Equine seminal plasma: its role in protecting stallion spermatozoa during cryopreservation

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Abstract

There is considerable variability in the ability of spermatozoa from different stallions to withstand the freezing process. As a result, stallions are usually categorized as good or bad freezers. In the equine industry, the demand for frozen semen for artificial insemination is increasing, resulting in a need for optimising cryopreservation techniques. Previously the removal of seminal plasma (SP) prior to cryopreservation was shown to improve post-thaw sperm quality, whereas the effect of adding SP on spermatozoa varied among studies. Single Layer Centrifugation (SLC) is a technique that has been used to select the most robust spermatozoa from the ejaculate and prolong their viability, as well as separating them from SP. The aims of this thesis were: 1) to study the effect of combining SLC with addition of SP from stallions of known freezability on sperm cryosurvival, including their ability to bind to heterologous oocytes; 2) To explore the differences in the major protein groups in SP between the breeding and non-breeding seasons in relation to sperm quality. Selecting robust spermatozoa using SLC prior to freezing improved post-thaw sperm quality, whereas addition of pooled SP from good or bad freezers to the selected sperm samples did not have an additional beneficial effect. Sperm quality was adversely impaired after restoring pooled SP to SLC-selected spermatozoa after thawing, independently of whether the SP came from a good or a bad freezer stallion, suggesting an inability of SP to repair stallion sperm cryoinjuries in the presence of an egg yolk-containing extender. Sperm binding affinity to the zona pellucida (ZP) of bovine oocytes was reduced after adding SP from good freezer stallions prior to cryopreservation. However, when SP was added after thawing, the mean number of bound spermatozoa was higher for the group treated with SP from good freezers than for the group treated with SP from bad freezers. Minor differences in protein composition were observed between seasons as well as between good and bad freezers. In the non-breeding season there was a higher content of heparin-binding proteins and also of a subset of non-heparin binding proteins compared to the breeding season; these proteins may be linked to fertility. Sperm quality did not decline during the non-breeding season and SP-testosterone levels did not vary between seasons. Customization of the cryopreservation protocol for individual stallions, including studying the effect of adding SP from individual stallions, would be required to optimize the effect on sperm cryosurvival. Global proteome investigation of SP proteins could help to identify cryosurvival biomarkers for stallion spermatozoa.

Keywords: equine, seminal plasma, spermatozoa, freezability, good freezer, bad freezer, zona pellucida, %DFI, cryoinjuries, ROS

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Abstract

Förmågan hos spermier från olika hingstar att tolerera frysningsprocessen varierar avsevärt. Detta resulterar i att hingstar vanligtvis kategoriseras som varande av bra eller dålig frysbarhet beroende på deras spermiers förmåga att utstå frysning. Efterfrågan för fryst sperma för användning vid artificiell insemination inom hästnäringen ökar, och detta leder till ett behov av att optimera tekniker för frysbevarande. Tidigare har borttagning av seminalplasma (SP) visat sig förbättra spermiekvaliteten efter upptining, medan effekten på spermierna av att tillsätta SP har varierat mellan studier. Single Layer Centrifugation (SLC) är en metod som har använts för att selektera fram de mest robusta spermierna från ejakulatet och förlänger deras viabilitet samt separerar dem från SP. Syftet med denna avhandling var att: 1) Studera effekten på spermieöverlevnad och spermiernas förmåga att binda till heterologa oocyter av att kombinera SLC med tillsats av SP från hingstar med känd frysbarhet. 2) Undersöka skillnader i de huvudsakliga proteingrupperna i SP i relation till spermiekvalitet mellan prover tagna under och utanför avelssäsong. Att selektera robusta spermier med hjälp av SLC innan nedfrysning förbättrade spermiekvaliteten efter upptining, medan tillsats av poolad SP från hingstar med god eller dålig frysbarhet till de selekterade spermieproverna inte hade någon ytterligare fördelaktig effekt. Spermiekvaliteten blev försämrad efter återförande av poolad SP till SLC-selekterade spermier efter upptining, oavsett om SP kom från en hingst med god eller dålig frysbarhet, vilket tyder på en oförmåga hos SP att reparera frysskador i närvaro av en spädningsvätska innehållande äggula. Spermiernas bindningsaffinitet till zona pellucida (ZP) hos bovina oocyter reducerades om SP från hingstar med god frysbarhet tillsattes innan nedfrysning. När SP istället tillsattes efter upptining var dock medelvärdet av antalet bundna spermier högre för gruppen som behandlats med SP från hingstar med god frysbarhet än för gruppen som behandlats med SP från hingstar med dålig frysbarhet. Smärre skillnader i proteinfördelningen observerades mellan årstider samt mellan hingstar med god och dålig frysbarhet. Utanför avelssäsongen innehöll SP en högre andel heparinbindande proteiner och också av en viss typ av icke-heparinbindande proteiner jämfört med under avelssäsongen, dessa proteiner kan vara länkade till fertilitet. Spermiekvaliteten minskade inte utanför avelssäsongen och testosteronnivåerna i SP varierade inte mellan årstiderna. Anpassning av infrysningsmetoden till enskilda hingstar samt att studera effekten av att tillsätta SP från individuella hingstar skulle krävas för att optimera effekten angående spermieöverlevnad. Globala undersökningar av proteomet gällande SP-proteiner kan bidra till att identifiera markörer för kryoöverlevnad hos hingstspermier.

Keywords: ekvin, seminalplasma, spermier, frysbarhet, god frysbarhet, dålig frysbarhet, zona pellucida, %DFI, frysskador, ROS

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Dedication

To my parents for their love and encouragement
My beloved family for their patience and support
My teachers for every single word I learned

“Research is to see what everybody else has seen and to think what nobody else has thought”

Albert Szent-Gyorgyi

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Al-Essawe, Essraa M.*; Johannisson, Anders; Wulf, Manuela; Aurich, Christine & Morrell, Jane M. (2018). Improved cryosurvival of stallion spermatozoa after colloid centrifugation is independent of the addition of seminal plasma. *Cryobiology*, 81, pp. 145-152.

II Al-Essawe, Essraa M.*; Johannisson, Anders; Wulf, Manuela; Aurich, Christine & Morrell, Jane M. (2018). Addition of seminal plasma to thawed stallion spermatozoa did not repair cryoinjuries. *Animal Reproduction Science*, 196, pp. 48-58.

III Al-Essawe, Essraa M.*; Wallgren, Margareta; Wulf, Manuela; Aurich, Christine; Macías-García, Beatriz; Sjunnesson, Ylva & Morrell, Jane M. (2018). Seminal plasma influences the fertilizing potential of cryopreserved stallion sperm. *Theriogenology*, 115 pp. 99-107.

IV Johannisson, Anders*; Al-Essawe, Essraa M.; Al-Saffar, Anas Kh.; Karkehabadi, Saeid; Lima-Verde, Isabel; Wulf, Manuela; Aurich, Christine & Morrell, Jane M. Season does not have a deleterious effect on proportions of stallion seminal plasma proteins. (*Submitted Manuscript*).

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The contribution of Essraa Al-Essawe to the papers included in this thesis was as follows:

I. Involved in hypothesis generation and contributed to the planning and design of the experiment, collected the samples, performed most of the laboratory work, performed the statistical analysis and analysed the results under supervision. She had the main responsibility for writing the manuscript, corresponding with the journal and revising the article under supervision.

II. Was involved in the experimental planning and design, collected the samples, performed most of the laboratory work and performed the statistical analysis. Analysed the results in collaboration with the supervisors and was responsible for writing the manuscript, corresponding with the journal and preparing the final version of the article.

III. Was involved in the experimental design, planned the work, collected the samples and performed most of the laboratory work. Performed the statistical analysis and analysed the results under supervision. Was responsible for writing the manuscript, corresponding with the journal and preparing the final version of the article.

IV. Was involved in the experimental planning and design, collected the samples and contributed to the laboratory work. Performed the statistical analysis and contributed to data analysis and writing the manuscript.

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Abbreviations

AI	Artificial insemination
ALH	Amplitude of lateral head deviation
BCF	Beat cross frequency
BSA	Bovine serum albumin
COCs	Cumulus oocyte complexes
CRISPs	Cysteine-Rich Secretory Protein
CRISP-3	cysteine-rich secretory protein-3
DFI	DNA fragmentation index
DGC	Density gradient centrifugation
Exp.	experiment
Fn-2	Fibronectin-type II modules
HSP	Horse Seminal Proteins
HZBA	Heterologous zona binding assay
IVF	*In vitro* fertilizing protocol
IVM	*In vitro* matured
LIN	Linearity
LN_2	Liquid nitrogen
MMP	Mitochondrial membrane potential
mROS	Mitochondria-specific reactive oxygen species
MW	Modified Whitten's medium
NS	Not significant
PBS-PVA	phosphate buffered saline containing 0.1% PVA
PI	Propidium iodide
PVA	Polyvinyl alcohol
ROS	Reactive oxygen species
RT	Room temperature
SCSA	Sperm chromatin structure assay
SLC	Single Layer Centrifugation

SP	Seminal plasma
STR	Straightness
TNE	Tris-sodium chloride-EDTA buffer
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight line velocity
WOB	Wobble
ZP	zona pellucida

1 Introduction

1.1 Background

Cryopreservation of semen is an essential biotechnology for assisted reproduction, allowing preservation of genetic material for an indefinite period. Sperm freezing technology has become an area of increasing interest for the equine industry (Samper *et al.,* 2007). As the equine industry continues to grow, artificial insemination (AI) with frozen thawed stallion semen has become critically important in horse breeding (Loomis, 2001; Metcalf, 2007; Miller, 2008). Progress has been associated with the use of new AI techniques, such as deep uterine insemination (Alvarenga *et al.,* 2016). Using frozen stallion semen minimizes the spread of disease, and eliminates geographic barriers (Alvarenga *et al.,* 2016). Furthermore, being able to use cryopreserved semen ensures the availability of spermatozoa when needed for insemination, particularly during periods of intensive demand for fresh semen (Loomis, 2001; Miller, 2008). Additionally, having a bank of cryopreserved semen allows the stallion to continue a career in competition without the necessity of being available for semen collection on specific days (Miller, 2008).

Although there are considerable advantages to mare and stallion owners from using cryopreserved semen in breeding, there are also several disadvantages, mainly decreased fertilizing ability and the expense of semen processing and storage (Watson, 2000; Miller, 2008; Battut *et al.,* 2017). One of the major disadvantages of equine sperm cryobiology is the inability to freeze all ejaculates successfully. There is considerable inter-individual variability in the ability of equine spermatozoa to survive cryopreservation. Approximately one third of stallions produce ejaculates that freeze well, one third of stallions produce ejaculates that can sometimes be frozen successfully and the remainder do not produce freezable ejaculates (Vidament *et al.,* 1997). Therefore, stallions are

usually classified as good or bad freezers according to the ability of their spermatozoa to survive cryopreservation (Tischner, 1979; Vidament *et al.,* 1997; Alvarenga *et al.,* 2005). Good freezers produced spermatozoa that had a post-thaw progressive motility >40% (Tischner, 1979; Moore *et al*., 2005b).

In the horse breeding industry, stallions are chosen for their individual athletic performance and pedigree (Loomis & Graham, 2008; Gibb & Aitken, 2016), with little regard to semen quality or the ability of their spermatozoa to survive cryopreservation. A champion stallion is not gelded or culled for poor semen quality or inability of his spermatozoa to withstand cryopreservation (Loomis, 2006); therefore, it is not possible to replace a stallion of bad freezability with one of good freezability. Not only does freezability vary among stallions, but there is also variation between ejaculates from a given stallion (Vidament *et al.,* 1997; Loomis & Graham, 2008). These facts have limited the widespread application of frozen semen in equine breeding, so that improvement in freezing protocols and composition of extenders is of great importance (Olaciregui *et al.,* 2014). Furthermore, maintaining sperm membrane stability and acrosome integrity, as well as avoiding the accumulation of reactive oxygen species (ROS) during storage, are all important factors for establishing a healthy pregnancy.

Seminal plasma (SP) is a complex mixture of secretions produced by the epididymis and the accessory sex glands. It plays an important role in sperm maturation, containing factors that modulate the fertilizing ability of ejaculated spermatozoa (Manjunath *et al.,* 1994). Removing SP prior to cryopreservation increases sperm motility, membrane integrity and fertility after thawing (Moore *et al.,* 2005a; Maxwell *et al.,* 2007). However, the effect of SP on spermatozoa during storage varies among studies. Some researchers claim beneficial effects of SP on ram sperm cryosurvival (Maxwell *et al.,* 1999) whilst others have seen detrimental effects on stallion sperm cryosurvival (Love *et al.,* 2005; Akcay *et al.,* 2006).

Adding defined amounts of SP before storage improved sperm motility during cooled storage and also after thawing (Katila *et al.,* 2002; Morrell *et al.,* 2012). Moore *et al.* (2005a) demonstrated a non-significant effect of short-term exposure of spermatozoa to SP prior to preservation, while prolonged exposure was deleterious to the motility after thawing. However, negative effects of SP during short and long term storage were reported in other studies (Jasko *et al.,* 1991; Braun *et al.,* 1994; Alghamdi *et al.,* 2002). An improvement in sperm quality after thawing of stallion spermatozoa with low tolerance to cryopreservation (bad freezer) was demonstrated when the samples were supplemented before freezing with SP from stallions with high cryosurvival rates (good freezer) (Aurich *et al.,* 1996). In contrast, adding SP from a stallion

with low post-thaw motility (≤ 20%) to sperm samples from a stallion with high post-thaw motility reduced sperm progressive motility without affecting the viability (Aurich *et al.,* 1996). Nevertheless, there is individual variation in response to the SP (Aurich *et al.,* 1998; Akcay *et al.,* 2006). In other species, SP was found to repair cryodamage, e.g. in ram (Bernardini *et al.,* 2011) and boar (Hernández *et al.,* 2007; Torres *et al.,* 2016) spermatozoa.

The use of frozen semen is increasing in spite of its lower fertility compared with fresh and cooled semen (Loomis, 2001). Therefore, there are increasing demands to improve stallion sperm freezability by enhancing cryopreservation technologies. Advanced methods for processing and selecting stallion semen provide valuable tools for handling stallion semen to achieve improved sperm cryosurvival. Traditional cryopreservation protocols using "washing" of spermatozoa by centrifugation in the presence of extender does not remove all the SP proteins coating the spermatozoa (Kruse *et al.,* 2011).

Single Layer Centrifugation (SLC) using a colloid is reported to select spermatozoa of superior quality and fertility from the rest of ejaculate, and separates them from SP and extender (Morrell *et al.,* 2009a; Morrell *et al.,* 2009b). In addition, it can remove SP proteins from the surface of ejaculated spermatozoa, thus enabling the addition of a known amount of SP to be studied to determine its effect on sperm cryosurvival (Kruse *et al.,* 2011). Thus, combining SLC with the addition of small quantities of SP from stallions of known freezability could help to improve cryosurvival of stallion spermatozoa (Hoogewijs *et al.,* 2011). *In vitro* tests that closely correlate with potential *in vivo* fertility to predict the fertility of cryopreserved spermatozoa without carrying out insemination trials are required for optimizing freezing techniques.

1.2 Stallion seminal plasma and its composition

Seminal plasma is a complex mixture of secretions produced by the epididymis and the accessory sex glands; its components include proteins, ions and organic substances of low molecular weight, such as free amino acids, monosaccharides, lipids, polyamines, prostaglandins and steroid hormones (Katila & Kareskoski, 2006). Seminal plasma serves as an aqueous medium for spermatozoa to act as a vehicle that flushes the urethra and simultaneously propels spermatozoa through the stallion's genital tract during ejaculation. Furthermore, SP increases the volume of the sperm-rich portion of the ejaculate that aids in depositing semen in the uterus and subsequent sperm transport through the female tract (Senger, 2003). It plays an important role in sperm maturation, containing factors that modulate the fertilizing ability of ejaculated spermatozoa (Manjunath *et al.*, 1994). It is also essential for protection and maintenance of

sperm viability as well as modulation of the female reproductive tract's immune response (Novak *et al.*, 2010; Kareskoski *et al.*, 2011). In addition, the vasodilatory components in the SP significantly increased uterine blood flow, which is thought to be one of the causes of uterine endometrial inflammation (Bollwein *et al.*, 2003).

The accessory sex glands secrete their content successively in a consistent manner. The pattern of seminal emission described by Kareskoski & Katila (2008) is as follows: the bulbourethral gland secretes fluid for the pre-sperm component that is followed by prostatic and ampullary secretions just before ejaculation occurs. Along with the secretion of epididymal cells, the ampulla and prostate continue to secrete their fluid to produce the first fraction of the ejaculate. The vesicular gland secretions start after the prostate gland stops releasing its secretion. The final ejaculatory fraction is primarily composed of fluid ejected from the seminal vesicles (Weber & Woods, 1993; Kareskoski & Katila, 2008). The stallion ejaculates in 6-9 jets; approximately 75-80% of the spermatozoa are ejaculated in the first three jets or "seminal fractions", and therefore, the term "sperm-rich" is used for the first part of the ejaculate (Tischner *et al.*, 1974; Katila & Kareskoski, 2006). The chemical composition of the ejaculate can be altered if any disruption in the sex gland function occurs.

The components of SP differ depending on the fraction of the ejaculate; the concentration of proteins and electrolytes vary between sperm-rich and sperm-poor fractions. Artificial insemination using epididymal spermatozoa previously exposed to SP yielded better pregnancy rates compared with using epididymal spermatozoa that had not been exposed (Heise *et al.*, 2010); this result supports the hypothesis that SP is one of many factors that contributes to maintaining sperm integrity for fertilization. Addition of SP prior to insemination with frozen/thawed spermatozoa may supply the semen with the protective qualities of the SP as well as assist in the transport of processed spermatozoa through the mare's reproductive tract (Troedsson *et al.*, 1998; Bollwein *et al.*, 2003; Heise *et al.*, 2010).

One of the most important components in SP are the proteins that attach to sperm cell membranes (Katila & Kareskoski, 2006) and are associated with full fertility (Kruse *et al.*, 2011). Seminal plasma proteins are separated into three main families, which include the fibronectin-type II modules (Fn-2), the cysteine-rich secretory proteins (CRISPs), and the spermadhesins (Töpfer-Petersen *et al.*, 2005). Horse Seminal Proteins (HSP) belong to the Fn-2 family and comprise the majority of proteins in stallion seminal plasma. These proteins help to coordinate the capacitation of spermatozoa based on their heparin-binding abilities and tight association with the sperm membrane (Sankhala *et al.*, 2012). The most abundant proteins in stallion SP are HSP-1 and HSP-2, which

are phosphorylcholine-binding proteins (Kareskoski *et al.*, 2011). The protein, HSP-1, may be the equine homologue of osteopontin, which has previously been identified as beneficial to fertility (Brandon *et al.*, 1999). In contrast, Novak *et al.* (2010) hypothesized that HSP-1 and HSP-2 indeed bind to spermatozoa and serve as protection from the female tract, thereby reducing their fertilizing ability. The positive correlation of HSP-1 and HSP-2 with stallion fertility observed by Brandon *et al.* (1999) was reinforced by the findings of Kareskoski *et al.* (2011).

At ejaculation, surface proteins (termed "decapacitation" factors), originating from the accessory sex glands associate with SP molecules and protect the spermatozoa from the environment prior to entry into the female tract (Maxwell *et al.*, 2007). In addition, epididymal proteins may provide protection against ROS (Dacheux *et al.*, 2003). Moreover, a family of Cysteine-Rich Secretory Protein (CRISPs) is found in the male genital tract. One of these, cysteine-rich secretory protein-3 (CRISP-3), constitutes a major fraction of equine SP protein, being secreted primarily from the ampulla, vas deferens and seminal vesicles (Schambony *et al.*, 1998). This unique species-specific expression profile indicates that CRISP-3 has an important role in equine reproduction, where it may bind to the sperm surface (Udby *et al.*, 2005; Hamann *et al.*, 2007). The CRISP-3 is found in the non-heparin binding fraction and does not bind to phosphorylcholine (Magdaleno *et al.*, 1997); it has been suggested that CRISP-3, and also HSP-2, could be a potential marker of equine semen freezability (Jobim *et al.*, 2011). Once inside the uterus, these SP and surface proteins are removed to facilitate binding with the zona pellucida (ZP) of the oocyte for the initiation of the acrosome reaction (Kareskoski *et al.*, 2011).

1.3 Stallion semen processing for cryopreservation

In dairy cattle, bulls have been selected for the AI industry based on the ability of their spermatozoa to withstand the stresses of standard cryopreservation protocols (Loomis & Graham, 2008). In contrast, breeding stallions are mostly not selected based on their fertility or sperm ability for cryopreservation (Colenbrander *et al.*, 2003). Therefore, advanced methods for processing and selecting stallion spermatozoa prior to cryopreservation provide valuable tools for handling poor quality semen to improve sperm cryosurvival. Removal of most of the SP helps to eliminate its detrimental effects on sperm survival during cryopreservation (Amann & Pickett, 1987). However, centrifugation of stallion semen can be harmful, especially when spermatozoa are packed tightly at the bottom of the tubes after vigorous centrifugation (Alvarenga *et al.*, 2016). Moreover, this technique does not improve the proportion of viable spermatozoa

in the sample or remove contaminants or other cells (Loomis, 2006; Morrell, 2012). In cryopreservation protocols, most (≥ 95%) SP is removed by centrifugation (Loomis, 2006). Moreover, semen samples contain a heterogeneous population of spermatozoa, (viable and non-viable spermatozoa suspended in secretions from various accessory sex glands). Semen may also contain other cells, such as leukocytes, epithelial cells, erythrocytes and immature germ cells, as well as contaminants e.g. bacteria, viruses, urine (Loomis, 2006; Morrell *et al.*, 2016). However, although the routine processing technique of sperm washing effectively separates the spermatozoa from most of the SP component of the ejaculate (Mortimer, 2000; Björndahl *et al.*, 2005), there is no selection of spermatozoa or removal of potential sources of ROS, which are detrimental to sperm viability (Morrell & Rodriguez-Martinez 2009).

In vivo, spermatozoa rapidly migrate away from SP at the site of semen deposition and make their way to the site of fertilization over a restricted period of time (Bedford, 2008). The desirable spermatozoa are thought to be selected by various mechanisms within the female reproductive tract (Holt & Fazeli, 2016; Morrell *et al.*, 2016). *In vitro* semen preparation techniques to mimic this selection have been reviewed by Morrell & Rodriguez-Martinez (2009).

Heterogeneous sperm populations can be separated into sub-populations using density gradient centrifugation. During this procedure, spermatozoa move to a point that matches their own density, known as the isopycnic point (Pertoft, 2000). Immotile, morphologically abnormal, acrosome reacted spermatozoa or those with damaged chromatin are mostly retained at the interface between the semen and the colloid (Morrell *et al.*, 2009a).

Single Layer Centrifugation through a ready-to-use colloid formulation for stallion semen based on silane-coated silica (Equicoll, formerly known as Androcoll-E) is a type of colloid centrifugation similar to density gradient centrifugation but with only one layer of colloid (Morrell, 2012). This technique has been shown to select robust spermatozoa from an ejaculate (Morrell *et al.*, 2008a; Morrell & Rodriguez-Martinez, 2009; Morrell *et al.*, 2009a; Morrell *et al.*, 2010b) and to separate them from SP. Even the SP proteins coating the sperm membrane are removed as the spermatozoa pass through the colloid (Kruse *et al.*, 2011). In addition, SLC has been shown to select stallion spermatozoa that live longer than unselected spermatozoa (Morrell *et al.*, 2010b) and are highly fertile (Morrell *et al.*, 2014a). Currently, SLC is the most practical technique for selecting spermatozoa to be used in animal breeding (Morrell & Rodriguez-Martinez, 2009). Since there is only one layer of colloid in the tube, the preparation time is shorter and the process is less complicated than for density gradient centrifugation (DGC), which requires at least two colloids of different

densities to be layered in the tube (Morrell & Rodriguez-Martinez, 2009). This SLC method is less time-consuming than other types of selection methods, and has been scaled-up to allow whole ejaculates to be processed in a practical manner (Morrell, 2012; Morrell *et al.*, 2016). It can also be scaled-down to accommodate small volumes (250 µL) of thawed semen (Abraham *et al.*, 2016). Some attention in conducting the layering process during SLC is required to avoid disrupting the interface integrity between the two layers (colloid and semen), which leads to reduced efficiency of the sperm selection process (Morrell & Rodriguez-Martinez, 2009).

The SLC method has been used to improve the quality of some problem ejaculates, allowing even spermatozoa from so-called "poor coolers" to be prepared to extend the shelf-life of sperm doses for AI (Morrell *et al.*, 2011b; Morrell, 2012; Morrell *et al.*, 2016), and to improve sperm survival during cryopreservation (Hoogewijs *et al.*, 2011; Hoogewijs *et al.*, 2012).

A modification of this technique was demonstrated to reduce bacterial contamination in stallion semen (Morrell *et al.*, 2014a) and improve stallion sperm quality (Al-Kass *et al.*, 2017). Physically separating spermatozoa from bacteria in semen could be an alternative to the use of antibiotics to avoid antibiotic resistance (Morrell *et al.*, 2019). Despite the presence of antibiotics in semen extenders, a large number of viable bacteria can still be detected (Althouse *et al.*, 2010; Zampieri *et al.*, 2013).

1.4 Prediction of the fertilizing potential of cryopreserved spermatozoa

Previously, sperm concentration, motility and morphology were used to evaluate sperm quality; however, the results of such tests do not necessarily correlate well with the final fertility rates achieved (Amann & Hammerstedt, 2002; Kuisma *et al.*, 2006). Nowadays, additional assays are available to evaluate sperm functionality indirectly; flow cytometric assays provide an objective means of analysing several thousand spermatozoa per sample, enabling an objective evaluation of sperm quality to be made (Gillan *et al.*, 2005; Hossain *et al.*, 2011). However, *in vitro* estimation of sperm fertilizing potential evaluated by different assays does not correlate well with fertility in a consistent manner. This lack of consistency between assays could be ascribed to the complexity of the spermatozoon itself (Graham & Mocé, 2005). Accurate evaluation of stallion fertility requires a large number of normal mares to be mated and is necessarily retrospective (Colenbrander *et al.*, 2003). However, AI trials are expensive, time consuming, and only a limited number of males can be tested (Loomis, 1999).

Sperm motility is readily identifiable and reflects several essential aspects of sperm metabolism (Katila, 2001). Although post-thaw motility appears to have little correlation with fertility (Katila, 2001; Kuisma *et al.*, 2006), it remains the only feasible method of assessment for most commercial semen collection enterprises in the absence of other reliable, rapid and cheap tests for more predictive parameters of sperm quality in the field (Morrell & Rodriguez-Martinez, 2009). Therefore, motility should be evaluated together with other parameters when estimating the fertilizing potential of spermatozoa (Katila, 2001). Graham (1996) reported that motility, membrane integrity, morphology and energy metabolism were the most important attributes to assess fertilizing capacity of the spermatozoa. Since each spermatozoon requires many attributes to be able to fertilize an oocyte, an assay measuring only a single attribute will fail to detect spermatozoa defective in a different attribute, resulting in an overestimation of the number of fertile spermatozoa in a semen sample (Graham & Mocé, 2005). Predicting sperm fertility without carrying out insemination trials demands *in vitro* tests that closely correlate with potential *in vivo* fertility.

The ZP of mammalian oocytes is a critical site for sperm-oocyte interaction. Therefore, assessment of the ability of the spermatozoa to bind to a homologous ZP is a useful test for prediction of sperm fertilizing ability (Hermansson *et al.*, 2006). This type of assay is assumed to be a reliable test to detect sperm damage at the molecular level, which is not visible by microscopic analysis, because binding is a receptor-ligand mediated reaction (Partyka *et al.*, 2012). However, for equine sperm function there are added complications with this type of assay due to the lack of an efficient *in vitro* fertilization (IVF) protocol in the horse, in addition to the difficulties in sourcing sufficient equine oocytes for *in vitro* fertilization. The heterologous zona binding assay (HZBA) using *in vitro* matured (IVM) bovine oocytes could be a better alternative to evaluate sperm fertilizing capacity *in vitro*. The HZBA has been used to assess fertilizing capacity in several species. The results have been correlated with *in vivo* fertility in stallion (Fazeli *et al.*, 1995; Meyers *et al.*, 1996). Thus, HZBA with bovine oocytes can be used to evaluate stallion sperm capacitation, the acrosome reaction, zona binding and penetration (Graham, 1997). The HZBA using salt-stored bovine oocytes could be a useful tool to evaluate stallion sperm fertilizing ability, serving as a preliminary screening when deciding whether to test the spermatozoa in AI trials.

1.5 The influence of season on stallion sperm cryosurvival

The horse is a long-day breeder species; reproductive seasonality in mares is important to ensure that their offspring are born at the appropriate time of the year (Nagy *et al.*, 2000). However, the annual changes in stallion reproductive function and fertility are less obvious than in the mare (Pickett *et al.*, 1970). The maximal reproductive activity occurs in late spring and summer. During the breeding season, semen collection centers focus on the production of cooled-stored semen with low production of cryopreserved semen (Aurich, 2016). Consequently, stallion semen is usually collected and cryopreserved during the non-breeding season in autumn and winter (Blottner *et al.*, 2001; Aurich, 2016). Seasonal influences occur in endocrine testicular function (Hoffmann & Landeck, 1999) as well as in the characteristics of fresh and frozen-thawed semen (Magistrini *et al.*, 1987; Wach-Gygax *et al.*, 2017).

Conflicting information regarding the influence of season on semen characteristics is available: some groups reported increased motility in ejaculates collected during the breeding season (Roser & Hughes, 1992; Janett *et al.*, 2003b; Gamboa *et al.*, 2010), whereas others observed lower motility at this time of the year (Janett *et al.*, 2003a) or reported no seasonal changes at all (Clay *et al.*, 1987; Schrammel *et al.*, 2016). Sperm morphology has also been reported to be influenced by season (Koyago *et al.*, 2009). Only minimal differences in the stability of chromatin between stallions and minor changes due to season or cryopreservation were shown (Blottner *et al.*, 2001). Sperm membrane integrity was affected by cryopreservation regardless of the season of semen collection (Wrench *et al.*, 2010), whereas the proportion of both motile and progressively motile sperm did not differ with cryopreservation in March (Wrench *et al.*, 2010). Differences among studies with regard to the time of the year best suited for cryopreservation may be due to differences in the methods applied to assess semen quality, or to specific stallions, and their location (Aurich, 2016).

Cryopreserving semen during the breeding season may result in sperm samples with greater post-thaw quality than those cryopreserved during the non-breeding season. It is generally assumed that the optimal time to collect semen for cryopreservation is in the spring, during the breeding season (Blottner *et al.*, 2001; Janett *et al.*, 2003a; Janett *et al.*, 2003b; Wrench *et al.*, 2010). However, sperm cryopreservation during December (non-breeding season) results in similar sperm survival rates as during the breeding season (Blottner *et al.*, 2001). Nevertheless, the differences among seasons are shown to be, at best, small and the much more important factor is the variation in the suitability of individual stallions for semen cryopreservation (Aurich, 2016). Furthermore, stallions are

fertile throughout the year and one of the prerequisites for successful preservation of stallion semen is high initial quality. Therefore, selection of suitable semen donors is a prerequisite for successful preservation (Blottner *et al.*, 2001; Aurich, 2016). Moreover, optimization of season will not change a bad freezer into a good one (Magistrini *et al.*, 1987).

2 Aims

The overall aims of this project were: 1) to develop protocols to enhance stallion sperm cryosurvival. 2) To explore the differences in SP major protein groups between the breeding and non-breeding seasons in relation to sperm quality in good and bad freezer stallions.

The specific objectives were:

- **Study I**: to investigate the effectiveness of applying SLC and adding pooled SP from good or bad freezer stallions prior to cryopreservation on post-thaw sperm quality.

- **Study II**: to evaluate whether adding pooled SP from stallions of known freezability to SP-free spermatozoa after thawing could minimize or repair cryoinjuries

- **Study III**: to examine the effect of adding SP on the functional capacity and fertilizing ability of cryopreserved stallion spermatozoa using a heterologous zona binding assay (*in vitro* matured bovine oocytes with intact ZP).

- **Study IV**: to determine the differences in the major protein groups in stallion SP between the breeding and non-breeding seasons in relation to sperm quality and testosterone levels between good and bad freezers.

3 Materials and Methods

3.1 Ethical permission

Ethical approval was not required for any of the experiments included in this thesis. Collecting semen samples using an artificial vagina does not compromise the animal's welfare.

3.2 Study design

Four studies were conducted:

In **study I**: the effect of adding a small proportion of SP prior to freezing was investigated in sperm samples prepared by SLC, compared with samples frozen by a standard freezing protocol with no SLC (Figure 1).
In **study II**: the role of SP in repairing cryoinjuries after thawing was investigated (Figure 2).
In **study III**: sperm functional capacity and fertilizing ability were evaluated using HZBA in samples treated with SP prior to cryopreservation or after thawing (Figure 3).
To evaluate the crossover effects of adding SP to sperm samples from good freezer stallions or bad freezer stallions, a retrospective study was performed by combining data from **study I-III**; the design of the statistical analysis is illustrated in Figure 4.
In **study IV**: the proportions of non-heparin-binding, phosphorylcholine-binding and heparin-binding proteins in SP were investigated between the breeding and non-breeding seasons, relating them to sperm quality and testosterone levels in good and bad freezers (Figure 5).

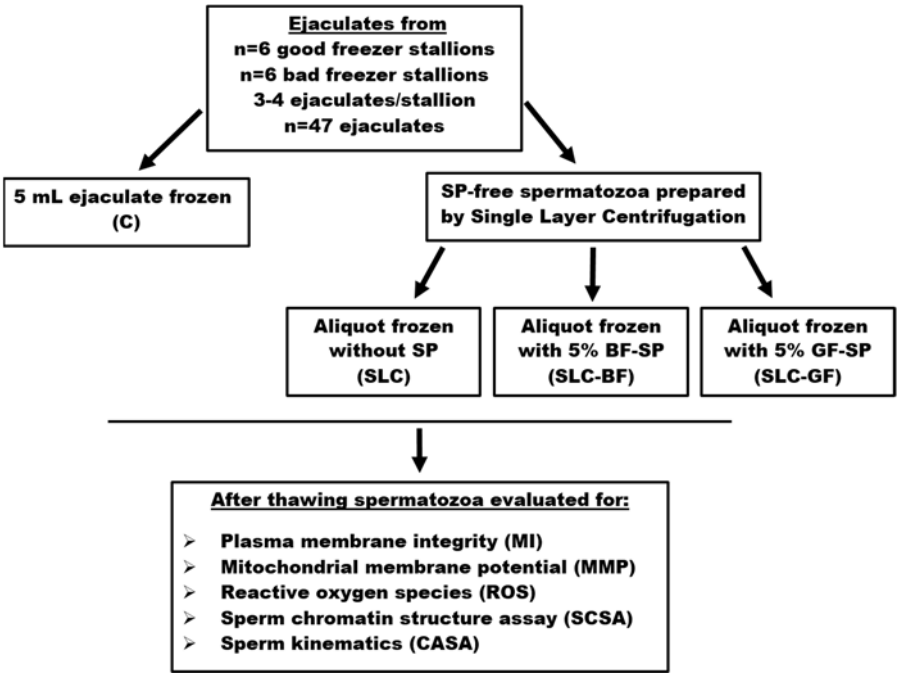

Figure 1. Experimental design for **study I**

Abbreviations: SLC, Single Layer Centrifugation; C, control; SP, seminal plasma; BF, bad freezer; GF, good freezer; CASA, computer assisted sperm analysis.

3.3 Animals and husbandry

Semen was obtained from twenty warmblood stallions of proven fertility, aged 4-18 years. The stallions were housed under standard husbandry condition at Brandenburg State Stud Neustadt/Dosse, Germany. The stallions were classified according to the freezability of their semen in previous years and a test freezing was conducted before the start of the study to confirm this classification.

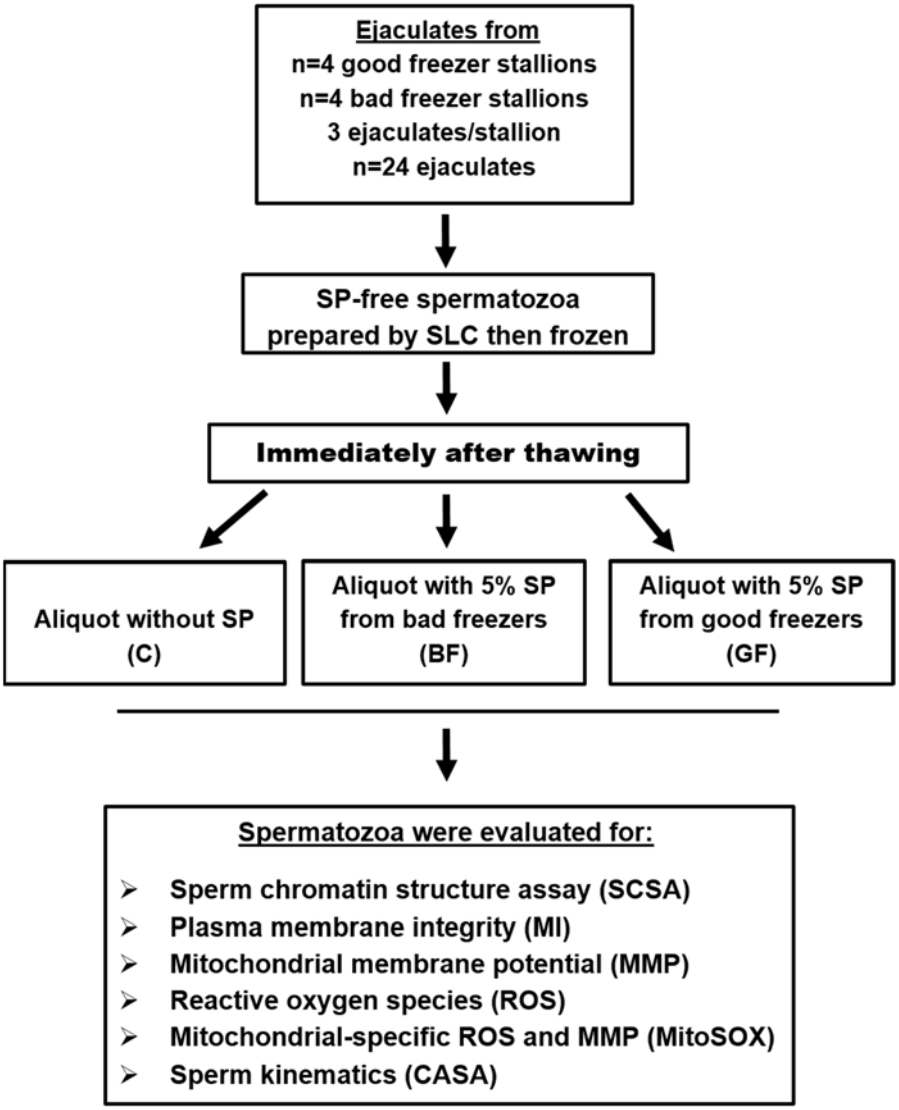

Figure 2. Experimental design for **study II**

Abbreviations: SP, seminal plasma; SLC, Single Layer Centrifugation; C, control; BF, bad freezer; GF, good freezer; CASA, computer assisted sperm analysis

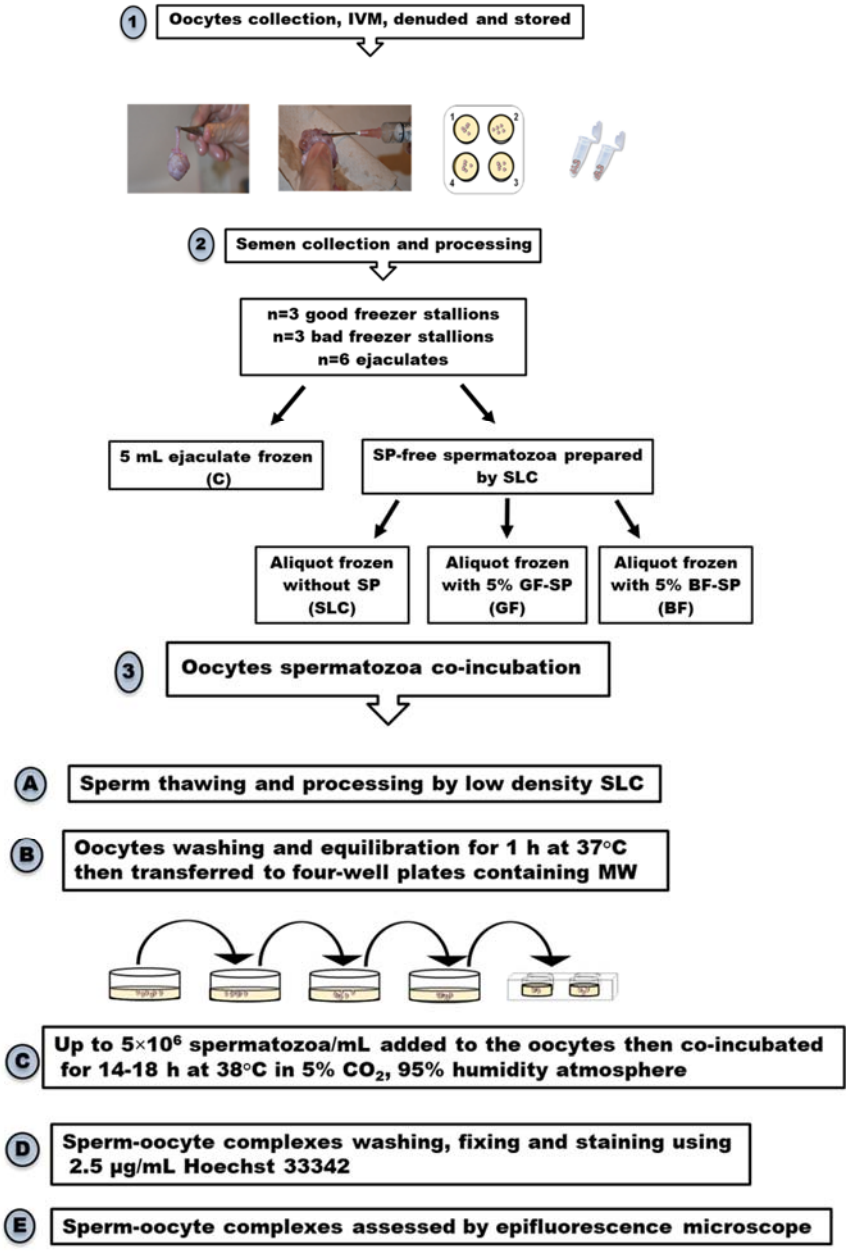

Figure 3. Experimental design for **study III**

Abbreviations: IVM, *in vitro* maturation; SP, seminal plasma; SLC, Single Layer Centrifugation; C, control; GF, good freezer; BF, bad freezer; MW, Modified Whitten's medium

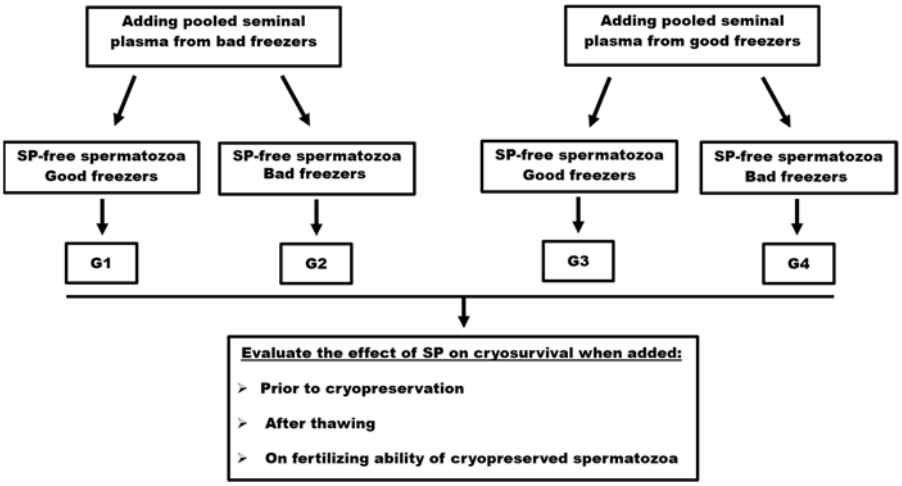

Figure 4. Crossover study design **study I-study III**

Abbreviations: SP, seminal plasma; G, group

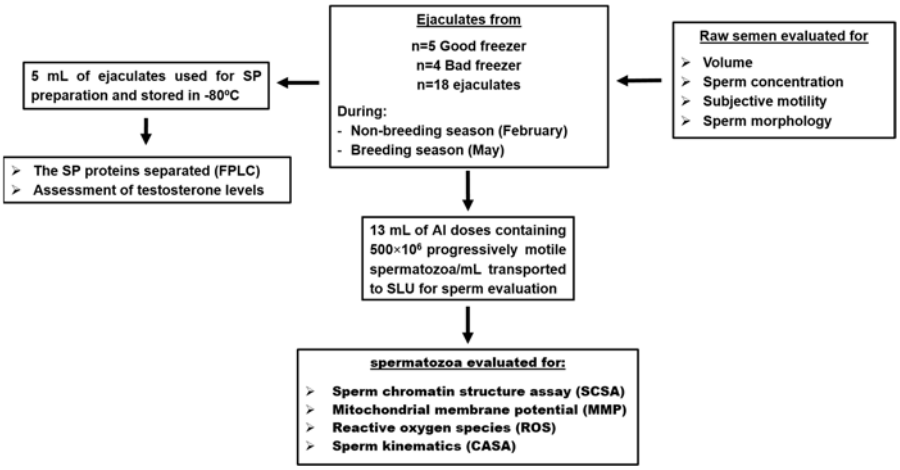

Figure 5. Experimental design for **study IV**

Abbreviations: SP, seminal plasma; FPLC, fast protein liquid chromatography; AI, artificial insemination; SLU, Swedish University of Agricultural Sciences.

3.4 Bovine oocytes collection, *in vitro* maturation and storage (**study III**)

All media and constituents were obtained from Sigma-Aldrich (Stockholm, Sweden) unless otherwise stated. Maturation medium was freshly produced in the laboratory.

3.4.1 Bovine oocytes collection and *in vitro* maturation

Bovine ovaries from a local abattoir were transported to the laboratory at the Swedish University of Agricultural Sciences (SLU) in 0.9 % sodium chloride at approximately 35ºC for oocyte recovery within 4 hours of collection. The protocol described by Laskowski *et al.* (2017) was used. In brief, cumulus oocyte complexes (COCs) were aspirated from 3-8 mm follicles using a 5mL syringe with an 18 gauge hypodermic needle. The aspirates were collected into TCM 199 (M7528) based search medium. Groups of up to 50 COCs were washed and moved into 500µL basic *in vitro* maturation medium, consisting of bicarbonate-buffer TCM199 (M2154) supplemented with 0.68 mM L-glutamine (G8540), 0.5µg mL^{-1} FSH and 0.1µg mL^{-1} LH (Stimufol; PARTNAR Animal Health, Port Huron, Canada), 50µg mL^{-1} gentamicin and 0.4% bovine serum albumin (BSA, w/v). The COCs were incubated for 22 h at 38.5ºC in a humidified 5% CO_2 atmosphere.

3.4.2 Storage of IVM bovine oocytes

In vitro matured bovine oocytes were mechanically denuded of cumulus cells by repeated pipetting. After washing 3 to 4 times with phosphate buffered saline containing 0.1% polyvinyl alcohol (PVA, P8136, Thermo Fisher Scientific, USA) (PBS-PVA), the washed oocytes were transferred to 500µL aliquots of salt storage solution (Lynham & Harrison, 1998; Herrick & Swanson, 2003), and were stored at 4ºC for up to 3 months (da Silva *et al.*, 2012).

3.4.3 Sperm capacitation medium

The basal medium was Modified Whitten's medium, (MW; McPartlin *et al.*, 2008). To achieve standard capacitation conditions according to Macías-García *et al.* (2015), the medium was supplemented with 25mM bicarbonate ($NaHCO_3$), 2.4 mM $CaCl_2$ and BSA (7mg/mL). The osmolarity was adjusted to 290-300 mOsm/kg with NaCl. The pH was adjusted to 7.4.
Bicarbonate was added 1h before the experiment; the medium was then incubated at 37°C in air.

3.5 Semen collection and evaluation

Study I, **II**, and **III**: Ejaculates were collected during the non-breeding season; for **study IV**, semen was collected during February (non-breeding season), as well as during May (breeding season). For semen collection, the stallions mounted a phantom in the presence of a mare and ejaculated into a warm (42-44ºC) lubricated Hannover artificial vagina (Minitube; Tiefenbach, Germany). The gel-fraction was removed. Semen volume and pH were measured immediately and the sperm concentration was evaluated by photometry (SpermaCue; Minitube). The fresh semen was immediately extended 1:1 (v/v) with either Equiplus extender (test freezing) or Gent extender at 38ºC (both from Minitube) depending on the study. Sperm motility was estimated subjectively using a phase contrast light microscope at 400X magnification. In addition, after further dilution up to 1:4 (v/v) with the same extender, sperm motility was assessed objectively using a computer-assisted sperm analyzer (AndroVision; Minitube) for **study I** and **II**.

3.6 Freezing protocol and classification of stallion semen freezability (**study I-IV**)

Sperm freezing was done as described previously by Schober *et al.* (2007) with slight modifications. Briefly, extended semen 1:2 (v/v) was centrifuged at 750×g for 10 min, the supernatant was removed and the sperm pellet was re-suspended with 1 mL of Gent freezing extender (Minitube). An equal volume of freezing extender was added to double the initial volume of the resuspended pellet. After filling straws using an automatic filling and sealing machine (MPP Uno; Minitube), they were frozen using a controlled freezer (Ice Cube 14S; Minitube). The protocol included a slow cooling phase (0.3°C/min) from 20°C down to 5°C, a rapid cooling phase (10°C/min) from 5°C to -25°C, followed by a second rapid phase (25°C/min) from -25°C to -140°C. After one hour the straws were plunged

into liquid nitrogen (LN_2, -196°C) and stored for one week before evaluation. One straw from each ejaculate was thawed by immersing in a 37°C water bath for 30 seconds. Post-thaw total and progressive motility were evaluated using the AndroVision (Minitube). According to the stud´s routine assessment protocol, good freezers have TM ≥ 60% and PM ≥ 40 whereas for bad freezers TM ≤ 50% and PM ≤ 30.

3.7 Preparation of seminal plasma

As a preparatory step for **study I-IV**, SP was obtained from fresh, gel-free raw semen by centrifugation at 2000×g for 10 minutes to pellet the spermatozoa. The supernatant was aspirated and re-centrifuged at 3500×g for 10 minutes if necessary. This process was repeated until the SP was free of spermatozoa. After filtering, SP aliquots were stored at -80ºC until required. Equal volumes of sperm-free SP from good freezer or bad freezer stallions were pooled prior to use in **study I-III**.

3.8 Preparation of artificial insemination doses (**study IV)**

Semen doses were prepared in Equiplus extender (Minitube) according to the stud´s usual procedure, i.e. each dose contained 500×10^6 motile spermatozoa/mL in a volume not exceeding 13mL. One AI dose from each ejaculate was transported to the laboratory at SLU in an insulated box at 5 to 7°C; sperm quality was evaluated within 24 hours of semen collection.

3.9 Semen processing and cryopreservation (**study I-III**)

3.9.1 Sperm concentration

In **study I**, **II** and **III** the sperm concentration before freezing was determined using the Nucleocounter SP-100 (ChemoMetec, Allerød, Denmark), as described by Morrell *et al.* (2010a).

3.9.2 Semen processing for control

The stud´s standard freezing protocol was used to prepare the control samples (C) for **study I** and **III**, as described in section 3.6.

3.9.3 Single Layer Centrifugation (SLC)

Seminal plasma free spermatozoa were prepared by SLC prior to cryopreservation (SLC group), in **study I**, **II** and **III**. The sperm concentration was adjusted to 100×10^6 spermatozoa /mL and SLC was performed according to the method described by Morrell *et al.* (2011a) using 15 mL of extended semen and 15 mL of Equicoll, previously equilibrated to room temperature (RT). After centrifugation at 300×g for 20 minutes, the supernatant and colloid were removed and the sperm pellet was transferred to 1 mL of fresh extender for freezing (Gent; Minitube).

For **study I** and **III**, the sperm suspension after SLC was divided into three aliquots: i) frozen without adding SP (SLC); ii) treated with 5% pooled SP from good freezer stallions (SLC-GF); iii) treated with 5% pooled SP from bad freezer stallions (SLC-BF).

3.9.4 Semen cryopreservation

For all groups, the sperm concentration was adjusted to 200×10^6 spermatozoa/mL with fresh extender for freezing (Gent; Minitube) before packaging in 0.5mL straws for freezing and storage as in section 3.6.

3.10 Preparation of frozen thawed samples

In **study I**, **II** and **III** the straws were thawed in a water bath at 37°C for 30s. After wiping with a tissue, the contents were dispensed into a clean dry tube. To assess the effect of adding SP after thawing in **study II** and **III**, the SLC samples were divided into three treatments: i) SLC-control without SP (C); ii) 5% pooled SP from bad freezer stallions (BF); iii) 5% pooled SP from good freezer stallions (GF). In **study III**, in order to remove the extender with the cryoprotectant and retrieve the entire sperm sample, the thawed samples for all groups were prepared by SLC using a low density colloid (Equicoll @ 40%). An aliquot (250 to 500µL) was layered over 1mL of the colloid at RT, and the preparation was centrifuged at 300×g for 20 min. The sperm pellet was aspirated from beneath the colloid and transferred to capacitation medium (100-200µL). The sperm concentration in the suspension was measured using the Nucleocounter SP-100 as described previously.

3.11 Sperm evaluation by flow cytometry (**study I**, **II** and **IV**)

The flow cytometric assessments in **study I** and **IV** were performed using a BD LSR flow cytometer (Becton Dickinson, San Jose, CA) equipped with standard optics. In **study II**, the flow cytometer was a FACSVerse, (BDBiosciences, San José, CA, USA).

3.11.1 Sperm chromatin structure assay (SCSA)

This assay was carried out as described previously (Evenson *et al.*, 2002; Morrell *et al.*, 2009b). Briefly, a 1:1 (v/v) sperm suspension with Tris-sodium chloride-EDTA buffer (TNE) was snap-frozen in LN_2 and stored at -80ºC. The samples were thawed on crushed ice and further diluted in TNE buffer up to 1:10 (v/v). After partial DNA denaturation with acid-detergent solution containing 0.17% Triton X-100, the spermatozoa were stained with 6µg/mL acridine orange and evaluated by flow cytometry within 3-5 minutes. Data were collected from 10,000 events for each sample and presented as the DNA fragmentation index (%DFI; ratio of the percentage of spermatozoa with denatured, single-stranded DNA to total spermatozoa acquired, i.e. both those with stable, double-stranded DNA, and denatured single-stranded DNA).

3.11.2 Mitochondrial membrane potential

In **study I**, **II** and **IV**: sperm mitochondrial membrane potential (MMP) was evaluated by labeling the spermatozoa with the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide, (JC-1; ThermoFisher, Waltham, MA, USA), according to the method used by Cossarizza *et al.* (1993). Briefly, sperm suspension at a final concentration of 1×10^6 spermatozoa/mL in CellWASH (BDBiosciences) was labeled with 3mM JC-1. The stained samples were analysed by flow cytometry; a total of 30,000 events was evaluated and the spermatozoa were classified as spermatozoa with high respiratory activity MMP-H (orange fluorescence) or those with low respiratory activity MMP-L (green fluorescence).

3.11.3 Sperm plasma membrane integrity

Sperm membrane integrity was evaluated in **study I** and **II** using SYBR-14 and propidium iodide (PI), (LIVE/DEAD® Sperm Viability Kit L-7011; Invitrogen™ Molecular Probes™, Eugene, Oregon, USA), according to the procedure described by Johannisson *et al.* (2009). A sperm suspension in

CellWASH was labeled with 0.02 mM SYBR-14 and 2.4 mM PI. The labeled samples were evaluated by flow cytometry; in total, 30,000 events were collected and quantified as proportions of the population. After gating, the spermatozoa were classified as membrane intact ($SYBR14^{+}$-PI^{-}), and membrane damaged ($SYBR14^{+}$-PI^{+}) or ($SYBR14^{-}$-PI^{+}).

3.11.4 Reactive oxygen species (ROS)

In **study I**, **II** and **IV**, ROS production was evaluated according to Johannisson *et al.* (2014). In brief, the sperm suspension in CellWASH was stained with 40µM Hoechst 33258 (HO; Sigma, Stockholm, Sweden), 40µM hydroethidine (HE; Invitrogen™ Molecular Probes™), and 2mM 2′, 7′-dichlorodihydrofluorescein diacetate (DCFDA; Invitrogen™ Molecular Probes™) was added. A further aliquot was prepared as above, with the addition of menadione (Sigma-Aldrich, Stockholm, Sweden) to a final concentration of 200µM, to determine whether the spermatozoa were capable of producing ROS when stimulated. A total of 30,000 events was evaluated after gating out debris, and the spermatozoa were classified as follows (%): viable, superoxide-negative; viable, superoxide-positive; non-viable, superoxide-positive; viable, hydrogen peroxide negative; viable, hydrogen peroxide positive; non-viable, hydrogen peroxide negative; and non-viable, hydrogen peroxide positive.

3.11.5 Simultaneous measurement of mitochondria-specific ROS and MMP (MitoSOX)

To evaluate mitochondria-specific reactive oxygen species (mROS) and MMP simultaneously (**study II**), a dual-staining method consisting of a mitochondria-specific superoxide fluorescent probe (MitoSOX Red; Invitrogen, Carlsbad, CA, USA) and JC-1 was used (Johannisson *et al.*, 2018). The sperm suspensions were prepared as in section 3.11.4, with the addition of Hoechst 33258 (40µM), MitoSOX Red (500µM) and JC-1 (0.9mM). A total of 30,000 events was evaluated for each sample. The spermatozoa were classified as follows (%): $MitoSOX^{+}$, $JC\text{-}1^{-}$ = high superoxide production and low mitochondrial membrane potential; $MitoSOX^{+}$, $JC\text{-}1^{+}$ = high superoxide production and high MMP; $MitoSOX^{-}$, $JC\text{-}1^{-}$ = low superoxide production and low MMP; $MitoSOX^{-}$, $JC\text{-}1^{+}$ = low superoxide production and high MMP.

3.12 Sperm kinematics after thawing

In **study I**, **II** and **IV**, sperm kinematics were analysed using the computer assessed sperm analysis instrument SpermVision®, version 3.5 (Minitube GmbH). The following parameters were measured: total motility (%), progressive motility (%), average path velocity (VAP; μm/s), curvilinear velocity (VCL; μm/s), straight line velocity (VSL; μm/s), straightness (STR; VSL/VAP), linearity (LIN; VSL/VCL), wobble (WOB; VAP/VCL), amplitude of lateral head deviation (ALH; μm) and beat cross frequency (BCF; Hz). Spermatozoa were considered to be immotile if VAP <20 and locally motile if VAP > 20 and <30, STR <0.5, VCL <9.

3.13 Binding assay (**study III**)

3.13.1 Co-incubation of spermatozoa and oocytes

The *in vitro* matured bovine oocytes were removed from salt-storage solution, equilibrated at RT for 30 minutes and washed in pre-warmed PBS-PVA prior to equilibration for one hour at 38°C in an atmosphere of 5% CO_2 in air. They were transferred into four-well plates containing 460μL MW in groups of 25 to 30 for insemination with sperm suspension at a final concentration of 5×10^6 spermatozoa/mL. The plates were incubated for 14 to18h in 38°C in 5% CO_2, with 100% humidity. Thereafter, the sperm-oocyte complexes were pipetted 3 to 5 times to remove loosely attached spermatozoa, rinsed gently using PBS-PVA and fixed in 2% (v/v) paraformaldehyde in PBS-PVA overnight at 4°C. The fixed oocytes were stored in Quench solution (Lynham & Harrison, 1998) and kept at 4°C for up to 5 days before assessment (Lynham & Harrison, 1998; Clulow *et al.*, 2010).

3.13.2 Assessment of sperm binding

The sperm-oocyte complexes were removed from storage Quench solution, washed three times with PBS-PVA, and moved to a 100μL PBS-PVA droplet containing 2.5μg/mL Hoechst 33342 (Sigma). After incubating for 20 to 30 min at RT in the dark, they were mounted on glass slides with 2μL anti-fade medium, Vectashield (Vector Laboratories, Burlingame, CA, USA), covered and sealed. The number of spermatozoa bound to each oocyte ZP was assessed using an epifluorescence microscope (LSM 510, Carl Zeiss, AB, Jena, Germany) with 200X magnification.

3.14 Sperm evaluation for artificial insemination doses (**study IV**)

Sperm samples were evaluated at SLU within 24 h of semen collection for sperm kinematics, sperm chromatin integrity (SCSA), MMP and ROS as previously described.

3.14.1 Sperm morphology

The morphological examinations were performed according to Lagerlöf (1934). Air-dried smears were prepared for assessment of the head shape of 500 spermatozoa at 1000X magnification, following staining. In addition, drops of sperm samples were fixed in buffered formaldehyde solution for evaluation of 200 spermatozoa in wet smears. The proportion of morphologically normal spermatozoa was calculated by subtracting abnormal spermatozoa.

3.15 Evaluation of SP proteins and testosterone (**study IV**)

3.15.1 Affinity liquid chromatography of SP (FPLC)

Aliquots of SP (350µL) were mixed with 40µL 0.5M Tris buffer and 630µL H_2O. Protein concentration was analysed using a Nanodrop 2000 spectrophotometer (Thermofisher Scientific). The SP proteins were separated on column HiPrep 16/10 Heparin FF, 20mL (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) by fast protein liquid chromatography (FPLC) according to Vařilová, *et al*. (2006) and Madej *et al*. (2013). The column was used directly on an ÄKTA Explorer™ system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The non-heparin-binding proteins, peak 1 and 2 (F1 + F2), were eluted with 0.02M Tris-HCl buffer containing 0.156M NaCl, pH 7.5. The phosphorylcholine-binding proteins, peak 3 (F3), were eluted with 0.02M Tris-HCl buffer containing 0.156M NaCl and 0.05M phosphorylcholine, pH 7.5. The proteins adsorbed on heparin, peak 4 (F4), were eluted using 0.02M Tris-HCl buffer, pH 7.5. Peak height (mAU), peak area (mAU*mL), and percentage of area for each peak in relation to the total area were recorded for each fraction, as well as ratios of the different fractions.

3.15.2 Assessment of testosterone concentration in stallion SP

Stallion SP testosterone concentration (ng/mL) was measured using a horse testosterone competition ELISA kit (catalog no. CSB-E13193Hs; Cusabio Biotech Co., China) with LOD 0.05ng/mL, according to the manufacturer's instructions. Testosterone concentrations were calculated using four parametric logistic curve-fit (https://www.myassays.com/). The values obtained from standard curve were used, the higher optical density measured represents lower (opposite) testosterone concentration in the sample. The intra-assay CV was 9.2% (manufacturer recommendation <15%), spiked sample percent recovery was 128 %.

3.16 Statistical analysis

In all studies (**I-IV**), statistical analyses were performed in SAS (SAS® 9.3, Cary, NC, USA) after checking normal distribution of the residuals; data with non-normal distribution of residuals were log-transformed to achieve normal or close to normal distribution. Post-hoc comparisons were adjusted for multiplicity using Tukey's method. All values were reported as Least Squares Means ± Standard Error (LSMEAN ± SEM), with $p \leq 0.05$ being considered to be significant. The comparisons among groups in each experiment were performed by the mixed model (Proc Mixed).

Study I: the statistical model included the fixed effects of treatments (four groups: C, SLC, SLC-BF and SLC-GF) and stallion status (good or bad freezer), in addition to groups*status interaction, and the random effects of interaction of stallion, ejaculates and stallion status.

Study II: the model included the fixed effects of treatment (three groups: C, GF and BF), and the random effects of interaction between (stallion*freezability).

Study III: the model included the fixed factors of treatment, stallion freezability, and the interaction between them, while the interaction among stallions and their freezability was considered as a random factor.

To evaluate the crossover effects of SP, in **study I-III** the model included the fixed effects of four groups and random effects of the interaction between the stallions and the ejaculates (stallions*ejaculates) for **study I** and **II**, and the stallions for **study III**.

Study IV: the statistical model included the fixed effects of seasons, and stallion status (good or bad freezer), in addition to seasons*status interaction, and the stallions were considered as a random factor.

4 Results

The results are presented in full in papers **I**–**IV**.

4.1 Selecting spermatozoa by SLC prior to cryopreservation

Preparing the samples by SLC prior to cryopreservation (Table 1) significantly reduced %DFI compared to control (**study I**). The SLC-selected group had a higher proportion of spermatozoa with high MMP, and a lower proportion of spermatozoa with low MMP than control. The proportions of the viable and non-viable hydrogen peroxide positive spermatozoa were higher in control than in the SLC-selected group.

No significant differences in kinematics were found among groups or ejaculates. In addition, the capability of the spermatozoa to bind to bovine oocytes ZP (**study III**) was not affected by processing the samples using SLC prior to cryopreservation (C, 21.23 ±2.93; SLC, 22.40±2.96; NS).

4.2 Sperm characteristics after thawing

The effects of adding pooled SP from good or bad freezer stallions to SLC-selected samples prior to freezing or after thawing on %DFI, MMP, MI, ROS, are shown in Table 1.

4.2.1 Chromatin integrity

Addition of SP from good or bad freezer stallions to SLC-selected spermatozoa prior to cryopreservation (**study I**) did not affect %DFI, whereas the post-thaw addition of SP to cryopreserved spermatozoa (**study II**) increased the proportion of spermatozoa with damaged chromatin. There were no differences between adding SP from good or bad freezer stallions either before freezing (**study I**) or after thawing (**study II**).

4.2.2 Mitochondrial Membrane Potential

The proportion of spermatozoa with high MMP was lower, and the proportion of spermatozoa with low MMP was higher, in both SP-treated groups than in the SLC-selected group, when SP was added prior to cryopreservation (**study I**). In contrast, the proportion of spermatozoa with low MMP was higher in samples treated by SP from good freezers after thawing (**study II**) compared with the SLC control. A higher proportion of spermatozoa with high MMP was seen in the SLC control.

No significant differences were found in both MMP categories between the SP-treated groups after thawing (**study II**).

4.2.3 Plasma membrane integrity

Adding SP before freezing to SP-free spermatozoa did not alter cell membrane integrity (**study I**), regardless of the origin of the SP. However, adding SP after thawing **(study II**) resulted in a decrease in the proportion of spermatozoa with intact plasma membranes in both treated groups.

4.2.4 Reactive Oxygen Species

The group treated with SP from good freezer stallions before freezing (**study I**) had a significantly lower proportion of viable, superoxide positive spermatozoa than SLC control without SP. In addition, the proportions of non-viable superoxide positive spermatozoa and non-viable hydrogen peroxide negative spermatozoa were different between the groups SLC-GF and SLC. Adding SP after thawing **(study II**) did not affect the proportion of viable superoxide negative, viable superoxide positive, viable hydrogen peroxide negative, or viable and non-viable hydrogen peroxide positive sperm populations.

Table 1. *Effect of applying SLC prior to cryopreservation and adding pooled seminal plasma from good or bad freezer stallions, either prior to freezing or after thawing*

	SP added before freezing				SP added after thawing		
Sperm parameters	C	SLC	GF	BF	SLC	GF	BF
%DFI	12.10±0.76[a]	5.44±0.76[b]	5.79±0.76[b]	5.61±0.76[b]	8.28±1.14[a]	9.5±1.14[b]	9.85±1.14[b]
MMP-L	79.15±3.23[a]	70.89±3.23[b]	78.85±3.23[a]	78.74±3.23[a]	70.58±4.1[a]	78.51±4.1[b]	76.14±4.1[a]
MMP-H	17.94±3.14[a]	26.56±3.14[b]	18.56±3.14[a]	18.75±3.14[a]	28.5±4.1[a]	20.5±4.1[b]	23.1±4.1[a]
Membrane intact	45.29±2.83	48.31±2.83	48.21±2.83	48.50±2.83	42.74±3.2[a]	41.33±3.2[b]	41.12±3.2[b]
Viable superoxide negative	27.08±2.17	28.78±2.17	28.24±2.17	28.82±2.17	32.63±2.22	30.05±2.22	31.64±2.22
Viable superoxide positive	24.96±1.71[a]	24.23±1.71[a]	21.48±1.71[b]	22.60±1.71[a,b]	15.46±1.61	15.92±1.61	14.48±1.61
Non-viable superoxide positive	48.04±2.11[a]	47.09±2.11[a]	50.39±2.11[b]	48.68±2.11[a,b]	51.14±1.59	53.36±1.59	52.69±1.59
Viable hydrogen peroxide negative	49.66±2.13	52.4.±2.13	49.08±2.13	50.26±2.13	47.75±2.21	46.11±2.21	46.77±2.21
Viable hydrogen peroxide positive	2.20±0.71[a]	0.57±0.71[b]	0.62±0.71[b]	1.09±0.71[b]	1.57±1.73	0.5±1.73	0.59±1.73
Non-viable hydrogen peroxide negative	46.34±2.16[a]	45.82±2.16[a]	49.56±2.16[b]	47.95±2.16[a,b]	47.1±1.5[a]	51.04±1.5[b]	50.31±1.5[a,b]
Non-viable hydrogen peroxide positive	1.34±0.52[a]	0.95±0.52[b]	0.49±0.52[b]	0.47±0.52[b]	2.89±1.65	1.6±1.65	1.53±1.65

Values are LSMEAN ± SEM.

Abbreviations: C, control group; SLC, spermatozoa selected by Single Layer Centrifugation before freezing without SP; GF, SLC-selected samples treated with 5% SP from good freezer stallions; BF, SLC-selected samples treated with 5% SP from bad freezer stallions; %DFI, DNA fragmentation index; MMP-L, low-mitochondrial membrane potential (%); MMP-H, high-mitochondrial membrane potential (%). Different letters indicate statistical difference within a row (between treatments within assay) in each class of treatment ($p \leq 0.05$).

The proportion of non-viable, hydrogen peroxide negative spermatozoa was increased in samples treated with SP from good freezer stallions.

4.2.5 Simultaneous measurement of mitochondria-specific ROS and MMP (MitoSOX)

The proportion of spermatozoa with high superoxide production and high MMP ($MitoSOX^+$, $JC\text{-}1^+$) (Table 2) was higher after treatment with SP from good freezer stallions than with SP from bad freezer stallions (**study II**). Adding menadione significantly increased the sperm population that had high superoxide production and low MMP ($MitoSOX^+$, $JC\text{-}1^-$), whereas the proportion of spermatozoa with low superoxide production and low MMP ($MitoSOX^-$, $JC\text{-}1^-$), and the sperm population with low superoxide production and high MMP ($MitoSOX^-$, $JC\text{-}1^+$) were decreased. Menadione did not affect the proportion of spermatozoa that showed high superoxide production and high MMP ($MitoSOX^+$, $JC\text{-}1^+$) in any treatment group.

Table 2. *Effect of adding seminal plasma from good or bad freezer stallions after thawing to spermatozoa selected by SLC prior to cryopreservation on simultaneous assessment of superoxide production and MMP*

(A) without menadione and (B) with menadione

	Treatment	$MitoSOX^+$, $JC\text{-}1^-$ (%)	$MitoSOX^+$, $JC\text{-}1^+$ (%)	$MitoSOX^-$, $JC\text{-}1^-$ (%)	$MitoSOX^-$, $JC\text{-}1^+$ (%)
A	C	11.3±3.63	5.53±2.17^{a,b}	21.55±4.73	61.63±4.13
	GF	12.1±3.63	8.27±2.17^{b}	22.83±4.73	56.82±4.13
	BF	11.85±3.63	4.47±2.17^{a}	27.01±4.73	56.66±4.13
B	C menadione	77.1±3.63***	10.49±2.17	11.88±4.73***	0.57±4.13***
	GF menadione	77.67±3.63***	10.83±2.17	9.15±4.73**	2.35±4.13***
	BF menadione	82.6±3.63***	7.69±2.17	9.08±4.73**	0.66±4.13***

Values shown are LSMEAN ± SEM.

Different superscript letters within the column indicate significant differences within group (A).

Different number of superscript stars within group (B) indicates different significance level compared with the corresponding group (A).

Abbreviations: C, SLC without SP; GF, SLC-selected samples treated with 5% SP from good freezer stallions; BF, SLC-selected samples treated with 5% SP from bad freezer stallions.

4.2.6 Sperm kinematics

There were no differences in sperm kinematics among the groups regardless of whether SP was added prior to cryopreservation (**study I**), or after thawing (**study II**).

4.2.7 Fertilizing ability

Adding SP from good freezer stallions prior to cryopreservation (**study I**), significantly reduced the sperm binding affinity to bovine oocyte ZP (Table 3). Adding SP from bad freezers before freezing did not change sperm binding ability compared with the SLC-selected group, whereas adding pooled SP from bad freezers after thawing significantly decreased the mean number of spermatozoa bound to bovine oocyte ZP. However, the mean number of bound spermatozoa was higher for the group treated with SP from good freezers than for the group treated with SP from bad freezer stallions.

Incorporation of SP from good or bad freezer stallions into co-incubation medium significantly reduced the mean number of spermatozoa bound to bovine oocyte ZP compared to the group incubated without any SP addition.

4.3 Crossover study

4.3.1 Post-thaw sperm quality

There was no significant alteration in post-thaw sperm quality irrespective of whether SP from bad or good freezer stallions was added to SP-free spermatozoa obtained from good or bad freezers, either before freezing (**study I**), or after thawing (**study II**).

4.3.2 Simultaneous measurement of mitochondria-specific ROS and MMP (MitoSOX)

In **study II**, the population of spermatozoa with high superoxide production and high MMP ($MitoSOX^{+}$, $JC\text{-}1^{+}$) was higher when SP-free spermatozoa obtained from good freezer stallions were treated with pooled SP from good freezer stallions, compared with the other group where spermatozoa obtained from bad freezer stallions received pooled SP from bad freezer stallions.

Table 3. *Effect of adding pooled seminal plasma from good or bad freezer stallions, prior to cryopreservation or after thawing, on sperm fertilizing capacity*

Groups	No. of bovine oocytes	Mean no. of spermatozoa bound to ZP
Experiment 1: adding SP prior to cryopreservation		
S	151	22.40±2.96[a]
GF	185	15.50±2.92[b]
BF	172	24.15±2.93[a]
Experiment 2: adding SP after thawing		
S	191	31.52±8.42[a]
GF	195	21.43±8.42[c]
BF	190	18.41±8.42[b]
Experiment 3: prolonged exposure to SP after thawing		
S	151	21.39±2.44[a]
GF	164	1.79±2.43[b]
BF	161	2.33±2.43[b]

Values are LSMEAN ± SEM.
Abbreviations: SP, seminal plasma; S, control without SP; GF, SLC-selected samples treated with 5% SP from good freezer stallions; BF, SLC-selected samples treated with 5% SP from bad freezer stallions.

4.3.3 Sperm fertilizing ability

The highest zona-binding ability was shown by SP-free spermatozoa from bad freezer stallions treated with pooled SP from bad freezers prior to cryopreservation, compared to SP from good freezer stallions (II vs. IV, Exp. 1). Sperm binding capability was significantly enhanced when SP-free spermatozoa from good freezer stallions were treated with SP from good freezers after thawing and before filtration by low density colloid (I vs. III, Exp. 2), compared to adding SP from bad freezer stallions. Incorporating SP from good or bad freezers into the oocyte medium dramatically reduced the ability of spermatozoa to bind to the ZP regardless of the source of the spermatozoa in Exp.3 (Figure 6, A & B).

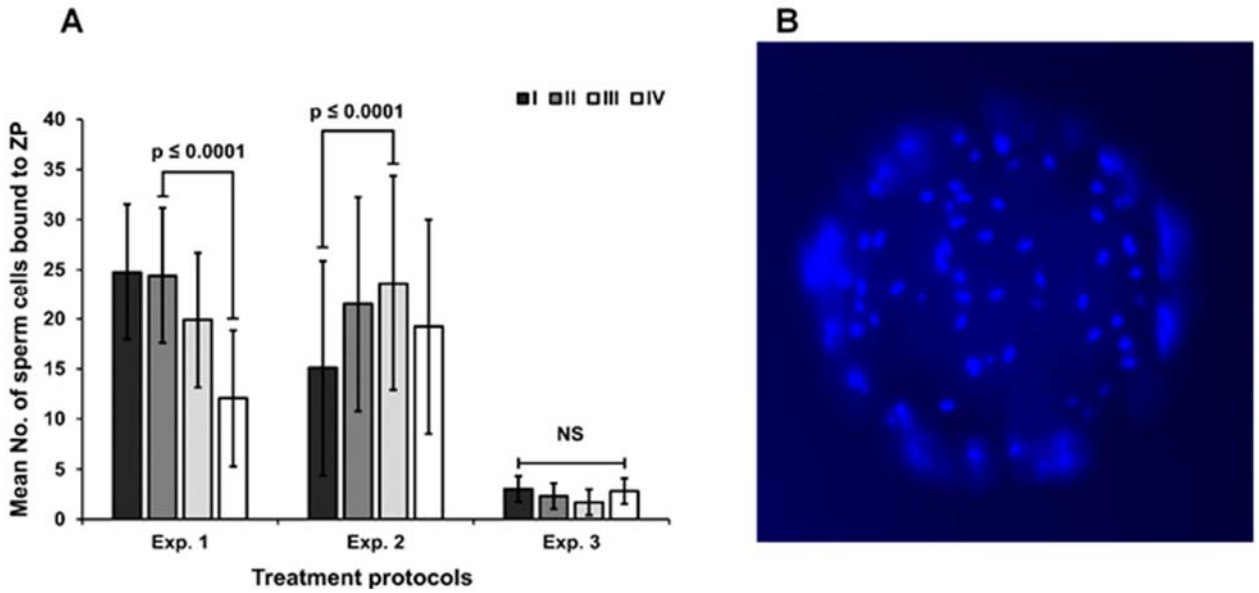

Figure 6. **(A)** Effect of adding pooled seminal plasma (SP) from good or bad freezer stallions to spermatozoa from stallions of known freezability on sperm binding ability to *in vitro* matured bovine oocytes with intact zona pellucida. Exp.1, adding SP prior to cryopreservation; Exp.2, adding SP after thawing and prior to colloid centrifugation; Exp.3, adding SP to the medium prior to co-incubation of spermatozoa with oocytes.

Values are Least Squares Means for the number of spermatozoa bound to zona pellucida.

Abbreviations: I, spermatozoa from good freezer + SP from bad freezers; II, spermatozoa from bad freezer + SP from bad freezers; III, spermatozoa from good freezer + SP from good freezers; IV, spermatozoa from bad freezer + SP from good freezers. NS, not significant. **(B)** Representative micrograph of a salt-stored bovine oocyte co-incubated with stallion spermatozoa showing spermatozoa bound to the zona pellucida.

4.4 Seminal plasma proteins and sperm characteristics in different seasons

4.4.1 Proportions of SP proteins

A typical chromatogram is shown in Figure 7, with four peaks where peak 1 and peak 2 represent non-heparin-binding proteins, peak 3 contains phosphorylcholine-binding proteins and peak 4 heparin-binding proteins. The amount of non-heparin binding proteins found in peak 2, as indicated by both peak height and area, was higher in the non-breeding season than in the breeding season (Table 4). In addition, the heparin-binding proteins in peak 4 were more abundant in the non-breeding season. The ratio of heparin-binding proteins to total proteins tended to be higher in the non-breeding season. The only difference found between the good and bad freezers in the different seasons was that the peak height for the heparin-binding proteins was higher in the non-breeding season for bad freezers.

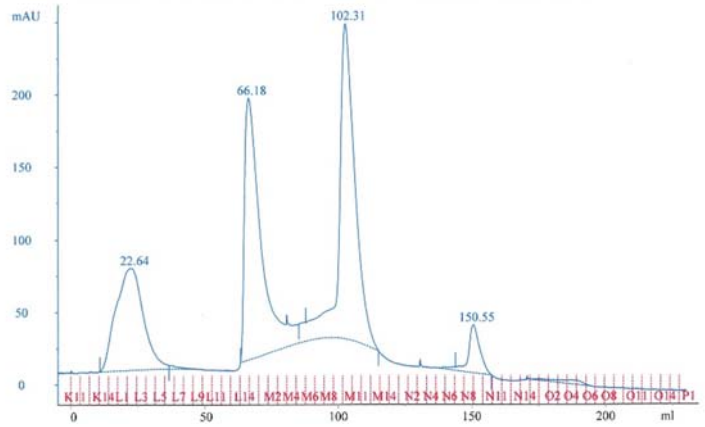

Figure 7. Typical FPLC chromatogram of stallion seminal plasma proteins (Non-heparin-, phosphorylcholine- and heparin-binding). Peak 1 and 2 contain proteins not interacting with heparin (Fraction F1) while peak 3 (Fraction F2) and peak 4 (Fraction F3) contains phosphorylcholine-binding and heparin-binding proteins, respectively.

Table 4. *Proportions of seminal plasma proteins by FPLC*

	All stallions		Good freezer stallions		Bad freezer stallions	
FPLC results	Breeding	Non-breeding	Breeding	Non-breeding	Breeding	Non-breeding
Peak 1 Area	192±31	138±31	229±41	150±41	156±46	126±46
Peak 1 Height	34.2±7.6	17.1±7.6	43.3±10.1	19.0±10.1	25.0±11.3	15.3±11.3
Peak 2 Area	128±25[a]	229±25[b]	119±33	212±33	137±37	246±37
Peak 2 Height	24.8±4.2[a]	40.2±4.2[b]	25.2±5.7	38.6±5.7	24.4±6.3	41.8±6.3
Peak 3 Area	358±39	386±39	336±52	386±52.	380±58	385±58
Peak 3 Height	62.8±6.8	69.1±6.8	59.5±9.0	67.0±9.0	66.1±10.1	71.2±10.1
Peak 4 Area	16.3±3.0[a]	25.1±3.0[b]	13.2±4.1	21.5±4.1	19.4±4.6	28.8±4.6
Peak 4 Height	4.34±0.76[a]	6.06±0.76[b]	3.57±1.01	4.54±1.01	5.12±1.13[a]	7.6±1.1[b]
Total Area	734±63	823±63	743±84	795±84	724±93	850±93
Area 1+2	320±35	367±35	348±46	362±46	293±52	372±52
Area3/Area4	374±39	411±39	349±52	407±52	400±58	414.±58
Area 1+2/total area	43.8±2.6	44.7±2.6	47.3±3.5	46.2±3.5	40.3±3.9	43.2±3.9
Area 3/total area	48.6±3.0	46.78±3.0	44.8±4.0	47.7±4.0	52.4±4.4	45.9±4.4
Area 4/total area	2.40±0.46	3.14±0.46	1.91±0.61	2.81±0.61	2.89±0.68	3.47±0.68

Values are LSMEAN ± SEM.

Different superscript letters indicate statistical difference within a row (between seasons within assay) in each class of stallion ($p \leq 0.05$). For the class all stallions P-values were as follows: Peak 2 Area, P=0.02; Peak 2 Height, P=0.04; Peak 4 Area, P=0.03; Peak 4 Height, P=0.01. For the class bad freezers P-value was as follows: Peak 4 Height, (BF), P=0.05.

4.4.2 Sperm quality parameters

Sperm concentration was higher in the non-breeding season than in the breeding season, whereas the proportion of spermatozoa with normal morphology was higher in the breeding season (Table 5). No differences in sperm morphology were seen between the two seasons in good freezer stallions. Furthermore, in the non-breeding season, good freezers had better morphology than bad freezers, whereas a higher proportion of spermatozoa with normal morphology was observed in bad freezers in the breeding season. Testosterone concentrations and ejaculate volume did not vary between seasons. For the kinematics, higher values for STR were seen in the non-breeding season (all stallions; breeding, 0.73±0.01; non-breeding, 0.78±0.01; P=0.04), while ALH was higher in the breeding season in all classes included in the study (all stallions; breeding,

4.31±0.13; non-breeding, 3.44±0.13; P=0.002; good freezer stallions; breeding, 4.30±0.17; non-breeding, 3.48±0.17; P=0.05; bad freezer stallions; breeding, 4.33±0.19; non-breeding, 3.41±0.19; P=0.04).

Table 5. *Semen characteristics and testosterone concentration between breeding and non-breeding seasons*

	All stallions		Good freezer stallions		Bad freezer stallions	
Semen values	Breeding	Non-breeding	Breeding	Non-breeding	Breeding	Non-breeding
Concentration (10^6/mL)	240±23[a]	348±23[b]	273±30	395±30	208±34	301±34
Ejaculate volume (mL)	22.2±2.2	20.8±2.2	21.2±3.0	20.0±3.0	23.2±3.3	21.5±3.3
Normal morphology (%)	71.4±3.0[a]	68.38±3.0[b]	78.4±4.0	79.0±4.0*	64.4±4.4[a]	57.8±4.4[b]
Testosterone (ng/mL)	1.46±0.34	0.81±0.34	1.41±0.46	0.55±0.46	1.51±0.51	1.06±0.51

Values are LSMEAN ± SEM.

Different superscript letters indicate statistical difference within a row (between seasons within assay) in each class of stallion ($p \leq 0.05$). For the class all stallions, P-values were as follows: Concentration, P=0.01; Morphology, P=0.05. For bad freezer stallions, P-value was as follows: Morphology P=0.04. An asterisk (*) indicates a difference in morphology between good freezer stallions and bad freezer stallions in the non-breeding season (P=0.04).

Results for flow cytometry parameters are shown in Table 6. Sperm chromatin integrity was not affected by season or whether the stallion was a good or bad freezer. In the non-breeding season, there was a higher proportion of spermatozoa with high MMP than in the breeding season, although no differences in MMP were found between good and bad freezers in different seasons. For ROS, there was a higher proportion of viable superoxide negative spermatozoa, lower proportions of viable and non-viable superoxide positive spermatozoa, and a lower proportion of non-viable hydrogen peroxide negative spermatozoa in the non-breeding season than in the breeding season. These differences occurred in both good and bad freezers.

Table 6. *Flow cytometry parameters in semen samples between breeding and non-breeding seasons*

	All stallions		Good freezer stallions		Bad freezer stallions	
Sperm parameters	Breeding	Non-breeding	Breeding	Non-breeding	Breeding	Non-breeding
%DFI	9.06±1.12	8.56±1.12	7.44±1.49	7.20±1.49	10.7±1.7	9.93±1.66
MMP-L	60.7±5.6[a]	44.5±5.6[b]	59.8±7.5	46.8±7.5	61.6±8.4	42.1±8.4
MMP-H	35.4±5.5[a]	52.2±5.5[b]	35.8±7.4	49.4±7.4	35.0±8.2	55.0±8.2
Viable superoxide negative	19.3±2.6[a]	55.3±2.6[b]	18.8±3.5[a]	57.4±3.5[b]	19.9±3.9[a]	53.2±3.9[b]
Viable superoxide positive	12.2±1.4[a]	4.48±1.41[b]	13.2±1.9[a]	4.70±1.89[b]	11.1±2.1	4.26±2.12
Non-viable superoxide positive	68.5±3.2[a]	39.8±3.2[b]	68.0±4.2[a]	37.4±4.2[b]	69.0±4.7[a]	42.1±4.7[b]
Viable hydrogen peroxide negative	30.0±5.8	38.6±5.8	29.2±7.8	37.9±7.8	30.8±8.7	39.4±8.7
Viable hydrogen peroxide positive	1.52±5.61	20.0±5.6	2.7±7.5	22.7±7.5	0.36±8.37	17.4±8.4
Non-viable hydrogen peroxide negative	63.4±5.8[a]	23.3±5.8[b]	59.4±7.8[a]	17.3±7.8[b]	67.4±8.7[a]	29.3±8.7[b]
Non-viable hydrogen peroxide positive	3.72±4.39	15.6±4.4	6.67±5.85	19.2±5.8	0.77±6.54	12.1±6.5

Values are LSMEAN ± SEM.

Different letters indicate statistical difference within a row (between seasons within assay) in each class of stallion ($p \leq 0.05$).

Abbreviations: %DFI, DNA fragmentation index; MMP-L, low-mitochondrial membrane potential (%); MMP-H, high-mitochondrial membrane potential (%).

For the class all stallions P-values were as follows: MMP-L, P=0.02; MMP-H, P=0.01; viable superoxide negative, P<0.0001; viable superoxide positive, P<0.001; non-viable superoxide positive, P<0.0001; non-viable hydrogen peroxide negative, P<0.0001. For good freezer stallions, P-values were as follows: viable superoxide negative, P<0.0001; viable superoxide positive, P=0.03; non-viable superoxide positive, P=0.0002; non-viable hydrogen peroxide negative, P=0.006. For bad freezer stallions, P-values were as follows: viable superoxide negative, P<0.0001; non-viable superoxide positive, P=0.004; non-viable hydrogen peroxide negative, P=0.04.

5 Discussion

5.1 Effect of SLC on post-thaw sperm quality

According to the results from **study I**, selecting spermatozoa by SLC prior to cryopreservation generally improved sperm quality after thawing compared with the non-selected control. Preserving sperm DNA integrity during freezing is an important factor in achieving a healthy pregnancy from insemination with cryopreserved spermatozoa (Evenson *et al.,* 2002). Colloid centrifugation prior to cryopreservation significantly reduced the amount of DNA fragmentation, in agreement with Hoogewijs *et al.* (2011). Moreover, the proportion of spermatozoa with high MMP was higher in the SLC group than control, which is consistent with a previous study on bull sperm samples, in which MMP was increased in the SLC-selected sperm population (Goodla *et al.,* 2014). A higher proportion of spermatozoa with high MMP was observed in human sperm samples prepared by a density gradient than in neat semen, which could be attributed to removal of most of the damaged cells (Marchetti *et al.,* 2002). Under physiological and biotechnological conditions, mitochondria are one of the major organelles with a crucial role in sperm function (Peña *et al.*, 2009; Peña *et al.*, 2011). Thus, it has been suggested that analysis of MMP is a sensitive test to determine sperm quality (Marchetti *et al.,* 2002), and could be a useful global marker for sperm fertilizability (Gallon *et al.*, 2006). Cryopreservation-induced mitochondrial damage was suggested to be a major cause of reducing stallion sperm quality after thawing (Schober *et al.,* 2007; Darr *et al.*, 2017). A high proportion of spermatozoa with high MMP was considered to be related to high fertilizing capacity (Gallon *et al.,* 2006).

Single Layer Centrifugation is a valuable technique to select good quality spermatozoa i.e. those with good morphology, viability and chromatin integrity, and hydrogen peroxide was reduced although superoxide was increased (Morrell *et al.*, 2017). Under physiological conditions, ROS in low levels are necessary for proper sperm function (Aitken *et al.*, 1997; Amaral *et al.*, 2013), whereas excessive ROS-formation can affect cell viability (Aitken, 1995; Baumber *et al.*, 2000), capacitation control (Agarwal *et al.*, 2014) and sperm-oocyte fusion (Aitken, 1995; Baumber *et al.*, 2000). Immature, morphologically abnormal spermatozoa and seminal leukocytes are the main sources of ROS in ejaculates (Gibb *et al.*, 2014); removing ROS-generating factors appears to be beneficial for sperm cryosurvival (Ball & Vo, 2001; Baumber *et al.*, 2002). In the present study, hydrogen peroxide production was decreased when SLC was applied prior to cryopreservation; since this free radical is toxic to spermatozoa during storage (Baumber *et al.*, 2000), its removal prior to freezing might lead to increased sperm survival during cryopreservation. Indeed, ROS production during semen storage was reported to reduce sperm quality and cause mitochondrial dysfunction (Nohl *et al.*, 1996). Moreover, pre-freeze exposure of spermatozoa to higher oxidative stress was shown to reduce cryosurvival (Ertmer *et al.*, 2017). Furthermore, it was suggested that excessive ROS-formation could negatively affect sperm cryo-tolerance due to the oxidative stress (Ball, 2008). The results of **study I** revealed a beneficial effect of SLC prior to cryopreservation in reducing the ROS production, which may have contributed to improved sperm cryosurvival.

Motility alone is considered to be a poor indicator of fertility in frozen–thawed samples (Samper *et al.*, 1990). Thus, when assessing sperm fertilizing potential, motility should be evaluated together with other parameters (Katila, 2001). No significant differences were detected for any of the sperm kinematics, regardless of whether SLC was conducted prior to cryopreservation or not. However, the progressive motility in the SLC samples met the suggested industry standard requirement for post-thaw sperm motility (≥30-35%) (Loomis & Graham, 2008; Miller, 2008).

5.2 Effect of adding SP on sperm cryosurvival

In **study I** and **II**, our aim was to gain a comprehensive view of the effects of adding SP to SP-free spermatozoa produced by SLC, either prior to cryopreservation or after thawing, on sperm cryo-tolerance. It has been observed

that approximately 0-5% of the original SP volume is left during semen processing for cryopreservation, depending on the sperm preparation method used (Alghamdi *et al.*, 2002; Alghamdi *et al.*, 2004; Moore *et al.*, 2005a; Barbacini & Squires, 2012). Therefore, 5% SP was added in our experiments.

In conventional freezing protocols, sperm washing and resuspension in cryopreservation medium is a common protocol used to prepare stallion semen for freezing. However, sperm washing alone does not remove all of the SP, or even SP proteins coating the sperm (Kruse *et al.*, 2011). Therefore, it is not possible to say how much SP remains in the washed sperm samples. Centrifugation through a colloid (SLC) has been utilized to prepare semen samples (**study I** and **II)**, to ensure complete removal of SP and most of the SP-proteins from the surface of ejaculated spermatozoa (Kruse *et al.*, 2011), thus, allowing the effect of adding a specific amount of SP on sperm cryosurvival to be investigated.

Pre-freezing addition of SP from a stallion of good or bad freezability to SP-free sperm samples did not add to the beneficial effects of SLC alone (0% SP) on stallion sperm cryosurvival (**study I**). However, post-thaw addition of SP (**study II**) significantly increased the proportion of spermatozoa with damaged chromatin compared with the SLC group. The sperm chromatin structure assay (SCSA) is a useful *in vitro* test used to measure sperm chromatin integrity, which is a significant biological parameter (Evenson *et al.*, 1980), and may provide an independent determinant of male fertility (Love *et al.*, 2002). The SCSA could be useful as an indicator for predicting sub-fertility of individual stallions (Morrell *et al.*, 2008b). Centrifugation through a single layer of colloid effectively selected spermatozoa with intact chromatin, reducing the incidence of spermatozoa with chromatin damage or single-stranded DNA in stallion sperm preparations to be used for AI (Morrell *et al.*, 2009b). Applying SLC prior to freezing decreased DNA damage in the sample after thawing (Hoogewijs *et al.*, 2011). These results are in agreement with those of Love *et al.* (2005), where the absence of SP was beneficial to DNA integrity compared to 10 or 20 % seminal plasma (Love *et al.*, 2005). In contrast, improvement in post-thaw sperm quality was observed when 20% SP was added before freezing (Katila *et al.*, 2002). Alghamdi *et al.* (2005) showed an improvement in fertility after extending thawed equine spermatozoa in SP. A deleterious effect of long-term exposure to SP prior to cryopreservation on stallion spermatozoa cryosurvival was reported (Moore *et al.*, 2005a). However, in their studies a different semen handling procedure was applied prior to freezing compared with the present study, and either the stallion´s own SP or donor stallion SP was used.

Adding SP prior to freezing (**study I**) reduced the proportion of spermatozoa with high MMP, and increased the proportion of spermatozoa with low MMP. Although adding SP to SLC-selected stallion spermatozoa prior to cryopreservation did not have an additional beneficial effect on sperm quality after thawing (Al-Essawe *et al.*, 2016a), it did not have a deleterious effect either, regardless of whether the SP came from good or bad freezers (**study I**). It should be noted that pooling the SP from several stallions might dilute the beneficial effects of SP from any individual stallion.

In **study II**, inclusion of pooled SP from either stallion category after thawing adversely affected sperm cell membrane integrity; thus, there was no indication that SP could repair cryoinjury. In contrast, De Andrade *et al.* (2011) observed an improvement in plasma and acrosomal membrane integrity when the thawing medium contained 20% SP. Furthermore, treating frozen-thawed boar semen with SP reversed cryocapacitation and protected the spermatozoa against spontaneous induction of the capacitation process (Vadnais *et al.*, 2005; Okazaki *et al.*, 2009).

Mitochondria generate a major part of the ATP required for sperm function, together with anaerobic glycolysis (Peña *et al.*, 2009). Adding SP from good freezers to SLC-selected samples after thawing (**study II**) appeared to impair mitochondrial function, as reflected in a higher proportion of spermatozoa with low MMP. Similarly, alterations in mitochondrial function were reported when autologous SP or homologous SP was added to spermatozoa after thawing (De Andrade *et al.*, 2011). In contrast, improved sperm quality, including mitochondrial respiration, was demonstrated after addition of SP to frozen-thawed ram spermatozoa (Ollero *et al.*, 1997; El-Hajj Ghaoui *et al.*, 2007a; Maxwell *et al.*, 2007). The beneficial effect was attributed to a vesicle-free protein fraction (El-Hajj Ghaoui *et al.*, 2007b). The differences in the results from these studies may be due to the different species, differences in processing procedures (washing or colloid centrifugation), the origin and amount of SP used (autologous, homologous or heterologous) and the evaluation technique used. Although SP from certain individuals was reported to have pronounced effects, this effect might be masked when pooling SP from several individuals (Morrell *et al.*, 2014b).

An imbalance between the generation and degradation of ROS induces oxidative stress (Baumber *et al.*, 2000). This effect may be augmented by removal of SP, which contains ROS scavengers such as catalase, superoxide dismutase and glutathione peroxidase (Katila & Kareskoski, 2006). Oxidative stress has a detrimental effect on sperm viability, motility and MMP, and increases DNA damage, morphology defects and lipid peroxidation, possibly

resulting in apoptosis-like phenomena (Kothari *et al.*, 2010; Mahfouz *et al.*, 2010; Aitken *et al.*, 2012). The results of **study I** indicated that adding SP from good freezers decreased the proportion of viable superoxide positive spermatozoa. This could be imputed to be due to the differences in SP composition between the two stallion categories including amounts of antioxidant enzymes which would be involved in regulating the ROS levels.

Recently, simultaneous measurement of mitochondria-specific ROS and MMP using JC-1 and MitoSOXRed fluorescence (MitoSOX) was used to provide additional information on stallion and bull sperm quality (Kumaresan *et al.*, 2017; Johannisson *et al.*, 2018). Adding SP from good freezer stallions to cryopreserved SLC-selected spermatozoa after thawing (**study II**) increased the proportion of spermatozoa with high superoxide production and high MMP ($MitoSOX^+$, $JC\text{-}1^+$ spermatozoa) indicating that the spermatozoa in this group were more metabolically active. Seminal plasma is known to possess antioxidant capacity to control the ROS levels in spermatozoa (Ball, 2008; Agarwal *et al.*, 2014). The differences in superoxide production between the two treatment groups may be related to differences in antioxidant levels in the SP of the good and bad freezers, although this was not tested in **study II**. Furthermore, high ROS levels of viable stallion spermatozoa after freezing were associated with good freezability ejaculates (Yeste *et al.*, 2015). In addition, a link between superoxide production, fertility, and selection by colloid centrifugation has been suggested for stallion spermatozoa (Morrell *et al.*, 2017).

Although stimulating ROS formation by adding menadione increased the sperm population that was stained with MitoSOXRed, as expected, indicating increased superoxide production from metabolic activity, JC-1 fluorescence was decreased indicating low mitochondrial membrane potential. This apparent paradox was also observed in a study by Johannisson *et al.* (2018). Similarly, a decrease in stallion sperm MMP was found using the xanthine/xanthine oxidative system to stimulate ROS production (Ertmer *et al.*, 2017). The latter authors reported that ROS accumulation occurred predominantly in the sperm midpiece region, where the mitochondria are located, in agreement with studies by Macías-García *et al.* (2015). Mitochondria are considered to be the most powerful intracellular source of ROS (Kothari *et al.*, 2010), and a sensitive target for the damaging effects of oxygen radicals (Orrenius *et al.*, 2007). Excessive ROS production was associated with a decrease of the mitochondrial membrane potential (Wang *et al.*, 2003; Morrell *et al.*, 2017). Cryopreservation exposes spermatozoa to osmotic stresses, both during the addition and removal

of cryoprotective agents, as well as during freezing and thawing (Hammerstedt *et al.*, 1990; Woods *et al.*, 2004). Mitochondrial damage induced by cryopreservation was suggested to be the major cause of post-thaw reduction in stallion sperm quality (Schober *et al.*, 2007) and might be the cause of the lower MMP in our study. However, an alternative explanation could be that the mitochondria were metabolically active, producing superoxide but depleting their energy reserves and thus lowering the membrane potential. To our knowledge, this is the first study where simultaneous measurement of JC-1 and MitoSOXRed fluorescence has been conducted in frozen stallion sperm samples. Further investigation is recommended to understand the association between MMP and ROS production by the mitochondria.

Motility is one of many attributes that a spermatozoon must possess to fertilize an oocyte. However, motility is not correlated with fertilizing capacity (Graham, 1996) and has a poor predictive value for fertility (Morrell *et al.*, 2017). It is commonly used as the criterion for accepting or rejecting a semen sample for artificial insemination (Fraser, 2017), possibly because it is the most easily measured parameter of sperm quality (Peña *et al.*, 2015). In **study I** and **II**, no differences were found in sperm motility, progressive motility or other kinematics either among treatments or among ejaculates, whether pooled SP was added prior to freezing (**study I**) or after thawing (**study II**). The threshold of acceptability was exceeded when SLC was conducted prior to cryopreservation, since sperm motility was increased compared to controls even in bad freezer stallions. Reducing the SP proportion to ≤ 5% prior to freezing or cooling was reported to increase motility compared to samples containing 10–30% (Alghamdi *et al.*, 2002; Alghamdi *et al.*, 2004). Furthermore, adding SP from stallions with good freezability to the semen of stallions with low cryosurvival rates prior to freezing was found to increase the number of spermatozoa with high motility and plasma membrane integrity (Aurich *et al.*, 1996). However, contradictory results regarding the influences of SP on post-thaw sperm motility have been reported in other studies (Hernández *et al.*, 2007; Okazaki *et al.*, 2009). The differences in the results can be explained by variations in research methodologies such as pre-freezing semen processing, the amount and the source of SP (whether from a good or bad freezer male, or if the SP originated from a certain fraction of the ejaculate), and also the exposure time to SP prior to cryopreservation. The response to SP could also depend on the species and the individual male. Moreover, differences in CASA instruments and settings used in each study can influence the results.

5.3 Effect of adding SP on sperm fertilizing capability

Selecting the best spermatozoa by SLC before freezing was reported to improve sperm quality after thawing (Katila *et al.,* 2002; Hoogewijs *et al.,* 2011; Al-Essawe *et al.,* 2016a). Processing the samples using SLC prior to cryopreservation did not affect the capability of the spermatozoa to bind to bovine oocytes ZP.

The results of this study indicated that adding pooled SP from good or bad freezer stallions to SP-free spermatozoa affected the sperm binding affinity according to the timing of exposure. It has been reported previously that the response to SP ranged from beneficial for some stallions to deleterious for others (Aurich *et al.*, 1998; Morrell *et al.*, 2014b). In this study, differences in response to both sorts of SP, whether added prior to freezing or after thawing, could be due to the tremendous variation in composition and quality of SP reported to exist among individuals (Katila & Kareskoski, 2006; Usuga *et al.*, 2017). Furthermore, differences in SP composition among stallions were found to be associated with the ability of stallion spermatozoa to withstand cryopreservation (Al-Essawe *et al.*, 2016b). The reduction in sperm binding affinity to the ZP of bovine oocytes when SP from good freezer stallions was added prior to cryopreservation (**study III**, experiment 1) may be due to the presence of SP proteins with decapacitating activity.

Only capacitated spermatozoa can bind to the ZP; non-capacitated or prematurely capacitated spermatozoa cannot bind to the oocyte or complete the fertilization process (Töpfer-Petersen *et al.,* 2000). Decapacitation factors present in SP, such as CRISP-1D form, have been reported to prevent premature capacitation (Roberts *et al.*, 2007), or even cause a reversion to the uncapacitated state, which in turn could prolong the fertilizing ability of cryopreserved spermatozoa (Moore *et al.*, 2005a). Delaying the capacitation process by adding SP from good freezer stallions prior to cryopreservation could be a practical means to decrease the susceptibility of spermatozoa to undergo the acrosome reaction. Moreover, the proportion of viable spermatozoa with non-reacted acrosome was increased after thawing in samples prepared by SLC prior to freezing, indicating an improvement in sperm cryosurvival (Al-Essawe *et al.*, 2016a). Such a delay in the acrosome reaction would allow more flexibility in the timing of insemination of thawed spermatozoa relative to ovulation, while still ensuring that sperm capacitation occurs at the optimal time. However, adding pooled SP from good freezer stallions to SP-free spermatozoa after thawing followed by SLC (**study III**, experiment 2), decreased the capability of spermatozoa to bind to bovine ZP compared with SLC-selection alone. Adding SP from bad freezers significantly reduced the mean number of spermatozoa

bound to ZP compared to the group treated with SP from good freezers. Selecting the sperm population after thawing using density gradient centrifugation was demonstrated to induce capacitation and improve the *in vitro* fertilization capacity assessed by heterologous sperm penetration assay using IVM zona-free bovine oocytes (Morató *et al.*, 2013).

Seminal plasma has been reported to repair sperm membrane cryodamage (Bernardini *et al.*, 2011); thus, adding SP after thawing could be beneficial for repairing damage induced by the freezing process, and maintaining the spermatozoa in a decapacitated state until ovulation occurs. Variation in SP composition among individuals, including decapacitation factors, might explain the differences in response to SP from each category. The dramatic impairment of ZP binding seen after prolonged exposure of spermatozoa to SP from either category (**study III**, experiment 3), could be due to decapacitation factors in SP inhibiting capacitation and the acrosome reaction. In addition, capacitation factors in SP, suggested to increase the proportion of the sperm population with a reacted acrosome (Therien *et al.*, 1998), could induce premature acrosome reaction, preventing primary binding (Balao da Silva *et al.*, 2013). Prolonged exposure to SP was previously observed to affect sperm DNA integrity (Love *et al.*, 2005; Morrell *et al.*, 2014b). The SP-proteins are involved in several essential steps preceding fertilization, such as capacitation, establishment of oviductal sperm reservoirs, modulation of uterine immune response, sperm transport to the fertilization site, and also in gamete interaction and fusion (Töpfer-Petersen *et al.*, 2005). Therefore, if a small amount of SP is added to the sperm sample after thawing shortly before insemination, the potential benefit of SP in repairing membrane damage could be obtained while avoiding any detrimental effect due to prolonged exposure. These possibilities should be investigated in an AI trial before any general recommendation can be made.

In our hands, the HZBA was a valuable method for evaluation stallion sperm fertilizing ability. Moreover, *in vitro* assessment of sperm fertilizing capability provides important information about the different attributes that spermatozoa must have in order to be capable of fertilization, especially in the absence of reliable and repeatable protocols for *in vitro* fertilization in the horse. The HZBA using salt-stored bovine oocytes, as in **study III**, could be a useful tool to evaluate stallion sperm fertilizing ability, serving as a preliminary screening when deciding whether to test the spermatozoa in AI trials.

5.4 Crossover study

The retrospective crossover study was designed to demonstrate the response of spermatozoa obtained from stallions of known freezability to the SP from good or bad freezer stallions on post-thaw sperm quality. Sperm kinematics, % DFI, MI and MMP (high and low categories) were not affected by adding either sort of SP, regardless of the origin of the spermatozoa or whether SP was added before freezing (**study I**), or after thawing (**study II**). Increases in the sperm population with high superoxide production and high MMP ($MitoSOX^+$, $JC\text{-}1^+$) were detected when SP-free spermatozoa obtained from good freezer stallions were treated with pooled SP from good freezers after thawing. The differences among the groups could be due to differences in SP composition between the good and bad freezer stallions (Al-Essawe *et al.*, 2016b), and also differences in the amount of antioxidants; in males. Antioxidants are found in the testis, epididymis, secretions of the male accessory organs and SP (Tremellen, 2008). In addition, it has been shown that antioxidant levels in SP vary greatly among stallions, and also among ejaculates for certain individual stallions (Usuga *et al.*, 2017).

In **study III**, the results indicated that the response of SP-free spermatozoa obtained from good or bad freezer stallions to SP is influenced by the origin of SP and the timing of exposure. Previous studies reported differences in the response of spermatozoa to various SP concentrations and origin (De Andrade *et al.*, 2011). In our study, pooling SP from several stallions could mask the beneficial effects of SP from certain individuals. Including SP from an individual stallion with known SP quality would be recommended to gain more insight into the effects of SP on sperm cryosurvival. Seminal plasma from certain individuals was found to have a less deleterious effect on sperm survival than SP from other individuals; the identification of "universal SP donors" i.e. individuals whose SP did not have a detrimental effect on sperm chromatin integrity, was suggested as a possibility to enhance the effects of SP during storage (Morrell *et al.*, 2014b). There are no standardized criteria applied to frozen stallion semen for AI, in respect of optimal sperm concentration or optimal number of spermatozoa for an insemination dose (Metcalf, 2007). Furthermore, each laboratory has its own freezing method, so that the number of spermatozoa packaged per straw, as well as the number of straws per insemination dose, varies greatly, not only for individual stallions, but also for each freezing center (Miller, 2008).

Therefore, customization is recommended to produce the highest quality semen from each individual stallion and reduce the huge variability in pregnancy rates. A single split-ejaculate test-freeze procedure (using protocols varying with respect to freezing extender, cooling rate, package size and thawing rate) results in selection of a protocol that could be better than the "conventional" protocol for as many as 30% of stallions (Loomis & Graham, 2008).

5.5 The effects of season on stallion SP proteins and sperm quality

In **study IV**, minor differences in the major protein groups in SP from good and bad freezer stallions were recognized in different seasons. A higher sperm quality was observed in the non-breeding season, as indicated by a shift towards more heparin-binding proteins. However, Novak *et al.* (2010) reported a negative association between the heparin-binding proteins SP1 and SP2 with fertility, although they found that CRISP-3 was positively related to first cycle conception rate. A possible explanation for this difference is that although SP1 and SP2 are identified as heparin-binding proteins in other studies, they also bind to phosphorylcholine (Kareskoski *et al.*, 2011) and would be eluted with phosphorylcholine-binding proteins in **study IV**.

In other studies, higher motility was observed in samples with high levels of CRISP-3, and differences in sperm quality parameters correlated to CRISP-3 genotype were detected (Usuga *et al.*, 2018; Restrepo *et al.*, 2019). In addition, CRISP-3, and also SP-2, were found to be potential markers of equine semen freezability (Jobim *et al.*, 2011).

The CRISP-3 protein is located in the non-heparin binding fraction and does not bind to phosphorylcholine (Magdaleno *et al.*, 1997). Hence, it is possible that CRISP-3 is the protein causing the excess of non-heparin, non-phosphorylcholine-binding proteins in the non-breeding season determined in **study IV**. The apparent discrepancy among studies may reflect a difference in terminology.

The observation that sperm quality was better in the non-breeding season could be due to more frequent sampling during the breeding season. In accordance with an earlier study by Wach-Gygax *et al.* (2017), sperm concentration was lower in the breeding season than in the non-breeding season. However, they showed a clear seasonal pattern in serum testosterone concentration, whereas we did not note a seasonal variation in SP testosterone concentration. This may be due to the low number of stallions included in the present study or to sampling at a different time relative to the start of the breeding

season. However, our finding is compatible with the results from a previous study where testosterone concentration in blood was not affected in stallions used frequently for semen collection (Aurich *et al.*, 1999).

In accordance with an earlier study by Blottner *et al.* (2001), chromatin integrity was unaffected by season. Janett *et al.* (2003b) showed that in warmblood stallions sampled in Switzerland, sperm viability and morphology were lowest in summer. This is somewhat in contrast to our study, where the bad freezers had better morphology in the breeding season than in the non-breeding season.

The findings of **study IV** suggest that stallion ejaculates could be used year-round for freezing, since sperm quality was not impaired during the non-breeding season. In addition, only minor differences in protein composition exist between the breeding and non-breeding seasons as well as between good and bad freezers. These observations are in agreement with those of Aurich (2016), who reported small variations in semen quality between seasons. The latter author concluded that the choice of stallion is more important than season of collection with regard to sperm quality.

6 Conclusions

This thesis demonstrates that handling and processing methods for stallion semen prior to cryopreservation could affect sperm cryosurvival. The response of stallion spermatozoa to SP is influenced by the ability of the spermatozoa to withstand cryopreservation and is affected by the timing of exposure and the origin of SP. Therefore, customization of freezing protocols for individual stallions is recommended to optimize this effect.

The main conclusions that can be drawn based on the results presented in this thesis are as follows:

- Selection of the robust spermatozoa population using SLC prior to cryopreservation improved post-thaw sperm quality.

- Adding SP prior to cryopreservation did not provide an additional beneficial effect, regardless of whether it originated from good or bad freezer stallions, and was not deleterious to DNA integrity.

- Adding SP from good or bad freezer stallions after thawing adversely impaired post-thaw sperm quality, suggesting an inability to repair or reverse stallion sperm cryoinjuries.

- MitoSOX is a useful test to evaluate mitochondrial functionality and ROS together, and to characterize the relationship between them.

- Adding SP from good freezer stallions prior to cryopreservation reduced sperm binding to ZP. Exposure of SP-free spermatozoa to SP from good or bad freezer stallions after thawing significantly decreased the sperm binding capability.

- Prolonged exposure to both sorts of SP dramatically impaired sperm capacitation and binding affinity.

- The heterologous ZBA using salt-stored bovine oocytes could be a useful tool to evaluate stallion sperm fertilizing ability.

- Minor differences in SP protein composition exist between breeding and non-breeding seasons as well as between good and bad freezers.

- Sperm quality is not impaired during the non-breeding season; therefore, stallion ejaculates could be used for freezing year-round without adversely affecting sperm quality after thawing.

7 Future perspectives

The findings reported in this thesis suggest several future lines of investigation.

- An interaction between the freezing extender and SP cannot be ruled out. In other studies, Gent extender was demonstrated to produce significantly better sperm parameters after thawing compared with other freezing extenders, although a potentially negative role of egg yolk on fertilization has been reported. Therefore, investigation of the effect of using non-egg yolk extenders with the addition of SP on post-thaw sperm quality should be carried out, including a fertility trial with frozen-thawed sperm samples.

- In the present study, sperm quality was evaluated within 10 min after thawing. More time intervals should be considered to study sperm longevity after thawing.

- In the present experiment, frozen–thawed spermatozoa were not removed from the freezing extender before analysis and have not been subjected to dilution after thawing. Reducing the osmotic stress in the treated samples by diluting the thawed sample in isotonic medium to mimic *in vivo* conditions in the mare's reproductive tract should be investigated.

- Adding lower concentrations of SP (1-3%) prior to cryopreservation to preserve sperm cell membrane during freezing, followed by adding more SP after thawing could be beneficial in repairing damage induced by the freezing process.

- In a previous study, SP from certain individuals was found to have a less deleterious effect during storage on sperm survival than SP from other individuals. Thus, more investigation is required to study the effects of adding different concentrations of SP from certain individual donors to SLC-selected spermatozoa prior to freezing in order to optimize cryosurvival.

- Differences in SP composition among individuals should be studied to identify which components are important in affecting sperm quality. Moreover, investigation of the global proteome of spermatozoa and SP of stallions with known freezability is recommended, to explore whether any of the identified proteins could serve as biomarkers for cryosurvival.

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Popular science summary

Stallions are selected for breeding according to their genotype and athletic skills with little regard to their fertility or the suitability of their spermatozoa for cryopreservation. Artificial insemination using frozen semen has become increasingly important in the horse breeding industry despite its lower pregnancy rates compared with raw, fresh, or chilled semen. The use of frozen stallion semen eliminates geographic barriers, minimizes the spread of disease, ensures semen availability independently of the stallion's collection schedule, and preserves the genetic material of the animal for an unlimited period. However, despite considerable research, it is still not possible to freeze all stallion ejaculates successfully. Stallions are classified as good or bad freezers based on the post-thaw sperm motility. To overcome the deleterious effects of cryopreservation, improved semen handling protocols prior to cryopreservation are required to achieve better sperm longevity and fertility after thawing. These improvements not only increase fertility rates but might also allow semen from bad freezer stallions to be frozen successfully.

Seminal plasma (SP) is the fluid portion of semen. Its main functions are to flush the urethra and propel spermatozoa through the stallion’s genital tract during ejaculation, as well as to transport and protect spermatozoa within the female genital tract. Seminal factors regulate sperm capacitation, the acrosome reaction and gamete interaction in the mare, whereas SP has an adverse effect on sperm viability, longevity and cryosurvival *in vitro*. Semen for cryopreservation is usually collected outside the normal breeding season, when it is no longer required for supplying AI doses. Thus, knowledge about the influence of season on sperm quality and SP composition is needed in order to develop semen handling procedure to optimise cryosurvival.

The present study investigated the possibility of enhancing stallion sperm cryosurvival by pre-freezing selection of the most motile and viable spermatozoa with good morphology and chromatin integrity using a colloid centrifugation technique (Single Layer Centrifugation, SLC). A precise amount of SP from stallions of known freezability was added to the selected sperm samples either prior to cryopreservation or after thawing. In addition, sperm quality, testosterone level in SP, and the major protein groups in SP in different seasons were measured.

According to our findings, selecting the robust sperm population by SLC prior to cryopreservation improved sperm quality after thawing, whereas adding SP from good or bad freezer stallions did not provide any additional benefit although it did not have a deleterious effect on sperm DNA integrity. Adding SP to SLC-selected spermatozoa after thawing had deleterious effects on sperm post-thaw quality regardless of SP origin. Sperm fertilizing capacity was evaluated using an *in vitro* technique that assesses the ability of spermatozoa to bind to the oocytes of another species. The results from this test indicated that sperm binding ability was affected by the origin of SP (whether the SP came from a good freezer stallion or a bad freezer stallion) and the timing of exposure to SP i.e. prior to cryopreservation or after thawing. Moreover, sperm quality and testosterone levels were not affected by season and only minor differences in SP-proteins were found, indicating that stallion semen can be used for freezing at any time during the year.

Populärvetenskaplig sammanfattning

Hingstar väljs ut för avel baserat på deras genotyp och atletiska förmåga med liten hänsyn till deras fruktsamhet eller lämpligheten hos deras spermier för frysbevarande. Artificiell insemination (AI) med fryst sperma har ökat i betydelse inom hästnäringen trots att detta ger lägre dräktighetskvoter jämfört med rå, färsk eller kyld sperma. Användningen av fryst hingstsperma eliminerar geografiska barriärer, minimerar smittspridning, garanterar tillgång till sperma oavsett hingstens insamlingsschema och bevarar djurets genetiska material under obegränsad tid. Dock är det trots avsevärda forskningsinsatser fortfarande inte möjligt att på ett framgångsrikt sätt frysa alla hingstejakulat. Hingstar klassificeras som varande av bra eller dålig frysbarhet baserat på spermiernas motilitet efter upptining. För att övervinna de skadliga effekterna av frysbevarande, krävs det förbättrade metoder för hantering av sperman för att uppnå bättre livslängd och fruktsamhet hos spermierna efter upptining. Dessa förbättringar öker inte bara fruktsamhetskvoterna utan kan också möjliggöra att sperma från hingstar med dålig frysbarhet kan frysas på ett framgångsrikt sätt.

Seminalplasma (SP) är den flytande delen av sperman. Dess huvudsakliga funktion är att spola rent urinröret och att framdriva spermier genom hingstens fortplantningsorgan under ejakulationen, samt att transportera och skydda spermierna i stoets fortplantningsorgan. Faktorer i sperman reglerar spermiernas kapacitering, akrosomreaktion och gameternas interaktion i honan, *in vitro* har SP en negativ effekt på spermiernas livsduglighet, livslängd och frysöverlevnad. Sperma för frysbevaring insamlas vanligtvis utanför avelssäsongen, när den inte längre behövs för att användas till AI-doser. Därför är kunskap om årstidernas påverkan på spermiekvalitet och innehållet i SP nödvändig för att utveckla metoder för hantering av sperma med syftet att optimera frysöverlevnaden. Denna studie undersökte om det är möjligt att öka spermiernas överlevnad efter frysning genom att före infrysning selektera fram de mest rörliga och livsdugliga spermierna, som också har bra morfologi och kromatinintegritet med hjälp av en

teknik för kolloidcentrifugering (Single Layer Centrifugation, SLC). En bestämd mängd SP från hingstar med bra frysbarhet tillsattes till de selekterade spermieproverna antingen före frysbevarande eller efter upptining. Dessutom mättes spermiekvalitet, testosteronnivåer i SP och de huvudsakliga proteingrupperna i SP under olika årstider.

Enligt våra resultat förbättrade selektion av den mest robusta spermiepopulationen med SLC innan frysbevarande spermiekvaliteten efter upptining, medan att tillsätta SP från hingstar med bra eller dålig frysbarhet inte någon ytterligare förbättring, dock hade tillsatsen ingen skadlig effekt på spermiernas DNA-integritet. Att tillsätta SP till SLC-selekterade spermier efter upptining hade en skadlig effekt på spermiernas kvalitet efter upptining oavsett ursprunget av SP. Spermiernas befruktningsförmåga utvärderades med en *in vitro*-teknik som mäter förmågan hos spermierna att binda till äggceller från en annan art. Resultaten från detta test antydde att spermiernas bindningsförmåga påverkades av ursprunget av SP (om SP kom från en hingst med bra eller dålig frysbarhet) och tidpunkten för tillsats av SP, dvs före frysbevarande eller efter upptining. Dessutom påverkades inte spermiekvalitet och testosteronnivåer av årstid och endast mindre skillnader i SP-proteiner hittades, vilket tyder på att hingstsperma kan användas till frysning oavsett tid på året.

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