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The Effects of Prostatic Fluid on Functional Characteristics of Cooled Canine Semen

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THE EFFECTS OF PROSTATIC FLUID ON FUNCTIONAL CHARACTERISTICS OF COOLED CANINE SEMEN

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Interdepartmental Program in
Veterinary Medical Sciences through the
Department of Veterinary Clinical
Sciences

by

Reto Fritsche

Dr. med. vet., Vetsuisse Faculty University of Bern, 2007
August 2015

The author would like to dedicate this thesis to his family, who enabled him to reach out for the world and pursue his dreams and training far away from home.

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ABSTRACT

The objectives of this study were to investigate concentration dependent effects of canine prostatic fluid (PF) on *in vitro* seminal parameters of cooled canine semen. Sperm motility parameters, plasma membrane integrity and stability, acrosome integrity and DNA fragmentation were measured after the addition of 0%, 10%, 25%, or 50% PF to extended semen of fertile dogs. Assessments were made at 0 h pre-cooling, at 24, and 48 h of cooled storage (4 °C), and after freezing and thawing followed by incubation (37 °C) at 0, 4 and 24 h. Our hypothesis was that lower dilutions of canine semen with PF in an egg yolk-Tris extender would improve plasma membrane stability and acrosome integrity, and preserve sperm kinetics and reduce DNA fragmentation in comparison with higher concentrations of PF in fertile dogs during cooling. Sperm motility parameters were assessed by computer assisted sperm analysis, and plasma membrane integrity by the hypo-osmotic swelling test. Flow cytometry was used after staining with YO-PRO-1/Ethidium Homodimer 1 (EthD-1) to evaluate membrane stability, fluorescent isothiocyanate-PNA (*Arachis Hypogaea*)/propidium iodide to assess acrosome integrity, and sperm chromatin structure assay to assess DNA fragmentation. The data was analyzed using a mixed linear model (ANOVA) and in case of significant effects of time, treatment, or treatment*time interaction ($P < 0.05$), least square means were used for pairwise comparisons. Acrosome integrity and DNA fragmentation were not affected by treatment with PF. During the cooling period motility parameters were not influenced by PF treatment. A lower proportion of early apoptotic and higher proportion of early necrotic cells was seen during cooling with 50% PF (YO-PRO-1/EthD-1). Although lower concentrations of PF did not improve the evaluated spermatozoal parameters, they did not seem to compromise sperm motility and plasma

membrane stability. The presence of 50% PF prior to cryopreservation decreased post thaw motility and produced a shift towards early necrotic cells after thawing. Therefore admixture with more than 10% PF should be avoided prior to cryopreservation of canine semen.

CHAPTER 1

INTRODUCTION

The first successful canine pregnancy using artificial insemination (AI) with cooled semen was reported by Harrop in 1954. Since then, this technique has found widespread use and allows for breeding of dogs at distant geographic locations. Transport of semen is less expensive and less stressful than either shipping of the male or the female dog (review: Kutzler, 2005). Diagnostics for optimal timing of AI, proper packaging of semen, prompt shipping, and the use of semen extenders contribute considerably to an optimal outcome. The prostate is the only accessory sex gland in the dog and contributes the largest fluid component to the ejaculate. Improper collection technique leads to a large proportion of prostatic fluid (PF) and dilution of the sperm rich fraction (SRF); concentration dependent effects of PF on seminal parameters has not been evaluated for cooled shipping of canine semen. In stallions, a minimal dilution ratio of the seminal plasma (SP) and optimal sperm concentrations for shipping of semen have been established (Jasko et al., 1991). Dog semen is considered relatively tolerant towards the detrimental effects of cooling (cold shock) and has been shown to maintain its fertility potential for up to 10 days under ideal circumstances (Johnson et al., 2001; Ponglowhapan et al., 2004; Verstegen et al., 2005). For cooled storage of canine semen, PF contamination is commonly avoided although studies have shown variable effects on sperm quality (Foote, 1963; Rota et al., 1995; Sirivaidyapong et al., 2001; Treulen et al., 2012).

Cryopreservation of canine semen allows for indefinite storage and preservation of genetic material for breeding purposes; first successful reports date back to 1969 (Seager). Techniques have evolved since then and whelping rates using frozen-thawed semen now

average around 60 to 70% (review: Linde-Forsberg, 2002). It is recommended to avoid PF contamination during semen collection or to remove PF by centrifugation due to its negative impact on post-thaw motility (Sirivaidyapong et al., 2001). Cryopreservation and thawing apply considerable stress on spermatozoa, through ice crystal formation, osmotic changes, reactive oxygen species, and the induction of apoptotic-like changes. It has been shown that cooling of canine semen for 1 to 2 days prior to cryopreservation results in comparable post-thaw motility, acrosome integrity and plasma membrane integrity compared to immediate cryopreservation immediately after collection (Hermansson and Linde-Forsberg, 2006).

In vivo studies have reported positive effects of PF on odds of conception, pregnancy rates and litter size when used as a flushing medium after vaginal or transcervical AI using frozen-thawed or cooled canine semen (Nöthling et al., 2005; England et al., 2012). The controversy surrounding canine PF originates from conflicting results regarding *in vivo* and *in vitro* applications of PF, and the use of variable PF concentrations for *in vitro* seminal parameter evaluations.

This study aimed to investigate concentration dependent effects of PF on *in vitro* seminal parameters and possibly determine an optimal dilution ratio of PF for use in cooled semen. The objective of this study was to measure sperm kinetics (motility parameters), plasma membrane integrity and stability, acrosome integrity, and DNA fragmentation after the addition of 0%, 10%, 25%, or 50% PF to extended semen of fertile dogs. Assessments were made at 0 h pre-cooling, at 24, and 48 h of cooled storage (4 °C), and after freezing and thawing followed by incubation (37 °C) at 0, 4 and 24 h.

The hypothesis that lower dilutions of canine semen with PF would improve seminal parameters in comparison with higher concentrations of PF in fertile dogs was based on the concept of progressive PF clearance in the female reproductive tract (England et al., 2006). After natural mating spermatozoa are initially exposed to high concentrations of PF, but due to vaginal and uterine clearance, and sperm reservoir establishment, exposure of spermatozoa to lower concentrations of PF over time might mimic physiological conditions more closely. A recent publication by Treulen et al. (2012) proposed beneficial effects of PF over a cooling period of 72 h on *in vitro* plasma membrane and acrosome integrity. This study is the first report investigating concentration dependent effects of PF on canine semen.

CHAPTER 2

LITERATURE REVIEW

2.1. Canine semen

The canine ejaculate consists of three distinct fractions, which contain a variable mixture of spermatozoa, prostatic fluid (PF) and fluid originating from the epididymides. Total sperm numbers range from 300 million to 2 billion cells dependent on testicle size, body weight, breed, age, frequency of ejaculation and collection technique (Boucher et al., 1958; review: Johnston, 1991). Canine spermatozoa measure approximately 68 μm in total length (11 μm midpiece length, 50 μm tail length) with a head width of approximately 4.1 μm (Bartlett, 1962; Dahlbom et al., 1997). Normal volume of the total ejaculate ranges from 1 to 80 mL and is dependent on the amount of PF collected by the operator (Johnston et al., 2001). Similarly, sperm concentration in an ejaculate is largely dependent on collection technique and amount of PF obtained; with a reported range of 4 to 400 $\times 10^6$ spermatozoa/mL (Johnston et al., 2001). Acceptable percentage of progressively motile sperm and normal percentage of morphologically normal spermatozoa are considered to be $\geq 70\%$ and $\geq 80\%$, respectively (Johnston et al., 2001). Percentage of progressively motile spermatozoa declines at a slower rate in samples held at room temperature (18 to 23 $^{\circ}\text{C}$) than in those held at body temperature (37 $^{\circ}\text{C}$) (Bartlett, 1962).

2.2. Ejaculation

The canine penis is of a vascular type and includes an os penis, a small corpus cavernosum (CCP), a corpus spongiosum (CSP) and a bulbus glandis (BG) at the proximal glans penis. Erection of the penis mainly affects the glans penis and is a result of parasympathetic

stimulation (Johnston et al., 2001). The os penis and CCP, which becomes a closed vascular system maintaining high pressures through activity of the ischiocavernosus muscles (Purohit and Beckett, 1976; Valji and Bookstein, 1987), allow for intromission before full erection occurs. Erection patterns further differ from other species in that a delayed detumescence of the BG occurs, which allows for the male and female to remain locked together (copulatory tie) for an extended period of time in the final phase of copulation. The CSP is not a closed vascular system but activity of the bulbospongiosus muscles partially occludes venous return and aids in filling of the BG after complete intromission (Purohit and Beckett, 1976). The constrictor vestibuli muscles of the bitch tighten caudal to the swollen BG and contribute to the copulatory tie by maintaining mechanical retention of the penis in the vagina (Grandage, 1972).

Ejaculation occurs in three distinct fractions, termed pre-sperm, sperm rich and PF fraction (Evans, 1933). The first or pre-sperm fraction originates from the prostate (England et al., 1990), is clear, acellular, and of small volume (0.5 to 5 mL) (Johnston et al., 2001). Its function is to flush the urethra of debris or retained urine and assist intromission by lubrication of the glans penis and female perineum. The second or sperm rich fraction (SRF) contains spermatozoa. It is normally opalescent, variable in volume (1 to 4 mL), and originates from the epididymal tail (Johnston et al., 2001). The third or PF fraction is the most voluminous. It is clear in the normal healthy dog, and originates from the prostate. It is ejected in a rhythmic pulsatile fashion facilitated by contractions of the urethralis muscle and prostate. This is outwardly visible by anal contractions and propulsion of the ejaculate under pressure. It occurs during the copulatory tie, which can occur over a period of 5 to 60 min (Johnston et al., 2001). Due to the engorged penis filling the entire vaginal space, the ejaculated fluid quickly gains entrance into

the uterus. Prostatic fluid may therefore facilitate cranial flushing of the previously ejaculated SRF in the female reproductive tract (Grandage, 1972; England et al., 2006). During the copulatory tie, step over behavior is often observed. The penis twists 180 degrees in a lateral plane, which is thought to assist with venous occlusion and maintenance of erection despite high intravaginal pressure during the copulatory tie (Johnston et al., 2001). Prior to the end of the copulatory tie the activity of the bulbospongiosus muscles subsides and pressures decreases, which facilitates detumescence of the bulbus glandis and terminates the copulatory tie (Hart, 1972).

2.3. Semen collection

Manual collection is the most common semen collection technique used for dogs. The two most commonly used variations of manual collection are the use of collection tubes or the use of a plastic cone (review: Kutzler, 2005). Electroejaculation has been reported, but is usually not necessary and is associated with urine contamination of the ejaculate (Johnston et al., 2001). The use of an artificial vagina is not necessary and prolonged contact with latex can be detrimental to semen (Boucher et al., 1958). Sexual arousal can be achieved solely by manual stimulation of the prepuce and penis, in the presence of an estrous teaser bitch, or with olfactory stimulation by use of a vaginal estrous swab. Manual stimulation alone can be sufficient with many dogs, whereas studs that have had more experience with natural mating may not ejaculate unless an estrous bitch is present. It is recommended to use a teaser bitch whenever possible as quantity and quality of semen has been demonstrated to improve (Boucher et al., 1958; Olar et al., 1983). A variety of receptacles can be used and it is important to avoid cold shock, contamination or contact with spermicidal substances, such as water,

detergent, or alcohol. Once initial engorgement of the glans penis has occurred, the prepuce is slid proximally over an enlarging BG and continuous encircling manual pressure is applied proximal to the BG. Careful technique and switching of receptacles during ejaculation allows the three fractions to be collected separately. The secretion of white fluid marks the beginning of the SRF, whereas the reappearance of clear fluid accompanied by pulsatory ejaculation marks the beginning of the third PF fraction. For terminology hereafter, prostatic fluid (PF) will refer to the sperm-free portion of the third fraction, and seminal plasma (SP) the sperm-free portion of the SRF. Centrifugation combined with filtration is necessary to harvest pure SP. Extraction of SP using high speed centrifugation has deleterious effects on spermatozoa (Rijsselaere et al., 2002; Len et al., 2010). Alternatively, PF can easily be obtained by separate collection of the third fraction during ejaculation (fractionated collection).

2.4. Sperm transport in the bitch reproductive tract

Transport of semen in the female canine reproductive tract occurs rapidly. Evans (1933) surgically created a uterine horn fistula in female dogs and found that within 25 to 50 s of natural mating, sperm suspension was present in the fistula. Abdominal straining further forced fluid to escape from these openings in streams, until the fluid seemed to be replaced by water-clear solution after 3 to 5 min (presumably PF). Tsutsui et al. (1989) repeated the experiment with similar findings, and reported delayed sperm transport with vaginal AI, and a lack of sperm transport when bitches were bred in late estrus, which could be explained by cervical closure (Verstegen et al., 2001). Vaginal and myometrial contractions, as well as abdominal musculature contractions are thought to contribute to sperm transport. England et al. (2006) reported an increase in myometrial contractions, measured by M-mode ultrasonography, in the

bitch in association with mating. Suggested causes for increased uterine contractions during coitus are vaginal stimulation and distension by an engorged BG causing oxytocin release, the large volume of the ejaculate, and the introduction of prostaglandins by constituents of SP (England et al., 2012). Coital stimulation can be simulated in the estrous bitch by digital palpation/dilatation of the vagina (Silva et al., 1996; England et al., 2006).

After copulation or AI, canine spermatozoa were found to be present in the bitch reproductive tract for a prolonged period of time. High concentrations of motile spermatozoa were found in the uterus 4 to 6 d after copulation, with diminished concentrations for as long as 11 days (Doak et al., 1967). At 6 h after mating a wide distribution of spermatozoa within the female reproductive tract as far as the distal utero-tubal junction (UTJ) was documented (England et al., 2013a). A large number of spermatozoa were found to bind to the uterine epithelium, crypts and glands (Doak et al., 1967; England et al., 2006; Rijsselaere et al., 2004; Karre et al., 2004; England et al., 2013a). Progressive clearance of spermatozoa, debris, bacteria, PF, and contents of any added semen extender from the female genital tract is facilitated by myometrial contractions and phagocytosis by polymorphonuclear leukocytes (PMN) (Ribeiro et al., 2006; England et al., 2013b).

Because of the delay from the onset of estrus to ovulation, and the ovulation of primary oocytes, which must mature into secondary oocytes prior to fertilization (Holst and Phemister, 1971), establishment of a sperm reservoir is thought to play an important role in maintaining spermatozoal competence until the time of fertilization. Furthermore, sperm binding to the reproductive epithelium (in all species) is considered to function as a selection process for maintenance of sperm viability and motility (Pacey et al., 2000), and delay of sperm membrane

destabilization as part of the capacitation process (Kawakami et al., 2001; Petrunkina et al., 2004; England et al., 2006; review: Rijsselaere et al., 2014). The site of fertilization in the dog, as in other species, is the uterine tube (oviduct) (Holst and Phemister, 1971). In most domestic species the UTJ and especially the distal segment of the isthmus of the oviduct are reported to function as sperm reservoirs (review: Rijsselaere et al., 2014). In the dog several studies document that uterine glands, along with the distal UTJ are significant sites of sperm storage (Doak et al., 1967; Karre et al., 2004; Rijsselaere et al., 2004; England et al., 2006; England et al., 2013a). No evidence has yet been provided that *in vivo* a sperm reservoir is established in the canine isthmus or ampulla (Rijsselaere et al., 2004; England et al., 2013a). England et al. (2013a) documented very low numbers of spermatozoa within the uterine tubes at any time points of study (6 h, 12 h, 24 h, and 48 h post insemination), and after 24 h the greatest number of bound and free luminal spermatozoa were present within the proximal uterine horns and distal UTJ.

In summary, spermatozoal lifespan is considerably longer in the dog than in many other domestic species. Fertile single matings three days before the LH peak provide evidence that the potential postcoital fertile longevity of canine semen is at least 6 days (Concannon et al., 1983). Even natural matings as early as 7 days before calculated LH surge (9 days before ovulation) have resulted in pregnancy (England et al., 1989).

2.5. Prostatic fluid

2.5.1. Characteristics and constituents

Prostatic fluid constitutes the third fraction of the canine ejaculate and is produced by the prostate, the sole accessory sex gland of the dog (England and Allen, 1990). The PF portion

of the ejaculate is clear and watery in a reproductively healthy dog. The volume depends on the duration of continued collection (1 to 80 mL) (Johnston et al., 2001). The pH averages 6.5, with a normal range of 6.1 to 7.2 (Bartlett, 1962b). Alterations in the pH of PF can occur with inflammatory conditions of the prostate, or excretory system, or could be due to urine contamination during ejaculation. Prostatic fluid (PF) is considered to be the sperm-free portion of the third fraction, and seminal plasma (SP) the sperm-free (post centrifugation) portion of the SRF. The physiological significance of differences is unclear as ejaculation of both portions is confluent and admixture occurs during ejaculation, or at the latest in the female reproductive tract. It is thought that most of the fluid component of the SRF, i.e. seminal fluid, is of epididymal origin, although it can be argued that SP obtained from centrifugation of a pure SRF would also contain certain components of prostatic origin. Dogs treated with finasteride, a type II 5 α -reductase inhibitor that inhibits the conversion of testosterone into dihydrotestosterone (metabolically active androgen in the prostate), were found to have a reduction in prostatic size and a decrease in prostatic secretions (Iguer-Ouada and Verstegen, 1997). The volume of the SRF decreases, concomitantly as sperm concentration in the SRF markedly increases, after treatment for 4 to 8 weeks and only minimal, if any, PF can be collected (Iguer-Ouada and Verstegen, 1997). It is therefore inherently difficult to clearly distinguish between SP fluid components originating from the epididymis or prostate.

The various components of PF and their functions have not been completely elucidated in dogs. Researchers found the presence of electrolytes (sodium, potassium, chloride, phosphate, magnesium) and trace minerals (zinc, copper, iron) in canine PF. They also found high concentrations of cholesterol, bicarbonate, fructose and lactic acid (Bartlett, 1962b;

Branam et al., 1984; England and Allen, 1990), which are assumed to function as energy substrates, and osmotic and pH buffers for spermatozoa as they are transported in the female reproductive tract. Most of the mentioned components were found to be present at a higher concentration in the SRF or SP than in PF (Table 2.1. and Table 2.2.).

Table 2.1. Mean (and range if given) biochemical concentrations in first, second and third fraction from 3 dogs, post centrifugation (Adapted from Bartlett, 1962b).

	1st fraction (Pre-sperm)	2nd fraction (SRF)	3rd fraction (PF)
Bicarbonate (mEq/l)	2.2 (1.7-2.5)	4.2 (2.3-5.4)	2.3 (1.7-2.7)
Ca (mEq/l)	1.4 (0.3-2.2)	0.4	0.5 (0.2-0.7)
Mg (mEq/l)	1.5 (1.3-1.7)	0.8	0.3 (0.1-0.6)
Na (mEq/l)	163.5 (146-195)	192	172
K (mEq/l)	5.8 (4.8-6.)	12.4	7.5
Cl (mEq/l)	159.8 (151.7-171.3)	206.3	160.1
Zn (mg/100 mL)	2.2 (1.71-2.81)	18.4 (16.6-20.74)	7.63 (5.88-9.39)
Fructose (mg/100 mL)	0.5 (0.4-0.6)	2.3 (1.2-3.0)	0.3 (0.2-0.6)
Lactic acid (mg/100 mL)	9.0 (5.8-10.7)	27.2 (20.6-41.2)	16.3 (7.9-21.0)
Total protein (g/100 mL)	21 (17.4-26.1)	37.2	23.4

Table 2.2. Mean (\pm SD) biochemical concentrations in first, second and third fraction of 20 dog ejaculates, post centrifugation (Adapted from England et al., 1990).

	1st fraction (Pre-sperm)	2nd fraction (SRF)	3rd fraction (PF)
Ca (mmol/l)	0.31 (0.14)	0.46 (0.35)	0.31 (0.26)
Mg (mmol/l)	0.85 (0.34)	1.12 (0.53)	0.84 (0.48)
P (mmol/l)	1.14 (1.06)	1.34 (1.09)	0.31 (0.19)
Na (mmol/l)	146 (7.6)	145 (12.4)	143 (6.7)
K (mmol/l)	7.57 (2.6)	10.78 (3.39)	10.27 (2.55)
Total protein (g/l)	14.4 (9.9)	42.5 (41.2)	30.7 (16.8)
Creatinine (μ mol/l)	468 (286)	145 (138)	103 (84)

Arginine esterase, an androgen dependent proteinase, is the most prominent component of the protein fraction, and accounts for more than 50% of total seminal proteins in dogs (Chapdelaine et al., 1984). One study comprehensively evaluated the protein composition

of SP (more accurately the entire second and a portion of the third fraction) by gel-electrophoresis in dogs of unknown fertility and identified 37 protein bands with a large variability in between dogs (De Souza et al., 2007). The majority of bands (85%) had molecular weights < 17 kDA. There was nearly a fourfold variation in SP protein concentration among individual dogs. No correlation between total protein content and seminal parameters was found. Two protein bands (B4 and B5) were positively correlated (r 0.46 to 0.76) with *in-vitro* spermatozoal parameters (sperm motility, vigor, percentage morphologically normal, and plasma membrane intact spermatozoa). This suggests a possible association between seminal or prostatic proteins, *in vitro* spermatozoal characteristics and potentially fertility, although the strength of correlation with spermatozoal parameters is weak to moderate. The presence of certain SP proteins has been found to be associated with high fertility or low fertility in bulls and stallions (Killian et al., 1993; Brandon et al., 1999), as well as post-thaw semen quality in bulls and stallions (Jobim et al., 2004; Jobim et al., 2011).

Capacitation is a reversible plasma membrane destabilization process, regulated by the removal of the glycoprotein layer, and is necessary in preparation for the acrosome reaction (Yanagimachi, 1988; Rota et al., 1999). Cholesterol, Ca^{2+} , and progesterone are involved in capacitation and the acrosome reaction of spermatozoa (Sirivaidyapong et al., 1999; review: Neild et al., 2005; Witte et al., 2009). It is generally accepted, in all species, that seminal fluids play an important role in delaying capacitation by coating sperm membranes with proteins and glycoproteins. In dogs, proteins and glycoproteins of prostatic origin have been suggested to play a role in coating sperm surface progesterone receptors, thereby preventing premature acrosome reaction (Sirivaidyapong et al., 1999).

Antioxidative enzymes have been identified in SP and PF and may play an important role in protecting spermatozoa from oxidative damage (Tulcan et al., 2004; Cassani et al., 2005; Kawakami et al., 2007; Strzezek et al., 2009; Koziorowska-Gilun and Strzezek, 2011; Neagu et al., 2011). Antioxidants are involved in detoxification of reactive oxygen species (ROS), which are byproducts of aerobic metabolism and form part of the respiratory oxidation at the inner mitochondrial membrane (Silva, 2006). Through peroxidation of plasma membrane phospholipids, ROS have been implicated to lead to a loss of sperm motility, viability, and cytosolic elements, as well as metabolic changes and structural alterations in spermatozoa (Cassani et al., 2005). Superoxide dismutase (SOD) is considered the first line defense mechanism against the harmful effects of ROS by removing the superoxide radical ($O_2^{\bullet-}$) from the cytosol of living cells; however H_2O_2 is generated as a consequence ($SOD: O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$). Catalase (CAT) and glutathione peroxidase (GPx) can potentially detoxify H_2O_2 to water, and GPx also removes lipid hydroperoxides (Silva, 2006). Furthermore, vitamins (A, C, and E), glutathione (GSH), ergothioneine (ERG) and other molecules are involved in the inhibition of oxidation pathways and non-enzymatic defense (Silva, 2006). Superoxide dismutase and CAT were found to be present in canine PF, although at a lower concentration, (28.3% and 48.6%, respectively), than in SP (Tulcan et al., 2004). Cassani et al. (2005) documented a substantial amount of SOD activity in canine semen, and a significantly lower concentration of SOD in PF than in SP of the SRF. Other researchers detected the presence of mainly SOD, and to a lesser extent GPx, GSH and ERG, but no CAT activity, in canine sperm homogenates and all three fractions separately (Strzezek et al., 2009; Koziorowska-Gilun and Strzezek, 2011). Kawakami et al. (2007) documented the presence of both SOD and CAT in SP of

Beagle dogs, with a significantly lower activity in five asthenozoospermic dogs. Neagu et al. (2011) found both SOD and GPx to be present in canine SP. Furthermore initial SOD activity in SP was positively correlated with percentage of linear motility after freezing and thawing of semen (Neagu et al., 2011).

Other prostatic fluid components and their functions are largely unknown. For example free zinc was identified as a prostatic antibacterial factor in normal human prostatic fluid and may play a role in natural resistance of the male urinary tract to infection (Branam et al., 1984; Cho et al., 2002; Goericke-Pesch and Hoffmann, 2008). It is likely that Zn^{2+} may have a similar effect in canine PF, but documentation is lacking.

2.5.2. Effects of PF on semen

Given the above-mentioned known properties of PF, one would assume that PF would have positive effects on spermatozoa, including longevity, motility, and fertility. However, the majority of the literature documents conflicting findings, with predominantly negative effects of PF on canine spermatozoa being reported with the use of advanced reproductive techniques (cooling and cryopreservation). Before further review it needs to be considered that not all publications clearly distinguish between PF and SP.

2.5.2.1. Effects of PF on *in vitro* seminal parameters

Early on, it was observed that untreated sperm in the second fraction survive longer when they were not combined with other fractions of the canine ejaculate, particularly the third PF fraction (Freiburg, 1935; Wales and White, 1963). England and Allen (1992) performed a similar experiment, the most prominent finding after incubation (37 °C) of the SRF with either the first or third fraction (PF) was a rapid decline of spermatozoal motility within 2 h.

Furthermore, a significant decrease of morphologically normal spermatozoa was noted. The control sample was incubated in Minimal Essential Medium (containing salts and sodium bicarbonate) and after 4 h of incubation there was no longer a significant difference in sperm motility in comparison with either treatment group. This finding is not surprising considering the absence of energy substrates. However, percentage morphologically normal and live spermatozoa (using an eosin nigrosin stain) remained significantly lower in both treatment groups, suggesting a possible direct detrimental effect of PF.

Foote (1963) found that the third fraction did not affect the survival (motility) of dog sperm when egg yolk or a milk-based extender was added. Rota et al. (1995) compared *in vitro* seminal parameters of spermatozoa in SP or three extenders during refrigeration (4 °C) over four days. The fractionated SRF was centrifuged and re-extended with autologous SP or three extenders (egg yolk-Tris, milk, or cream extender; all containing antibiotics). At day one sperm motility and sperm membrane integrity (using fluorescent stain C-FDA/PI and HOST) were significantly lower in the SP treatment group. A significant decrease in normal acrosome morphology was observed after day three. Antibiotics were not added to SP, which may explain why a significant increase in pH by day three was found with the treatment group with SP. The investigators could not conclude that SP was detrimental, but the addition of semen extenders proved to be beneficial. Sirivaidyapong et al. (2001) compared *in vitro* seminal parameters of the SRF in extender and in PF with extender. Storage at 4 °C for 6 h did not affect seminal parameters (motility, viability, acrosome integrity); however, centrifugation (600 g x 10 min) of semen followed by re-suspension in PF before freezing had an adverse effect on post-thaw progressive motility and viability. Rota et al. (2007) incubated frozen-thawed semen with

autologous PF (1:2) over four hours at 38 °C and their findings suggested an initial reduction of hyperactive motility with PF treatment, with no effect on sperm longevity or acrosome status. A similar experiment carried out by Milani et al. (2010) found no positive effects on motility patterns post-thawing. In 2009, Koderle et al. compared post-thaw spermatozoal motility and chromatin structure (using a sperm chromatin structure assay; SCSA) between cryopreserved semen, which either had SP removed by centrifugation (700 *g* x 5 min) or had not been centrifuged. They found decreased motility and normal morphology in the treatment group in which centrifugation and SP removal had occurred prior to freezing. Chromatin denaturation increased after three hours of post-thaw incubation but there was no significant difference between treatments.

Ejaculated spermatozoa necessarily have experienced exposure to SP and PF, whereas spermatozoa obtained from the epididymides will not have had contact with PF. For the evaluation of frozen-thawed epididymal sperm it was found that the addition of 20% PF before cooling decreased the number of bent principal pieces and cytoplasmic droplets, and the addition of PF after thawing increased progressive motility using a Tris-based extender (Biladyl®; Nöthling et al., 2007). Another study exposed epididymal sperm during the sperm recovery process (slicing of epididymal tail with swim up of spermatozoa) to PF for 20 min, and found an increase in motility parameters immediately after extraction and dilution in PF but no significant differences after freezing-thawing. However, there was an increased proportion of spermatozoa with DNA damage in the PF treatment group at 4 h after thawing than in the Tris treatment group (15.8% vs. 6.7%) suggesting a negative impact of PF on chromatin integrity (Korochkina et al., 2014).

A recent study (Treulen et al., 2012) compared *in vitro* seminal parameters (motility, membrane and acrosome integrity, mitochondrial membrane potential and phosphatidylserine translocation) of cooled semen (5 °C over 72 h) extended after centrifugation with either the addition of autologous SP or PF (1:1) to extender. Motility was found to decrease significantly after 48 h with both SP and PF treatment groups, whereas membrane integrity and acrosome integrity were increased after 48 h and 72 h, in both treatment groups. Phosphatidylserine translocation (a marker for non-return point of cell death) was only significantly reduced with SP and no significant difference was found in mitochondrial membrane potential between all treatments. The authors theorized that there might be natural inhibitors in SP and PF that allow for transient inhibition of motility until sperm reach the female genital tract. A reduction in motility could reduce energy consumption and preserve the functionality of semen for a prolonged period of time (Günzel-Apel and Ekrod, 1991). This could have clinical applications for the cooling and shipping of canine semen. If PF had positive effects on sperm longevity it could be beneficial to include a certain proportion of PF in preparation of the SRF for shipping with extender. To prove the theory of a transient motility inhibition, subsequent dilution of PF extended SRF should ameliorate motility parameters.

Based on above mentioned reports, PF in the absence of semen extenders has been shown to have deleterious *in vitro* effects on canine spermatozoa (Freiburg, 1935; Wales and White, 1963; England and Allen, 1992), whereas admixture of PF with semen extender during a cooling period was shown to have variably positive (Treulen et al., 2012) or negative (Foote, 1963; Rota et al., 1995; Sirivaidyapong et al., 2001) effects. No study to date has investigated concentration dependent effects of PF on seminal parameters. It is also unclear if there is a

critical ratio of PF to SRF that could explain positive or negative effects on cooled shipped canine semen. For cryopreservation, the presence of prostatic fluid is generally avoided (Sirivaidyapong et al., 2001), and established protocols include a centrifugation step with removal of most PF contaminants (review: Linde Forsberg, 2002).

2.5.2.2. Effects of PF on semen *in vivo*

All of the mentioned studies evaluated different aspects of the effects of PF on *in vitro* seminal parameters. There are few published studies that have investigated *in vivo* fertility data with the use of PF as a variable. Nöthling and Volkmann (1993) reported the first *in vivo* study on the effect of PF on fertility. Twenty bitches were vaginally inseminated with frozen-thawed semen; half of the bitches received frozen-thawed semen (vaginal AI) diluted in 7 to 10 mL of PF (treatment group) and the other half were inseminated with frozen thawed semen without PF addition (control group). The treatment group had 100% pregnancy rate, whereas the control group only had 60%. This study did not account for the difference in volume between the treatment and control groups. A subsequent study accounted for this difference by adding an afTALP (albumine free Tyrode's albumine lactate pyruvate) medium to the control group for vaginal insemination of frozen-thawed semen (n = 12 for each group) (Nöthling et al., 2005). The control medium afTALP was chosen as albumin has previously been shown to induce acrosome reaction in dogs (Sirivaidyapong et al., 2000), and as the authors found in a trial experiment that afTALP maintained post-thaw motility better than saline or PF over a period of 150 to 210 min (Nöthling, et al., 2005). The bitches were spayed three weeks after the beginning of diestrus and conception rates were 66% for the PF treatment group and 83% for the control group (no significant difference). A logistic regression model was used to evaluate

the odds of conception. After adjustment for the number of progressively motile spermatozoa per day and the random effect of the bitch, the addition of PF resulted in an increased odds of conception compared to afTALP.

England et al. (2012) found an increase in pregnancy rate and litter size, after transcervical insemination with fresh and frozen semen, when using PF as a flush medium in comparison with phosphate-buffered saline (PBS). Flushing with 1 mL of pooled homologous PF or PBS (control) was performed following insemination with fresh or frozen canine semen. An examination of *in vitro* effects of PF or PBS on spermatozoal parameters was not performed in this study. Schäfer-Somi et al. (2005), documented lower spermatozoal motility and membrane integrity after post-thaw dilution with PBS in comparison with PF, Tris extender, or saline. England et al. (2012) theorized that the increase in pregnancy rate and litter size with the use of PF as a flushing media might relate to a reduction of sperm attachment to PMN in the presence of PF. It was shown that PMN reduce the ability of sperm to attach to uterine epithelium, and this effect was ameliorated by SP and PF, both of which appear to reduce the attachment of PMN to sperm (England et al., 2013b). Canine SP had previously been described to have an immunomodulatory action, with a reported decrease in luminal PMN after insemination of semen in extender with SP in comparison to semen in extender without SP (Ribeiro et al., 2006).

2.5.3. Effects of seminal fluids in other species

In other species the constitution of seminal fluids is inherently different, presumably due to a different complement of the accessory sex glands across species. Research has focused on direct effects on spermatozoa and also indirect effects on the female reproductive tract.

Bovine SP proteins (BSP proteins) have been shown to be involved in sperm membrane lipid modification events that occur during sperm capacitation (Manjunath and Thérien, 2002) and to promote binding of sperm to oviductal epithelial cells (Gwathmey et al., 2003; Gwathmey et al., 2006). In the pig, spermadhesins, protein products of the SP, have not only been found to associate with the spermatozoal surface and modulate capacitation and acrosome reaction, but also to contribute to zona pellucida binding of the oocyte (Töpfer-Petersen et al., 1998). It is theorized that BSP's and spermadhesins, which attach onto the sperm surface during ejaculation, increase membrane stability prior to capacitation and sperm transport (Manjunath and Thérien, 2002). Gradual removal of these compounds in the female reproductive tract subsequently allows capacitation and the acrosome reaction to occur, and for fertilization to take place. In the female reproductive tract exposure to SP has been associated with enhanced sperm transport (review: de Graaf, 2013), hastening of ovulation (Weitze et al., 1990), and increased blood to the uterus and oviduct in the horse and the pig (Bollwein et al., 2001; review: de Graaf, 2013). In the mare, SP has been shown to modulate sperm-induced endometritis (Troedsson et al., 2001), protect viable spermatozoa and promote phagocytosis of dead spermatozoa (Troedsson et al., 2005). It was also postulated that prostaglandins found in SP may aid uterine contractions and sperm transport following mating (Claus, 1990; Troedsson et al., 2005). In camelids, SP induces ovulation through one of its components, identified as beta nerve growth factor (Kershaw-Young et al., 2012; Ratto et al., 2012).

2.5.4. Summary of PF effects in dogs

Overall, the action, effects and importance of canine PF remains controversial and has not been comprehensively evaluated in dogs. Additional complexity results from differentiation between ejaculated sperm versus epididymal sperm, PF versus SP, differences between *in vitro* and *in vivo* conditions, and fresh versus cooled or frozen-thawed sperm. Ejaculated sperm has experienced exposure to SP and PF, whereas epididymal spermatozoa will have had no contact with PF (Nöthling et al., 2007; Korochkina et al., 2014). It has been suggested that during *in vivo* conditions spermatozoa might escape prolonged deleterious PF exposure (Freiburg, 1935; Wales and White, 1963; England and Allen, 1992) by rapidly leaving the uterine lumen and collecting into uterine crypts and glands (England and Allen, 1992; England et al., 2006; Rota et al., 2007). Interactions of PF with the female reproductive tract, such as uterine vasodilation, immunomodulation, influx and function of PMN have been reported (Ribeiro et al., 2006; England et al., 2013b). Clearance of spermatozoa, debris, bacteria, PF, and or semen extender components from the female genital tract is facilitated by myometrial contractions and phagocytosis by PMN (Ribeiro et al., 2006; England et al., 2013b). With progressive clearance, exposure of canine spermatozoa to PF components may decrease. Hypothetically exposure may be increased in comparison with other species, as the canine sperm reservoir has been implicated to be the proximal uterine epithelium and distal UTJ in dogs (Doak et al., 1967; Karre et al., 2004; Rijsselaere et al., 2004; England et al., 2006; England et al., 2013a).

Based on the above assumptions, *in vitro* conditions for cooling and cryopreservation procedures of canine spermatozoa are inherently different from *in vivo* conditions.

Reproductive techniques such as cooling and cryopreservation affect the lifespan, viability and

fertility of semen. Cooling and especially cryopreservation are major "stressors" on spermatozoa as will be further explained later in this review. Prostatic fluid may increase, decrease or have no effect on spermatozoal integrity, function and fertility during such conditions; a concentration dependent effect can be assumed but has yet to be documented.

2.6. Fertility

Fertility is the natural capability to produce offspring. Fertility is an output measurement and can only be established by effective production of a litter (i.e. pregnancy), which is influenced by male fertility, female fertility, time of mating, time and type of AI, and type of semen used. The cause of an absence of pregnancy, e.g. infertility or subfertility, is therefore difficult to elucidate and is often multifactorial. Furthermore, it is hard to quantify fertility. Litter size or fecundity would be the most obvious parameter to measure fertility quantitatively; however, litter size is affected by the same multitude of factors that affect the presence or absence of pregnancy. Fertility trials in canine reproductive research are difficult to carry out for a number of reasons including fewer breeding opportunities due to a monoestrus physiology compared with polyestrous species, and the variability between and within estrous cycles in bitches.

2.6.1. Stud dog fertility

Seminal parameters, ejaculatory frequency, mating capabilities, uninterrupted spermatogenesis, and semen handling or processing influence male fertility in all species when assisted reproductive techniques are used. Despite extensive research, an unknown element of inherent fertility persists, as no single or comprehensive *in vitro* seminal parameter has been established to have a one to one correlation with *in vivo* fertility. This is especially true in dogs,

spermatozoal motility, morphology, viability, longevity, plasma membrane-, acrosome- and DNA-integrity, and total sperm numbers may influence male fertility (Oettle, 1993; Mickelsen et al., 1993; review: Lopate, 2009; Hollinshead and Hanlon, 2012). Correlations between some factors have been identified, such as motility and viability (Foster et al., 2011), and normal morphology and progressive motility (Ellington et al., 1993).

Suggested normal minimal values for a breeding soundness evaluation in the dog are 70% progressive motility (review: Johnston, 1991), at least 70 to 80% morphologically normal (review: Johnston, 1991; review: Lopate, 2009), and > 10 million spermatozoa/kg bodyweight (review: Lopate, 2009). For vaginal artificial insemination in the bitch minimal numbers of 100 to 200 million total motile sperm cells (Gill et al., 1970) or at least 220 million total morphologically normal cells (Mickelsen et al., 1993) have been suggested. For intrauterine insemination with frozen-thawed semen, a minimum dose of 100 million motile sperm cells is generally accepted (Farstad and Andersen Berg, 1989). Semen quality often exceeds these parameters yet fertility of the dog may still be suboptimal (review: Lopate, 2009). Conversely, dogs with as low as 10% progressive motility and 9% normal morphology and total numbers as low as 36 million have been demonstrated fertile by breeding (England and Allen, 1989; Mickelsen et al., 1993).

2.6.2. Effect of prostatic disease on fertility

The influence of canine prostatic disease on fertility is insufficiently described in the literature. Painful or inflammatory prostatic diseases can negatively affect breeding behavior with refusal of copulation, or failure of ejaculation, or can have deleterious effects on semen quality due to bacterial or inflammatory components in the ejaculate. Benign prostatic

hyperplasia (BPH) is the most common prostatic disease in intact male dogs (Johnston et al., 2001) and is also thought to affect fertility (Krawiec and Heflin, 1992; Johnston et al., 2001; Goericke-Pesch and Hoffmann, 2008; review: Lopate, 2012). Hemospermia is a common clinical sign of BPH, although red blood cells are not thought to affect spermatozoa negatively. During a natural mating blood components are physiologically present in the reproductive tract of the bitch and will mix with the ejaculate without affecting fertility. Therefore changes in the composition of the prostatic fluid have been postulated to explain possible infertility or subfertility in dogs with BPH (Goericke-Pesch and Hoffmann, 2008).

The reduction of prostatic fluid volume during treatment of BPH with finasteride did not negatively affect fertility (Iguer-Ouada and Verstegen, 1997; Sirinarumitr et al., 2001). Possible conclusions are that PF is not absolutely necessary for a fertile mating, or that very small amounts of prostatic secretions are sufficient to exert specific beneficial effects on spermatozoa.

2.7. Semen evaluation

The basic parameters for the evaluation of an ejaculate consist of volume, motility, morphology and concentration. Volume and color are routinely recorded immediately after collection.

2.7.1. Motility

Motility can be evaluated using bright field or phase contrast microscopy by placing a drop of fresh or extended semen on a pre-warmed microscope slide covered with a coverslip and evaluating several fields at 100 to 400x magnification on a warmed stage. Subjective assessment of the percentage of total motility and progressive motility is recorded. Motility is

artificially increased near air bubbles and decreased at the edge of a cover slip (Johnston et al., 2001).

Objective systems for evaluation of spermatozoal motility are available, e. g. computer assisted sperm analysis (CASA) systems. They offer a rapid, accurate and simultaneous assessment of different seminal parameters. Such as total motility (TM), progressive motility (PM), linearity of sperm movement (LIN), straightness (STR), wobble (WOB), beat cross frequency (BCF), amplitude of lateral head displacement (LHD), in addition to different velocity parameters (average path velocity, VAP; curvilinear velocity, VCL; straight line velocity, VSL). Parameter settings (frame rate, image settings), software, and concentration can influence results and require further standardization for reproducible results (review: Verstegen et al., 2002).

2.7.2. Morphology

The evaluation of spermatozoal morphology, is considered a standard part of a routine semen evaluation. As is the case for other species, sperm morphology can affect fertility but minimal values have not been conclusively established for the dog. A breeding trial in dogs suggested the normal morphology percentage below which fertility was adversely impacted to be 60% (Oettle, 1993), whereas others recommend a minimum of 70% or 80% normal morphology in a breeding soundness evaluation (review: Johnston, 1991; review: Lopate, 2009).

Sperm morphology can be evaluated using phase contrast or differential interference contrast (DIC) microscopic evaluation of wet mount preparations, or bright field microscopic evaluation of stained preparations (eosin-nigrosin or Wright-Giemsa stain). Visualization of the

various specific morphological abnormalities depends on microscopic method. For contrast microscopy a small semen sample is fixed in formalin buffered saline (FBS), a drop (5 to 10 μ l) is placed on a microscope slide, covered by a coverslip, and evaluated at 1000x with immersion oil under the microscope. At least 100 to 200 spermatozoa are counted under 1000x and according to morphology classified as normal, acrosome -, head shape -, or midpiece abnormalities, detached head, or tail defects (kinked or coiled), retained proximal or distal cytoplasmic droplets. Spermatozoa can have more than one abnormality in which case both may be counted or the more severe abnormality is counted, depending on operator preference. Classification systems from other species can be applied to canine semen, such as primary and secondary (occurring during or after spermatogenesis), minor and major (based on effect on fertility), or compensable and non-compensable defects (Blom, 1972; review: Saacke, 1990; review: Chenoweth, 2005). Background or cellular stains can be used but iatrogenic artifacts can be induced during preparation (e.g. detached heads, bent or coiled tails, or reflex midpieces) (Johnston et al., 2001).

2.7.3. Concentration

Determination of sperm concentration involves enumerating spermatozoa contained in a defined volume of semen. It is essential for calculation of total sperm numbers, which is obtained by multiplication of the number of spermatozoa per mL with the total ejaculate volume. The most commonly used method to evaluate sperm concentration is a hemacytometer (Neubauer) counting chamber (Root Kustritz et al., 2007); alternative automated systems are a fluorescence-based system ("Nucleocounter"; NucleoCounter®SP-

100™; ChemoMetec A/S, Denmark), a spectrophotometer (Batista et al., 2012), CASA, or flow cytometry.

To perform a sperm count with a hemacytometer, a 1:100 dilution of semen in FBS is prepared and both sides of the counting chamber are loaded with 10 µl of the dilution under the coverslip. The spermatozoa need to settle for 5 min in a humidified container to prevent evaporation before counting. Using bright field or contrast microscopy and a 20x to 40x objective, the number of sperm heads within the central 25 squares is counted. The count is performed on both sides of the hemacytometer and the average result calculated, which represents the sperm concentration in millions per mL. If less than 200 cells are counted, a lower dilution should be prepared and an appropriate correction factor applied to calculate concentration. Variations can occur due to different hemacytometer design and due to operator error (counting method, semen handling, and sampling technique). To establish consistency at least two chambers should be counted with no greater than 10% difference between the counts. To achieve high precision a duplicate count by two technicians has been recommended (Christensen et al., 2005).

The NucleoCounter is an automatic instrument that uses an integrated fluorescent microscope system to detect signals from a fluorescent dye (propidium iodide) bound to the nuclei of sperm. Results from studies using boar and bull semen have demonstrated that results obtained with the NucleoCounter agree with those obtained with flow cytometry (Hansen et al., 2005; Anzar et al., 2009).

2.7.4. Ancillary semen tests

2.7.4.1. Plasma membrane integrity

Various methods exist to evaluate sperm plasma membrane integrity, which is considered an important measurement of spermatozoal viability. An easy to use clinical test is the hypo-osmotic swelling test (HOST) (England and Plummer, 1993; Kumi-Diaka, 1993; Pinto and Kozink, 2008), which evaluates the functional intactness of the sperm membrane by incubating a semen sample in a hypo-osmotic solution. Spermatozoa with intact plasma membrane experience an influx of water and expansion of the sperm membrane under hypo-osmotic conditions (Kumi-Diaka, 1993). Ten μl of semen are added to 100 μl of a 100-mmol hypo-osmotic sucrose solution and incubated at 37 °C for 1 min (Pinto and Kozink, 2008). Using bright field or contrast microscopy, 200 spermatozoa are counted as curled (intact plasma membrane) or straight (impaired plasma membrane). The percentage of intact spermatozoa is calculated by dividing the number of curled spermatozoa by the total number of spermatozoa.

Fluorescent probes are used to evaluate a number of different sperm characteristics, and can be used either with epifluorescence microscopy or flow cytometry. Flow cytometry has the advantage of being able to assess a great number (> 10,000) of spermatozoa in a short period of time (review: Nizanski et al., 2012). A general disadvantage of flow cytometry is considered to be a systematic overestimation of the proportion of unstained cells where the non-sperm particles are not excluded from analysis by additional identification other than light-scatter characteristics (Petrunkina and Harrison, 2010).

The sperm membrane is directly or indirectly related to many sperm functions, e.g., it is essential to maintain homeostasis, motility and the capacity to interact with the environment.

Sperm membrane integrity can be assessed with flow cytometry by use of various fluorescent probes. The combination of SYBR-14 and propidium iodide (PI) is one of the most widely used (review: Martinez-Pastor et al., 2010). SYBR-14 is membrane permeable dye, staining viable sperm heads green. The probes PI or ethidium homodimer (EthD) are membrane impermeable probes, that only gain access to cells with a compromised plasmalemma, emitting red fluorescence when binding to nucleic acids and quenching SYBR-14 fluorescence (Garner and Johnson, 1995).

Membrane stability and permeability are two closely interlinked functions (review: Hossain et al., 2011). Increased membrane permeability is considered an indicator of apoptosis. Apoptosis-like changes are thought to occur with sperm processing, freezing/thawing, or other stresses, and is believed to impair sperm function and fertilizing potential (Martin et al., 2004; review: Nizanski et al., 2012). The impermeable membrane probe YO-PRO-1 iodide, is a green carbocyanine dye and amphipathic small molecule, that can leak in after destabilization of the membrane, most likely as a result of silencing of a multidrug transporter involved in actively transporting the dye out of intact cells. Subviable cells lack appropriate amounts of ATP to transport YO-PRO-1 back out of the cell. In the early stages of apoptosis, a modification of membrane permeability occurs, which selectively allows entry of some non-permeable DNA-binding molecules. This subpopulation may show early damage or a shift to another physiological state. Using YO-PRO-1 in combination with EthD-1, four cell populations can be distinguished (Pena et al., 2005). First, an unstained population with stable plasma membranes. Second, an early apoptotic population, unable to pump out YO-PRO-1, but still impermeable for other dead-cell discriminatory dyes like EthD-1 (YO-PRO-1 positive, EthD-1 negative). EthD-1

penetrates through damaged membranes only, emitting red fluorescence when binding to DNA. With increasing loss of membrane permeability two additional sub-populations are distinguished, termed early and late necrotic spermatozoa. Early necrotic cells emit both red and green fluorescence (YO-PRO-1 positive, EthD-1 positive), whereas late necrotic cells emit only red fluorescence as EthD-1 quenches or displaces YO-PRO-1 (YO-PRO-1 negative, EthD-1 positive) (Pena et al., 2005). This stain has previously been used in dogs to evaluate the use of antioxidants during cryopreservation (Neagu et al., 2010).

2.7.4.2. Acrosome integrity

The acrosome reaction is a prerequisite for successful penetration of the oocyte. By fusion of the plasma membrane with the outer acrosomal membrane, spermatozoa release their acrosomal enzymatic content (hydrolytic enzymes), which together with hyper-activated motility facilitates penetration of the zona pellucida. Cells undergoing the acrosome reaction before reaching the isthmus and before zona binding are rendered infertile (Silva and Gadella, 2006). Certain stains, like a Giemsa stain, provide good detail for evaluation of the acrosome under bright field microscopy (review: Lopate, 2009). Fluorescent-conjugated lectins such as *Pisum sativum* agglutinin (green pea, PSA), or *Arachis hypogaea* agglutinin (peanut, PNA) conjugated to fluorescein isothiocyanate (FITC) can be used for evaluation under fluorescent microscopy or with flow cytometry (Cross and Watson, 1994). The number of acrosome-reacted cells is calculated by dividing the number of acrosome-reacted spermatozoa by the total number of spermatozoa. While PSA binds to acrosomal contents (saccharide groups of the glycoprotein pro-acrosin), PNA interacts with the glycoconjugates (β -galactose moieties) of the outer acrosomal membrane (Cross and Watson, 1994; Pena et al., 1999; Szasz et al., 2000; Silva

and Gadella, 2006). Acrosomal integrity of canine sperm has been previously assessed using flow cytometry and staining with FITC-PSA or FITC-PNA and membrane impermeable propidium iodide (PI) or ethidium homodimer1 (EthD-1) (Kawakami et al., 1993; Pena et al., 1999; Sirivaidyapong et al., 2000; Szasz et al., 2000; Cheuqueman et al., 2012). The absence of the fluorescence on the living sperm is indicative of an intact acrosome, and fluorescence is indicative for acrosome disruption or acrosome reaction (Silva and Gadella, 2006). Binding sites may disappear after extensive acrosomal damage, producing false negative staining (review: Martinez-Pastor et al., 2010). Some workers favor PNA over PSA, as PNA is believed to display less non-specific binding to other areas of the spermatozoon (review: Graham, 2001), and it is the lectin of choice when evaluating sperm extended in egg yolk containing media, as PSA has a nonspecific binding affinity to egg yolk (Thomas et al., 1997; review: Hossain et al., 2011). Other binding sites could easily be distinguished using epifluorescence microscopy, whereas flow cytometry only identifies one signal from the entire spermatozoon (review: Hossain et al., 2011).

2.7.4.3. Sperm chromatin structure assay (SCSA)

Sperm chromatin structure assay (SCSA) is the most commonly used and a relatively simple test for assessing sperm chromatin by flow cytometry (review: Martinez-Pastor et al., 2010). It was originally developed by Evenson et al. (1980) and has since been applied to a variety of species. It determines the extent of DNA denaturation following acid treatment in each individual spermatozoon, by measuring the metachromic shift from green to red fluorescence for acridine orange (AO). Acridine orange intercalates in the DNA and fluoresces green when associated with double-stranded DNA (dsDNA) and red when associated with

single-stranded DNA (ssDNA). The in-situ denaturation step induces the formation of ssDNA from breakages, therefore each sperm head yields a mixture of green and red fluorescence when interrogated with a 488-nm laser, depending on the DNA fragmentation (number of nicks) and the susceptibility to denaturation (Figure 2.1.).

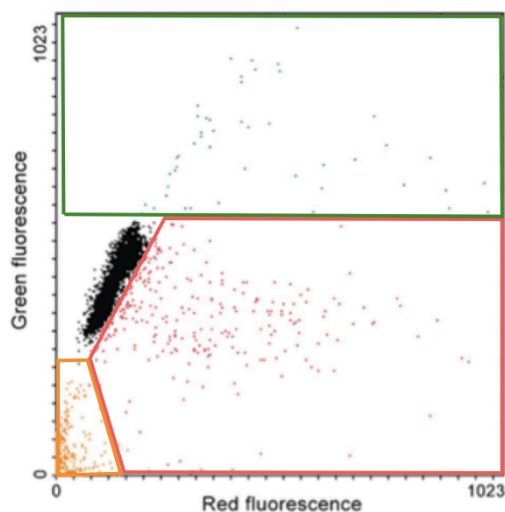


Figure 2.1. Sperm chromatin analysis by acridine orange staining. Intact spermatozoa are indicated in black, cells with increased amount of single-stranded DNA (indicating DNA damage) are indicated in red, and spermatozoa with immature chromatin are indicated in green. Cell debris is indicated in orange and is gated out during analysis. Adapted from Hossain et al., 2011. Copyright © 2011 by Shanghai Materia Medica, Shanghai Jiao Tong University. Reprinted by permission of Shanghai Materia Medica, Shanghai Jiao Tong University.

Causes for the detection of DNA damage include residual apoptotic cells passing through the seminiferous tubules, apoptosis occurring in late mature cells, oxidative stress, heat stress, radiation injury, or protamine deficiency. One of the major DNA damaging agents is considered to be reactive oxygen species (ROS) (Evenson and Wixon, 2006). The DNA fragmentation index (DFI) is calculated for each spermatozoon (ratio red/total fluorescence). It is used to refer to both individual fluorescence ratio (DFI, formerly termed α_t) and to the total percentage of sperm with moderate and high DFI (%DFI, formerly termed $\text{COMP}\alpha_t$). The %DFI therefore expresses the percentage of cells with high susceptibility to low pH-induced DNA

denaturation. A high %DFI has been related to reduced fertility, low blastocyst rate after intracytoplasmic sperm injection (ICSI), longer times to pregnancy and higher spontaneous miscarriage rates in humans (Virro et al., 2004; Evenson and Wixon, 2006). Spermatozoa with damaged DNA may be able to fertilize an oocyte, but could disturb epigenetic regulation of the early embryo, block further development (incompensable defect) and result in early embryonic loss (Lewis and Aitken, 2005; Everson and Wixon, 2006).

In domestic species a high %DFI has been connected to lower fertility in boars (Evenson et al., 1994; Boe-Hansen et al., 2008), bulls (Ballachey et al., 1987; Waterhouse et al., 2006), stallions (Love and Kenney, 1998; review: Love, 2005; Morrell et al., 2008), and rams (Nordstoga et al., 2013). It appears that the threshold for subfertility in bulls (10 to 20%) and boars (8%) is much lower than for humans (>30%, Everson and Wixon, 2006).

The SCSA has also been successfully used for the assessment of dog semen (Garcia-Macias et al., 2006). Kim et al. (2010a) found the %DFI of frozen-thawed dog semen to be on average 6.47%, while that of fresh canine semen was 1.59%. Koderle et al. (2009) found no difference in %DFI before and immediately after cryopreservation for centrifuged and non-centrifuged (containing SP) canine semen, but noted an increase in %DFI after 3h of incubation of frozen-thawed semen for both treatment groups.

2.8. Cooling of canine semen

Preservation of semen by cooling and subsequent storage is an important technology used in canine reproductive management. The first successful use of chilled canine semen dates back to 1954 (Harrop). Cooling reduces the metabolic activity of spermatozoa, thus preserving sperm quality parameters, such as motility and viability. The maintenance of fertility is limited

but it has been reported that the quality (motility) of cold stored canine semen (4 to 5 °C) is maintained for up to 8 (Ponglowhapan et al., 2004) to 16 d (Verstegen et al., 2005), while fertility potential has been conserved for up to 10 d (Verstegen et al., 2005). Cold shock is cellular injury that occurs during cooling to 1 °C, either directly by disrupting cellular structures or indirectly by altering cellular function. Cold shock results in a lower proportion of motile spermatozoa, circular movements, diminished energy production, leakage of ions and molecules from a more permeable plasma membrane, and cell death (review: Amann and Pickett, 1987). Canine semen is considered to be relatively tolerant to cold shock in comparison with other species (Johnston et al., 2001). Two different cooling rates (-2.25 °C/min and -0.2 °C/min) were recently compared for cooling canine semen from 23 to 5 °C. No difference in seminal parameters was found when semen was subsequently stored for 96 h at 5 °C (Rodenas et al., 2014). The ability of spermatozoa to resist cold shock is thought to relate to the lipid composition of the sperm membrane, specifically the amount of unsaturated fatty acyl chains and cholesterol content, which differs for each species (Darin-Bennett et al., 1974; review: Farstad, 2009).

The fluid mosaic model (Singer and Nicolson, 1972) describes the cell membrane as a dynamic bilayer of polar phospholipids, with their hydrophilic head groups orientated to the outside and hydrophobic fatty acyl chains to the inside of the membrane. The fatty acyl chains are the major determinant of whether the membrane is in a fluid or gel phase state at any particular temperature (Quinn, 1989). Other important components of the cell membrane are proteins and cholesterol, which are intermingled within the lipids. Cholesterol specifically intercalates in the fatty acyl portion of the membrane and helps to stabilize the membrane at

body temperature (review: Graham, 2011). With cooling, lipids undergo a phase transition, from fluid into a gel or solid state, in which the fatty acyl chains are increasingly rigid and parallel to each other. Longer fatty acyl chains transition into a solid state at higher temperatures. Fatty acids with more unsaturated bonds exhibit lower transition temperatures. Parts of a membrane with the highest transition temperature will undergo phase transition earlier and cluster as a solid lipid 'iceberg' in a sea of fluid lipids (Hammerstedt et al., 1990); with continued cooling, eventually the entire membrane will turn into a gel-like state. Cholesterol stabilizes cell membranes in that it decreases fluidity at higher temperatures and increases fluidity at low temperatures (review: Graham, 2011). During the cooling period the borders between these initial 'iceberg' microdomains create ion-permeable gaps, which are unstable and can lead to membrane fusion or rupture. Furthermore proteins associated with these domains may cease to function. Also during cooling or upon rewarming, the non-bilayer preferring lipids within any microdomain may not return into apposition with the proper protein but aggregate in a hexagonal-II phase. This may also induce excessive membrane permeability, instability, or even cause membrane disruption and capacitation-like changes.

Another effect of cooling is a reduction of enzyme and transport activities of proteins at lower temperatures, with an accumulation of sodium and calcium ions within the cell. All of these processes can have detrimental effects on cell homeostasis, can induce acrosome reaction, affect cell motility or morphology, and ultimately cause a loss of fertilizing potential.

To counteract the damaging effects of cooling, semen extenders with cryoprotective properties are commonly added to semen. Components of semen extenders facilitate the following functions: nutrition, buffering capability, osmotic stability, antimicrobial activity, and

cryoprotection. Commonly used cryoprotective additives to reduce cold-shock injury are low-density lipoproteins found in egg yolk, skim milk, or cream. Others, less commonly used additives are ethylenediaminetetraacetic acid (EDTA), butylated hydroxytoluene (BHT), and phosphatidylserine. The exact mechanism and mode of interaction of these cryoprotectants is not completely understood, but it is generally accepted that they exert a stabilizing effect on spermatozoal plasma membranes during cooling.

2.9. Cryopreservation

Successful cryopreservation of semen allows for indefinite storage of semen and is a popular method to preserve genetic potential for future use. Freezing (liquid nitrogen -196 °C) and thawing of semen is considerably more 'stressful' to spermatozoa than cooling to 4 to 5 °C. The cryopreservation process can cause irreversible damage to spermatozoa. There are four main causes of spermatozoal damage that occur during cryopreservation: ice crystal formation, reactive oxygen species generation, osmotic stress, and apoptotic-like changes.

Cryoprotective agents are added to semen in order to minimize processing damage. Egg yolk (EY) protects cell membranes against cold shock. It prevents or restores the loss of phospholipids from the membrane; however, the exact mechanism is unknown. Egg yolk is not a defined entity, but a complex biological fluid containing proteins, vitamins, phospholipids, glucose, and antioxidants, which are all potentially useful for cell membrane integrity (review: Farstad, 2009). Cryoprotectants, such as glycerol, ethylene glycol, or dimethylsulfoxide, are membrane-permeable compounds that form strong bonds with water molecules, competing with hydrogen bonds between water molecules, thus lowering the freezing point and decreasing crystal formation during the freezing process. Glycerol is the most commonly used

cryoprotectant in canine freezing extenders. There are a variety of freezing protocols using different extenders, cryoprotective agents, sperm and cryoprotectant concentrations, cooling rates, freeze methods, freeze rates, storage methods, and thawing protocols.

2.9.1. Ice crystal formation

During freezing, ice crystals will form from water molecules, while remaining solutes and ions will subsequently increase osmolality of the surrounding fluid. It was proposed that when intracellular ice crystals form during rapid cooling, cells are killed by chemical toxicity or osmotic stress, and by mechanical damage caused by phase separation of solution and crystal growth (review: Mazur, 1984). To avoid formation of intracellular ice crystals, specific cooling rates and cryoprotective agents, such as glycerol, ethylene glycol, or dimethylsulfoxide, are used in extenders. Intracellular ice formation is unlikely to occur with currently used cryopreservation protocols and cryoprotectants (review: Morris et al., 2012).

Extracellular ice crystal formation still occurs causing a considerable increase in the osmolality in the fluid surrounding spermatozoa within developing ice channels. The loss of spermatozoal viability encountered with rapid cooling is likely due to osmotic imbalance and recrystallisation during thawing (review: Morris et al., 2012).

2.9.2. Osmotic stress

During cryopreservation spermatozoa are exposed to periods of hypo- and hyperosmotic extra-cellular environments. The addition of cryoprotective agents exposes cells to a hypertonic environment. This causes an initial decrease in cell volume, with subsequent re-expansion to the initial volume as the cryoprotectant enters the cell and equilibrates across the plasma membrane (Figure 2.2.). The membrane permeability of the cryoprotectant determines

the time required for equilibration but is expected to occur at a slower rate than that for water. During freezing, the cells again shrink as ice crystals form in the extracellular space. The increased concentration of ions and solutes in the shrinking fluid filled ice channels enhances osmotic stress and cell dehydration, until the remaining fluid and the cells vitrify (freeze in a glass-like state without crystal formation).

A second period of osmotic stress occurs during thawing (Figure 2.2). Spermatozoa still in thawed freezing extender are initially exposed to a hypo-osmotic environment created by melting ice crystals, and as a result, there is an increase in cell volume until solute concentration equilibrates.

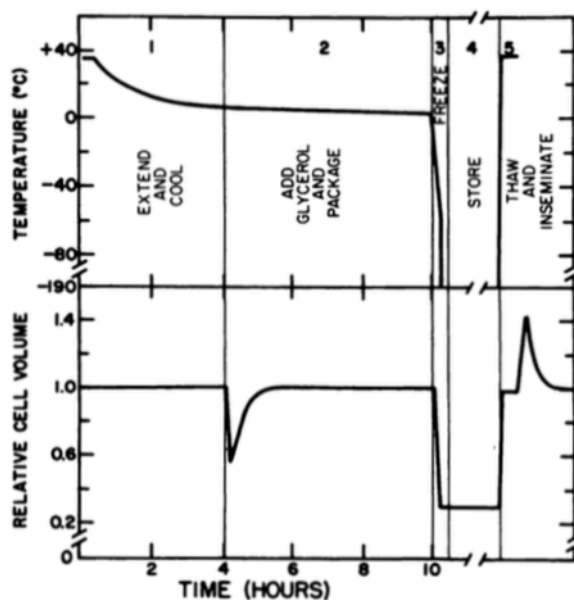


Figure 2.2. Changes in temperature and cell volume associated with a cryoprocessing cycle for bull sperm. A time-event profile associated with a successful (but perhaps not optimum) cryopreservation process for bovine sperm is provided, with change in temperature (top panel) and change in cell volume (bottom panel) over time. This representation, adapted from a presentation by Schneider (1986) for embryos, divides the process into five stages and emphasizes both the dynamic stage of the membrane and the short time-period over which the sperm are expected to respond to changes in their environment. From Hammerstedt et al., 1990. Copyright © 1990 by Wiley. Reprinted by permission of Wiley.

While the effects of osmotic stress have been investigated in other species, there has been little information regarding the effects of osmotic stress on canine spermatozoa during cryopreservation (review: Eilts, 2005). Spermatozoal total motility and membrane integrity decreased in hyperosmotic fluid (> 500 mOsm) dependent on the type of extender used. Despite their sensitivity to osmotic stress, canine spermatozoa were found to tolerate cell shrinkage and re-expansion with exposure to hypertonic solutions of permeating cryoprotectants well (especially glycerol and ethyleneglycol) (Songsasen et al., 2002).

2.9.3. Reactive oxygen species

The extent of molecular oxidation or peroxidation is increased when sperm cells are subjected to centrifugation and or cryopreservation (Agarwal et al., 2003; Silva, 2006; Kim et al., 2010a; Chatdarong et al., 2012). Molecular oxidation affects cell function and can lead to cell degeneration or death. Free radicals are a major factor in inducing peroxidation and impaired function of proteins, lipids and DNA. Some molecular substrates must be oxidized for spermatozoa to acquire fertilizing capability; free radicals are involved in capacitation, acrosome reaction, and penetration of the oolemma (Silva, 2006). Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and the cellular capacity to detoxify ROS or to repair damage caused by ROS. Reactive oxygen species are products of aerobic metabolism, derive from the reduction of oxygen, and include radicals and other reactive products. Radicals are atomic or molecular species that have unpaired electrons in their orbits, making them unstable, as they will be involved in cellular reactions in order to displace or share these electrons.

Spermatozoa are sensitive to lipid peroxidation due to their high content of polyunsaturated fatty acids, limited cytoplasmic enzymatic scavenging consortium, and inability to resynthesize membrane components (Agarwal et al., 2003; Michael et al., 2007). Peroxidation of proteins and carbohydrates can impair cell function and homeostasis. Oxidation of DNA may result in protein to DNA crosslinking which may interfere with chromatin unfolding, DNA repair, replication and transcription, leading to single or double strand breaks, which in turn, may inactivate genes, lead to protein deactivation and accelerate apoptosis (Agarwal et al., 2003; Silva, 2006). Spermatozoal mitochondrial DNA is not coated with histone proteins, and has a 10 to 100 fold higher mutation rate (Silva, 2006). Spermatozoa have a minimal cytoplasmic compartment with limited intracellular scavenging enzymatic consortium, and rely predominantly on extracellular sources (SP). Cells become more susceptible to oxidative stress when separated from the anti-oxidative activity of SP (processing with centrifugation) (Silva, 2006).

2.9.4. Apoptotic-like changes

Apoptosis is defined as the process of programmed cell death that affects single cells and induces a series of cellular, morphological and biochemical alterations, leading to cell suicide, without any related inflammation in the surrounding tissue. Apoptosis seems to be strictly regulated by extrinsic or intrinsic factors and can be triggered by a variety of stimuli. It occurs as a normal consequence of development and as a result of cellular stress. As described for numerous other species (Ball, 2008), cryopreservation has been implicated to cause apoptotic like changes in canine spermatozoa (Sokolowska et al., 2009; Kim et al., 2010b; Neagu et al., 2010; review: Nizanski, 2012).

Apoptosis is thought to be a key regulator of spermatogenesis and responsible for the control of male gamete overpopulation. Ejaculated spermatozoa have been shown to demonstrate changes consistent with apoptosis; it is unclear whether this process is triggered during spermatogenesis or at a post-testicular level (Agarwal et al., 2003). Initiation of apoptosis can result from extrinsic factors that bind to cell-surface death receptors (Fas) and switch on the caspase cascade. Intrinsic factors like high levels of ROS disrupt the inner and outer mitochondrial membrane, resulting in a release of cytochrome-C protein that activates caspases and induces apoptosis (Agarwal et al., 2003; Silva, 2006). Other intrinsic factors implicated to induce apoptosis are osmotic stress, heat, hypoxia, DNA damage, or lack of pro-survival factors.

Apoptotic like changes in canine spermatozoa after cryopreservation have been implicated to relate to detectable changes like phosphatidylserine translocation across the plasma membrane (Kim et al., 2010b), increased membrane permeability (Sokolowska et al., 2009; Neagu et al., 2010), decreased mitochondrial membrane potential (Kasimanickam et al., 2012), increased DNA fragmentation (Kim et al., 2010a), and increased caspase activity (Sokolowska et al., 2009). However, controversy remains concerning the significance of these findings, the role of apoptotic changes subsequent to cryopreservation, and the effect of apoptosis on fertility (Ball, 2008).

CHAPTER 3

THE EFFECTS OF PROSTATIC FLUID ON FUNCTIONAL CHARACTERISTICS OF COOLED CANINE SEMEN

3.1. Introduction

The goal of this research project was to investigate the effect of prostatic fluid (PF) in cooled canine semen using *in vitro* semen parameters; specifically, to determine the optimal dilution ratio of PF for use in cooled semen. The objective of this study was to measure sperm kinetics (motility parameters), plasma membrane integrity and stability, acrosome integrity, and DNA fragmentation after the addition of 0%, 10%, 25%, or 50% PF to extended semen from fertile dogs. Assessments were made at 0 h pre-cooling, at 24, and 48 h of cooled storage (4 °C); and after freezing and thawing followed by incubation (37 °C) at 0, 4 and 24 h.

Our hypothesis was that extending semen from fertile dogs with lower dilutions of PF in an egg yolk-Tris extender would improve plasma membrane stability and acrosome integrity, and preserve sperm kinetics and reduce DNA fragmentation in comparison with higher concentrations of PF during cooling.

3.2. Materials and methods

Animals included in the study were four healthy, client-owned dogs with established fertility (produced a litter within the last 6 mo): two Labrador retrievers, one boxer and one dachshund (age 2.2 to 2.8 y). All procedures were in accordance with the Louisiana State University Institutional Animal Care and Use Committee and Clinical Protocols Committee and all owners signed informed consent forms approved by the Clinical Protocol Committee.

3.2.1. Semen and PF collection

Prior to the experiments, nine dogs with established fertility were initially examined to evaluate minimal inclusion criteria. A clinical exam and trans-abdominal ultrasound evaluation of the prostate were performed. Semen collection was facilitated by manual stimulation of the bulbus glandis in the presence of an estrous bitch. Fractionated collection of the sperm rich (SRF) and PF fractions of the ejaculate were performed by digital manipulation and collection into separate sterile collection cones. The dogs were monitored for normal penile detumescence and retraction within the prepuce. A routine semen evaluation (volume, color, motility, morphology and concentration) was performed on each ejaculate. Exclusion criteria were signs indicative of prostatitis (heterogeneous parenchyma with or without prostatic enlargement on ultrasound examination or neutrophilia on a cytological specimen of the PF), or a low total sperm count ($< 600 \times 10^6$ spermatozoa), or low motility or normal morphology ($< 70\%$ for both parameters). Two dogs were excluded from participation in the study due to low semen quality (motility and morphology), one dog due to low total sperm numbers, one dog due to blood tinged PF containing neutrophils and red blood cells, and one dog that had been enrolled was not presented by the owner on the day of the experiments.

The PF from the four fertile dogs meeting all inclusion criteria was individually centrifuged ($5,000 \times g$ for 10 min at 4°C ; Sorvall Legend X1, Thermo scientific; Pittsburg, PA), the supernatant was removed and filtered (Millex-GS Syringe Filter, SLGS033SB; pore size $0.22 \mu\text{m}$, EMD Millipore; Billerica, MA). The pH of the PF supernatant was measured (Corning 440 pH meter; Corning, NY); all samples were within the acceptable range of 6.1 to 7.2, and were stored at -20°C for later use in the experiments.

3.2.2. Semen processing overview

On three days (replicates), at least three weeks apart, the four fertile dogs were presented sequentially within 30 min from each other. The SRF was collected individually as described above and evaluated for volume, color and motility. Each ejaculate was immediately extended 1:1 (v/v) with a pre-warmed (37 °C) egg yolk-Tris extender (EYT extender part 1) (Hermansson and Linde Forsberg, 2006). During the first two experimental days, the PF fraction was also collected, centrifuged, filtered and frozen as described above, and used for the subsequent replicate.

Immediately after semen collection and evaluation of the last individual dog (within 2 h from the collection of the first dog), the four extended ejaculates were pooled in 50-mL polypropylene tubes (Corning®, NY) and analyzed for sperm motility, morphology, concentration (NucleoCounter®SP-100™; Chemometec; Allerød, Denmark), volume, and pH. The previously frozen PF samples were thawed in a 37 °C water bath (Isotemp 2340; Fisher-Scientific; Waltham, MA) and pooled, with the PF from each dog contributing an equal volume (2 mL). Pooling of the sperm rich fractions and PF was done to reduce the effect of individual variations in response to cooling and freezing, and to provide adequate numbers of spermatozoa per treatment.

Five aliquots of the pooled SRF were prepared and centrifuged (Centrifuge 5804; Eppendorf; Hauppauge, NY) in 15-mL tubes (Corning®, NY) at 700 x *g* for 8 min. The previously pooled PF was mixed with EYT extender part 1 at room temperature (21 °C) to create 0%, 10%, 25% and 50% PF-extender solutions (treatment groups PF0, PF10, PF25 and PF50, respectively). After centrifugation of the SRF and removal of the supernatant, each sperm pellet was

extended with one of the prepared PF-extender solutions. Concentration was measured with integrated fluorescence microscopy. Each aliquot was further extended to a final spermatozoal concentration of 200×10^6 cells/mL and stored in 4.5-mL polypropylene vials (Nunc®; Sigma-Aldrich; MO).

The 4.5-mL vials were then placed in 50-mL tubes containing water at room temperature (water jackets) for additional insulation and temperature stability. Cooling was performed in a refrigerator at 4 °C. Equilibration to 4 °C was achieved after 1.5 h, at which time one of two control treatments (0% PF-extender solution) was processed for immediate freezing (PF0-frz), whereas the remaining four aliquots were stored at 4 °C for 48 h (PF0, PF10, PF25 and PF50). Cooling rate (-0.2 °C per min on average), time to equilibration, and storage temperature were monitored with a thermocoupler (Atkins T Thermocouple; Atkins Technical Inc.; Gainesville, FL). At 0, 24, and 48 h of cooled storage, 150-μL aliquots were removed from each treatment to evaluate sperm kinetics, plasma membrane integrity and stability, acrosome integrity, and DNA fragmentation. After 48 h, the remaining samples were frozen in 0.5-mL French straws (MOFA Global; Verona, WI) and stored in liquid nitrogen until thawed. Two straws of semen were thawed for each treatment group. Five minutes after thawing, the semen was slowly mixed with an equal volume of thaw medium over 5 min (Hermansson and Linde Forsberg, 2006) in 1.8-mL polypropylene vials (Nunc®; Sigma-Aldrich; MO) and held at 37 °C in a water bath for 24 h. Aliquots (150-μL) were removed at 0, 4, and 24 h and analyzed for the described semen characteristics.

3.2.3. Concentration

Sperm concentration was determined by use of an integrated fluorescence microscope (NucleoCounter®SP-100™; Chemometec; Allerød, Denmark), which detects signals from propidium iodide (PI) bound to sperm DNA.

3.2.4. Morphology

Sperm morphology was assessed from a sample fixed with formaldehyde buffered saline 1:10 (v:v), under DIC (Differential Interference Contrast) microscopy at 1000x (Olympus BX41; Tokyo, Japan). One hundred cells were counted and classified according to Johnston et al. (2001).

3.2.5. pH

Measurements of the pH of PF, semen extender and mixtures of both were performed using a pH meter (Corning 440; Corning; NY).

3.2.6. Cryopreservation

To avoid variation, the EYT extender (Uppsala-Equex I) was prepared in one batch as previously described (Hermansson and Linde Forsberg, 2006), clarified from particles by centrifugation at 10,000 x *g* for 15 min (Avanti J-25; Beckman Coulter; Brea, CA), the pH and osmolality (5004 Micro Osmette; Precision Systems Inc.; Natick, MA) were measured, and stored in 10-mL aliquots at -20 °C until used. The EYT extender part 1 containing 20% egg yolk and 0% glycerol (pH 6.78; osmolality 333 mOsm) was used for cooling and storage of semen at 4 °C. For freezing, the EYT extender part 2 containing 10% glycerol and 1% Equex STM paste (pH 6.74; osmolality above the detection limit of instrumentation due to the presence of glycerol and a detergent (Equex STM paste; Nova Chemical Sales Inc.; Scituate, MA)), was equilibrated

to 4 °C over 1.5 h, and slowly added to the extended semen at a ratio of 1:1 (v/v) over 10 min, before loading into 0.5-mL straws. Straws were placed horizontally on a floating rack, 4 cm above liquid nitrogen (LN₂), in a closed Styrofoam box for 10 min, and then plunged directly into LN₂. The straws were thawed (1 to 6 months after freezing) at 37 °C for 30 s in a water bath. The recovered semen was mixed with thaw medium (Tris buffer, 0% egg yolk, 0% glycerol) at a ratio of 1:1 (v/v) (pH 6.76, osmolality 315 mOsm).

3.2.7. Motility

Prior to analysis, aliquots of 50-µL from each treatment group were allowed to warm to 37 °C for 5 min and diluted with Tris buffer to a concentration of 25×10^6 spermatozoa/mL. Assessment of sperm motility was performed using computer assisted sperm analysis (CASA) (Sperm Vision® 3.0.; Minitube; Verona, WI; Olympus BX41; Tokyo, Japan) using chambered slides (Standard Count Analysis Chamber Leica 20 micron; MOFA Global; Verona, WI) as previously described (Schäfer-Somi and Aurich, 2007). Spermatozoa were identified based on defined parameters (Appendix A). After recording 5 fields, each image was manually edited for misidentified cells. The following parameters were measured: total motility (TM, %); progressive motility (PM, %); amplitude of lateral head displacement (ALH, µm); linear coefficient (LIN, %); straightness coefficient (STR, %); beat cross frequency (BCF, Hz); and wobble coefficient (WOB, %). Results rounded to one decimal place for statistical analysis.

3.2.8. Hypo-osmotic swelling test (HOST)

The hypo-osmotic swelling test (HOST) identifies spermatozoa with a functional intact membrane. A 10-µl aliquot of semen was added to 100 µl of a 100-mmol hypo-osmotic sucrose solution and incubated at 37 °C for 1 min (Pinto and Kozink, 2008). Using phase contrast

microscopy at 400x (Olympus BX41; Tokyo, Japan), 200 spermatozoa were counted as curled (intact plasma membrane) or straight (impaired plasma membrane). The percentage of intact spermatozoa was calculated by dividing the number of curled spermatozoa by the total number of spermatozoa. The HOST was only performed for all treatments at 24 h and 48 h of the cooling period.

3.2.9. Flow cytometry

Flow cytometry was used to evaluate cells after staining with YO-PRO-1/EthD-1 and PNA-FITC/PI (BD FACSCalibur; BD Bioscience; San Jose, CA) or after staining with acridine orange (BD FACSScan; BD Bioscience; San Jose, CA). Plasma membrane stability and acrosome integrity data were analyzed with associated software (Appendix B; Cellquest Pro; BD Bioscience; San Jose, CA). The SCSA output data were analyzed with Microsoft Excel (Microsoft® Excel® for Mac 2011; Redmond, WA) or SAS 9.4 (Cary, NC).

3.2.9.1. Plasma membrane stability

The cell-impermeant carbocyanine dye YO-PRO-1 iodide (YO-PRO-1; Y3603; Molecular Probes by Life Technologies, Inc.; Eugene, OR) gains access to cells after destabilization of the membrane and emits green fluorescence after binding to DNA (excitation maximum 491 nm, emission maximum 509 nm). Ethidium Homodimer-1 (EthD-1; E1169; Molecular Probes by Life Technologies, Inc.; Eugene, OR), which has a high affinity for DNA, penetrates only through damaged membranes and emits red fluorescence after binding to DNA (excitation maximum 582 nm, emission maximum 617 nm).

The staining protocol was adjusted from Neagu et al. (2010). Briefly, 250 µl of a sperm suspension (1×10^6 spermatozoa/mL) was loaded with 5 µl of 20-µM YO-PRO-1 and 1 µl of

1.167-mM EthD-1, thoroughly mixed, and incubated at 37 °C in the dark for 16 min. After staining, the sample was kept light protected and immediately evaluated by flow cytometry; 40,000 events were recorded. Using this stain combination four cell populations were distinguished (Appendix B.2.): an unstained live population with stable plasma membrane (YO-PRO-1 negative, EthD-1 negative), an early apoptotic population (YO-PRO-1 positive, EthD-1 negative), an early necrotic population with increased permeability (YO-PRO-1 positive, EthD-1 positive), and a late necrotic population when EthD-1 quenches or displaces all green fluorescence (YO-PRO-1 negative, EthD-1 positive) (Pena et al., 2005). The percentage of each subpopulation was calculated by dividing the number of cells per gated region by the total number of cells in all four gated regions.

3.2.9.2. Acrosome reactivity

Arachis hypogaea agglutinin (PNA) is a lectin from the peanut plant, and is membrane impermeable. It binds to the outer acrosomal membrane of spermatozoa (glycoconjugates, β -galactose moieties), thereby labeling those cells with damaged or reacted acrosomes. The PNA probe is conjugated to fluorescein isothiocyanate (FITC) (PNA- FITC; F-2301-5; EY Laboratories Inc.; San Mateo, CA) and emits green fluorescence (excitation maximum 492 nm, emission maximum 517 nm). It was combined with the viability dye propidium iodide (PI; P-3566; Molecular Probes by Life Technologies, Inc.; Eugene, OR), which is membrane impermeable and gains access to cells with compromised cell-membranes, fluorescing red (excitation maximum 535 nm, emission maximum 617 nm) when associated with DNA.

The use of this stain for flow cytometry was previously described for stallion sperm (Len et al., 2010). Briefly, 250 μ L of a sperm suspension (1×10^6 spermatozoa/mL) was loaded with

2 µl FITC-PNA (1 mg/ml), thoroughly mixed and incubated for 10 min at 37 °C in the dark. Following the addition of 4.2 µl of 50-µM PI, the sample was mixed and incubated for another 10 min at 37 °C in the dark. After staining, the sample was kept light protected, immediately evaluated by flow cytometry; 40,000 events were recorded. This stain combination distinguishes four cell populations (Appendix B.3.): an unstained, acrosome intact and viable population (LL: FITC-PNA negative, PI negative), an acrosome reacted and viable population (LR: FITC-PNA positive, PI negative), an acrosome intact and non-viable population (UL: FITC-PNA negative, PI positive), and an acrosome reacted and non-viable population (UR: FITC-PNA positive, PI positive). The percentage of each subpopulation was calculated by dividing the number of cells per quadrant by the total number of cells.

3.2.9.3. Sperm chromatin structure assay (SCSA)

The Sperm Chromatin Structure Assay (SCSA) determines the extent of DNA denaturation following acid treatment by measuring the metachromic shift from green to red fluorescence for acridine orange (AO; A3568, Molecular Probes by Life Technologies, Inc., Eugene, OR, USA). Acridine orange intercalates in the DNA and fluoresces green when associated with double-stranded DNA (dsDNA) and red when associated to single-stranded DNA (ssDNA).

The protocol employed was in accordance with Evenson and Jost (2000) and had previously been used with dog spermatozoa (Garcia-Macias et al., 2006). Briefly, 10 µl of sperm suspension was diluted with 1 mL of TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM disodium EDTA, pH 7.4; Evenson and Jost, 2000) to 1 to 2 x 10⁶ spermatozoa/mL, snap-frozen in

polypropylene cryovials (Brand Cryogenic tube; Sigma-Aldrich; MO) and stored in LN₂ until analysis.

All samples were analyzed using a flow cytometer (BD-FACScan; BD Bioscience; San Jose, CA). Before use, the flow cytometer tubing was equilibrated over 30 min with acridine orange (AO) equilibration buffer (Evenson and Jost, 2000), and several reference samples were run to adjust and standardize red and green fluorescence levels. After thawing in ice water, 200 μ l of thawed sperm solution was mixed with 400 μ l of freshly made acid-detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton-X 100; pH 1.4; Evenson and Jost, 2000), followed in 30 s by the addition of 1.2 mL of AO solution (containing 6 μ g/ml of AO; Evenson and Jost, 2000). Samples were analyzed after 3 min. All samples were run in duplicate. After 5 to 10 samples a thawed reference sample was run to verify and account for drift of fluorescence level standards. Samples were run on high fluid control at a cell flow of 200 to 250 cells/s; 5000 counts were recorded.

The DNA fragmentation index (DFI) was calculated for each spermatozoon (ratio red/total fluorescence) (Evenson et al., 1995). It is typical to refer to both individual fluorescence ratio (DFI, formerly termed α_t) and to the total percentage of sperm with moderate and high DFI (%DFI, formerly termed $\text{COMP}\alpha_t$). The DFI for each sperm cell was calculated (Microsoft® Excel® for Mac 2011; Redmond, WA), %DFI was calculated based on a DFI histogram (number of cells with high DFI / total number of cells; Microsoft® Excel® for Mac 2011; Redmond, WA). The average of duplicates was used for data analysis.

3.2.10. Statistical analyses

Data was compared over time and between treatments using a mixed linear model (SAS 9.4; Cary, NC) with significance level set at $P < 0.05$. The ANOVA mixed effects model consisted of a factorial arrangement of treatments which contained the fixed effects: treatment, time, and treatment*time interaction. SampleID was the random effect. If the overall fixed effect was significant, comparisons between groups were done using pairwise t tests of least-squares means (lsm). Cooling and post-thaw time periods were compared separately from each other.

3.3. Results

Initial TM and PM of the pooled semen for the three replicates was 92.8% and 86%, 89.3% and 82.7%, and 77.4% and 57%, respectively. The pH of the pooled ejaculates was 6.45, 6.36 and 6.84, respectively. Normal morphology was 79%, 82% and 71%, respectively.

The percentages of total motile cells are presented in Figure 3.1. During cooling, TM decreased over time, irrespective of treatment groups. In comparison with 0 h, TM was lower at 24 h ($P = 0.018$) and at 48 h ($P < 0.00010$), while 24 h and 48 h were not significantly different. After thawing, TM across all treatments was lower at 4 h post-thaw (PT) than at 0 h PT ($P < 0.00010$). Motility was zero at 24 h PT. No treatment*time effect was seen before or after cryopreservation, but post-thaw comparison of treatments, irrespective of time, was significant, with PF0, PF0-frz, PF10 and PF25 higher than PF50 ($P = 0.0021$, 0.00010 , 0.011 and 0.011 ; respectively), and PF0-frz higher than PF10 and PF25 ($P = 0.048$ and 0.048 ; respectively).

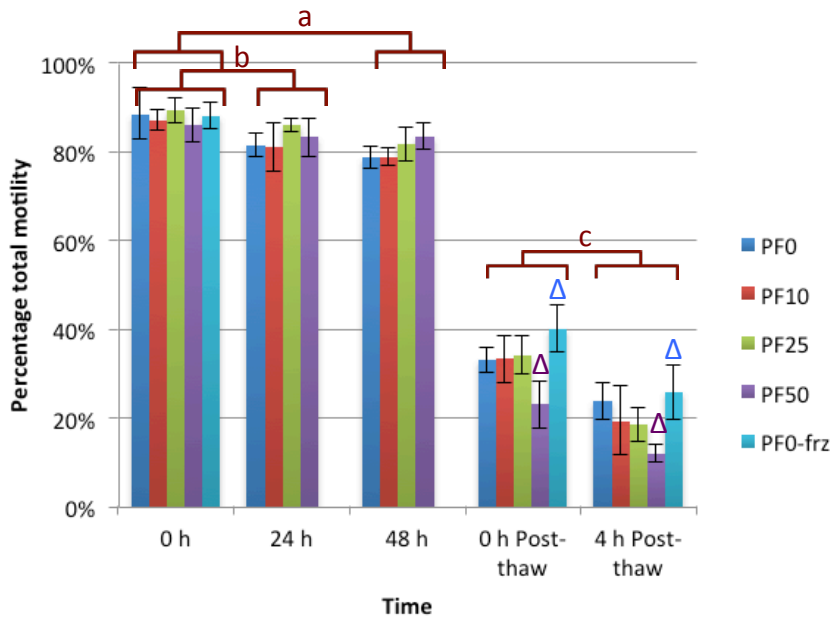


Figure 3.1. Percentages of total motility for canine semen extended in an egg yolk-Tris extender modified with 0% PF (PF0), 10% PF (PF10), 25% PF (PF25), 50% PF (PF50) after 0, 24, and 48 h of cooling (4 °C); 0% PF without a 48 h cooling period (PF0-frz); and after cryopreservation and thawing at 0 and 4 h post-thaw. No significant differences of treatment within time pre-freeze or treatment*time interaction were observed. Brackets indicate significant differences of time effects; a: 0 h > 48 h ($P < 0.00010$), b: 0 h > 24 h ($P = 0.018$), c: 0 h PT > 4 h PT ($P < 0.00010$). Δ Mean post-thaw PF50 was significantly lower than other post-thaw treatments ($P \leq 0.011$). Δ Mean post-thaw PF0-frz was significantly higher than mean PF10 or PF25 treatments ($P = 0.048$).

The percentages of progressively motile cells are presented in Figure 3.2. During cooling, PM decreased over time, irrespective of treatment groups; at 48 h PM was lower compared to 0 h ($P < 0.00010$) and 24 h ($P = 0.0052$), but 0 h and 24 h were not significantly different from each other. After thawing, PM across all treatments was lower at 4 h PT than at 0 h PT ($P = 0.00020$). Progressive motility was zero at 24 h PT. No treatment*time effect was seen before or after cryopreservation, but post-thaw comparison of treatments, irrespective of time, was significant, with PF50 lower than PF0, PF0-frz, PF10 and PF25 ($P = 0.0024, 0.00060, 0.012, 0.012$; respectively).

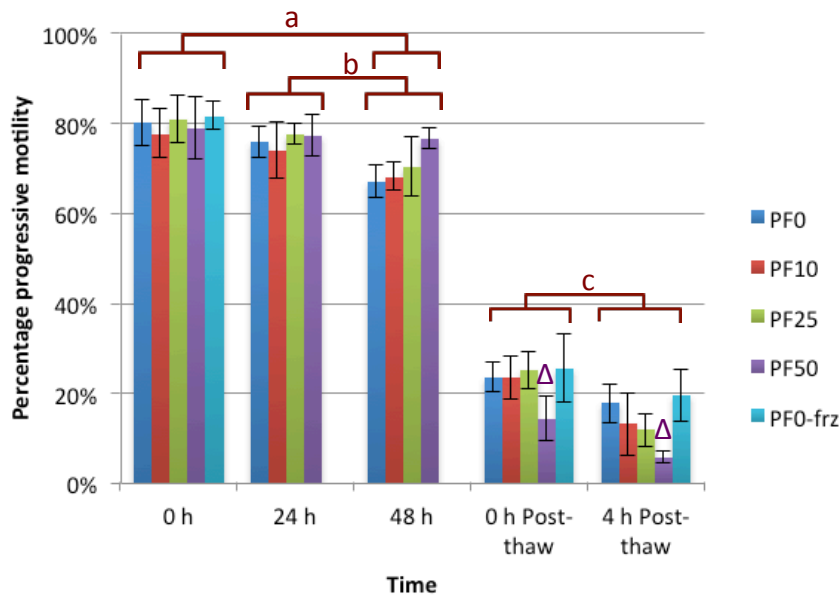


Figure 3.2. Percentages of progressive motility for canine semen extended in an egg yolk-Tris extender modified with 0% PF (PF0), 10% PF (PF10), 25% PF (PF25), 50% PF (PF50) after 0, 24 and 48 h of cooling (4 °C); 0% PF without a 48 h cooling period (PF0-frz); and after cryopreservation and thawing at 0 and 4 h post-thaw. No significant differences of treatment within time pre-freeze or treatment*time interaction were observed. Brackets indicate significant differences of time effects; a: 0 h > 48 h ($P < 0.00010$), b: 24 h > 48 h ($P = 0.0052$), c: 0 h PT > 4 h PT ($P = 0.00020$). Δ Mean post-thaw PF50 was significantly lower than other post-thaw treatments ($P \leq 0.012$).

Of the additional motion parameters evaluated, the following significant differences were found: during the cooling period, an effect of treatment independent of time was seen for BCF; it was increased with PF50 in comparison with PF0 ($P = 0.016$) and PF10 ($P = 0.0059$). Also during the cooling period, an effect of time independent of treatment was seen for ALH; with ALH being larger at 0 h than at 48 h ($P = 0.0021$); and an effect of treatment, independent of time; with ALH being lower in PF50 than in PF0 ($P = 0.00010$), PF10 ($P < 0.00010$) or PF25 ($P = 0.0014$). After cryopreservation and thawing, ALH increased from 0 h PT to 4 h PT ($P = 0.0046$), irrespective of treatment, and was higher in the PF0-frz treatment than in PF0, PF10, PF25 and PF50 ($P = 0.021, 0.0036, 0.017$ and 0.0016 , respectively), independent of time.

For STR, LIN and WOB a time effect, independent of treatment, was seen after cryopreservation and thawing, with all parameters decreasing from 0 h PT to 4 h PT ($P = 0.015$, $P = 0.0024$, $P = 0.0017$, respectively).

The percentages of plasma membrane intact cells as assessed by HOST are presented in Figure 3.3. During the cooling period, no significant differences were seen between 24 h and 48 h or among treatments. After cryopreservation and thawing, irrespective of treatment the percentage of plasma membrane intact cells was lower at 4h PT than at 0 h PT ($P = 0.0099$), lower at 24 h PT than at 0 h PT ($P < 0.00010$) and lower at 24 h PT than at 4 h PT ($P = 0.021$).

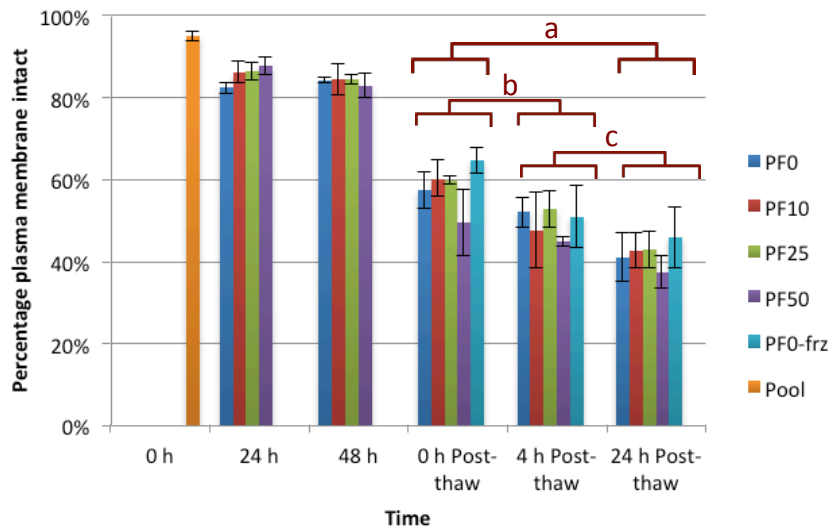


Figure 3.3. Percentages of plasma membrane intact cells (hypo-osmotic swelling test) for canine semen extended in an egg yolk-Tris extender modified with 0% PF (PF0), 10% PF (PF10), 25% PF (PF25), 50% PF (PF50) after 0, 24 and 48 h of cooling (4 °C); 0% PF without a 48 h cooling period (PF0-frz); and after cryopreservation and thawing at 0, 4 and 24 h post-thaw. No significant differences of treatment within time, time pre-freeze, or treatment*time interaction were observed. Brackets indicate significant differences of time effects; a: 0 h PT > 24 h PT ($P < 0.00010$), b: 0 h PT > 4 h PT ($P = 0.0099$), c: 4 h PT > 24 h PT ($P = 0.021$).

The percentages of live cells (YO-PRO-1 negative / EthD-1 negative) are presented in Figure 3.5. During the cooling period, no effect of time, or treatment*time was seen on percentages of live (unstained) cells. Treatments differed significantly over 48 h of cooling, irrespective of time. Percentage of unstained cells was greater in PF0 than in PF10 ($P = 0.048$) or PF25 ($P = 0.00060$), and PF0 was approaching significance for being greater than PF50 ($P = 0.052$). No differences were detected for the post-thaw period.

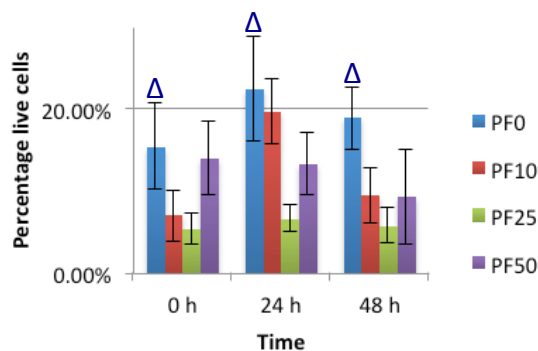


Figure 3.4. Percentages of live cells (YO-PRO-1 negative / EthD-1 negative) in relation to the total number of gated cells for canine semen extended in an egg yolk-Tris extender modified with 0% PF (PF0), 10% PF (PF10), 25% PF (PF25), 50% PF (PF50) after 0, 24 and 48 h of cooling (4 °C); and 0% PF without a 48 h cooling period (PF0-frz). No significant differences of time pre-freeze, or treatment*time interaction were observed. Δ Mean pre-freeze PF0 was significantly higher than mean PF10 or PF25 treatments ($P = 0.048$ or $P = 0.00060$, respectively).

The percentages of early apoptotic cells (YO-PRO-1 positive / EthD-1 negative) over total of stained cells (without unstained cells) are presented in Figure 3.4. During the cooling period, no effect of time or treatment*time was seen on the percentages of early apoptotic cells. Treatments differed significantly over 48 h of cooling, irrespective of time. Percentage of early apoptotic cells was lower in PF50 than in PF0 ($P = 0.0014$), PF10 ($P = 0.0023$), or PF25 ($P = 0.025$). An overall decrease of early apoptotic cells was obvious after cryopreservation and thawing. Irrespective of treatment, the percentage of early apoptotic cells was higher at 0 h PT

than at 4 h PT , higher at 0 h PT than at 24 h PT and higher at 4 h PT than at 24 h PT ($P < 0.00010$ for each).

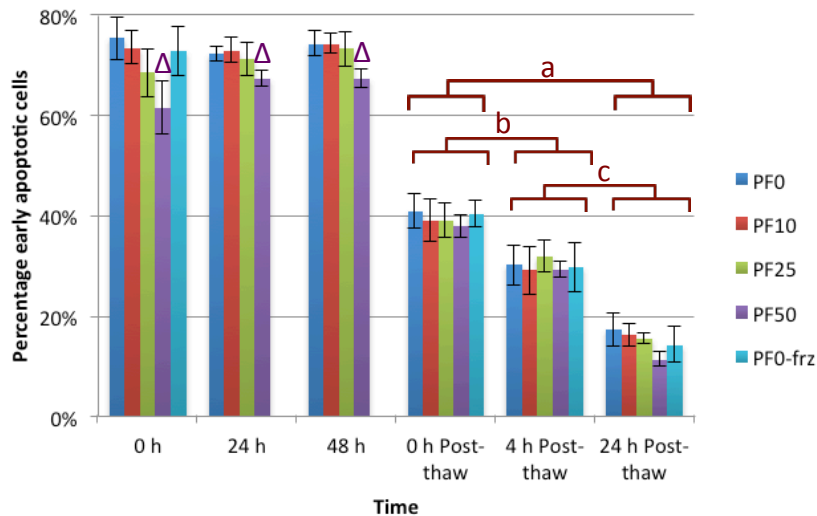


Figure 3.5. Percentages of early apoptotic cells (YO-PRO-1 positive / EthD-1 negative) in regards to the total of stained cells for canine semen extended in an egg yolk-Tris extender modified with 0% PF (PF0), 10% PF (PF10), 25% PF (PF25), 50% PF (PF50) after 0, 24 and 48 h of cooling (4 °C); 0% PF without a 48 h cooling period (PF0-frz); and after cryopreservation and thawing at 0, 4 and 24 h post-thaw. No significant differences of time pre-freeze, treatment within time post-thaw, or treatment*time interaction were observed. Δ Mean PF50 was significantly lower than all other pre-freeze treatments ($P \leq 0.025$). Brackets indicate significant differences of time effects; a: 0 h PT > 24 h PT, b: 0 h PT > 4 h PT, c: 4 h PT > 24 h PT ($P < 0.00010$ for each).

The percentages of early necrotic cells (YO-PRO-1 positive / EthD-1 positive) over the total of stained cells (without unstained live cells) for treatments at all times are presented in Figure 3.5. During the cooling period, no effect of time or treatment*time was seen. Treatments differed significantly over 48 h of cooling, irrespective of time. Percentage of early necrotic cells was higher in PF50 than in PF0 ($P < 0.00010$), PF10 ($P = 0.00080$), or PF25 ($P = 0.021$). An overall increase of early necrotic cells was obvious after thawing and increased over time, irrespective of treatment; the percentage of early necrotic cells was higher at 4 h PT than at 0 h PT ($P = 0.0070$), higher at 24 h PT than at 0 h PT and higher at 24 h PT than at 4 h PT

($P < 0.00010$ for both). Irrespective of time, percentage of early necrotic cells was higher in PF25 than PF0 ($P = 0.012$), PF0-frz ($P = 0.0015$), or PF10 ($P = 0.042$); and higher in PF50 than PF0, PF0-frz, PF10 ($P < 0.00010$ for each) or PF25 ($P = 0.0076$).

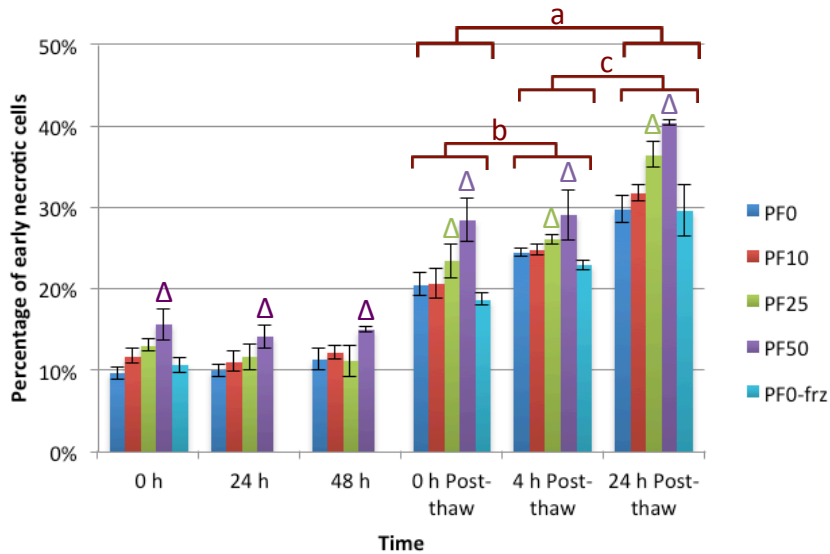


Figure 3.6. Percentages of early necrotic cells (YO-PRO-1 positive / EthD-1 positive) in relation to the total of stained cells for canine semen extended in an egg yolk-Tris extender modified with 0% PF (PF0), 10% PF (PF10), 25% PF (PF25), 50% PF (PF50) after 0, 24 and 48 h of cooling (4 °C); 0% PF without a 48 h cooling period (PF0-frz); and after cryopreservation and thawing at 0, 4 and 24 h post-thaw. No significant differences of time pre-freeze, or treatment*time interaction were observed. Δ Mean PF50 was significantly higher than all other pre-freeze treatments ($P \leq 0.021$). Brackets indicate significant differences of time effects; a: 0 h PT < 24 h PT ($P < 0.00010$), b: 0 h PT < 4 h PT ($P = 0.0070$), c: 4 h PT < 24 h PT ($P < 0.00010$). Δ Mean PF50 post-thaw was significantly higher than all other post-thaw treatments ($P \leq 0.0076$). Δ Mean PF25 post-thaw was significantly higher than PF10, PF0 and PF0-frz treatments ($P \leq 0.042$).

The percentages of late necrotic cells (YO-PRO-1 negative / EthD-1 positive) over the total of stained cells (without unstained live cells) for treatments at all times are presented in Figure 3.6. No significant effects of time, treatment, or treatment*time were seen during the cooling period. An overall increase of late necrotic cells was apparent after cryopreservation and thawing and increased over time, irrespective of treatment; the percentage of late necrotic cells was higher at 4 h PT than at 0 h PT, higher at 24 h PT than at 0 h PT and higher at 24 h PT

than at 4 h PT ($P < 0.00010$ for each). Irrespective of time, the percentage of late necrotic cells was lower in PF25 than PF0-frz ($P = 0.0025$) or PF10 ($P = 0.038$); and lower in PF50 than PF0, PF0-frz or PF10 ($P = 0.014$, 0.00030 or 0.0064 , respectively).

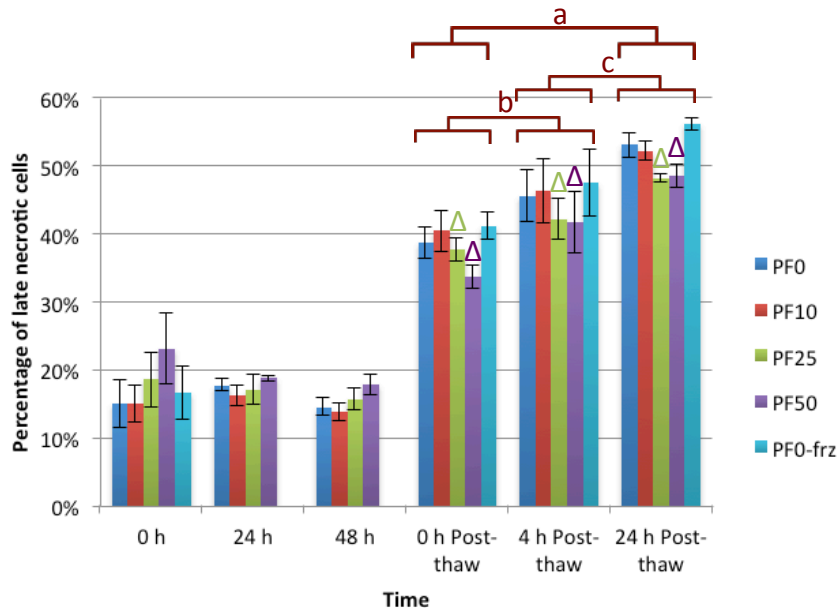


Figure 3.7. Percentages of late necrotic cells (YO-PRO-1 negative / EthD-1 positive) in regards to total of stained cells; for canine semen extended in an egg yolk-Tris extender modified with 0% PF (PF0), 10% PF (PF10), 25% PF (PF25), 50% PF (PF50) after 0, 24 and 48 h of cooling (4 °C); 0% PF without a 48 h cooling period (PF0-frz); and after cryopreservation and thawing at 0, 4 and 24 h post-thaw. No significant differences of time pre-freeze, treatment within time pre-freeze, or treatment*time interaction were observed. Brackets indicate significant differences of time effects; a: 0 h PT < 24 h PT, b: 0 h PT < 4 h PT, c: 4 h PT < 24 h PT ($P < 0.00010$ for each).

△ Mean post-thaw PF50 was significantly lower than all other post-thaw treatments ($P \leq 0.014$).

△ Mean post-thaw PF25 was significantly lower than PF0-frz or PF10 treatments ($P \leq 0.0076$).

Mean post-thaw PF25 and PF50 were not significantly different from each other ($P = 0.45$).

The percentages of dead cells (PI positive) over the total of gated cells for treatments at all times are presented in Figure 3.7. During the cooling period, no effect of time or treatment*time were seen. Treatments differed significantly over 48 h of cooling, irrespective of time. Percentage of PI positive cells was lower in PF0 than PF10, PF25 and PF50 ($P = 0.031$, 0.00050 and < 0.00010 , respectively); and higher in PF50 than in PF10 ($P = 0.0020$). After

cryopreservation and thawing, irrespective of treatment the percentage of PI positive cells was higher at 4 h PT than at 0 h PT ($P < 0.00010$), higher at 24 h PT than at 0 h PT ($P < 0.00010$) and higher at 24 h PT than at 4 h PT ($P = 0.017$).

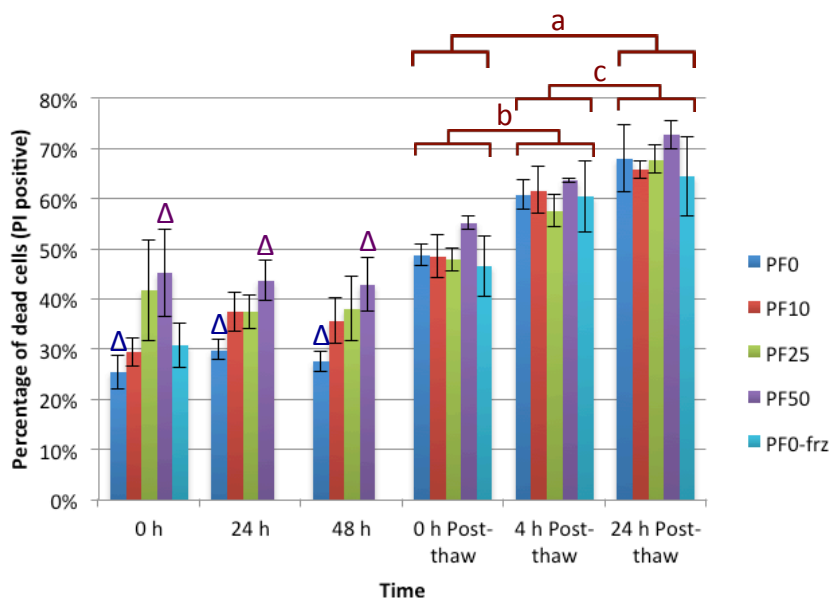


Figure 3.8. Percentages of dead cells (PI positive) for canine semen extended in an egg yolk-Tris extender modified with 0% PF (PF0), 10% PF (PF10), 25% PF (PF25), 50% PF (PF50) after 0, 24 and 48 h of cooling (4 °C); 0% PF without a 48 h cooling period (PF0-frz); and after cryopreservation and thawing at 0, 4 and 24 h post-thaw. No significant differences of time pre-freeze, or treatment*time interaction were observed. Δ Mean pre-freeze PF0 was significantly lower than all other treatments ($P \leq 0.031$). \triangle Mean pre-freeze PF50 was significantly higher than the mean PF10 treatment ($P = 0.0020$). Brackets indicate significant differences of time effects; a: 0 h PT < 24 h PT ($P < 0.00010$), b: 0 h PT < 4 h PT ($P < 0.00010$), c: 4 h PT < 24 h PT ($P = 0.017$).

The percentages of acrosome intact live cells (PNA-FITC negative / PI negative cells; unstained) over the total of gated cells for treatments at all times are presented in Figure 3.8. During cooling, a significant effect of time and treatment were observed. Percentage of acrosome intact live cells, irrespective of treatment, was higher at 48 h than at 0 h ($P = 0.014$) and higher at 48 h than at 24 h ($P = 0.017$). Irrespective of time, percentage of acrosome intact live cells was higher in PF0 than in PF25 or PF50 ($P < 0.00010$ for both); higher in PF10 than in

PF25 or PF50 ($P = 0.0017$ or < 0.00010 , respectively) and higher in PF25 than in PF50 ($P = 0.035$).

After cryopreservation and thawing, irrespective of treatment, the percentage of acrosome

intact live cells was higher at 0 h PT than at 4 h PT, higher at 0 h PT than at 24 h PT and higher at

4 h PT than at 24 h PT ($P < 0.00010$ for each).

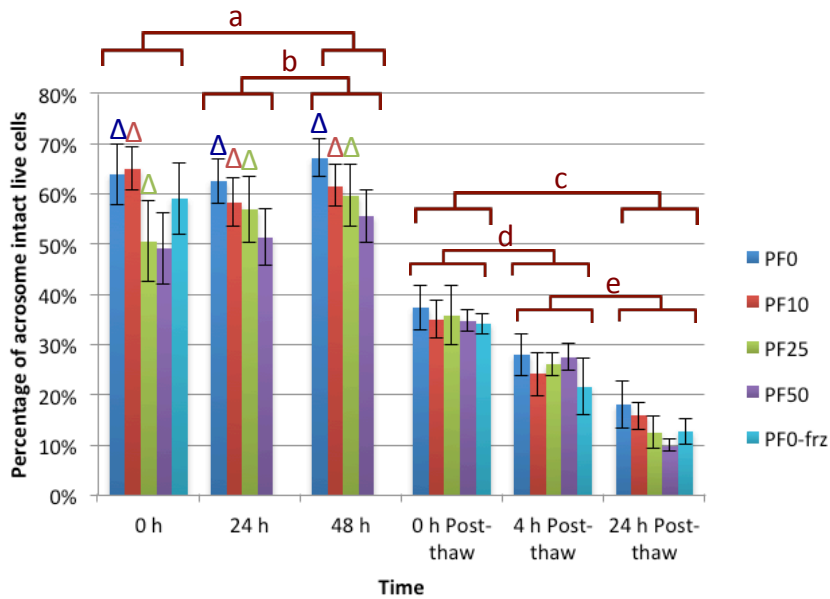


Figure 3.9. Percentages of acrosome intact live cells (PNA-FITC negative / PI negative cells) for canine semen extended in an egg yolk-Tris extender modified with 0% PF (PF0), 10% PF (PF10), 25% PF (PF25), 50% PF (PF50) after 0, 24 and 48 h of cooling (4 °C); 0% PF without a 48 h cooling period (PF0-frz); and after cryopreservation and thawing at 0, 4 and 24 h post-thaw. No significant differences of treatment within time post-thaw, or treatment*time interaction were observed. ΔMean pre-freeze PF0, and Δmean pre-freeze PF10 were significantly higher than mean PF25 or PF50 treatments ($P < 0.00010$, and $P \leq 0.0017$, respectively). ΔMean pre-freeze PF25 was significantly higher than mean PF50 treatment ($P = 0.035$). Brackets indicate significant differences of time effects; a: 48 h > 0 h ($P = 0.014$), b: 48 h > 24 h ($P = 0.017$), c: 0 h PT > 24 h PT ($P < 0.00010$), d: 0 h PT > 4 h PT ($P < 0.00010$), e: 4 h PT > 24 h PT ($P < 0.00010$).

The percentages of acrosome reacted cells (PNA-FITC positive) over the total of gated cells for treatments at all times are presented in Figure 3.10. During cooling, no significant differences of time, treatment, or treatment*time were observed. After cryopreservation and thawing, irrespective of treatment, the percentage of acrosome reacted cells was higher at 0 h PT than at 4 h PT ($P = 0.00010$) and higher at 0 h PT than at 24 h PT ($P = 0.00070$).

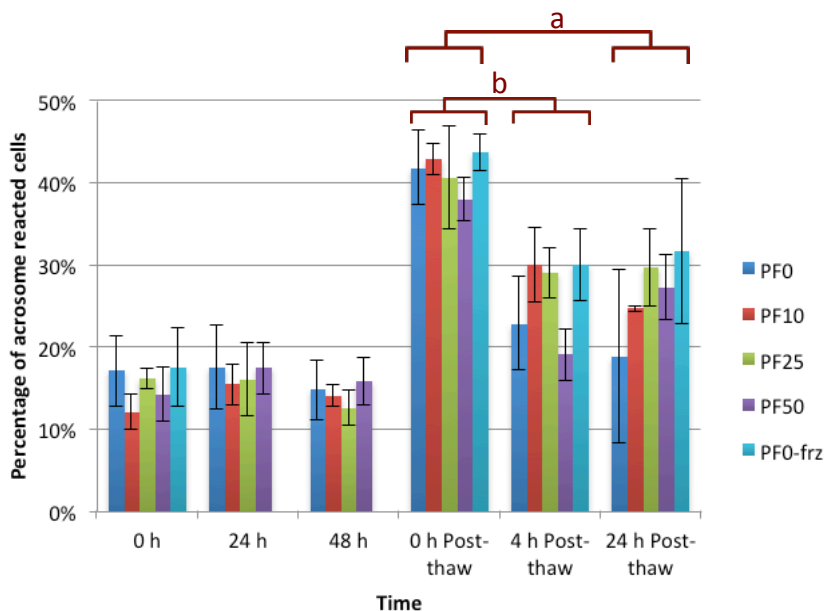


Figure 3.10. Percentages of acrosome reacted cells (PNA-FITC positive) for canine semen extended in an egg yolk-Tris extender modified with 0% PF (PF0), 10% PF (PF10), 25% PF (PF25), 50% PF (PF50) after 0, 24 and 48 h of cooling (4 °C); 0% PF without a 48 h cooling period (PF0-frz); and after cryopreservation and thawing at 0, 4 and 24 h post-thaw. No significant differences of treatment within time, time pre-freeze, or treatment*time interaction were observed. Brackets indicate significant differences of time effects; a: 0 h PT > 24 h PT ($P = 0.00070$), b: 0 h PT > 4 h PT ($P = 0.00010$).

The percentages of cells with moderate to high DFI (%DFI) for treatments at all times are presented in Figure 3.10. During the cooling period, no significant effects of treatment or time were seen, but a treatment*time interaction effect was significant ($P = 0.029$). Evaluation of least square means at each time point revealed the following differences: at 24h, PF25 was higher than PF0 ($P = 0.048$), PF10 ($P = 0.0097$) and PF50 ($P = 0.042$). At 48h, PF10 was higher

than PF0 ($P = 0.0036$). After cryopreservation and thawing, an effect of time independent of treatment, was seen with an increase at 24h PT in comparison with 0h PT or 4h PT ($P < 0.00010$ for both).

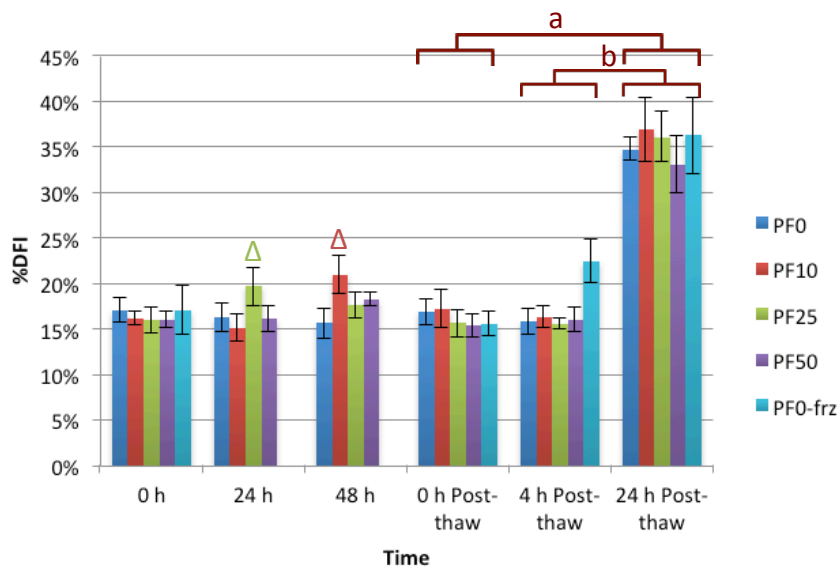


Figure 3.11. Percentages of cells with moderate to high DFI (%DFI) for canine semen extended in an egg yolk-Tris extender modified with 0% PF (PF0), 10% PF (PF10), 25% PF (PF25), 50% PF (PF50) after 0, 24 and 48 h of cooling (4 °C); 0% PF without a 48 h cooling period (PF0-frz); and after cryopreservation and thawing at 0, 4 and 24 h post-thaw. No significant differences of treatment within time, or time pre-freeze were observed. A significant treatment*time interaction was observed during cooling with PF25 (Δ) higher than other treatments at 24 h, and PF10 (Δ) higher than other treatments at 48 h. Brackets indicate significant differences of time effects; a: 0 h PT < 24 h PT, b: 4 h PT < 24 h PT.

3.4. Discussion

Shipment of cooled semen for artificial insemination (AI) across geographical distances remains one of the most commonly used reproductive techniques for canine breeding management. Prostatic fluid (PF) is a natural component of the canine ejaculate and PF contamination of the sperm-rich fraction (SRF) during semen collection is sometimes unavoidable. The controversy surrounding canine PF originates from conflicting results regarding *in vivo* and *in vitro* applications of PF, where PF was shown to elicit positive or

negative effects or both. The use of variable PF concentrations for *in vitro* seminal parameter evaluations in these studies might contribute to the conflicting results. This study aimed to investigate concentration dependent effects of PF on *in vitro* seminal parameters and possibly determine an optimal dilution ratio of PF for use in cooled semen.

The hypothesis that lower dilutions of canine semen with PF would improve seminal parameters in comparison with higher concentrations of PF in fertile dogs was based on the concept of progressive PF clearance of spermatozoa after deposition in the female reproductive tract (England et al., 2006). After natural mating spermatozoa are initially exposed to high concentrations of PF, but due to vaginal and uterine clearance, and sperm reservoir establishment, contact with PF decreases over time *in vivo*. Therefore exposure of spermatozoa to lower concentrations of PF *in vitro* over time might mimic physiological conditions more closely. In support of this theory, a recent publication by Treulen et al. (2012) proposed beneficial effects of PF over a cooling period of 72 h on *in vitro* plasma membrane and acrosome integrity. Concentration dependent effects of PF on canine semen have not been investigated prior to this study.

In order to evaluate concentration dependent effects of canine PF on seminal parameters, the PF and SRF portions of four fertile stud dogs were pooled to minimize individual variation among samples. The pooled SRF was centrifuged to remove most seminal fluids (SP and PF) that had been admixed during ejaculation and semen collection. Treatments (PF0, PF10, PF25 and PF50) were applied over a 48 h cooling period. Cryopreservation was then performed in order to further stress spermatozoa, as canine semen has been shown to be relatively tolerant to cooling, with reported maintenance of semen quality for up to 16 days

(Verstegen et al., 2005). In addition, treatments and controls were incubated post-thawing at 37 °C over 24 h to mimic *in vivo* conditions.

Total and progressive motility declined over time during the cooling period, irrespective of treatment. The presence of PF did not compromise nor enhance TM or PM during the cooling period. The findings in this study are different from those of Treulen et al. (2012) who reported a significant decrease in progressive motility from $88.7 \pm 1.1\%$ to $38.5 \pm 1.7\%$ in the presence of 50% PF during a 48 h cooling period. Methodological differences between these studies were the use of objective motility assessment (CASA) in this study and the addition of fructose in the EYT extender rather than glucose. Fructose has been reported to be superior for maintaining sperm motility during cooled storage (Ponglowhapan et al., 2004). Further differences were a cooling period of 72 h at a low spermatozoal concentration of $40 \times 10^6/\text{mL}$ and centrifugation of aliquots at $720 \times g$ for 5 min in a TRIS buffer as a washing step before assessment of seminal parameters (Treulen et al., 2012) in comparison to a cooling period of 48 h at 200×10^6 spermatozoa/mL with no additional centrifugation for the results reported herein. Canine spermatozoa were reported to exhibit significantly higher post-thaw motility and maintain plasma membrane integrity longer during post-thaw incubation (38 °C) when frozen at 200×10^6 spermatozoa/mL in comparison with 50×10^6 spermatozoa/mL (Pena and Linde-Forsberg, 2000). Spermatozoal parameters were documented to remain unchanged after centrifugation at $720 \times g$ for 5 min in canine semen (Rijsselaere et al., 2002) or $900 \times g$ for 10 min in equine semen (Len et al., 2010). But repeated centrifugation with low sperm recovery rates could potentially affect spermatozoal parameters, therefore additional centrifugation after the initial aliquot preparation was avoided in the present study.

Post-thaw motility (total and progressive) was lower than anticipated. Post-thaw TM and PM were not different between the PF0 control treatment that had been cooled for 48 h and the immediately frozen control treatment (PF0-frz) ($P = 0.1836$, $P = 0.538$, respectively). After thawing, a significant decrease in TM and PM was seen between 0 h and 4 h; after 24 h of incubation sperm motility was zero for all treatment groups. Post-thaw TM and PM were significantly lower for the PF50 treatment than any other. This finding is similar to the report by Sirivaidyapong et al. (2001), in which ejaculates (SRF) were extended either without centrifugation (T1) or centrifuged and resuspended either in extender (T2) or in PF (1:1) before dilution with extender (T3). All three treatments were cooled for 6 h before cryopreservation and post-thaw motility was significantly lowered by the addition of PF (T3: $12 \pm 9\%$ compared with T2: $50 \pm 12\%$ and T1: $41 \pm 8\%$). It is unclear whether the treatment group with PF in the study by Sirivaidyapong et al. contained 50% PF or some lower percentage. In the present study, each treatment and control group received an equivalent amount of freezing extender (1:1, v/v) containing 10% glycerol and 1% detergent (Equex STM paste); therefore post-thaw results cannot be explained by a decreased presence of the cell-penetrating cryoprotectant glycerol in treatments with increasing PF concentration. However, treatments with increasing PF in this study were exposed to less EYT extender part 1 over the 48 h cooling period, during which no treatment effect on TM or PM was seen.

Analysis of other motion parameters revealed a decline of ALH during the 48 h cooling period in all groups, and significantly lower ALH for the PF50 treatment group in comparison with other treatments. This could indicate a decrease of hyperactive motility over time in the presence of 50% PF. Increased ALH, in conjunction with a decrease in LIN and increase in VCL,

has been used to estimate hyperactive motility (Rota et al., 2007). Tris buffer, which has been shown to have minimal influence on motility parameters in comparison to saline or phosphate buffered saline (Schäfer-Somi et al., 2005), was used in the present study as a post-thaw diluent and samples were incubated at 37 °C for 5 min before motility assessment. Prostatic fluid has also been evaluated as a possible post-thaw additive (unspecified dilution ratio), with an intent to stimulate sperm motility patterns after cryopreservation and thawing, and was found to lower ALH parameters during incubation when compared to agents (pentoxifylline, caffeine, and 2'-deoxyadenosine) that stimulate motility by increasing cellular levels of cAMP (Milani et al., 2010). In the present study post-thaw results revealed an increase of ALH between 0 h and 4 h, with significantly higher values observed in the PF0-frz control treatment. After thawing, STR, LIN and WOB also significantly decreased from 0 h to 4 h. It has previously been reported that incubation of cryopreserved semen with PF post-thawing (1:2, v/v) may decrease hyperactive motility initially, as indicated by lower ALH, VCL and higher LIN (Rota et al., 2007). This effect was not observed in this study, which could be attributed to PF addition pre-cryopreservation in this study, or the use of a different CASA system. The addition of Tris buffer (1:1, v/v) after thawing diluted concentrations of PF in all treatments. Whether an increase of hyperactive motility (increased ALH with decreased STR and LIN) in this study was related to cryopreservation-induced damage or due to dilution of PF content remains unclear. The co-existence of different kinematic subpopulations of sperm cells within an ejaculate has been described for dogs (Nunez-Martinez et al., 2006; Pena et al., 2012) as well as for other species (Quintero-Moreno et al., 2002). Placement of sperm into three or more predefined classes for each attribute based on select thresholds or data-defined clustering, would be considered more

appropriate than comparison of means for each attribute across all cells (review: Amann and Waberski, 2014).

Sperm membrane integrity, stability or viability was assessed by hypo-osmotic swelling test (HOST) and the fluorescent probes YO-PRO-1, EthD-1 and PI. The sperm plasma membrane consists of three distinct membrane compartments, one covering the outer acrosomal membrane, one covering the post-acrosomal portion of the sperm head and one covering the middle and principle piece; HOST assesses integrity of the plasma membrane covering the middle and principle piece (review: Mocé and Graham, 2008). The percentage of plasma membrane intact cells did not differ between treatments or time during the cooling period. Initial HOST assessment prior to cooling (0 h) was not performed for all replicates and is therefore shown (Figure 3.3.) for the pre-centrifugation pooled samples. The percentage of plasma membrane intact cells was found to decrease over time after thawing independent of PF treatment. The HOST is an easy to use clinical test, but lacks sensitivity in comparison to flow cytometry, where 100-fold more cells are rapidly assessed.

The combination of the fluorescent probes YO-PRO-1 and EthD-1 for use with flow cytometry has been reported to be a useful tool to detect sperm membrane changes (Pena et al., 2005). Comparison of live unstained cells over time and treatments pre-freeze revealed a decrease in the percentage of intact cells in treatments with PF. Despite clarification of the egg yolk used in the extenders by high-speed centrifugation, it remains possible that egg yolk particles interfered with light scatter identification of sperm cells. This effect seemed to become more pronounced after cryopreservation, as a drastic increase of intact live cells after freezing and thawing occurred, which is not logical. Therefore, all further comparisons were

done among stained cell populations only. A treatment effect on sperm membrane stability was seen during the cooling period. The PF50 treatment group contained significantly lower percentages of early apoptotic cells and higher percentages of early necrotic cell populations than any other treatment, which could indicate a shift from early apoptotic to early necrotic cells in the treatment with 50% PF. After thawing, irrespective of treatment, the ratio of early apoptotic cells decreased over time, whereas the percentages of early and late necrotic cells increased. Furthermore a significant treatment effect was seen post-thawing in the PF25 and PF50 groups; both contained higher percentages of early necrotic and lower percentages of late necrotic cells than most of the other treatments.

Propidium iodide (PI) has similar characteristics as EthD-1, and is thought to evaluate the intactness of the membrane compartment covering the post-acrosomal portion of the sperm head (review: Mocé and Graham, 2008). Staining with PI was used in combination with the acrosome stain for evaluation of cell membrane integrity or viability. Evaluation of PI positive cells alone (irrespective of acrosome reaction) revealed similar findings as the combination stain YO-PRO-1/EthD-1. The control group (PF0) contained fewer PI positive (dead cells) than any of the other treatment groups. A significant increase over each time point irrespective of treatment was observed after thawing.

A limitation of this study was the lack of fluorescent labeling of intact cells. Petrunkina et al. (2010) demonstrated that there is a systematic overestimation in the proportion of unstained (viable) cells detected by flow cytometry in cases where the non-sperm particles are not excluded from analysis by additional identification other than light-scatter characteristics. A triple stain combination of YO-PRO-1, EthD-1 and SNARF-1 (which stains intact viable cells) for

flow cytometry has been described but requires availability of a triple-laser cytometer (Pena et al., 2005).

The results of the YO-PRO-1 / EthD-1 and PI stains using flow cytometry suggest a concentration dependent, detrimental effect of PF on plasma membrane stability and integrity during the cooling period. Lower concentrations of PF did not seem to compromise sperm membrane stability. The stress of cryopreservation enhanced the effect of higher concentrations of PF, with an overall increase of early and late necrotic cells. Specifically, in treatments with high PF content (PF50 and PF25) a relative increase of early necrotic cells and concurrent decrease of late necrotic cells was observed post-thawing. This change in ratio may be explained by death of additional viable and early apoptotic cells, contributing to a relative increase of the early necrotic cell population. As discussed above for sperm motility, it remains unclear whether the concentration dependent effects were due to a direct negative impact on spermatozoa by PF or could be attributed to a dilution of cryoprotective extender properties with increasing PF. Additional controls using a solution with equivalent pH and osmolality as PF and using the same dilution ratio (10%, 25%, and 50%) might have brought clarification to this question. Irrespective of the cause, it is relevant from a practical aspect that collection of significant amounts of PF with the SRF, or a lack of fractioning, might compromise spermatozoal plasma membrane stability, integrity and viability for cooled storage or shipping of cooled semen in EYT extender.

The percentage of acrosome intact live cells significantly increased over the 48 h of cooling. Furthermore, a concentration dependent effect was seen with a lower percentage of acrosome intact live cells in treatments with higher PF content; only PF0 and PF10 were not

significantly different from each other. Such an effect was not apparent when percentages of FITC-PNA stained or acrosome reacted cells were compared amongst treatments. It is therefore likely that the live-dead component of the stain combination explains the treatment effect on acrosome intact live cells. The percentage of acrosome reacted cells increased after cryopreservation and thawing and then significantly decreased over the 24 h incubation period, a finding which could be explained by the loss of all acrosomal binding sites with extensive acrosomal damage (review: Martinez-Pastor et al., 2010). The presence of PF did not exert a protective effect on acrosome status during the cooling period or after cryopreservation in this study. Previous studies reported no difference in the percentage of acrosome intact live cells (FITC-PNA / EthD-1) over a cooling period of 6 h or after cryopreservation of canine semen in the presence or absence of PF (Sirivaidyapong et al., 2001; Rota et al., 2007). There was no effect of differing centrifugation forces on the percentage of acrosome reacted cells (FITC-PSA) after 72 h of cooled storage at 4 °C (Rijsselaere et al., 2002). Treulen et al. (2012) reported a higher proportion of acrosome intact cells (FITC-PSA) in the presence of 50% PF at 72 h of cooling in comparison to a 0% control. The medium used to dilute sperm (specifically the Ca^{2+} content) and the incubation temperature have been shown to influence *in vitro* acrosome reaction (Sirivaidyapong et al., 2000). The lack of live positive identification of sperm cells for use with these stains with flow cytometry is not desirable. Similar to plasma membrane integrity the development of triple stains would provide more conclusive data.

The %DFI expresses the percentage of cells with high susceptibility to low pH-induced DNA denaturation. Korochkina et al. (2014) investigated DNA fragmentation in association with the addition of PF to canine epididymal sperm. Harvested epididymal spermatozoa were

exposed to pure PF or a Tris solution before cryopreservation. After thawing and 4 h of incubation at 37 °C in a thaw medium the PF treatment group exhibited significantly higher %DFI. SCSA was used in this study to evaluate an influence of PF concentration on DNA fragmentation of ejaculated canine spermatozoa. Although a significant treatment*time interaction revealed differences of %DFI at certain time points and PF concentrations (significantly increased after 24 h and 48 h of cooling for PF25 and PF10, respectively), the lack of DNA repair mechanism in mature sperm cells (Silva, 2006) would suggest this finding to be due to methodological error in samples at these time points or statistical variability. A higher number of replicates might have been useful to more clearly evaluate this finding. An increase in %DFI after cryopreservation of ejaculated spermatozoa, thawing and 3 h of storage at 37 °C, was not affected by centrifugation and the removal of PF and SP (Koderle et al., 2009). Similarly, an increase of %DFI after cooling-cryopreservation-thawing and 24 h of incubation at 37 °C was found in the present study, suggesting DNA damage, which could be due to oxidative stress, storage temperature, extender, handling conditions or bacterial growth, but seemed to be independent of PF treatment. Kim et al. (2010b) described an increase of %DFI after cryopreservation and thawing in comparison with fresh semen, attributing the observed DNA fragmentation to cryoinjury. An investigation of SCSA and canine fertility data is lacking. Studies in other species (Love and Kenney, 1998; Virro et al., 2004; Lewis and Aitken, 2005; review: Love, 2005; Evenson and Wixon, 2006; Waterhouse et al., 2006; Nordstoga et al., 2013) would suggest that individuals with high DNA fragmentation in freshly ejaculated sperm have compromised fertility. Love et al. (2002) suggested that in properly processed stallion semen no change in %DFI after 24 h of cooled storage is typically seen; however, failure of appropriate SP

removal can impact DNA fragmentation even in highly fertile stallions. It can be hypothesized that effects of canine PF on DNA-fragmentation might be more pronounced in individual dogs, which was not investigated with this study.

The present study evaluated concentration dependent effects of PF using pooled semen and pooled PF; individual effects might have been more variable as has been reported for the stallion. Removal of SP by centrifugation benefits individual stallions with poor tolerance to cooling (Brinsko et al., 2000; Varner et al., 2008) and transfer of SP prior to cryopreservation from stallions with high PT semen quality to those with low PT motility improved PT motility (Aurich et al., 1996). Some subfertile stallions have been reported to show an improved fertility after SP transfer from a fertile stallion (Varner et al., 2008). A recent study investigated different freezing protocols for storage of SP of stallions to be used with SP allotransfer (Whigham et al., 2014) and found no difference in the effect of SP freezing protocols on seminal parameters. Similar studies have not been done for dogs. It would be interesting to investigate differences and effects of transfer of PF between stud dogs with high or low fertility.

Based on research in other species no single *in vitro* seminal parameter can be expected to explain *in vivo* fertility (review: Mocé and Graham, 2008). There are limitations of both *in vitro* assays and estimates of fertility, which hinders the ability to predict fertilizing potential of a semen sample (review: Amann, 2005). The multi-factorial nature of sperm function with each of its essential compartments, the heterogeneity of sperm populations and inaccuracies of *in vitro* assays contribute to variability. The use of several different *in vitro* assays in combination is recommended (review: Mocé and Graham, 2008) and may enhance our insight and management of semen to enhance animal fertility.

CHAPTER 4

CONCLUSIONS

Based on the results of this study we propose a concentration dependent effect of canine prostatic fluid (PF) on certain *in vitro* seminal characteristics during cooled storage of semen. The hypothesis that lower dilutions of canine semen with PF in an egg yolk-Tris extender would improve plasma membrane stability and acrosome integrity, and preserve sperm kinetics and reduce DNA fragmentation in comparison with high concentrations of PF in fertile dogs, was not supported by the findings in this experiment. In fertile dogs PF did not negatively impact motility parameters during the cooling period, but the presence of 50% PF had a negative impact after cryopreservation and thawing. Motility parameters could potentially be more adversely affected by PF during cooling in sub- or infertile dogs. Plasma membrane stability and integrity were negatively impacted in a concentration dependent manner during cooling; this effect was amplified after applying a stressor in the form of cryopreservation. This was shown by use of the fluorescent stains YO-PRO-1/EthD-1 with higher proportions of live (unstained) cells in absence of PF during cooling, lower proportions of early apoptotic and higher proportions of early necrotic cells with 50% PF during cooling, and a shift towards early necrotic cells after cryopreservation and thawing with both 25 and 50% PF. Staining with propidium iodide showed a decrease in membrane integrity in any treatment containing PF. Acrosome integrity and DNA fragmentation were not significantly influenced by PF. Although lower concentrations of PF did not improve the evaluated spermatozoal parameters, they did not seem to compromise sperm motility, plasma membrane stability, acrosome integrity or DNA fragmentation. Therefore, during manual semen collection, large

volumes of PF should be excluded, but the collection of small amounts of PF, which marks the end of the sperm-rich fraction (SRF), would not seem to be detrimental for shipping cooled canine semen. The presence of large amounts of PF pre-freezing were shown to have a detrimental effect on spermatozoal post-thaw motility and membrane stability. Therefore admixture with more than 10% PF should be avoided prior to cryopreservation of canine semen. However, to maximize sperm recovery rate after centrifugation, avoiding disturbance of the sperm pellet during the removal of the supernatant by allowing small amounts of PF to remain should not dramatically affect sperm cooling or freezing results.

Semen extender likely provides important plasma membrane stabilizing effects on spermatozoa during cooling or freezing, which may be more beneficial than any effects of PF. Further studies are required to investigate whether PF elicits direct negative effects on spermatozoa or whether dilution of cryoprotective extender properties by PF could explain some of the observed results. It can be speculated that semen extender might be a more suitable flushing media during artificial insemination procedures when compared to PF.

All samples were pooled for this study, therefore it remains to be demonstrated if individual effects may be more pronounced and if PF of individual dogs could negatively or positively affect fertility potential as has been previously reported with SP of the stallion. Future work should investigate individual differences and specifically examine the influence of PF from dogs with prostatic disease or subfertility.

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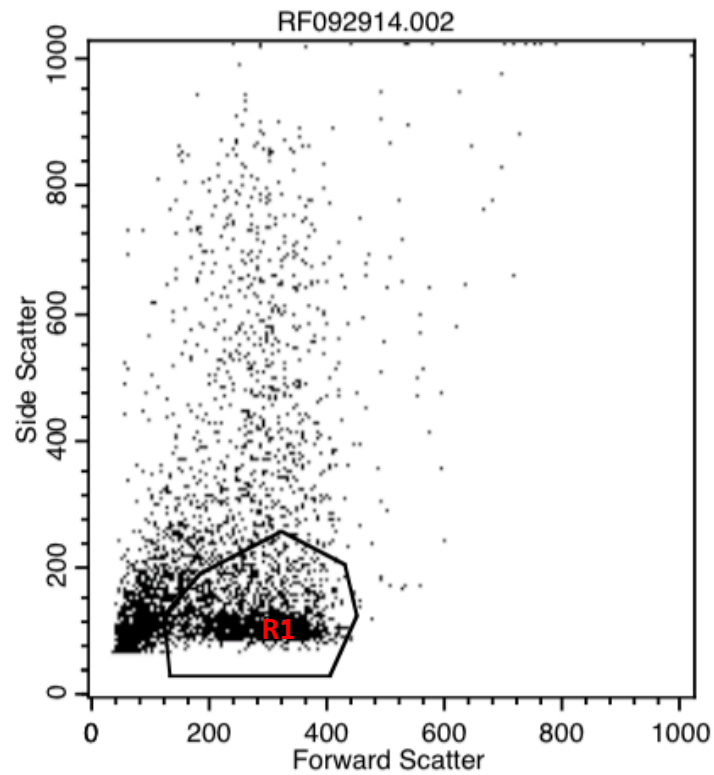
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APPENDIX A
THE CASA SPERM VISION® 3.0. PARAMETERS USED FOR CANINE SPERMATOZOAL ANALYSIS

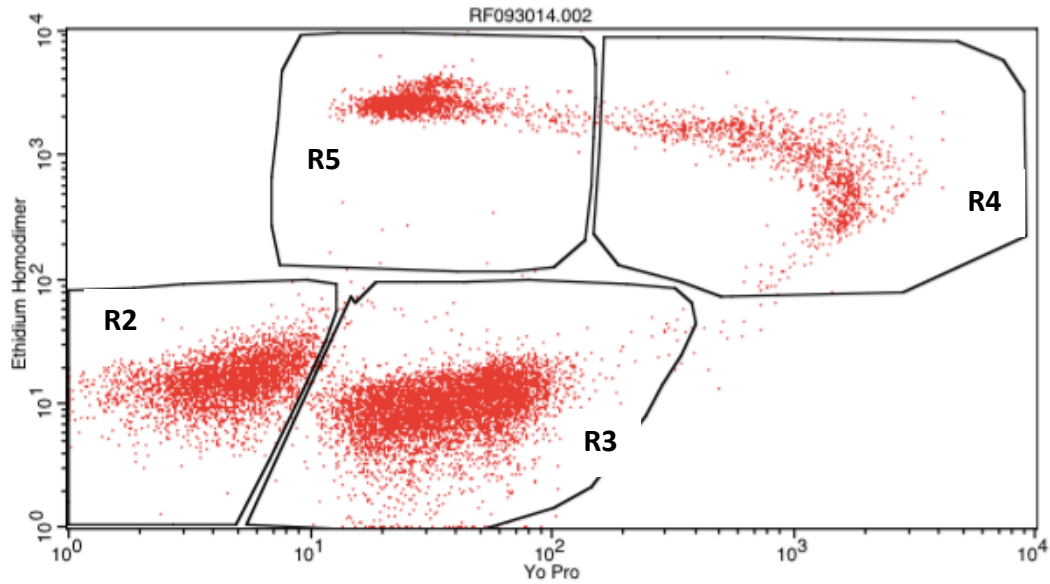
- Depth of sample chamber: 20 μM
- Pixel to micron ratio: 140 to 100
- Number of fields evaluated: 5
- Cell identification area: 14 to 80 μM^2
- VCL = Curvilinear velocity ($\mu\text{m/s}$): The instantaneously recorded sequential progression along the entire trajectory of the spermatozoon.
- VSL = Linear velocity ($\mu\text{m/s}$): The straight trajectory of the spermatozoa per unit of time.
- VAP = Mean velocity ($\mu\text{m/s}$): The mean trajectory of the spermatozoa per unit of time.
- LIN = Linear coefficient (%): VSL/VCL
- STR = Straightness coefficient (%): VSL/VAP
- WOB = Wobble coefficient (%): VAP/VCL
- ALH = Amplitude of lateral head displacement (μm): Mean width of sperm head oscillation.
- BCF = Frequency of head displacement (Hz): The number of lateral oscillatory movements of the sperm head around the mean trajectory.

APPENDIX B FLOW CYTOMETRIC GATING

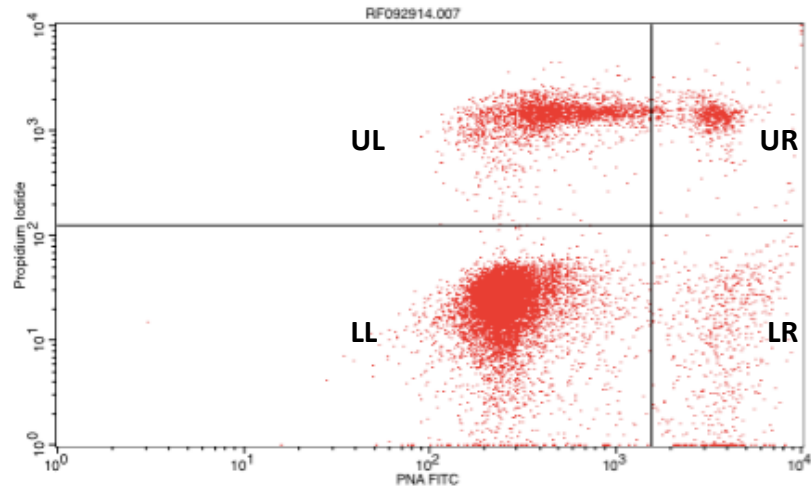
Appendix B.1. An example of flow cytometric analysis (Cellquest Pro; BD Bioscience, San Jose, CA). The distribution of recorded events based on side-scatter and forward-scatter characteristics is shown, with the population R1 (red) being the location of spermatozoa.



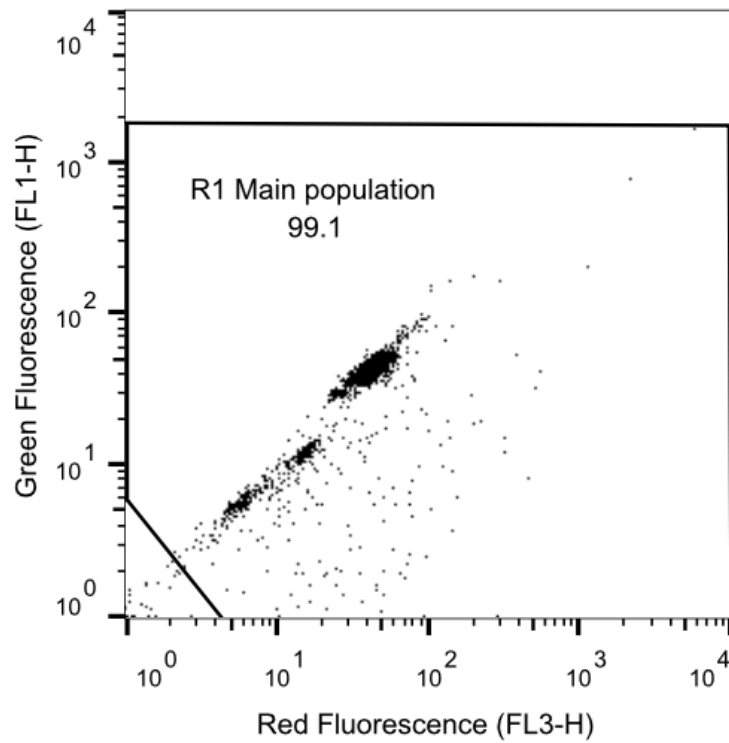
Appendix B.2. Scatter dot plot of a plasma membrane stability-stained (YO-PRO-1/EthD-1) canine semen sample analyzed with a flow cytometer (Cellquest Pro; BD Bioscience, San Jose, CA). The gated regions are identified as R2: Live cells (YO-PRO-1 negative / EthD-1 negative); R3: early apoptotic cells (YO-PRO-1 positive / EthD-1 negative); R4: early necrotic cells (YO-PRO-1 positive / EthD-1 positive); and R5: late necrotic cells (YO-PRO-1 negative / EthD-1 positive).



APPENDIX B.3. Scatter plot of an acrosome integrity-stained (PNA-FITC/PI) canine semen sample analyzed with a flow cytometer. Quadratic gating was used to analyze the cell population (Cellquest Pro; BD Bioscience, San Jose, CA). Upper left (UL): dead-nonreacted acrosome; upper right (UR): dead-reacted acrosome; lower left (LL): live-nonreacted acrosome; lower right (LR): live-reacted acrosome.



APPENDIX B.4. Scatter plot of an acridine orange-stained (AO) canine semen sample analyzed with a flow cytometer (Cellquest Pro; BD Bioscience, San Jose, CA) with red fluorescence detected on FL-3 on horizontal axis (single stranded DNA) and green fluorescence detected on FL-1 on vertical axis (double stranded DNA).



APPENDIX C
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Appendix C.1. Figure 2.1. Hossain, M.S., Johannisson, A., Wallgren, M., Nagy, S., Siqueira, A.P., and H. Rodriguez-Martinez. 2011. Flow cytometry for the assessment of animal sperm integrity and functionality: state of the art. *Asian J Androl* 13:406–419.

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Thank you so much

Best wishes

Danqing (aja@sibs.ac.cn)

Appendix C.2. Figure 2.2. Hammerstedt, R.H., Graham, J.K., and J.P. Nolan. 1990. Cryopreservation of mammalian sperm: What we ask them to survive. *J Androl* 11:73–88.

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VITA

Reto Fritsche was born in Appenzell, Switzerland, in 1983. He studied veterinary medicine at the Vetsuisse Faculty, University of Bern, Switzerland, and obtained his veterinary degree (med. vet.) in 2007. He then worked as an associate veterinarian in a mixed animal practice for 2.5 years (Clinica Alpina, Sent, Switzerland). In 2012 he attained his Swiss doctorate degree in veterinary medicine (Dr. med. vet.) after working for 1.5 years as a clinical assistant and doctorate student at the Section of Small Animal Reproduction, Vetsuisse Faculty, University of Zurich, Switzerland. The title of his doctoral thesis was "Inheritance of ectopic ureters in Entlebucher Mountain Dogs". In July 2012, he began a combined residency/master of science program in Theriogenology at the School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA. He became a Diplomate of the American College of Theriogenologists in 2014.