		2.5	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
0	187.6	1.9	186.1	1.8	183.3	1.9	185.0	1.9	185.9[h]	1.9	185.0	2.1
1	225.4[a]	1.3	219.2	1.4	213.7	1.5	223.2	1.5	223.3[i]	1.4	210.7	1.8
2	210.8	1.7	213.9	1.4	204.9[b]	1.5	203.8	1.9	217.9	1.7	204.9	1.8
3	191.4[c]	1.7	183.2[c]	1.6	200.4[c]	1.4	197.4	1.9	202.4	1.6	200.3	1.8
4	194.8	1.5	199.8[d]	1.4	193.7	1.5	207.6	1.7	211.0	1.8	219.8[j]	1.6
5	211.1	1.3	208.9	1.4	210.1	1.3	215.2	1.5	218.6	1.7	222.0[k]	1.3
6	203.2	1.4	200.6	1.3	200.0	1.2	212.3	1.5	206.8[l]	1.6	213.8	1.4
7	190.4	1.3	192.7	1.4	190.5	1.1	199.2[m]	1.5	189.9	1.6	193.3	1.5
8	175.2	1.3	177.0[e]	1.3	172.5	1.0	178.0	1.5	179.9	1.2	184.1[n]	1.2
9	165.4	0.9	166.4	1.2	163.6	1.0	163.1	1.3	170.8[o]	1.4	166.9	1.2
10	151.6	1.4	151.5	1.5	159.5[f]	1.1	160.5	1.4	157.1	1.4	161.9[q]	1.4
11	151.3	2.0	167.1[g]	2.7	155.2	1.8	151.9[r]	1.9	162.1	1.7	165.5	1.7

Significant differences

Single-factor ANOVA:

a) without Equex pasta (0 %)			b) with Equex pasta (1 %)	
1 hour after thawing	0.5 h / 1.0h, 2.5 h	a: P < 0.01		
	1.0 h / 2.5 h	s: P < 0.01		
2 hours after thawing	2.5 h / 0.5 h, 1.0 h	b: P < 0.01	1.0 h / 0.5 h, 2.5 h	i: P < 0.01
3 hours after thawing	0.5 h / 1.0 h	c: P < 0.01		
	2.5 h / 0.5 h, 1.0 h	t: P < 0.01		
4 hours after thawing	1.0 h / 0.5 h, 2.5 h	d: P < 0.01	2.5 h / 0.5 h, 1.0 h	j: P < 0.01
5 hours after thawing			2.5 h / 0.5 h	k: P < 0.01
6 hours after thawing			1.0 h / 0.5 h, 2.5 h	l: P < 0.01
7 hours after thawing			0.5 h / 1.0 h, 2.5 h	m: P < 0.01
8 hours after thawing	1.0 h / 2.5 h	e: P < 0.05	2.5 h / 0.5 h, 2.5 h	n: P < 0.01
9 hours after thawing			1.0 h / 0.5 h	o: P < 0.01
10 hours after thawing	2.5 h / 0.5 h, 1.0 h	f: P < 0.01	2.5 h / 1.0 h	q: P < 0.05
11 hours after thawing	1.0 h / 0.5 h, 2.5 h	g: P < 0.01	0.5 h / 1.0 h, 2.5 h	r: P < 0.01

Table 9.3.14: Straightness (VSL/VAP; %) of frozen-thawed Mexican wolf semen cooled for different times (0.5 h, 1.0 h, 2.5 h) without (0 %) or with (0 %) Equex pasta during a longevity test evaluated for 11 hours after thawing

hours after thawing	0 % Equex pasta						1 % Equex pasta					
	0.5		1.0		2.5		0.5		1.0		2.5	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
0	87.6[a]	0.4	86.9	0.3	86.0	0.4	85.9	0.4	87.3[k]	0.4	85.0	0.5
1	82.1	0.3	83.2[b]	0.3	83.0	0.3	81.2	0.3	79.4[l]	0.4	81.6	0.4
2	82.0	0.4	82.5	0.3	84.5[c]	0.3	82.4	0.4	81.4	0.3	84.7[m]	0.4
3	84.3	0.3	83.7	0.3	82.9[d]	0.3	82.4[n]	0.4	80.8	0.4	81.8	0.4
4	79.7	0.3	79.1	0.3	78.8	0.3	75.7	0.4	76.3	0.4	75.3	0.4
5	69.2	0.4	70.6[e]	0.4	69.7	0.4	67.7	0.4	67.0	0.4	67.7	0.3
6	58.6	0.4	58.5	0.4	60.0[f]	0.4	56.2[o]	0.4	60.4	0.4	59.7	0.4
7	52.9[g]	0.4	54.3	0.4	54.7	0.3	53.8	0.4	53.6	0.5	53.9	0.4
8	49.4	0.4	49.4	0.4	51.7[h]	0.3	52.1	0.4	52.8	0.4	53.9[q]	0.4
9	47.8[i]	0.3	49.5	0.4	50.1	0.4	51.9	0.4	53.0	0.5	52.5	0.4
10	47.9[j]	0.5	51.6	0.6	50.3	0.4	51.9	0.5	52.1	0.6	52.9	0.5
11	50.3	0.7	51.9	1.0	52.1	0.7	52.5[r]	0.8	51.0	0.6	50.1	0.6

Significant differences:

Single-factor ANOVA:

a) without Equex pasta (0 %)			b) with Equex pasta (1 %)	
0 hours after thawing	0.5 h / 2.5 h	a: $P < 0.05$	1.0 h / 0.5 h, 2.5 h	k: $P < 0.01$
1 hour after thawing	1.0 h / 0.5 h	b: $P < 0.05$	1.0 h / 0.5 h, 2.5 h	l: $P < 0.01$
2 hours after thawing	2.5 h / 0.5 h, 1.0 h	c: $P < 0.01$	2.5 h / 0.5 h, 1.0 h	m: $P < 0.01$
3 hours after thawing	2.5 h / 0.5 h	d: $P < 0.01$	0.5 h / 1.0 h	n: $P < 0.01$
5 hours after thawing	1.0 h / 0.5 h, 2.5 h	e: $P < 0.05$		
6 hours after thawing	2.5 h /0.5 h, 1.0 h	f: $P < 0.01$	0.5 h / 1.0 h, 2.5 h	o: $P < 0.01$
7 hours after thawing	0.5 h / 1.0 h, 2.5 h	g: $P < 0.01$		
8 hours after thawing	2.5 h / 0.5 h, 2.5 h	h: $P < 0.01$	2.5 h / 0.5 h	q: $P < 0.01$
9 hours after thawing	0.5 h / 1.0 h, 2.5 h	i: $P < 0.01$		
10 hours after thawing	0.5 h / 1.0 h, 2.5 h	j: $P < 0.01$		
11 hours after thawing			0.5 h / 2.5 h	r: $P < 0.05$

Table 9.3.15: Linearity (VCL/VAP; %) of frozen-thawed Mexican wolf semen cooled for different times (0.5 h, 1.0 h, 2.5 h) without (0 %) or with (1 %) Equex pasta during a longevity test evaluated for 11 hours after thawing

hours after thawing	0 % Equex pasta						1 % Equex pasta					
	0.5		1.0		2.5		0.5		1.0		2.5	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
0	53.1[a]	0.5	51.1	0.5	51.2	0.5	50.6	0.6	52.6[h]	0.5	49.5	0.6
1	42.6[b]	0.3	45.1	0.4	44.6	0.4	41.6	0.3	41.3	0.4	42.6[i]	0.4
2	44.5	0.4	45.5	0.4	48.2[c]	0.4	45.3	0.5	43.0	0.4	47.5	0.5
3	49.8	0.4	50.5	0.5	46.9[d]	0.4	46.7	0.5	45.7	0.4	45.8	0.5
4	42.3[e]	0.4	41.7	0.4	40.7	0.4	38.0	0.4	37.6	0.4	36.2[j]	0.4
5	30.8	0.3	31.3	0.3	31.5	0.3	29.3	0.3	28.6	0.3	29.8[k]	0.3
6	23.6	0.2	24.3	0.2	24.4[f]	0.2	22.5[l]	0.2	24.6	0.2	24.0	0.2
7	21.8	0.2	21.8	0.2	21.6	0.2	21.9	0.2	21.5	0.2	20.7[m]	0.2
8	20.2	0.2	20.3	0.2	20.4	0.2	20.8	0.2	20.7	0.2	20.8	0.2
9	19.5	0.1	19.7	0.2	19.7	0.2	20.5	0.2	22.0[n]	0.3	20.1	0.2
10	19.1	0.3	20.7[g]	0.3	19.2	0.2	20.1	0.2	21.7[o]	0.3	19.7	0.2
11	20.3	0.3	20.7	0.5	21.0	0.4	21.0[q]	0.4	20.4	0.3	19.9	0.3

Significant differences

Single-factor ANOVA:

a) without Equex pasta (0 %)			b) with Equex pasta (1 %)	
0 hours after thawing	0.5 h / 1.0 h, 2.5 h	a: P < 0.01	1.0 h / 0.5 h, 2.5 h	h: P < 0.01
1 hour after thawing	0.5 h / 0.5 h, 2.5 h	b: P < 0.01	2.5 h / 1.0 h	i: P < 0.05
	0.5 h / 1.0 h	r: P < 0.01	2.5 h / 0.5 h, 1.0 h	s: P < 0.01
			0.5 h / 1.0 h	t: P < 0.01
2 hours after thawing	2.5 h / 0.5 h, 1.0 h	c: P < 0.01		
3 hours after thawing	2.5 h / 0.5 h, 1.0 h	d: P < 0.01		
4 hours after thawing	0.5 h / 2.5 h	e: P < 0.01	2.5 h / 0.5 h, 1.0 h	j: P < 0.01
5 hours after thawing			2.5 h / 1.0 h	k: P < 0.01
6 hours after thawing	2.5 h / 0.5 h	f: P < 0.05	0.5 h / 1.0 h, 2.5 h	l: P < 0.01
7 hours after thawing			2.5 h / 0.5 h, 1.0 h	m: P < 0.01
9 hours after thawing			1.0 h / 0.5 h, 2.5 h	n: P < 0.01
10 hours after thawing	1.0 h / 0.5 h, 2.5 h	g: P < 0.01	1.0 h / 0.5 h, 2.5 h	o: P < 0.01
11 hours agter thawing			0.5 h / 2.5 h	q: P < 0.05

Table 9.3.16: Average head displacement (ALH; µm) of frozen-thawed Mexican wolf semen cooled for different times (0.5 h, 1.0 h, 2.5 h) without (0 %) or with (1 %) Equex pasta during a longevity test evaluated for 11 hours after thawing

hours after thawing	0 % Equex pasta						1 % Equex pasta					
	0.5		1.0		2.5		0.5		1.0		2.5	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
0	7.3	0.1	7.4	0.1	7.4	0.1	7.4	0.1	7.4	0.1	7.5	0.1
1	9.3[a]	0.1	8.9	0.1	8.7	0.1	9.2	0.1	9.2	0.1	8.7[g]	0.1
2	8.8	0.1	8.8[b]	0.1	8.5	0.1	8.4	0.1	9.1	0.1	8.5[h]	0.1
3	8.0	0.1	7.9[c]	0.1	8.5	0.1	8.3	0.1	8.6	0.1	8.4[i]	0.1
4	8.4[d]	0.1	8.5	0.1	8.2	0.1	8.8	0.1	9.1[j]	0.1	9.3	0.1
5	9.2[e]	0.1	9.2	0.1	9.3	0.1	9.3	0.1	9.6	0.1	9.5	0.1
6	9.6[f]	0.1	9.6	0.1	9.3	0.1	9.8	0.1	9.5	0.1	9.6[k]	0.1
7	9.6	0.1	9.4	0.1	9.2	0.1	9.7[l]	0.1	9.3	0.1	9.4	0.1
8	9.2	0.1	9.3	0.1	8.8	0.1	9.2	0.1	9.1	0.1	9.0	0.1
9	9.1	0.1	8.9	0.1	8.6	0.1	9.0	0.1	8.9	0.1	8.5	0.1
10	8.5	0.1	8.3	0.1	8.4	0.1	8.9	0.1	8.6	0.1	8.5	0.1
11	8.6	0.1	9.2	0.1	8.5	0.1	8.5	0.1	9.1	0.1	9.1	0.1

Significant differences

Single-factor ANOVA:

a) without Equex pasta (0 %)			b) with Equex pasta (1 %)	
1 hour after thawing	0.5 h / 1.0 h, 2.5 h	a: P < 0.01	2.5 h / 0.5 h, 1.0 h	g: P < 0.01
2 hours after thawing	2.5 h / 0.5 h, 1.0 h	b: P < 0.01	1.0 h / 0.5 h, 2.5 h	h: P < 0.01
3 hours after thawing	2.5 h / 0.5 h, 1.0 h	c: P < 0.01	1.0 h / 2.5 h, 0.5 h	i: P < 0.01
4 hours after thawing	1.0 h / 2.5 h	d: P < 0.01	0.5 h / 1.0 h, 2.5 h	j: P < 0.01
5 hours after thawing			0.5 h / 1.0 h, 2.5 h	s: P < 0.01
6 hours after thawing	2.5 h / 0.5 h, 1.0 h	f: P < 0.01	0.5 h / 1.0 h	k: P < 0.01
7 hours after thawing	0.5 h / 2.5 h	m: P < 0.01	0.5 h / 1.0 h, 2.5 h	l: P < 0.01
8 hours after thawing	2.5 h / 0.5 h, 1.0 h	n: P < 0.01		
9 hours after thawing	2.5 h / 0.5 h, 1.0 h	o: P < 0.01	2.5 h / 0.5 h, 1.0 h	t: P < 0.01
	1.0 h / 0.5 h	q: P < 0.01		
10 hours after thawing			0.5 h / 1.0 h, 2.5 h	u: P < 0.01
11 hours after thawing	1.0 h / 0.5 h, 2.5 h	r: P < 0.01	0.5 h / 1.0 h, 2.5 h	v: P < 0.01

Table 9.3.17: Beat cross frequency (BCF; Hz) of frozen-thawed Mexican wolf semen cooled for different times (0.5 h, 1.0 h, 2.5 h) without (0 %) or with (1 %) Equex pasta during a longevity test evaluated for 11 hours after thawing

hours after thawing	0 % Equex pasta						1 % Equex pasta					
	0.5		1.0		2.5		0.5		1.0		2.5	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
0	31.4	0.3	31.8	0.3	30.9	0.3	31.7[j]	0.3	30.0	0.3	30.2	0.4
1	29.2	0.2	29.4	0.2	29.6	0.2	28.9[k]	0.2	29.5	0.2	29.4	0.2
2	29.4	0.2	29.0	0.2	29.0	0.2	29.4	0.3	29.0	0.2	28.4	0.2
3	27.6	0.2	27.0	0.2	28.4[a]	0.2	28.6	0.3	27.8	0.2	28.2	0.3
4	28.9	0.2	28.6	0.2	30.0[b]	0.2	30.1[l]	0.3	29.1	0.3	29.7	0.2
5	30.0[c]	0.2	29.6	0.2	28.9	0.2	31.2[m]	0.2	29.9	0.2	30.5	0.2
6	28.0	0.2	27.6	0.2	28.7[d]	0.2	28.3[n]	0.2	29.3	0.2	29.4	0.2
7	26.3[e]	0.2	27.2	0.2	27.7	0.2	27.3	0.2	27.7	0.3	28.2	0.2
8	25.9	0.2	26.0	0.3	28.0[f]	0.2	25.8[o]	0.3	27.3[o]	0.2	28.2[o]	0.2
9	25.6	0.2	26.0	0.3	27.9[g]	0.3	24.2[q]	0.3	25.9[q]	0.3	27.2[q]	0.3
10	26.9	0.4	26.5	0.4	28.3[h]	0.3	24.1[r]	0.3	25.5[r]	0.3	27.0[r]	0.3
11	25.1	0.5	23.6	0.6	25.6[i]	0.4	24.1	0.4	22.7[s]	0.4	24.5	0.4

Significant differences

Single-factor ANOVA:

a) without Equex pasta (0 %)			b) with Equex pasta (1 %)	
0 hours after thawing			0.5 h / 1.0 h, 2.5 h	j: P < 0.01
2 hours after thawing			0.5 h / 2.5 h	k: P < 0.05
3 hours after thawing	2.5 h / 0.5 h, 1.0 h	a: P < 0.01		
4 hours after thawing	2.5 h / 0.5 h, 1.0 h	b: P < 0.01	0.5 h / 1.0 h	l: P < 0.05
5 hours after thawing	0.5 h / 2.5 h	c: P < 0.01	0.5 h / 1.0 h	m: P < 0.01
6 hours after thawing	2.5 h / 1.0 h	d: P < 0.01	0.5 h / 1.0 h, 2.5 h	n: P < 0.01
7 hours after thawing	0.5 h / 1.0 h, 2.5 h	e: P < 0.01		
8 hours after thawing	2.5 h / 0.5 h, 1.0 h	f: P < 0.01	2.5 h / 0.5 h, 1.0 h	o: P < 0.01
			1.0 h / 0.5 h	u: P < 0.01
9 hours after thawing	2.5 h / 0.5 h, 1.0 h	g: P < 0.01	2.5 h / 0.5 h, 1.0 h	q: P < 0.01
			1.0 h / 0.5 h	v: P < 0.01
10 hours after thawing	2.5 h / 0.5 h, 1.0 h	h: P < 0.01	2.5 h / 0.5 h, 1.0 h	r: P < 0.01
			1.0 h / 0.5 h	w: P < 0.01
11 hours after thawing	2.5 h / 1.0 h	i: P < 0.01	1.0 h / 0.5 h, 2.5 h	s: P < 0.01

Table 9.3.18: Sperm velocities (VAP, VSL, VCL, µm/s) of frozen-thawed Mexican gray wolf spermatozoa cooled for different times (0.5 h, 1.0 h, 2.5 h) without (0 %) or with (1 %) Equex pasta during a longevity test evaluated for 11 hours after thawing [values with corresponding letters and subscripts are considered significantly different]

	VAP				VSL				VCL			
	0 %		1 %		0 %		1 %		0 %		1 %	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
0	103.6	0.6	102.7	0.6	92.8	0.6	91.2	0.7	185.8	1.1	185.3	1.1
1	108.2^{b1}	0.4	105.0^{b1}	0.4	91.9^{b2}	0.4	87.0^{b2}	0.4	219.8	0.8	220.1	0.9
2	106.9^{c1}	0.4	104.6^{c1}	0.5	91.9^{c2}	0.4	89.1^{c2}	0.5	210.1	0.9	209.6	1.1
3	102.8	0.4	102.8	0.4	88.2^{d2}	0.4	86.1^{d2}	0.5	192.4^{d3}	0.9	200.2^{d3}	1.0
4	94.9^{e1}	0.4	96.5^{e1}	0.4	76.8^{e2}	0.4	74.3^{e2}	0.4	196.1^{e3}	0.8	213.2^{e3}	1.0
5	88.2^{f1}	0.3	90.7^{f1}	0.3	62.2	0.3	61.5	0.3	210.0^{f3}	0.8	219.0^{f3}	0.9
6	80.2^{g1}	0.3	83.9^{g1}	0.4	46.9^{g2}	0.3	48.4^{g2}	0.3	201.2^{g3}	0.8	211.1^{g3}	0.9
7	76.1	0.3	76.7	0.4	40.1	0.2	39.8	0.3	191.0^{h3}	0.7	194.5^{h3}	0.9
8	70.7	0.3	71.2	0.4	34.2^{i2}	0.2	36.1^{i2}	0.2	174.5^{i3}	0.7	180.9^{i3}	0.7
9	66.9	0.3	66.7	0.4	31.4^{j2}	0.2	33.6^{j2}	0.2	165.0	0.6	166.8	0.7
10	61.4^{k1}	0.3	63.2^{k1}	0.4	29.3^{k2}	0.2	32.1^{k2}	0.3	154.8^{k3}	0.7	159.7^{k3}	0.8
11	62.9	0.6	64.3	0.5	30.5^{l2}	0.3	31.4^{l2}	0.3	155.7^{l3}	1.2	160.3^{l3}	1.0

Significant differences

Single-factor ANOVA

Dilution without or with Equex pasta

1 h after thawing	b_1: $P < 0.01$	b_2: $P < 0.01$	
2 h after thawing	c_1: $P < 0.01$	c_2: $P < 0.01$	
3 h after thawing		d_2: $P < 0.01$	d_3: $P < 0.01$
4 h after thawing	e_1: $P < 0.01$	e_2: $P < 0.01$	e_3: $P < 0.01$
5 h after thawing	f_1: $P < 0.01$		f_3: $P < 0.01$
6 h after thawing	g_1: $P < 0.01$	g_2: $P < 0.01$	g_3: $P < 0.01$
7 h after thawing			h_3: $P < 0.01$
8 h after thawing		i_2: $P < 0.01$	i_3: $P < 0.01$
9 h after thawing		j_2: $P < 0.01$	
10 h after thawing	k_1: $P < 0.01$	k_2: $P < 0.01$	k_3: $P < 0.01$
11 h after thawing		l_2: $P < 0.05$	l_3: $P < 0.01$

Table 9.3.19: Straightness (%, STR) and linearity (%, LIN) of frozen-thawed Mexican gray wolf spermatozoa cooled for different times (0.5 h, 1.0 h, 2.5 h) without (0 %) or with (1 %) Equex pasta during a longevity test evaluated for 11 hours after thawing

	STR				LIN			
	0 %		1 %		0 %		1 %	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
0	86.9[a]	0.2	86.2[a]	0.2	51.8	0.3	51.0	0.3
1	82.7[b]	0.2	80.6[b]	0.2	44.0[i]	0.2	41.7[i]	0.2
2	83.0	0.2	82.7	0.2	46.1[j]	0.2	45.0[j]	0.3
3	83.6[c]	0.2	81.6[c]	0.2	48.9[k]	0.2	46.0[k]	0.3
4	79.2[d]	0.2	75.7[d]	0.2	41.6[l]	0.2	37.2[l]	0.2
5	69.8[e]	0.2	67.5[e]	0.2	31.2[m]	0.2	29.3[m]	0.2
6	59.1	0.2	58.8	0.2	24.1[n]	0.1	23.7[n]	0.1
7	54.0	0.2	53.8	0.2	21.7[o]	0.1	21.4[o]	0.1
8	50.4[f]	0.2	53.0[f]	0.2	20.3[q]	0.1	20.8[q]	0.1
9	49.0[g]	0.2	52.4[g]	0.3	19.6[r]	0.1	20.8[r]	0.1
10	49.9[h]	0.3	52.3[h]	0.3	19.6[s]	0.1	20.5[s]	0.2
11	51.3	0.5	51.1	0.4	20.7	0.2	20.4	0.2

Significant differences

Single-factor ANOVA

Dilution without or with Equex pasta

0 h after thawing	a: $P < 0.05$	
1 h after thawing	b: $P < 0.01$	i: $P < 0.01$
2 h after thawing		j: $P < 0.01$
3 h after thawing	c: $P < 0.01$	k: $P < 0.01$
4 h after thawing	d: $P < 0.01$	l: $P < 0.01$
5 h after thawing	e: $P < 0.01$	m: $P < 0..01$
6 h after thawing		n: $P < 0.05$
7 h after thawing		o: $P < 0.05$
8 h after thawing	f: $P < 0.01$	q: $P < 0.01$
9 h after thawing	g: $P < 0.01$	r: $P < 0.01$
10 h after thawing	h: $P < 0.01$	s: $P < 0.01$

Table 9.3.20: Lateral head displacement (µm, ALH) and beat cross frequency (Hz, BCF) of frozen-thawed Mexican gray wolf spermatozoa cooled for different times (0.5 h, 1.0 h, 2.5 h) without (0 %) or with (1 %) Equex pasta during a longevity test evaluated for 11 hours after thawing

	ALH				BCF			
	0 %		1 %		0 %		1 %	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
0	7.4	0.1	7.4	0.1	31.4[h]	0.2	30.7[h]	0.2
1	9.0[a]	0.1	9.1[a]	0.1	29.4	0.1	29.3	0.1
2	8.7	0.1	8.7	0.1	29.1	0.1	28.9	0.1
3	8.2[b]	0.1	8.5[b]	0.1	27.7[i]	0.1	28.2[i]	0.2
4	8.4[c]	0.1	9.1[c]	0.1	29.1[j]	0.1	29.7[j]	0.1
5	9.2[d]	0.1	9.5[d]	0.1	29.5[k]	0.1	30.6[k]	0.1
6	9.5[e]	0.1	9.6[e]	0.1	28.1[l]	0.1	29.0[l]	0.1
7	9.4	0.1	9.5	0.1	27.1[m]	0.1	27.7[m]	0.1
8	9.1	0.1	9.1	0.1	26.8	0.1	27.2	0.1
9	8.9	0.1	8.8	0.1	26.5[n]	0.1	25.8[n]	0.2
10	8.4[f]	0.1	8.7[f]	0.1	27.4[o]	0.2	25.5[o]	0.2
11	8.7[g]	0.1	8.9[g]	0.1	25.1[q]	0.3	23.8[q]	0.2

Significant differences

Single-factor ANOVA

Dilution without or with Equex pasta

0 h after thawing		h: $P < 0.01$
2 h after thawing	a: $P < 0.05$	
3 h after thawing	b: $P < 0.01$	i: $P < 0.05$
4 h after thawing	c: $P < 0.01$	j: $P < 0.01$
5 h after thawing	d: $P < 0.01$	k: $P < 0.01$
6 h after thawing	e: $P < 0.01$	l: $P < 0.01$
7 h after thawing		m: $P < 0.01$
9 h after thawing		n: $P < 0.01$
10 h after thawing	f: $P < 0.01$	o: $P < 0.01$
11 h after thawing	g: $P < 0.01$	q: $P < 0.01$

Table 9.3.21 : Live spermatozoa (Eosin-Nigrosin, %) in frozen-thawed semen of the domestic dog, generic gray and Mexican wolf cooled for different times (dog: 0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h; wolves: 0.5 h, 1.0 h, 2.5 h). Diluted without (0%) or with (1%) Equex pasta (wolves).

species	cooling time (h)	0 % Equex pasta		1 % Equex pasta	
		mean	SEM	mean	SEM
domestic dog (n=4)	0.5 (m=8)	86.9	2.1	---	---
	1.5 (m=8)	84.0	3.1	---	---
	2.5 (m=8)	82.3	4.1	---	---
	3.5 (m=8)	81.8	3.2	---	---
	4.5 (m=8)	78.5	5.5	---	---
generic gray wolf (n=7)	0.5 (m=7)	54.9	8.6	33.6	9.6
	1.0 (m=8)	37.6 (m=7)	6.8	46.4	7.1
	2.5 (m=6)	67.4 (m=7)	10.1	54.5	13.4
Mexican wolf (n=4)	0.5 (m=23)	78.6 (m=26)	2.7	75.8	4.4
	1.0 (m=23)	79.2 (m=26)	2.4	77.6	4.3
	2.5 (m=21)	81.4 (m=24)	2.8	81.2	2.3

Table 9.3.22: Spermatozoa with intact plasma membranes (%, HOST) of frozen-thawed generic gray (n = 7) and Mexican wolf (n = 4) semen cooled in different cooling times (0.5 h, 1.0 h, 2.5 h) diluted without (0 %) or with (1 %) Equex pasta

species	Equex pasta	cooling time (h)	intact plasma membrane	
			mean	SEM
generic gray wolf	0 %	0.5 (m = 7)	57.4	7.6
		1.0 (m = 7)	53.7	6.4
		2.5 (m = 7)	39.1	5.5
	1 %	0.5 (m = 7)	39.0	7.5
		1.0 (m = 8)	34.6	8.1
		2.5 (m = 6)	45.2	3.1
Mexican wolf	0 %	0.5 (m = 26)	62.3	2.5
		1.0 (m = 26)	62.7	2.7
		2.5 (m = 24)	61.4	2.8
	1 %	0.5 (m = 23)	51.8	3.4
		1.0 (m = 23)	52.0	3.9
		2.5 (m = 21)	51.6	2.8

Table 9.3.23: Spermatozoa with normal morphology (%) in frozen-thawed domestic dog, generic gray and Mexican wolf semen cooled for different times (dog: 0.5 h, 1.5 h, 2.5 h, 3.5 h, 4.5 h; wolves: 0.5 h, 1.0 h, 2.5 h) without (0 %) or with (1 %) Equex pasta

species	cooling time (h)	0 % Equex pasta		1 % Equex pasta	
		mean	SEM	mean	SEM
domestic dog (n=4)	0.5 (m=8)	60.6	6.2	-----	-----
	1.5 (m=8)	61.8	7.4	-----	-----
	2.5 (m=8)	60.4	9.0	-----	-----
	3.5 (m=8)	59.4	8.2	-----	-----
	4.5 (m=8)	56.3	7.6	-----	-----
generic gray wolf (n=7)	0.5 (m=7)	61.6	10.6	63.9	9.8
	1.0 (m=7)	64.1 (m=8)	10.0	51.0	12.7
	2.5 (m=7)	57.9 (m=6)	10.7	72.0	7.9
Mexican wolf (n=4)	0.5 (m=23)	77.3 (m=26)	2.0	69.3	1.8
	1.0 (m=23)	73.9 (m=26)	2.0	70.7	2.4
	2.5 (m=21)	70.0 (m=24)	2.5	68.7	2.9

Teilergebnisse dieser Dissertation wurden wie folgt zur Veröffentlichung eingereicht und befinden sich zur Zeit im Druck / parts of results of this thesis are in press at

Zeitschrift / journal ‚Theriogenology':

ZINDL, C., C.S. ASA and A.-R. GÜNZEL-APEL:
Influence of cooling rates and addition of Equex pasta on cooled and frozen-thawed semen of generic gray (*Canis lupus*) and Mexican gray wolves (*Canis lupus baileyi*)

Acknowledgement

I would like to thank Prof. Dr. Anne-Rose Günzel-Apel for providing me with the possibility to confer a doctor's degree with this thesis; for the enriching company during conferences, for uncountable hours to review the thesis and especially for having faith in me to work through the experimental part far away from the reproductive department in Hanover.

I also would like to thank my supervisor in the USA, Dr. Cheryl Asa, for her assistance, advice and support, in every minute either in the field with the wolves or in the laboratory and for all I learned from her about reproduction in wolves and other canids. I especially thank her for the obviousness how she included me into her team for the months I spent during the experimental part of my work, and to allow me to use the laboratory.

Special thanks to Karen Bauman, for incredible organization of field trips to the WCSRC, to Minnesota and TESCO; and her invaluable technical knowledge of all instruments ever used in the project.

I wish to thank Dr. Gerardo Camillo, Department of Biology, St. Louis University for his statistical expertise and patience during the statistical analysis.

I thank Carol Fiesler and Kathy Roeder for their 'pipetting assistance' in Minnesota.

Special thanks to the staff from the Wild Canid Survival and Research Center (Eureka, Missouri), especially Dr. Sue Lindsey the Executive Director and the staff of the Wildlife Science Center (Forest Lake, Minnesota), who permitted us to work with their animals.

A special thanks to the dogs 'Cochice', 'Jake', 'Maui' and 'Schooner' and their owners who beared with me to make the pre-trials possible.

I thank Annabel Musson, Dr. Monica Hall-Woods, Dr. Karen DeMatteo, Karen Bauman, Chris Dutton and Luis Padilla for their encouragement, happy hours, crazy projects and that they always had an open ear. I also thank Jane Merkel for 'tolerating' me in her house and always having time to talk when things didn't go like they should have gone.

I also thank Joan Bauman for the leisure time in the opera away from the laboratory and her passing on to me unvaluable knowledge about reproductive endocrinology.

I would like to deeply thank Dr. Isabelle von Richthofen and Dr. Sonja Hopf for always being there as my best friends, bearing good and bad times and helping review this thesis.

I thank Dr. Monika Ott and Dr. habil Michael Mähler for reviewing parts of this thesis. Monika, thank you for being as crazy as I am and helping me to compensate this final struggle with so much laughter.

I wish to thank the team of the veterinary practice Dr. Wilkes, Bahde, Nathaus and Schmidt to have helped me generously to organize my working schedule as flexible as possible to be able to finish writing this thesis.

I wish to thank my parents for her support financially and mentally during the time of my veterinary studies and years of completing the thesis. Without them I would have not been able to come that far. I thank my sister, Sabine for those endless hours of encouragement on the phone.

Ich danke ganz besonders meinen Eltern für ihre finanzielle und mentale Unterstützung während meiner Studienjahre und der Anfertigung der Doktorarbeit. Ohne sie hätte ich es nicht so weit geschafft.

I big thank you to my boyfriend Tillmann Dworok, who had to put up with me during many frustrating hours and always supported and encouraged me to bring this thesis to an end.

ISBN 3-938026-89-8

Verlag: Deutsche Veterinärmedizinische Gesellschaft Service GmbH
35392 Gießen Frankfurter Straße 89 Tel. 0641/24466 Fax: 0641 / 25375
e-mail: Geschätftsstelle@dvg.net Homepage: http://www.dvg.net