

**Induced immotility during long-term  
storage at +5°C does not prolong survival  
of dog spermatozoa**

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The present thesis is a partial fulfilment of the requirements for Master of Science Degree (MSc) in Veterinary Medicine for International Students at the Swedish University of Agricultural Sciences (SLU), in the field of Animal Reproduction.

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**The days are not so far away when sex sorted preserved liquid semen will be available in drug stores saying “Let me stay cool, I will give you beautiful puppies”.**

To my parents (Abdus Satter and Jamela Khatun )

## ABSTRACT

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Preservation of canine semen by extension and chilling has provided the AI veterinarians with an opportunity to overcome limitations of both time and space. Several parameters are assessed to predict the fertility of canine spermatozoa for a given sample. Motility is one of them. Spermatozoa stay immotile while preserved in CLONE (Cryogenetic Laboratories of New England, Inc, USA) Chilled Semen Extender whereas they maintain motility over the whole 23 days period of study while preserved in a Tris egg yolk glucose extender at 5°C.

The aim of this study was to investigate whether the immotility induced by the CLONE chilled semen extender prolongs the lifespan of dog spermatozoa stored at 5°C, compared with a Tris-egg yolk-glucose (TG) extender, which maintains motility. Semen from eleven dogs was pooled, split in four aliquots, centrifuged, and the supernatants removed. The four sperm pellets were mixed with a TG extender; with the CLONE chilled semen (CL) extender; with TG extender mixed with TG but without egg yolk and with less glucose (TG+A<sup>TG</sup>); or with the CLONE extender mixed with the CLONE activator (CL+A<sup>CL</sup>) to a final sperm concentration of 200 x 10<sup>6</sup> spermatozoa / mL. The samples were stored at 5°C for 23 days and examined twelve times for sperm motility by computer-assisted sperm analysis (CASA), as well as for plasma membrane and acrosome integrity, glucose consumption, and DNA fragmentation index (DFI). The experiment was performed in triplicate. The third replicate was examined for presence of bacteria. For the statistical calculations, the 23 experimental days were divided into three periods, the first period comprising days 1–8, the second period days 9–14, and the third period days 15–23. Total motility (TM%) did not differ between extenders in the first period, but was higher in TG and TG+A<sup>TG</sup> compared with CL+A<sup>CL</sup> in the second and third periods, and with CL in the third period (P<0.05). Progressive motility (PM%) was higher in TG (P<0.0001), TG+A<sup>TG</sup> (P=0.0007) and CL+A<sup>CL</sup> (P=0.0176) than in CL in the first period. Both TG and TG+A<sup>TG</sup> maintained higher PM% than did CL and CL+A<sup>CL</sup> in the second and third period. Tris-egg yolk-glucose and TG+A<sup>TG</sup> maintained higher straight line velocity (VSL) and average path velocity (VAP) compared with CL and CL+A<sup>CL</sup> during the whole experimental period. Curvilinear velocity (VCL) was higher in TG, CL, and TG+A<sup>TG</sup> than in CL+A<sup>CL</sup> samples (P<0.05) in the first and third periods. No differences in lateral head displacement (LHD) were observed between extenders or over time. Acrosome and plasma membrane integrity decreased over time, but there were no differences among the extenders in the first and second periods (P>0.05). In the third period, however, TG, TG+A<sup>TG</sup>, and CL maintained acrosome and plasma membrane integrity better than CL+A<sup>CL</sup>. Glucose consumption was not significantly different between extenders until the third period, when it was higher in CL and CL+A<sup>CL</sup> than in TG (P=0.0055) and TG+A<sup>TG</sup> (P=0.0010). No breakdown of DNA chromatin (P>0.05) occurred until day 14. A lower DFI was observed in TG+A<sup>TG</sup> than in TG and CL in the third period. Microorganisms found were mainly a sparse growth of *Escherichia coli* (days 1, 8, and 14) and a mixed flora (days 1, 8, 14, and 23), but also included yeast (days 14 and 23) and *Pseudomonas fluorescens* (day 23). In conclusion, spermatozoa preserved in TG or TG+A<sup>TG</sup> showed better values for all the different parameters throughout the experiment compared with sperm subjected to CL or CL+A<sup>CL</sup>. Consequently, the immotility induced by the CLONE chilled semen extender during long-term cold storage at 5°C did not prolong the lifespan of spermatozoa compared with the lifespan following storage in Tris-egg yolk-glucose. In addition, our results indicate that good quality dog semen may possibly be stored for up to 14 days in

TG extender at 5°C, with retained fertilizing capacity. *In vivo* studies should, however, be performed to further support this conclusion.

Keywords: chilled semen, computer-assisted sperm analysis (CASA), sperm chromatin structure assay (SCSA), DNA fragmentation index (DFI).

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## **General Background (A review of the literature)**

### **Artificial insemination in the dog**

Artificial insemination in the dog is recommended when natural mating is not possible due to anatomical, physiological or behavioral causes or geographical distance when the dog and bitch reside in different places, or when one ejaculate from a valuable stud dog is to be divided for insemination of several bitches. AI technology has enabled the breeders to increase the quality of their breed by inseminating semen collected from an exceptional male, and even after its death a valuable stud dog can continue to produce offspring with semen preserved frozen in a canine semen bank.

The first documented artificial insemination in the dog was successfully performed by the Italian natural philosopher Lazzaro Spallanzani in 1778 (see Heape, 1897) who obtained a litter of 2 pups 63 days after the insemination. Artificial insemination in the dog has been performed many times since the findings of Spallanzani but was done for experimental purposes, until in 1954 when Harrop et al. reported that they had produced a litter of 2 healthy male pups from a maiden Greyhound bitch inseminated with chilled extended semen. This report drew the attention of veterinarians to put emphasis on canine reproduction research. In 1969, Seager was the first to report that he had performed AI successfully in the dog with frozen semen. In the last 25 years, a lot of research has been conducted on different aspects of canine reproduction, and AI technology has been well developed. Especially the technique of intrauterine insemination using the Scandinavian catheter (Andersen, 1975; Linde-Forsberg, 2001) without any surgical intervention increased the success rate of AI in the dog (Linde-Forsberg and Forsberg, 1989; Rota et al., 1999, Linde-Forsberg 1995; 2001). Today AI in the dog is a common procedure in veterinary clinics.

### **Preservation of canine semen**

The discovery that bull semen could be frozen and still retain its fertilizing capacity (Smith et al., 1950) encouraged other workers to conduct experiments with canine semen and that of other animals (Bouchard et al., 1958; Foote, 1964). Sperm rich 2<sup>nd</sup> fractions of ejaculates are normally preferred for preservation. Semen can be collected easily by digital manipulation (Linde-Forsberg, 1995). The ejaculates are then subjected to centrifugation to remove the seminal plasma. High centrifugation speed may cause damage to the plasma membrane of spermatozoa (Rijsselaere et al., 2002). However, centrifugation at 700g for 6-10 min was found to be suitable for removing the seminal plasma from canine semen (Peña and Linde-Forsberg, 2000) and is the routine procedure at the Division of Reproduction at SLU. Thereafter, the sperm pellets are mixed with diluents to a

final concentration of  $200 \times 10^6$  spermatozoa / mL for preservation (Peña and Linde-Forsberg, 2000).

Cryopreservation techniques have been adapted for dog semen and semen can be preserved for an unlimited period in liquid nitrogen (Nagase et al., 1954; Mixner, 1969; Seager, 1969) but cryopreserved semen has got some limitations in using for AI and international shipment (such as a somewhat lower conception rate, and that thawing of semen and performing AI is more time consuming and thus more expensive, the risks involved with the handling of liquid nitrogen, problems with carrying of the large nitrogen containers, international rules and regulations pertaining to the transportation of nitrogen containers in air craft etc). Therefore, chilled extended semen is often the best option for AI in veterinary clinics. A temperature of 4°C to 5°C (39°F to 41°F) is found to be the most suitable for cold storage of canine semen (Morton et al., 1989; Bouchard, 1990; Brown, 1992). The rate of cooling is important to prevent the spermatozoa from cold shock. Slow cooling allows the cellular adjustment related to phase transition changes in lipids and altered functional state of the sperm membrane (Salisbury et al., 1978). The optimal cooling rate for chilling of canine semen has been suggested to be from -0.3 to -1°C/min (Bouchard et al., 1990). Recent studies showed that canine semen can be stored for up to around 3 weeks if it is diluted with a Tris egg yolk extender with 70mM glucose or 70mM fructose (Ponglowhapan et al., 2004) and preserved at 5°C.

### **Extenders for the preservation of canine semen**

A good extender is expected to have nutrients as a source of energy, buffer to prevent against harmful changes in pH and to provide a physiologic osmotic pressure and concentration of electrolytes to protect cells from cold shock during the cooling process, and cryoprotectants to reduce sperm cell damage during freezing and subsequent thawing, as well as antibiotics to prevent growth of bacteria (see Concannon et al., 1989).

Seminal fluid has been found to shorten the survival of dog spermatozoa (Freiberg, 1935; Rota et al., 1995), thus it was recommended to remove seminal fluid and mix with a diluent consisting of glucose, potassium, sodium tartrate and salt free peptone (Freiberg, 1935). The first successful use of chilled extended dog semen in artificial insemination was reported by Harrop in 1954, who extended the semen with pasteurized milk and preserved it at 4°C for several hours before artificially inseminating a bitch. Since then many types of extenders have been formulated and tested for their capability to preserve semen chilled or frozen, such as skim milk extender (Oloufa et al., 1956; Province et al., 1984), non fat dried milk solid glucose extender (Bouchard, 1990), tomato juice and coconut milk (Chieffi and Masotti, 1959 cited by Seager and Fletcher, 1972), glycerol, egg yolk and lactose (Nagase et al., 1964), Caprogen Egg Yolk extender (Province et al., 1984), Tris buffered Egg Yolk extender (Gill et al., 1970), Citrate Egg Yolk extender (Christiansen, 1984), Citrate-Bicarbonate Egg Yolk extender (Foote 1964; Province et al., 1984), homogenized, pasteurized cream containing 12% fat

(Linde-Forsberg, 1995; Rota et al., 1995), Tris-fructose, Tris-glucose and EDTA extender (Iguer-Ouada et al., 2001), Tris citrate egg yolk glucose with Equex STM paste (Rota et al., 1997) and Tris citrate egg yolk with 70mM fructose/glucose, in which spermatozoa can be preserved chilled for up to 3 weeks (Ponglowhapan et al., 2004).

Following the increased demand from breeders, several commercial companies are marketing extenders for dog semen preservation. However, the compositions of these commercial extenders are in most cases a trade secret. This includes CLONE (Cryogenetic Laboratories of New England Inc, Philadelphia, USA), Biladyl and Triladyl (Minitüb, Tiefenbach, Germany), Green extender, Fresh-phos extenders (IMV, France); and, Androhep (Minitüb of America), Puppy Pak (ICSB, Oregon, USA), Kenny's Canine extender, Canine Express Semen Shipper (Exodus Breeders Corporation, York, PA 17406, USA) and Fresh Kooled Canine Semen extender (Camelot Farms, Texas, USA).

### **Composition of semen extenders**

The main components of semen extenders are sugars that provide metabolic energy to spermatozoa and maintain proper osmotic pressure of the media (Salisbury et al., 1978). The most commonly used sugars are glucose and fructose. Other sugars that may be used are mannose, lactose, sucrose, trehalose, raffinose and arabinose (Seager 1969; England 1993; Aisen et al., 2002). Egg yolk is an important ingredient of semen extenders that prevents the spermatozoa from cold shock during the chilling process (Watson and Martin, 1975) and improves the sperm quality of chilled dog semen (Foote, 1964). The osmolarity of fresh canine semen ranges from 294.1 to 337.8 (Rota et al., 1995) and the average pH of dog semen is 6.4, and ranges from 5.8 to 7.2 in different fractions of the ejaculate (Bartlett et al., 1962). Spermatozoa use glucose and produce lactic acid thereby decreasing the pH during storage (Foote, 1963). To maintain optimum pH of the semen some buffers are used in the extenders. The most frequently used buffer is Tris (hydroxymethyl) aminomethane (Tris) and others are Sodium bicarbonate, N-2-hydroxyethylpiperazine-N-ethanesulphonic acid (HEPES), basic salts (NaCl, KCl) (Kuster and Althouse 1999). Egg yolk and sugars are enriched media for microorganisms. Common enteropathogenic bacteria isolated from semen are *Pasteurella multocida*,  $\beta$ -hemolytic *Streptococci* and *E coli* (Bjurström and Linde-Forsberg, 1992). These organisms are able to use sugar and produce acid and gas, change the pH of the medium and may be detrimental to spermatozoa (Brenner et al., 2005). However, to prevent the bacterial growth a combination of benzyl penicillin (1000IU/mL) and dihydrostreptomycin (1mg/mL) is usually used for canine semen extenders (Linde-Forsberg, 1991).

## **In vitro assessment of semen fertility**

To date a lot of effort has been put into the development of a reliable technique for *in vitro* evaluation of canine semen with the aim to enable the maximum fertility from a valuable sire, but no single method is yet available which can provide an accurate prediction of the fertility of semen (Amann et al., 1993). The evaluation of semen includes assessment of the physical characteristics of the ejaculate such as color, volume, concentration of spermatozoa, pH and osmolarity (Seager, 1972) and morpho-physiology studies of spermatozoa. It should be noted that the basic requirements for the laboratory assays are objectivity, repeatability and accuracy. Combinations of several assays are needed to predict the fertility of a given semen sample (Graham et al., 1990; Rodriguez-Martinez, 2003). Januskauskas et al. (2001) found a higher correlation between predicted value with known fertility when motility assessments were combined with other parameters of sperm function ( $r^2=0.83$ ) in AI bulls. However, several procedures have been used to evaluate the physiological and morphological characteristics thereby differentiating viable cells from dead cells, such as plasma membrane integrity (Mayer et al., 1951; Swanson and Bearden, 1951; Hackett and Macpherson, 1965) and acrosomal integrity (Wells and Awa, 1970; Chacarov and Mollova, 1976; Cross and Meizel, 1989; Ericsson, 1989; Axner et al., 2002) but the number of cells examined by these two procedures are small. To make an accurate prediction of the fertility of a semen sample a maximum number of cells should be examined. Flow cytometry offers the possibility to analyze thousands of cells in a very short time (<1 min) with precision and gives an objective assessment of sperm chromatin structure (Evanson et al., 1980), sperm morphology (Pinkel et al., 1979) and cell viability (Garner et al., 1986; Peña et al., 1999a; 1999b). Motility of spermatozoa is an important indicator of the viability of the cells. Computer assisted semen analysis (CASA) has become a widely used tool that gives objective information about the cell characteristics: motion, velocities, morphology and concentration of cells. The potential for CASA in clinical applications is very high (Mortimer, 1997). Studies have shown that there are significant relationships between aspects of sperm movement and cervical penetration, as determined using a cervical mucus substitute (Mortimer et al., 1990), and fertilization *in vitro* (Holt et al., 1985; Sukcharoen et al., 1996) and *in vivo* (Barratt et al., 1993; Macleod and Irvine, 1995).

## **Introduction to the research report**

The idea of extension and preservation of canine semen was first generated by Freiberg, in 1935 when he found that seminal fluid is detrimental for canine semen preservation. Now it is widely accepted that the extender plays a vital role to preserve semen and provides spermatozoa with nutrients for energy, proper pH, osmolarity to maintain its cellular integrity and fertility. The first successful artificial insemination with chilled dog semen was reported by Harrop in 1954, and that with frozen semen by Seager in 1969. Many attempts have been made over the years to prepare a suitable extender for long term preservation of semen. Cryopreservation methods have already been developed for dog semen and the semen can be preserved for unlimited periods of time in liquid nitrogen at  $-196^{\circ}\text{C}$  (Nagase et al., 1954; Mixner, 1969; Seager, 1969; Rota et al., 1997; 1999; Linde-Forsberg, 2002). However, considering the high cost of AI, shipment and handling of frozen semen a method was tested showing that semen can be stored chilled and shipped to a semen bank for freezing (Hermansson and Linde Forsberg, 2006). For this reason AI veterinarians are getting interested in preservation of semen in chilled liquid state at  $5^{\circ}\text{C}$  for the convenient use of semen for AI at the optimal period of estrus in the bitch without facing any obstacle of time and place.

The use of extender for chilled dog semen preservation was initiated by Harrop in 1954. Since then many types of extenders have been tried over the ensuing 50 years but Tris citrate egg yolk extenders with glucose or fructose have proved to be among the best laboratory made extenders (Linde-Forsberg, 1995; Rota et al., 1995; Iguer-Ouada and Versteegen 2001; Peña et al., 2006).

Following the increased demand of AI in the dog, several commercial companies are providing extenders that are widely used in AI clinics in different countries, although their elemental and other composition is not known due to proprietary ownership. The CLONE Inc (Cryogenetic Laboratories of New England Inc, Philadelphia, USA) is one of them. An interesting thing is that the spermatozoa become immotile when mixed with the CLONE Chilled Semen Extender and preserved at  $5^{\circ}\text{C}$ .

### **Aim of the Study**

The aim of this study was to find out whether immotility induced by the CLONE chilled semen extender prolonged the lifespan of spermatozoa compared to when they were stored in Tris-egg yolk-glucose extenders which maintained the motility, as assessed by glucose consumption, sperm motility, plasma membrane and acrosomal integrity and sperm chromatin structure during 23 days of storage at  $5^{\circ}\text{C}$ .

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## Research Report

# Induced immotility during long-term storage at +5°C does not prolong survival of dog spermatozoa

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### Abstract

The aim of this study was to investigate whether the immotility induced by the CLONE chilled semen extender prolongs the lifespan of dog spermatozoa stored at 5°C, compared with a Tris-egg yolk-glucose (TG) extender, which maintains motility. Semen from eleven dogs was pooled, split in four aliquots, centrifuged, and the supernatants removed. The four sperm pellets were mixed with TG extender; with the CLONE chilled semen (CL) extender; with TG extender mixed with a TG without egg yolk and with less glucose (TG+A<sup>TG</sup>); or with the CLONE extender mixed with the CLONE activator (CL+A<sup>CL</sup>) to a final sperm concentration of 200 x 10<sup>6</sup> spermatozoa / mL. The samples were stored at 5°C for 23 days and examined twelve times for sperm motility by computer-assisted sperm analysis (CASA), as well as for plasma membrane and acrosome integrity, glucose consumption, and DNA fragmentation index (DFI). The experiment was performed in triplicate. The third replicate was examined for presence of bacteria. For the statistical calculations, the 23 experimental days were divided into three periods, the first period comprising days 1–8, the second period days 9–14, and the third period days 15–23. Total motility (TM%) did not differ between extenders in the first period, but was higher in TG and TG+A<sup>TG</sup> compared with CL+A<sup>CL</sup> in the second and third periods, and with CL in the third period (P<0.05). Progressive motility (PM%) was higher in TG (P<0.0001), TG+A<sup>TG</sup> (P=0.0007) and CL+A<sup>CL</sup> (P=0.0176) than in CL in the first period. Both TG and TG+A<sup>TG</sup> maintained higher PM% than did CL and CL+A<sup>CL</sup> in the second and third period. Tris-egg yolk-glucose and TG+A<sup>TG</sup> maintained higher straight line velocity (VSL) and average path velocity (VAP) compared with CL and CL+A<sup>CL</sup> during the whole experimental period. Curvilinear velocity (VCL) was higher in TG, CL, and TG+A<sup>TG</sup> than in CL+A<sup>CL</sup> samples (P<0.05) in the first and third periods. No differences in lateral head displacement (LHD) were observed between extenders or over time. Acrosome and plasma membrane integrity decreased over time, but there were no differences among the extenders in the first and second periods (P>0.05). In the third period, however, TG, TG+A<sup>TG</sup>, and CL maintained acrosome and plasma membrane integrity better than CL+A<sup>CL</sup>. Glucose consumption was not significantly different between extenders until the third period, when it was higher in CL and CL+A<sup>CL</sup> than in TG (P=0.0055) and TG+A<sup>TG</sup> (P=0.0010). No breakdown of DNA chromatin (P>0.05) occurred until day 14. A lower DFI was observed in TG+A<sup>TG</sup> than in TG and CL in the third period. Microorganisms found were mainly a sparse growth of *Escherichia coli* (days 1, 8, and 14) and a mixed flora (days 1, 8, 14, and 23), but also included yeast (days 14 and 23) and *Pseudomonas fluorescens* (day 23). In conclusion, spermatozoa preserved in

TG or TG+A<sup>TG</sup> showed better values for all the different parameters throughout the experiment compared with sperm subjected to CL or CL+A<sup>CL</sup>. Consequently, the immotility induced by the CLONE chilled semen extender during long-term cold storage at 5°C did not prolong the lifespan of spermatozoa compared with the lifespan following storage in Tris-egg yolk-glucose. In addition, our results indicate that good quality dog semen may possibly be stored for up to 14 days in TG extender at 5°C, with retained fertilizing capacity. *In vivo* studies should, however, be performed to further support this conclusion.

*Keywords:* chilled semen, computer-assisted sperm analysis (CASA), sperm chromatin structure assay (SCSA), DNA fragmentation index (DFI).

## 1. Introduction

Preserving canine semen by adding an extender and chilling it ensures that the semen can be available for artificial insemination (AI) of the bitch at the optimum time during estrus even if the male is not present, or lives far from where the bitch lives. Handling and shipping of chilled semen is both easier and cheaper than when using frozen semen. It has also been demonstrated that dog semen can be successfully frozen after having being cold stored for 1–2 days [1]. Semen can consequently be collected nearer to the dog's home and sent chilled to a semen bank to be frozen. Furthermore, the success rates of AI are higher for chilled semen than for frozen semen when equally good methods for timing of the estrous cycle, and for AI, are used [2], [3], [4] and [5].

A limitation for the use of chilled semen is the survival time of the preserved spermatozoa. Means of prolonging their survival are the addition of suitable extenders, providing energy, maintaining pH and osmolarity, and protecting the acrosome and plasma membrane integrity against damage, while chilling lowers sperm metabolism. For storage of chilled dog semen, 4–5°C has been found to be the most suitable temperature [6], [7] and [8]. No study testing whether inducing immotility of the spermatozoa during cold storage would further extend the survival time of dog semen has so far been published.

Many studies have been performed on chilled dog semen, but the periods of cold storage have usually been only 1–4 days; e.g. [2], [9] and [10]. In a previous study, however, it was found that the viability of dog spermatozoa stored at +5°C in Tris-egg yolk-fructose or Tris egg yolk-glucose extenders lasted for about 3 weeks [11]. The *in vivo* fertilizing capacity of the stored semen was, however, not tested. The first successful pregnancy using chilled semen was reported by Harrop in 1954 [12]. Later, Tsutsui et al. (2003) [13] obtained 83.3% [five bitches out of 6 (5/6)] pregnancies with an average litter size of 3.2 pups by inseminating semen that had been preserved chilled for 4 days. Their conception rates obtained with semen stored for 5, 6, 7, and 9 days were 50.0% (1/2), 0% (0/5), 33.3% (1/3), and 0% (0/1), respectively, while the numbers of pups obtained with semen stored for 5 and 7 days were two and one. Versteegen et al. (2005) [14] report a pregnancy rate of 60% with semen that had been preserved chilled for 7–11 days. The average litter size was 2.3±1.4 by single AI (five out of ten bitches pregnant) and 4.6±2.8 by two AIs at an interval of 48 hours (seven out of ten bitches pregnant). The longevity of the spermatozoa was increased by centrifuging the chilled semen, removing the supernatant, and adding fresh extender [14].

Sperm heads are tightly packed with DNA materials. Components of the diluting media, centrifuging, and prolonged cold storage cause damage to the plasma membrane, acrosome, and DNA chromatin [15], [16] and [17]. Perez-Llano et al. (2006) [18] found that sperm extenders delay, or partially prevent, sperm DNA fragmentation in boar spermatozoa. A strong correlation between the sperm chromatin structure assay (SCSA) and sperm head morphometry in dog spermatozoa has been found [19], but so far, only one study has been published on DNA fragmentation of dog semen [20]. Epididymal sperm and ejaculated semen from Iberian deer, dogs, and rams was collected, diluted with Tris Sodium Chloride Ethylene-diamine-tetra-acetic Acid (TNE) buffer and preserved in LN<sub>2</sub> in an ultra cold freezer (-80°C). Sperm chromatin structure assay was performed and significantly higher DNA fragmentation index (DFI) values were found in spermatozoa from the caput compared with sperm from the corpus and cauda epididymis. No differences were seen between any of the epididymal spermatozoa and ejaculated dog spermatozoa. The present study is the first to investigate DFI in dog spermatozoa, preserved using ordinary dog semen extenders and cooling procedures.

Among the available extenders for chilled canine semen, the commercial CLONE Chilled Semen Kit (Cryogenetic Laboratories of New England, Inc., Philadelphia, PA, USA) and various Tris-egg yolk extenders, e.g., the Uppsala Chilled Semen Extender [10], are well known and widely used in practice. According to the manufacturer's instructions, semen preserved using the CLONE Chilled Semen Kit should be used within 36–48 hours, while for the Tris-egg yolk extenders, a time limit of 1–4 days is usually recommended, although the results of Ponglowhapan et al. (2004) [11] suggest that a longer period of preservation may be possible. When preserved in CLONE extender spermatozoa are rendered immotile, and remain immotile throughout the period of storage at 5°C. They regain motility after addition of the activator in the CLONE Chilled Semen Kit and rearming at 37°C for 8–10 minutes. By contrast, when stored in Tris extenders the spermatozoa maintain their motility during storage at 5°C.

The aim of this study was to find out whether immotility induced by the CLONE chilled semen extender would prolong the survival of spermatozoa compared with storage in a Tris-egg yolk-glucose extender which maintained motility, as assessed by sperm motility, plasma membrane and acrosomal integrity, glucose consumption, and sperm chromatin structure during 23 days of storage at 5°C.

## **2. Materials and Methods**

### **2.1. Animals**

A total of eleven privately owned, clinically healthy dogs aged between 2 and 5 years were used in this study, seven or eight in each replicate: five Golden Retrievers, five Labrador Retrievers, and one German Shepherd Dog. For the ejaculates to be included in the study, the percentage of motile spermatozoa, assessed by subjective evaluation, and of morphologically normal spermatozoa of the fresh semen had to be  $\geq 85\%$ .

## 2.2. Preparation of extenders

The CLONE chilled semen extender (CL) and activator were analyzed at the Division of Clinical Chemistry at the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, for some of their elemental components and sugars. Their full composition was, however, not known. The compositions of the Tris-egg yolk-glucose (TG) and the TG activator, and the glucose content, pH, and osmolarities of TG, CL, and both activators are shown in Table 1, and what is known of the chemical contents of the two extenders and two activators is shown in Table 2.

The CL extender and the CL activator were found to contain glucose but not fructose. The Tris-egg yolk extenders, therefore, were also made with glucose. In a preliminary study, an activator for TG was made, based on what was known about the composition of the CLONE activator, i.e., that it contains only sodium, chloride, and glucose. The results of using this activator were poor (data not shown), possibly due to the low osmolarity and Tris concentration, resulting from mixing TG and this activator. In order for all samples to be treated similarly during this study, another TG extender was instead made without the egg yolk, and with a glucose content similar to that in the CLONE activator, and was used as activator for the TG samples.

According to the instructions for the CLONE Chilled Semen Kit, CLONE activator should be added to the chilled extended semen at the time of the AI and the sample should be left at 37°C for 10–20 min for reactivation before use. To further study the effects of the CLONE activator the experiment was designed to compare four different extenders: (1) the TG extender; (2) the CL extender; (3) the TG extender mixed 1:1 with the TG activator of the same basic composition as TG but without egg yolk and with a lower glucose content (TG+A<sup>TG</sup>); and (4) the CL extender mixed 1:1 with the CLONE activator (CL+A<sup>CL</sup>). The final concentrations of glucose in the four extenders were 58mM/L (TG), 49.4mM/L (CL), 43.5mM/L (TG + A<sup>TG</sup>), and 35.9mM/L (CL+A<sup>CL</sup>), respectively.

Table 1.

Composition, pH, and osmolarity of the Tris-egg yolk-glucose (TG) extender and TG activator (A<sup>TG</sup>), and glucose contents, pH, and osmolarity of the CLONE chilled semen (CL) extender and CL activator (A<sup>CL</sup>).

Extenders	Constituents	/100 ml	pH	Osmolarity
TG (extender 1)(58mM glucose/L)	Trishydroxyaminomethane	3.025 g	6.8	326
	Citric acid monohydrate	1.7 g		
	Benzympenicillin	0.06 g		
	DHS	0.1 g		
	Egg yolk	20% (v/v)		
	D-glucose	1.261 g		
	Distilled water	to 100 ml		
TG activator (A <sup>TG</sup> ) (23.1mM glucose/L)	Trishydroxyaminomethane	3.025 g	6.9	268
	Citric acid monohydrate	1.7 g		
	Benzympenicillin	0.06 g		
	DHS	0.1 g		
	D-glucose	0.45 g		
	Distilled water	to 100 ml		
*CL (extender 2)(48.5 mM glucose /L)			6.3	571
*CL activator (A <sup>CL</sup> ) (23.3 mM glucose /L)			6.3	199
TG+A <sup>TG</sup> (extender 3)			6.8	289
CL+A <sup>CL</sup> (extender 4)			6.3	354

\*The full composition of this extender/activator is not known.

The TG+A<sup>TG</sup> and CL+A<sup>CL</sup> extenders (extenders 3 and 4) were made on day 1 at room temperature before cooling to 5°C. Thereafter, on each of the twelve experimental days before evaluation of the samples, the protocol involved adding the A<sup>TG</sup> to the TG (extender 1) and the A<sup>CL</sup> to the CL (extender 2) before evaluation, in accordance with the CLONE instructions, while no such additions were made to extenders no. 3 and 4, that already contained the respective activators (Fig. 1). Using this design, the four samples were pairwise subjected to the same treatments, i.e., TG and CL, and TG+A<sup>TG</sup> and CL+A<sup>CL</sup>.

Table 2.

Some components of the Tris-egg yolk-glucose (TG) and CLONE chilled semen (CL) extenders and the two activators.

Elements	TG	*CL	A <sup>TG</sup>	*A <sup>CL</sup>
Calcium	3.8 mmol/L	4.5 mmol/L	<0.7 mmol/L	<0.1 mmol/L
Magnesium	1.24 mmol/L	1.22 mmol/L	<0.05 mmol/L	<0.05 mmol/L
Phosphate	2.8 mmol/L	2.7 mmol/L	<0.1 mmol/L	<0.1 mmol/L
Sodium	19 mmol/L	17 mmol/L	7 mmol/L	69 mmol/L
Potassium	4.6 mmol/L	3.8 mmol/L	0.2 mmol/L	0.3 mmol/L
Chloride	35 mmol/L	34 mmol/L	32 mmol/L	69 mmol/L
Zink	149 µmol/L	117 µmol/L	0 µmol/L	0 µmol/L
Protein	43 g/L	34 g/L	8 g/L	2 g/L
Albumin	8 g/L	7 g/L	0 g/L	0 g/L
Triglycerides	10.3 mmol/L	276.4 mmol/L	<0.1 mmol/L	<0.1 mmol/L
Cholesterol	63.7 mmol/L	8.4 mmol/L	<0.1 mmol/L	<0.1 mmol/L
Egg yolk	20%	Not known	None	Not known

\*The full composition of this extender/activator is not known.

### 2.3. Semen collection and processing

The sperm-rich fraction of the ejaculates was collected by digital manipulation into prewarmed, calibrated polypropylene vials (Linde-Forsberg, 1995) [5]. The semen samples were pooled in a 50mL test tube, the volume was recorded, the sample mixed well by turning the tube upside down several times, and the concentration then measured in a calibrated photometer (SpermaCue, Minitüb, Tiefenbach, Germany). Motility was immediately determined subjectively under a phase contrast microscope. An aliquot was sent to the sperm laboratory at the Department of Clinical Sciences, Division of Reproduction, SLU, Uppsala, for routine morphological analysis [21] [22] and [23].

### 2.4. Experimental design

The experimental design is illustrated in Fig. 1. The experiment was repeated in triplicate. A 500 µl sample was taken from the pooled semen to check its quality (see Section 2.5). The pool was then divided into four equal aliquots, which were centrifuged at 700 x g for 8 min. The supernatants were transferred into four separate test tubes and recentrifuged, at the same speed and time, to recover any remaining spermatozoa, and these pellets were added to the ones from the first centrifugation. An aliquot of 100 µl supernatant was collected from each of the four samples to measure the glucose content.

In order to calculate the correct dilution rate, the volume of the sperm pellets was determined before addition of extender to each sample. The sperm pellets were then resuspended with TG, CL, TG+A<sup>TG</sup>, or CL+A<sup>CL</sup> to produce a final sperm concentration of  $200 \times 10^6$  cells/mL. The four aliquots were placed in a cooling bench at room temperature for 45 minutes until the temperature reached 5°C. The cooling rate was -0.5°C/min. The samples were then stored at 5°C throughout the 23 days of the study.

The samples were evaluated for glucose consumption, motility, plasma membrane, and acrosomal integrity daily for the first 4 days, every second day from day 6 to day 14, and then on days 17, 20, and 23. Deoxyribonucleic acid

fragmentation index and bacteriological findings (third replicate) were assessed on days 1, 8, 14, and 23.

## ***2.5. Semen evaluation***

A 500 µl aliquot of semen was taken from each chilled semen sample on each experimental day for evaluation. Immediately after being taken out of the refrigerator, each sample was divided into three aliquots: 155 µl for glucose analysis, 20 µl for SCSA, and 325 µl for assessment of motility, and plasma membrane and acrosomal integrity.

### **2.5.1. Glucose analysis**

The glucose concentration of the samples was measured by spectrophotometry using an autoanalyzer (Konelab 30; Thermo Clinical Labsystems Oy, Vantaa, Finland). All the commercial reagents used in this study were obtained from Thermo Electron Corporation (Vantaa, Finland). Glucose concentration was expressed as mM/L. The minimum detection limit was 0.1 mM/L (2 mg/dL) and the measuring range was 0.3–40.0 mM/L (5–720 mg/dL). The extending measuring range after secondary dilution was 0.3–120.0 mM/L (5–2,160 mg/dL). An aliquot of 100 µl supernatant was collected by centrifuging 155 µl semen from each sample on each experimental day, lowered into liquid nitrogen for 30 seconds, and then preserved at -80°C until glucose analysis.



### **2.5.2. Sperm chromatin structure assay**

Following the protocol described by Januskauskas et al. (2001) [24], 20  $\mu$ l semen was mixed with 180  $\mu$ l TNE buffer and again 20 $\mu$ l of this diluted semen was mixed with 180 $\mu$ l TNE buffer to a concentration of  $2 \times 10^6$  spermatozoa/mL. An amount of 200 $\mu$ l of this diluted semen was frozen for 1 min in liquid nitrogen vapor and then for 1 min in liquid nitrogen. It was then preserved at  $-80^{\circ}\text{C}$  in an ultra freezer until flow cytometric analysis (FCA).

Semen samples were thawed on crushed ice and mixed with 400 $\mu$ l of low pH TRITON acid detergent solution which causes partial denaturation of DNA in situ. After 30 sec, 1,200 $\mu$ l of acridine orange (AO) solution was added and the samples analyzed by a FacsStar plus flow cytometer (Becton Dickinson Immunochemistry Systems, San Jose, CA, USA) equipped with standard optics, excited with an argon ion laser (Inova 90; Choerent, Santa Clara, CA, USA) tuned at 488 nm, and running at 200 mW.

Acridine orange intercalated to double-stranded DNA fluoresces green ( $530 \pm 30$  nm) and AO associated with single-stranded DNA fluoresces red ( $>630$  nm). From each sample, a total of 10,000 events were measured, with a flow rate of around 200 cells/sec [24]. The fluorescence stability of the flow cytometer was monitored daily using standard beads (Fluoresbrite plain YG 1.0  $\mu\text{M}$ ; Polysciences, Inc., Warrington, PA, USA).

All the samples were run at a time, with the same instrument settings. Data were collected using Cellquest, version 3.3 (Becton Dickinson). Scattergram analysis on raw data, with each point representing the coordinate of red and green fluorescence intensity values for every individual spermatozoon, was carried out using FCS Express, version 2 (DeNovo Software, Thornthill, Ontario, Canada). Events accumulated in the lower left corner correspond to sample debris and were excluded from the analysis. The results were expressed as DFI (%).

### **2.5.3. Addition of activator**

The rest of the samples (325 $\mu$ l) were left at room temperature for 15 minutes. Activator from the CLONE Chilled Semen Kit was added to the samples preserved with CL extender, and TG activator was added to the sample preserved with TG extender. All four samples were placed in a heating cupboard at  $37^{\circ}\text{C}$  for 10 min, and then evaluated for motility, and plasma membrane and acrosomal integrity.

### **2.5.4. Evaluation of sperm motility**

Sperm motility was evaluated using computer-assisted sperm analysis (CASA) (Strömberg-Mika Cell motion analyzer; Mika Medical, Bad Feilnbach, Germany) at  $37^{\circ}\text{C}$ . The semen samples were diluted four times with phosphate-buffered saline (PBS) to make a readable concentration for CASA. At least 200 sperm cells were counted from four randomly selected fields. The following parameters were measured: the percentage of total motility (TM%), the percentage of linearly motile

spermatozoa (progressive motility, PM%), average path velocity (VAP,  $\mu\text{m/s}$ ), straight line velocity (VSL,  $\mu\text{m/s}$ ), curvilinear velocity (VCL,  $\mu\text{m/s}$ ), and lateral head displacement (LHD,  $\mu\text{m}$ ). The percentage of progressive motility was calculated from among the motile spermatozoa and the velocities were calculated from spermatozoa showing linear motility. The motion analyzer was set up following the protocol described by Ponglowhapan et al. (2004) [11].

#### **2.5.5. Assessment of plasma membrane integrity**

A dual fluorescent staining technique was used to evaluate sperm plasma membrane integrity [10]. A fluorescent staining medium was prepared with saline, formalin, propidium iodide (PI), and 6-carboxyfluorescein diacetate (C-FDA) (Calbiochem, LabKemi, Stockholm, Sweden). At least 200 spermatozoa were assessed for each sample and classified into two categories (intact and damaged plasma membranes). The results are presented as percentages.

#### **2.5.6. Assessment of acrosomal integrity**

Acrosomal integrity was evaluated by staining the spermatozoa with a fluorescent probe made from PBS, fluorescent isothiocyanate-conjugated peanut agglutinin (FITC-PNA) and propidium iodide (PI) (Calbiochem, LabKemi, Stockholm, Sweden), following the modified protocol of Axnér et al. (2002) [25]. At least 200 spermatozoa were counted in each smear and categorized according to their FITC-PNA patterns (intact or damaged acrosome). The results are expressed as percentages.

### **2.6. Bacteriological study**

In the last replicate of the experiment, bacteriological cultures were made from all samples on days 1, 8, 14, and 23. Samples were taken from the extended preserved semen using sterile culture swabs with Amies medium (Copan International, Copanitalia S.p.a., Brescia, Italy) and sent to the National Veterinary Institute, Uppsala, Sweden for microbiological culturing. The samples were cultured within 6 hours. The swab was streaked onto horse blood agar and Bromocresol purple lactose agar and incubated at 37°C. The samples were read after 1, 2, and 7 days. The term “mixed flora” was used for a group of three or more different non-pathogenic bacteria found to grow in the samples.

### **2.7. Statistical analysis**

Statistical analysis of the data was performed using the SAS software, version 9 (SAS Institute, Inc., Cary, NC, USA). Analysis of variance was applied to analysis of the data using the GLM procedure. For the statistical analysis, the study was divided into three time periods: days 1–8 = the first period (six observations); days 9–14 = the second period (three observations); and days 15–23 = the third period (three observations). The statistical model for all parameters included the fixed

effects of extender, replicate, and time period, and the interaction between extender and time period. Values are presented in graphs/tables as means and standard error of the mean (SEM) and the differences are considered to be statistically significant when  $P < 0.05$ .

### 3. Results

#### 3.1. Pooled semen

The mean concentration of spermatozoa in freshly pooled semen was  $264.7 \pm 26.8 \times 10^6/\text{mL}$ . Subjective motility ranged from 85% to 95%, and  $\geq 85\%$  of spermatozoa were morphologically normal. The mean volume of the sperm pellet was  $466.7 \pm 21.5 \mu\text{l}$ . In pooled semen, the mean TM% was  $85.9 \pm 3.1\%$ , PM%  $53.8 \pm 2.4\%$ , plasma membrane integrity  $83.7 \pm 2.4\%$ , acrosomal integrity  $76.0 \pm 3.8\%$ , and DFI  $4.3 \pm 2.4\%$ . The characteristic movement patterns of VSL, VAP, VCL, and LHD averages were  $81.9 \pm 20.1 \mu\text{m}/\text{sec}$ ,  $91.4 \pm 20.7 \mu\text{m}/\text{sec}$ ,  $150.8 \pm 5.8 \mu\text{m}/\text{sec}$ , and  $4.9 \pm 1.2 \mu\text{m}$ , respectively.

#### 3.2. Motility

The TM% of the spermatozoa in the four extenders over time is shown in Figure 2 and Table 3. Immediately after mixing of the sperm pellets with the extenders the mean TM% in TG, CL, TG+A<sup>TG</sup>, and CL+A<sup>CL</sup> samples was  $90.9 \pm 2.1$ ,  $80.2 \pm 4.6$ ,  $87.5 \pm 1.1$ , and  $85.8 \pm 2.3$ , respectively. During the first 8 days, the TM% did not differ between the four samples ( $P > 0.05$ ). Thereafter, it was higher in TG and TG+A<sup>TG</sup> than in CL+A<sup>CL</sup> ( $P < 0.0001$ ) throughout the experiment. The TM% was slightly lower in the second period ( $P = 0.06$ ) and significantly lower in the third period in CL samples compared with the TG ( $P < 0.0001$ ) and TG+A<sup>TG</sup> ( $P = 0.014$ ) samples. Overall, TG and TG+A<sup>TG</sup> samples showed a higher TM% than CL and CL+A<sup>CL</sup> samples throughout the experimental period.

The percentage of progressive motility on day 1 (immediately after addition of each extender) was  $80.1 \pm 13.4$  in TG,  $89.4 \pm 3.1$  in CL,  $82.8 \pm 12.2$  in TG+A<sup>TG</sup>, and  $82.3 \pm 7.5$  in CL+A<sup>CL</sup>. The PM% was higher in the TG ( $P < 0.0001$ ), TG+A<sup>TG</sup> ( $P = 0.0007$ ), and CL+A<sup>CL</sup> ( $P = 0.0176$ ) samples than in the CL sample in the first period. Both TG and TG+A<sup>TG</sup> maintained a higher PM% ( $P < 0.05$ ) compared with the CL and CL+A<sup>CL</sup> samples over the last two periods.

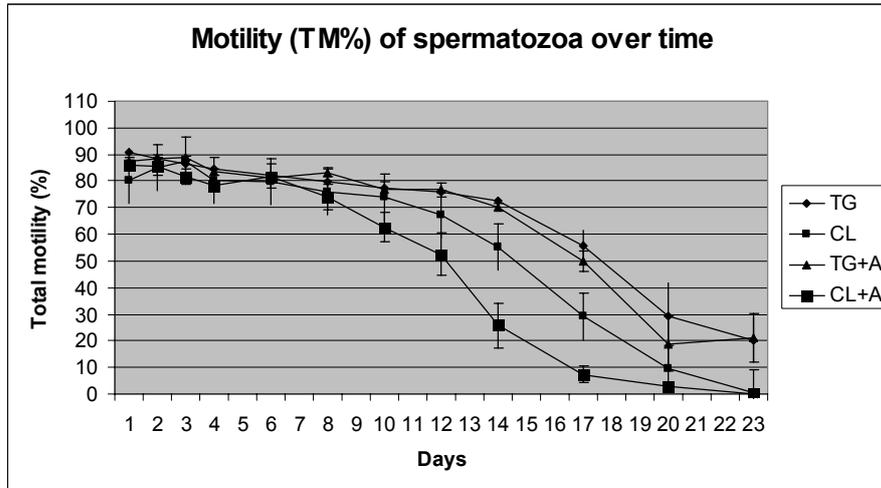


Figure 2. Total motility percentages (TM%) of spermatozoa over time (means ± S.E.M).

Table 3.

Percentage of total motility (TM%) and progressive motility (PM%), and straight line velocity (VSL) of spermatozoa over the three time periods (P-1–P-3). Least square means (LSMs) were used to analyze the data.

	TM%			PM%			VSL		
	P-1	P-2	P-3	P-1	P-2	P-3	P-1	P-2	P-3
TG	85.3 <sup>a</sup>	75.3 <sup>a</sup>	35.1 <sup>a</sup>	65.6 <sup>a</sup>	50.1 <sup>a</sup>	23.4 <sup>a</sup>	130.9 <sup>a</sup>	120.5 <sup>a</sup>	72.5 <sup>a</sup>
CL	81.4 <sup>a</sup>	65.5 <sup>a</sup>	13.1 <sup>b</sup>	41.7 <sup>b</sup>	26.1 <sup>b</sup>	7.8 <sup>b</sup>	102.6 <sup>b</sup>	89.6 <sup>b</sup>	33.2 <sup>b</sup>
TG+A <sup>TG</sup>	85.4 <sup>a</sup>	74.5 <sup>a</sup>	30 <sup>a</sup>	61.9 <sup>ac</sup>	48.3 <sup>a</sup>	25.4 <sup>a</sup>	129.8 <sup>a</sup>	115.8 <sup>a</sup>	67.7 <sup>a</sup>
CL+A <sup>CL</sup>	81.2 <sup>a</sup>	47 <sup>b</sup>	3.5 <sup>cb</sup>	55.7 <sup>ac</sup>	22.1 <sup>cb</sup>	8.1 <sup>b</sup>	97.8 <sup>cb</sup>	75.4 <sup>cb</sup>	33.2 <sup>cb</sup>

Period 1 included days 1–8; period 2 included days 9–14; and period 3 included days 15–23. Different superscript letters (a–d) indicate differences ( $P < 0.05$ ) among the extenders.

On day 1, the VSL, VAP, VCL, and average lateral head displacement (LHD<sub>avg</sub>) were  $127.9 \pm 29.5 \mu\text{m/s}$ ,  $150 \pm 4.6 \mu\text{m/s}$ ,  $187.9 \pm 3.5 \mu\text{m/s}$ , and  $3.9 \pm 0.6 \mu\text{m}$  in the TG,  $122.8 \pm 16.6 \mu\text{m/s}$ ,  $126.5 \pm 17.8 \mu\text{m/s}$ ,  $151.9 \pm 19.4 \mu\text{m/s}$ , and  $2.7 \pm 0.4 \mu\text{m}$  in the CL,  $147.7 \pm 9.6 \mu\text{m/s}$ ,  $156.1 \pm 4.3 \mu\text{m/s}$ ,  $196.7 \pm 10.1 \mu\text{m/s}$ , and  $4.0 \pm 0.3 \mu\text{m}$  in the TG+A<sup>TG</sup>, and  $120 \pm 11.3 \mu\text{m/s}$ ,  $125.9 \pm 15.3 \mu\text{m/s}$ ,  $153.3 \pm 16.1 \mu\text{m/s}$ , and  $2.9 \pm 0.5 \mu\text{m}$  in the CL+A<sup>CL</sup> sample. Spermatozoa in TG samples maintained higher VSL and VAP than sperm in CL and CL+A<sup>CL</sup> samples during the whole experimental period ( $P < 0.05$ ). Higher VCL was observed in TG, CL, and TG+A<sup>TG</sup> than in CL+A<sup>CL</sup> samples ( $P < 0.05$ ) in the first and third period, but there were no differences between the four samples in the second period. No differences were observed in LHD between the four extenders or over time during the experiment.

Table 4.

Average path velocity (VAP), curvilinear velocity (VCL), and lateral head displacement (LHD) of spermatozoa over the three time periods (P-1–P-3). Results were analyzed using least square means (LSMs).

Period 1 included days 1–8; period 2 included days 9–14; and period 3 included days 15–

	VAP			VCL			LHD		
	P-1	P-2	P-3	P-1	P-2	P-3	P-1	P-2	P-3
TG	144.1 <sup>a</sup>	131.3 <sup>a</sup>	84.7 <sup>a</sup>	206.7 <sup>a</sup>	216.4 <sup>a</sup>	169.5 <sup>a</sup>	5.2 <sup>a</sup>	6.0 <sup>a</sup>	6.4 <sup>a</sup>
CL	118.9 <sup>b</sup>	108.7 <sup>b</sup>	45.9 <sup>b</sup>	199.1 <sup>a</sup>	211.1 <sup>a</sup>	115.2 <sup>bc</sup>	5.9 <sup>a</sup>	6.7 <sup>a</sup>	5.0 <sup>a</sup>
TG+A <sup>TG</sup>	142.0 <sup>a</sup>	127.3 <sup>ab</sup>	76.8 <sup>a</sup>	205.7 <sup>a</sup>	212.0 <sup>a</sup>	151.3 <sup>ab</sup>	5.4 <sup>a</sup>	6.0 <sup>a</sup>	4.7 <sup>a</sup>
CL+A <sup>CL</sup>	110.3 <sup>cb</sup>	89.4 <sup>c</sup>	34.6 <sup>cb</sup>	168.8 <sup>b</sup>	189.1 <sup>a</sup>	95.8 <sup>c</sup>	4.7 <sup>a</sup>	6.8 <sup>a</sup>	5.1 <sup>a</sup>

23. Different superscript letters (a–c) indicate differences ( $P < 0.05$ ) among the extenders.

Table 5.

Plasma membrane integrity, acrosomal integrity, daily glucose consumption (glucose per day, GPD), and DNA fragmentation index (DFI) of spermatozoa over the three time periods (P-1–P-3), analyzed using least square means (LSMs).

	Plasma membrane			Acrosome			GPD			DFI		
	P-1	P-2	P-3	P-1	P-2	P-3	P-1	P-2	P-3	P-1	P-2	P-3
TG	77.2 <sup>a</sup>	65.8 <sup>a</sup>	37.2 <sup>a</sup>	65.1 <sup>a</sup>	49.7 <sup>a</sup>	28.6 <sup>a</sup>	0.9 <sup>a</sup>	0.5 <sup>a</sup>	0.8 <sup>b</sup>	3.3 <sup>a</sup>	3.8 <sup>a</sup>	14.1 <sup>a</sup>
CL	78.2 <sup>a</sup>	64.7 <sup>a</sup>	31.5 <sup>a</sup>	49.5 <sup>a</sup>	49.5 <sup>a</sup>	26.8 <sup>a</sup>	0.6 <sup>a</sup>	0.7 <sup>a</sup>	2.6 <sup>a</sup>	3.9 <sup>a</sup>	6.4 <sup>a</sup>	11.9 <sup>a</sup>
TG+A <sup>TG</sup>	76.3 <sup>a</sup>	66.2 <sup>a</sup>	36.2 <sup>a</sup>	64.3 <sup>a</sup>	48.7 <sup>a</sup>	26.4 <sup>a</sup>	0.9 <sup>a</sup>	0.4 <sup>a</sup>	0.5 <sup>c</sup>	3.3 <sup>a</sup>	3.5 <sup>a</sup>	4.4 <sup>b</sup>
CL+A <sup>CL</sup>	76.3 <sup>a</sup>	59.1 <sup>a</sup>	21.1 <sup>b</sup>	64.1 <sup>a</sup>	42.8 <sup>a</sup>	15.9 <sup>b</sup>	0.6 <sup>a</sup>	0.9 <sup>a</sup>	1.6 <sup>abc</sup>	3.7 <sup>a</sup>	4.3 <sup>a</sup>	6.7 <sup>b</sup>

Period 1 included days 1–8; period 2 included days 9–14; and period 3 included days 15–

23. Different superscript letters (a–c) indicate differences ( $P < 0.05$ ) among the extenders.

### 3.3. Plasma membrane integrity

On day 1, immediately after mixing with extenders, the mean percentage of spermatozoa with an intact plasma membrane was  $86.5 \pm 2.3$  in TG,  $85.3 \pm 2.6$  in CL,  $82.6 \pm 4.2$  in TG+A<sup>TG</sup>, and  $86.4 \pm 3.1$  in CL+A<sup>CL</sup> samples. Extension, chilling, and long-term preservation induced damage to the plasma membrane over time. For the first 14 days, losses of plasma membrane integrity were observed in all samples, but there were no significant differences between the extenders. Spermatozoa in TG and TG+A<sup>TG</sup> maintained plasma membrane integrity better than CL+A<sup>CL</sup> samples in the third period ( $P < 0.05$ ). Plasma membrane integrity results can be seen in Figure 3 and Table 5.

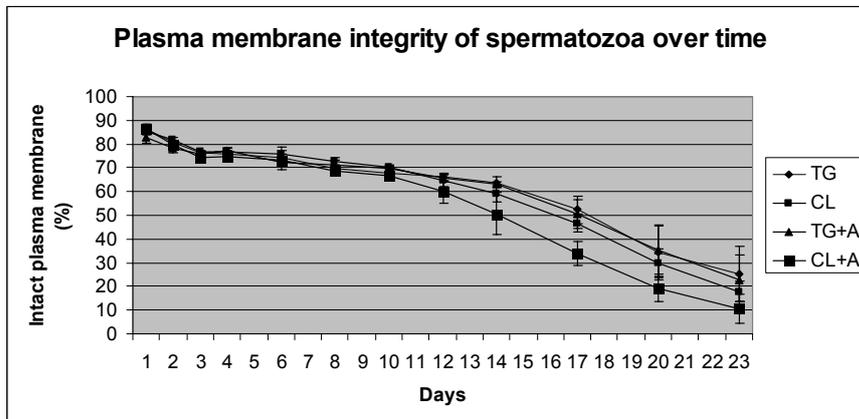


Figure 3. Plasma membrane integrity of spermatozoa over time (means  $\pm$  S.E.M).

### 3.4. Acrosome integrity

Figure 4 and Table 5 show acrosome integrity. After extension on day 1, the mean percentage of spermatozoa with an intact acrosome was  $74.5 \pm 6.6$  in TG,  $75.4 \pm 7.7$  in CL,  $73.8 \pm 4.6$  in TG+A<sup>TG</sup>, and  $74.3 \pm 7.9$  in CL+A<sup>CL</sup> samples. Acrosome integrity decreased gradually over time in all samples, but there were no differences between the extenders ( $P > 0.05$ ) in the first and second period. During the third period of the experiment, higher frequencies of intact acrosomes were observed in spermatozoa in TG ( $P = 0.020$ ) and in TG+A<sup>TG</sup> ( $P = 0.0101$ ) than in spermatozoa in CL+A<sup>CL</sup>.

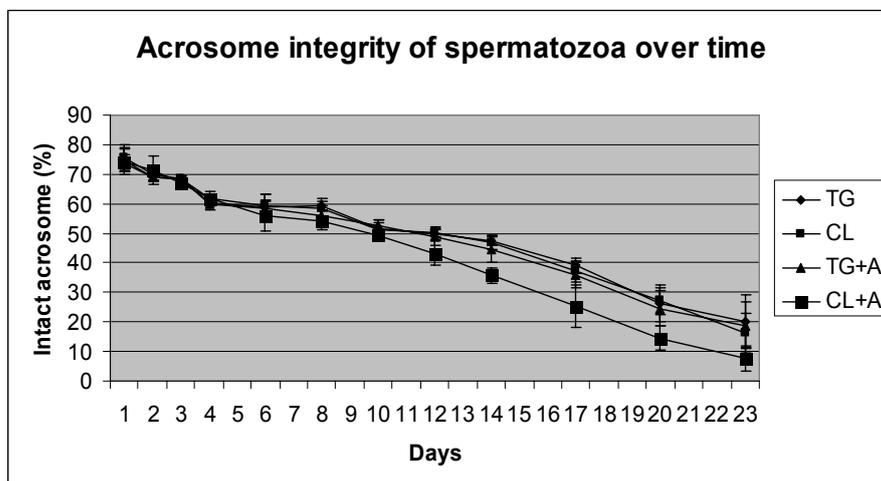


Figure 4. Acrosome integrity over time (means  $\pm$  S.E.M).

### 3.5. Glucose consumption

On day 1, immediately after mixing sperm samples with extender, the glucose concentration was  $53.4 \pm 0.4$  mM/L,  $44.9 \pm 0.1$  mM/L,  $39.0 \pm 0.5$  mM/L, and  $33.2 \pm 1.2$  mM/L in TG, CL, TG+A<sup>TG</sup>, and CL+A<sup>CL</sup>, respectively. Spermatozoa consumed  $6.2 \pm 0.1$  mM,  $3.7 \pm 0.5$  mM,  $5.7 \pm 1.7$  mM, and  $3.3 \pm 0.8$  mM glucose, respectively, in the TG, CL, TG+A<sup>TG</sup>, and CL+A<sup>CL</sup> extenders in the first 8 days ( $P > 0.05$ ). In the third period, significantly higher consumption of glucose was observed in CL and CL+A<sup>CL</sup> than in TG ( $P = 0.0055$ ) and TG+A<sup>TG</sup> ( $P = 0.0010$ ) (Figures 5 and 6, and Table 5).

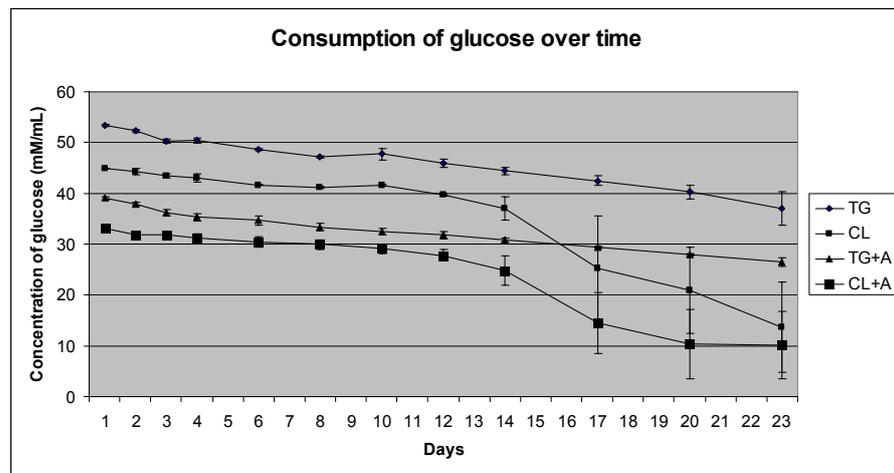


Figure 5. Glucose consumption (mM) by spermatozoa over time (means  $\pm$  S.E.M).

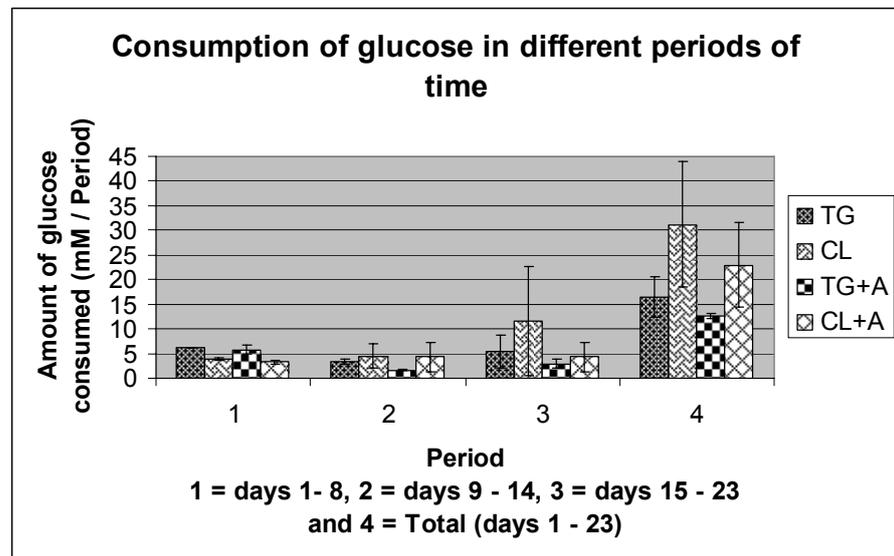


Figure 6. Glucose consumption (mM) by spermatozoa during different periods of time (means  $\pm$  S.E.M).

### 3.6. Sperm chromatin structure assay

Sperm chromatin structure assay results can be seen in Table 5 and Figure 7. On day 1, the mean DFI (%) was  $3.3 \pm 1.6$  in TG,  $4.2 \pm 1.7$  in CL,  $3.3 \pm 1.2$  in TG+A<sup>TG</sup>, and  $3.6 \pm 2.0$  in CL+A<sup>CL</sup>. There were no changes in the breakdown of DNA chromatin ( $P > 0.05$ ) until day 14 in any of the samples.

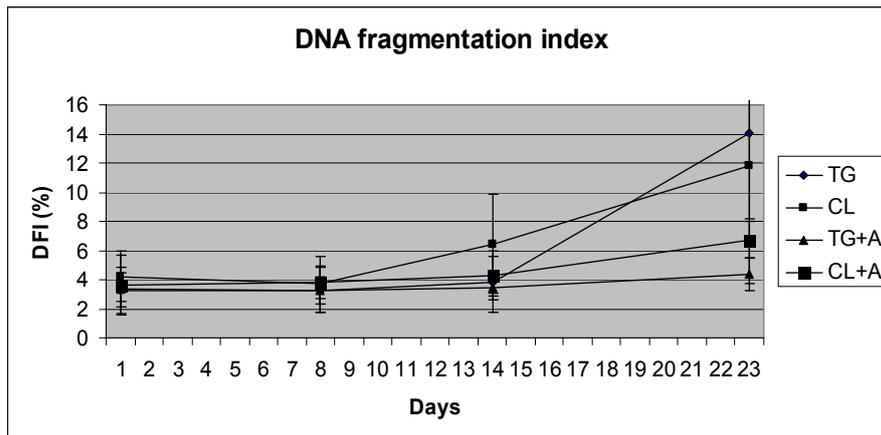


Figure 7. Deoxyribonucleic acid fragmentation index (DFI, %) of spermatozoa over time in the four extenders. Results are expressed as means  $\pm$  S.E.M.

During the last period of the experiment, we observed more DNA fragmentation in TG ( $P=0.0001$ ) and CL ( $P=0.0023$ ) than in CL+A<sup>CL</sup> and TG+A<sup>TG</sup> samples. The lowest fragmentation was observed in TG+A<sup>TG</sup> samples.

### 3.7. Bacteriological study

The third semen pool harbored a sparse growth of *Escherichia coli*, and a sparse mixed flora of non-pathogenic bacteria. The *E. coli* persisted to day 14 in TG and TG+A<sup>TG</sup> samples, and a mixed flora to day 14 in CL+A<sup>CL</sup> and day 23 in CL samples. Yeast occurred from day 14 in TG and *Pseudomonas fluorescens* was found on day 23 in CL+A<sup>CL</sup> samples (Table 6). According to the sensitivity tests, the *E. coli* was sensitive only to gentamicin, enrofloxacin, and nitrofurantoin, while *Ps. fluorescens* was sensitive to gentamicin, streptomycin, trimetoprim-sulfamethoxazole and tetracycline.

Table 6.  
Growth of microorganisms at 37°C in the samples over time.

	Day 1	Day 8	Day 14	Day 23
Pooled semen	Ec+, M+			
TG		Ec+	Ec+, Y+, M+	Y+ +
CL		M+	M++	M+
TG+A <sup>TG</sup>		Ec+	Ec+	M+
CL+A <sup>CL</sup>		M+	M++	Pf++

Ec = *E. coli*; M = mixed flora; Pf = *Pseudomonas fluorescens*; Y = yeast.  
+ = little growth; ++ = moderate growth; and +++ = profuse growth of microorganisms.

## 4. Discussion

We tested the hypothesis that immotility during storage increases the longevity of dog spermatozoa. Our results, however, clearly demonstrate that under the conditions of the present study, immotility induced by the CLONE chilled semen extender during long-term cold storage at 5°C did not improve longevity of dog spermatozoa compared with preservation in a TG extender in which they maintained motility. On the contrary, all the tested parameters were better preserved over time in the TG extenders. As expected, the extenders to which the activators were added on day 1 preserved the spermatozoa less well. The exception was DNA fragmentation, which was lower in TG+A<sup>TG</sup> and CL+A<sup>CL</sup> than in the samples without activators during period 3. Early addition (i.e., on day 1) of A<sup>CL</sup> resulted in a higher PM% during the first period, but a higher VCL was found in CL in the first and third period and the glucose consumption did not differ between CL and CL+A<sup>CL</sup>. Consequently, there was no clearly measurable difference in sperm activity and metabolism. The lower preservation capacity observed may have been due to the lower egg yolk and glucose content of the TG+A<sup>TG</sup> compared with the TG, and to a diluting effect of the A<sup>CL</sup> on the CL. According to the manufacturer's instructions, semen preserved in the CLONE chilled semen extender should be used within 36–48 hours and the A<sup>CL</sup> should be added at the time of AI. From our results, however, it seems probable that spermatozoa may be preserved in this extender for a longer period of time, although not for as long as with the Tris extenders.

A previous study [11] demonstrated that dog spermatozoa preferentially consume glucose over fructose when both sugars are present. Motility was, however, better maintained when the Tris-egg yolk extender contained fructose instead of glucose, and a higher sugar concentration (70 mM) was better than a lower concentration (10 mM). For chilled semen to be used for AI, a Tris-egg yolk 70 mM fructose extender is therefore routinely used. In the present study, however, the energy consumption in the tested extenders was to be compared and as the CL and the A<sup>CL</sup> extenders contain only glucose the Tris extenders were made accordingly. Because the full compositions of the CL and A<sup>CL</sup> were not known to us, the glucose content of the TG was not exactly the same as that of CL. However, as the TG and TG+A<sup>TG</sup> samples consumed similar amounts of glucose in the first period, and as the CL and CL+A<sup>CL</sup> samples were similar in their glucose consumption during that period, it seems that, at the concentrations used in this study, the slightly differing amounts of glucose in the extenders did not influence the amounts consumed by spermatozoa. Although non-significant, the somewhat

lower consumption of glucose in the CL and CL+A<sup>CL</sup> samples compared with the TG and TG+A<sup>TG</sup> samples during days 1–8 may have been due to immotility of spermatozoa during storage. In a previous study of sugar consumption in chilled dog semen over 23 days, Ponglowhapan et al. (2004) [11] observed that glucose consumption was higher in the first 3 days than in the following periods. The authors suggested that the slowing down of the sperm metabolic rate in the early stage of preservation was not as efficient as during the later periods. Another reason for this result may be that the number of live spermatozoa in the samples decreases over time and the consumption of glucose would follow this trend. Such a lowering of glucose consumption was found in our study in TG and TG+A<sup>TG</sup> samples between periods 1 and 2. By contrast, glucose consumption was seen to increase from period to period in CL and CL+A<sup>CL</sup> samples. However, the results in periods 2 and 3 may have been influenced by microbial growth, causing fermentation of glucose.

Fructose has been shown to induce a higher PM%, while glucose was seen to be better at inducing hyperactivated motility in dog spermatozoa, which is considered of importance for sperm oocyte interaction and fertilization [26] and [27]. In the present study using extenders with only glucose, however, LHD was not found to differ between extenders or over time.

Motility is an important criterion for the assessment of the fertilizing potential of semen and it provides valuable information about the spermatozoa's reaction to adverse environments [7]. We do not know how the CL extender induces sperm immotility, or how the activator restores motility. Spermatozoa cold stored in CL will regain their motility also without addition of the A<sup>CL</sup> when placed at room temperature or at 37°C, but at a somewhat slower rate (Linde Forsberg, unpublished observation).

The osmolarity of CL is high, 571 mOsm/kg, which indicates that it may contain glycerol, but the high osmolarity is unlikely in itself to interfere with motility. The reactivation could possibly have been due to the dilution effect, the osmolarity of the A<sup>CL</sup> being only 199 mOsm/kg, or to the addition of more glucose. Versteegen et al. (2005) [14] report how cold-stored spermatozoa that had become non-motile after a certain period of preservation were reactivated and motility parameters restimulated by centrifuging the sample and adding more of the extender.

Spermatozoa are immotile when they enter the caput epididymidis through the ductuli efferentes, but as they mature during epididymal transit they acquire the capacity for motility. This capacity is, however, suppressed in the cauda epididymidis where the spermatozoa are stored in a quiescent, energy-saving state [28], [29] and [30]. Several mechanisms have been described to be involved in the suppression of sperm motility in the epididymis, through conditions including decreased Na<sup>+</sup> concentration, and increased K<sup>+</sup> concentration, pH, and fluid viscosity [31] and [32]. Einarsson (1971) [33] observed that the ratio of potassium/sodium influences epididymal sperm motility in the boar, a high ratio being inhibitory to motility. In our study, this ratio was found to be higher in the inhibitory CL than in the activator. The difference, however, is likely to have been due to an egg yolk component in the CL, which was lacking in the A<sup>CL</sup>, and therefore is probably of no consequence for motility in this case.

The simultaneous decrease in pH [34] and in bicarbonate concentration [35] and [36] in the epididymal fluid that occurs along the epididymal duct is considered to

be of major importance for immobilization of the spermatozoa. Acott and Carr (1984) [37] immobilized bull spermatozoa by incubating them with fluid from the cauda, and Okamura et al. (1985; 1987) [38] and [39] activated sperm cells by adding bicarbonate and increasing the pH. Carr et al. (1985) [40] found species differences in the relative importance of pH, lactate, and viscoelastic drag to reduce sperm motility. In the dog, a species with a low pH and presence of lactate in the fluid of the cauda epididymidis, they suggested that sperm intracellular pH was the most important factor. In the present study, however, pH was similar (6.3) in CL and A<sup>CL</sup>, and consequently, this appears not to be the regulatory factor in this case.

Both carbon dioxide and nitrogen gassing have been found to be effective transient inhibitors of motility of ejaculated spermatozoa and many diluents have been formulated using these methods to optimize sperm survival and prolonging the shelf life of chilled bull semen; [see, e.g. 41]. Whether such gassing has been applied to the CL extender is not known to us and was not tested in the present study.

Harrison (1976) [42] inactivated motility of ejaculated bull spermatozoa by two washings with Ficoll, thereafter resuspending the spermatozoa in a buffer. Motility was restored by addition of seminal plasma from vasectomized bulls and also of bovine serum albumin (BSA) and theophylline. In preliminary tests, we found this principle to apply also to dog semen (data not shown). A suspension of Ficoll 70 in a Tris-glucose extender without egg yolk was carefully mixed with dog semen and left overnight at +5°C, which rendered the spermatozoa immotile. The sample was split in half and resuspended either in the TG extender or in a Tris extender without egg yolk. The samples were again split in half and one of each left at room temperature for 15 min and checked for motility and the other group was left first at room temperature for 15 min and then at 37°C for 10 min. The TG extender proved superior to the Tris extender without egg yolk in restoring motility, and 37°C was better than room temperature. This, apparently, is a way to effectively inhibit and reactivate motility of dog spermatozoa. We do not know whether this is in fact the method used for the CL extender. Although the induced immotility, in our study, did not prove advantageous for the preservation of chilled dog semen, further studies should be done attempting to identify methods that will effectively lower the metabolism of dog spermatozoa in order to further prolong their survival in a liquid state.

The integrity of the plasma membrane is an important indicator of the viability of sperm cells. Chilling and cryopreservation induce changes in the components of the sperm plasma membrane [43] and dilution of semen in a Tris buffer is not enough to prevent the cold-induced redistribution of intramembranous particles [44]. Egg yolk, present in TG (20%) and TG+A<sup>TG</sup> (10%), is believed to maintain the colloid pressure of the medium and protect the plasma and acrosomal membranes against seminal cationic peptides that have been shown to bind to the sperm-negative charges of bovine and ram spermatozoa [45] and [46]. Dog spermatozoa may, however, respond differently to chilling due to species differences in cold resistance and different lipid composition of the membrane. It is likely that the CL extender also contains egg yolk (Table 2). The A<sup>CL</sup>, by contrast, is a transparent fluid, like the A<sup>TG</sup>, which contains no egg yolk. For the first 10 days of this study, plasma membrane integrity was lost at the same rate in all the

samples, and the difference was significant only for the CL+A<sup>CL</sup> sample in the third period.

Acrosomal integrity was not different between the TG, TG+A<sup>TG</sup>, and CL samples until day 14 ( $P>0.05$ ). It was lower in CL+A<sup>CL</sup> samples only in the third period. In the present study, it was more common for spermatozoa to lose their acrosome integrity than to lose the integrity of their plasma membrane. This finding was in contrast to the findings of Ponglowhapan et al. (2004) [11]. This difference may be due to the fact that different protocols for evaluation of chilled semen were used in the two studies. In our study, semen samples were stored at room temperature for 15 min and then placed in a heating chamber at 37°C for 20 min (10 min' protocol time + 8 min for running of the CASA, plus 2 min for preparing the samples for assessment of plasma membrane integrity) before starting the evaluation of the acrosome integrity. Ponglowhapan et al. (2004) [11] rewarmed the semen samples to room temperature for 15 min before evaluation, but did not incubate the samples at 37°C for 10–20 min. Moreover, glucose has been reported to induce the acrosome reaction in dog spermatozoa [47] and spermatozoa undergoing the acrosome reaction rapidly lose their plasma membrane, although dead or dying cells can show similar acrosomal changes [48]. The incubation of the semen samples at 37°C before assessments possibly induced capacitation-like changes or some enzymatic reaction that resulted in more acrosomal losses, although this was not reflected in hyperactivated motility.

The SCSA has recently been established as an important tool for evaluating the fertilizing potential of a semen sample, since it provides more objective information about the integrity of the DNA chromatin [19] and [49]. There were no significant differences in chromatin integrity among the samples in the first and second period in our studies. Similar findings were reported previously in boar semen by Perez-Llano et al. (2006) [18], who found that sperm extenders may delay or partially prevent sperm DNA fragmentation. However, in our study, higher denaturation of DNA chromatin was observed in the third period in TG and CL samples compared with the TG+A<sup>TG</sup> and CL+A<sup>CL</sup> samples. The only difference in composition between TG and TG + A<sup>TG</sup> is that the concentrations of egg yolk and glucose are lower in the latter extender. Besides, CL+A<sup>CL</sup> contains less glucose than CL, but there are also other major differences (see Tables 1 and 2). Only for the DNA fragmentation were the extenders with activators found to be more beneficial, during the third period. One reason may be that these samples were not further diluted before each assessment, as was done with the TG and CL samples on each experimental day, and that this dilution may have induced damages in spermatozoa as they were aging during the third period.

We selected clinically healthy dogs for our study, but after the collection and pooling of the semen for the third replicate of the experiment, it was discovered that the semen of one of the dogs contained *E. coli*. It is often reported that Gram-negative urinary tract bacteria such as *E. coli* are found in canine ejaculates. The bacteria most frequently isolated from the prepuce and semen of dogs are *Pasteurella multocida*, *B-hemolytic streptococci*, and *E. coli* [50]. These bacteria are transferred between the dog and bitch on several occasions during mating, but in a population of healthy dogs, the fertility of neither the dog nor the bitch is affected [50]. Benzyl penicillin and dihydrostreptomycin were used in TG and A<sup>TG</sup>, but we do not know whether CL and A<sup>CL</sup> contain any antibiotics. The bacteria

found in our study were, however, resistant to benzyl penicillin, and the *E. coli*, but not the *Ps. fluorescens*, were also resistant to DHS. *Escherichia coli* was found in both TG extenders, but not in the two CL extenders, while *Ps. fluorescens* was found only in the CL+A<sup>CL</sup> extender. The mixed flora was more commonly isolated from the two CL extenders. It therefore seems likely that if CL and CL+A<sup>CL</sup> do contain antibiotics, these are different from the ones used for TG and A<sup>TG</sup>.

Egg yolk is an enriched medium for bacteria, and bacterial growth is common in Tris egg yolk extenders at 22°C and 35°C. It has been suggested that motility parameters may be influenced by bacterial growth in such extenders at higher storage temperatures [51] and [52]. Diemer et al. (2003) [53] demonstrated that human sperm incubated with *E. coli* bacteria at 37°C had significant alterations in motility within 4–6 hours. Aurich and Spersger (2007) [54] performed a control study of stallion sperm infected with six different bacterial strains isolated from the genital tracts of mares with clinical signs of endometritis. Only two, *Ps. aeruginosa* and *Streptococcus equisimilis*, were found to have detrimental effects on semen quality during cool storage, and addition of gentamicin to the semen extender did not totally block these bacterial effects. Furthermore, gentamicin was shown to be detrimental to spermatozoal function. However, the presence of bacteria may not have the same effect on dog sperm preserved at 5°C as our study did not show any significant alteration in motility parameters before 1 week of preservation at +5°C. Further investigations are required to explore the effects of microbial growth on chilled canine semen and to find the best suited antibiotics to be used for dog semen extenders.

The bacteria isolated in our study are able to use glucose as their energy source. Microbial fermentation of glucose should therefore be considered during studies of the spermatozoal consumption of glucose, as it is not possible to separate the spermatozoal consumption of glucose from the glucose consumption of microbial fermentation. Moreover, the end products of glucose fermentation may also have altered the pH and osmolarity of the samples in our study. *Pseudomonas fluorescens* and *E. coli*, in addition, produce acid and gas [55] and [56], further negatively influencing sperm viability.

In conclusion, our results show that the immotility induced by the CLONE chilled semen extender does not further prolong the lifespan of dog spermatozoa stored at +5°C. In addition, our results indicate that good quality dog semen may possibly be stored for up to 14 days in TG extenders at +5°C with retained fertilizing potential. *In vivo* studies should, however, be done to further support this conclusion.

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