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Influence of seminal plasma on fresh and post-thaw parameters of stallion epididymal spermatozoa

by

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Dedicated to my loving parents, Waltraut und Rüdiger Heise, who have always given wings to my dreams

Diese These ist meinen Eltern, Waltraut und Rüdiger Heise, gewidmet, die meinen Träumen immer Flügel verliehen haben

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DECLARATION

I, Annett Heise, do hereby declare that the research presented in this thesis, was conceived and executed by myself, and apart from the normal guidance from my supervisor, I have received no assistance.

Neither the substance, nor any part of this thesis has been submitted in the past, or is to be submitted for a degree at this University or any other University.

This thesis is presented in fulfillment of the requirements for the degree MSc (PAS).

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Signed …….

Annett Heise

Date …….

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ABSTRACT

Cryopreservation of epididymal spermatozoa may be the only opportunity to preserve valuable genetics of males in cases of unforeseen injury or death. Stallion epididymal spermatozoa have been cryopreserved successfully and it has been demonstrated that stallion epididymal spermatozoa are fertile, and pregnancies as well as live foals have been produced. As spermatozoal quality parameters like motility, morphology and viability have a major influence on fertility and pregnancy rates, it is of great interest to describe these and investigate the influence of seminal plasma on these parameters.

Fresh and post-thaw parameters (motility, morphology and viability) of stallion epididymal spermatozoa that have been and that have not been exposed to seminal plasma were evaluated, and directly compared to fresh and post-thaw parameters of ejaculated spermatozoa from the same stallions.
Six sperm categories of each stallion (n= 4) were evaluated for motility, morphology and viability. These categories were fresh ejaculated spermatozoa (Fr-E), fresh epididymal spermatozoa that had been exposed to seminal plasma (Fr-SP+), fresh epididymal spermatozoa that had never been exposed to seminal plasma (Fr-SP-), frozen-thawed ejaculated spermatozoa (Cr-E), frozen-thawed epididymal spermatozoa that had been exposed to seminal plasma prior to freezing (Cr-SP+) and frozen-thawed epididymal spermatozoa that had never been exposed to seminal plasma (Cr-SP-).

Results show that seminal plasma stimulates initial motility of fresh epididymal stallion spermatozoa while this difference in progressive motility is no longer present post-thaw; and that progressive motility of fresh or frozen-thawed ejaculated stallion spermatozoa is not always a good indicator for post-thaw progressive motility of epididymal spermatozoa.
This study shows that seminal plasma has a positive influence on the incidence of overall sperm defects, midpiece reflexes and distal cytoplasmic droplets in frozen-thawed stallion epididymal spermatozoa while the occurance of midpiece reflexes is likely to be linked to distal cytoplasmic droplets. Furthermore, we could show that seminal plasma does not have an influence on viability of fresh and frozen-thawed morphologically normal epididymal spermatozoa.

In conclusion, we recommend the retrograde flushing technique using seminal plasma as flushing medium to harvest and freeze stallion epididymal spermatozoa.

SAMEVATTING

Bevriesing van epididimale spermatozoa mag die enigste geleentheid wees om waardevolle genetika van manlike diere te bewaar in die geval van onverwagse dood of besering. Epididimale spermatozoa van hingste is al suksesvol bevries, is bewys vrugbaar te wees en dragtighede sowel as lewendige vullens is deur die gebruik daarvan gelewer. Aangesien maatstawwe van spermgehalte soos beweeglikheid, morfologie en lewensvatbaarheid ‘n beduidende invloed uitoefen op vrugbaarheid en dragtigheidsyfers, is dit van groot belang om hierdie maatstawwe te omskryf en die invloed van seminale plasma daarop te ondersoek.

Vars en ontdooide maatstawwe (beweeglikheid, morfologie en lewensvatbaarheid) van hings epididimale spermatozoa wat of aan seminal plasma blootgestel is of nie, is vergelyk met vars en ontdooide maatstawwe van geejakuleerde spermatozoa.
Ses spermkategorieë van elke hings (n=4) is geevalueer vir beweeglikheid, morfologie en lewensvatbaarheid. Die kategorieë was vars geejakuleerde spermatozoa (Fr-E), vars epididimale spermatozoa wat blootgestel is aan seminale plasma (Fr-SP+), vars epididimale spermatozoa sonder blootstelling aan seminale plasma (Fr-SP-), ontdooide geejakuleerde spermatozoa (Cr-E), ontdooide epididimale spermatozoa blootgestel aan seminale plasma voor bevriesing (Cr-SP+) en ontdooide epididimale spermatozoa wat nooit blootgestel is aan seminale plasma nie (Cr-SP-).

Uitslae toon dat seminale plasma die aanvanklike beweeglikheid van vars epididimale hings spermatozoa stimuleer, terwyl die verskil in lynbeweeglikheid nie meer teenwoordig is na ontdooiing nie; dat lynbeweeglikheid van vars epididimale spermatozoa of ontdooide geejakuleerde hings spermatozoa nie altyd ‘n goeie aanduiding is vir lynbeweeglikheid na ontdooiing nie. Hierdie studie toon dat seminale plasma ‘n positiewe invloed het op die voorkoms van algehele spermdefekte, midstukreflekse en distale sitoplasmiese druppeltjies in ontdooide hings epididimale spermatozoa terwyl die voorkoms van midstuk reflekse waarskynlik verband hou met distale sitoplasmiese druppletjies. Verder kon ons ook aantoon dat seminale plasma geen invloed het op die lewensvatbaarheid van vars en ontdooide morfologies normale epididimale spermatozoa nie.

Ten slotte beveel ons aan dat retrograadse spoeling met seminale plasma as spoelmedium gebruik word wanneer hings epididimale spermatozoa versamel en bevries word.

Chapter 1

1 Introduction and Justification

Cryopreservation of epididymal spermatozoa may be the only opportunity to preserve valuable genetics of males in cases of unforeseen injury or death. Stallion epididymal spermatozoa have been cryopreserved successfully (Bruemmer *et al.* 2002; Cary *et al.* 2004; Heise *et al.* 2010; Jimenez 1987; Melo *et al.* 2008; Morris *et al.* 2002; Neild *et al.* 2006; Papa *et al.* 2008; Tiplady *et al.* 2002). Different methods for collection of stallion epididymal spermatozoa have been described (Bruemmer 2006; Cary *et al.* 2004) and their cryopreservation after 24 hours of ground transportation has been shown to be successful (Neild *et al.* 2006). It has been hypothesised that epididymal spermatozoa are more resistant to cold shock and should therefore be better suitable for cryopreservation than ejaculated spermatozoa (Johnson *et al.* 1980) but only one report has shown post-thaw motility of epididymal spermatozoa that was equal to or better than that of frozen-thawed ejaculated spermatozoa (Volkmann *et al.* 2001).

It has been demonstrated that stallion epididymal spermatozoa are fertile and pregnancies as well as live foals have been produced (Barker and Gandier 1957; Heise *et al.* 2010; Melo *et al.* 2008; Morris *et al.* 2002; Papa *et al.* 2008). Pregnancy rates after conventional AI with fresh and frozen-thawed epididymal spermatozoa have been low (Morris *et al.* 2002). Higher pregnancy rates using frozen-thawed epididymal spermatozoa have only been reported recently (Heise *et al.* 2010; Melo *et al.* 2008; Papa *et al.* 2008). The effects of seminal plasma on pre-freeze and post-thaw spermatozoal motility of epididymal spermatozoa and on the fertility of epididymal spermatozoa have been investigated but the results were contradictory. To compare existing publications with regards to post-thaw spermatozoal motility, exposure or no exposure of equine epididymal spermatozoa to

seminal plasma is difficult as experimental designs differ significantly which may have an influence on the outcome and explain the contradictory findings.

There are no publications at present giving a general overview about stallion epididymal spermatozoal quality parameters with or without exposure to seminal plasma in direct comparison to ejaculated stallion spermatozoa. As spermatozoal quality parameters like motility, morphology and viability have a major influence on fertility and pregnancy rates, it is of great interest to describe these and investigate the influence of seminal plasma on these parameters.

2 Research question

The aims of this study were 1) to investigate the effect of seminal plasma on spermatozoal motility, morphology and viability of equine epididymal spermatozoa and 2) to directly compare sperm parameters of fresh and frozen-thawed stallion epididymal spermatozoa to sperm parameters of fresh and frozen-thawed ejaculated spermatozoa.

3 Hypothesis

Exposure of stallion epididymal spermatozoa to equine seminal plasma improves spermatozoal motility, morphology and viability of fresh and frozen-thawed stallion epididymal spermatozoa.

4 Literature review

4.1 The equine epididymis

The equine epididymis (McKinnon and Voss 1992) is anatomically divided into 3 parts: caput, corpus and cauda. The caput lies at the cranial pole of the testis and lateral of the spermatic cord and continues as the corpus of the epididymis. The caput is J shaped, rather flat and is closely attached to the testis. The corpus epididymis is a cylindrical structure loosely attached to the dorsal surface of the testis. The cauda epididymidis is large, bulbous and loosely attached to the caudal pole of the testis.

The proximal caput epididymidis contains the distal ends of 13 to15 highly coiled efferent ducts originating from tubules of the extra testicular rete testis (McKinnon and Voss 1992). Within the caput epididymis efferent ducts fuse into a single duct termed the epididymal duct. The single duct, possibly 45 m long, is folded in pleats and continues in a tortuous pattern through caput, corpus and cauda epididymidis and is continuous with the deferent duct. Based on cellular structure, six to eight regions can be distinguished in the stallion epididymis, and the function of each region is probably different.

From a functional point of view, the epididymis has 3 segments (McKinnon and Voss 1992). Epithelia of efferent ducts plus the initial segment of the caput are involved in resorption of most of the fluid and solutes entering from the testis and also secrete some compounds. The middle segment includes major portions of the caput and corpus epididymidis and is involved in spermatozoal maturation, a process that depends on specific secretions of the epithelium. The terminal segment is composed of the cauda epididymidis and proximal deferent duct and is involved in storage of fertile spermatozoa.

4.2 Sperm maturation in the horse

Mammalian spermatozoa are highly differentiated cells when they leave the testes but they lack forward motility and the capacity to fertilise oocytes. It is generally accepted that spermatozoa become functionally mature, acquiring both functions, during the transit through the cauda epdidymidis (Gatti *et al.* 2004).

After leaving the testes, spermatozoa enter the ductuli efferentes testis and are then transported through several zones of the epididymes. These include the initial segment of the epididymal duct, 2 or 3 spermatozoal maturation zones in the caput epididymis and corpus epididymis and a zone where fertile spermatozoa are stored in the cauda epididymis until ejaculated. Progressive motility acquisition is gradual as the spermatozoa move along the epididymis. Thus the number of motile cells seen after dilution increases in the corpus and reaches a maximum when they enter the cauda epididymis, while their type of movement changes from wiggling to straight-line displacement (Yanagimachi 1994). In the majority of species spermatozoal motility in the epididymis is balanced by two important components: one concerning the maturation of the flagellar machinery like axonema, dense fibres, and the other controlling this machinery to maintain the spermatozoa in an immotile status within the epididymis(Gatti *et al.* 2004). Fertile spermatozoa result from a successive series of complex morphological and surface modifications. Fertility of maturing spermatozoa can be measured by artificial insemination or in vitro fertilisation. These studies also show an increasing fertility gradient between the caput and the corpus with a maximum in the cauda epididymis. The number of spermatozoa able to recognise and bind to the oocyte increases in parallel to the number of motile spermatozoa, but the increase in binding is observed even at 4°C when spermatozoal motility is strongly reduced suggesting that these physiological properties are unrelated (Gatti *et al.* 2004).

4.3 Harvesting of equine epididymal spermatozoa

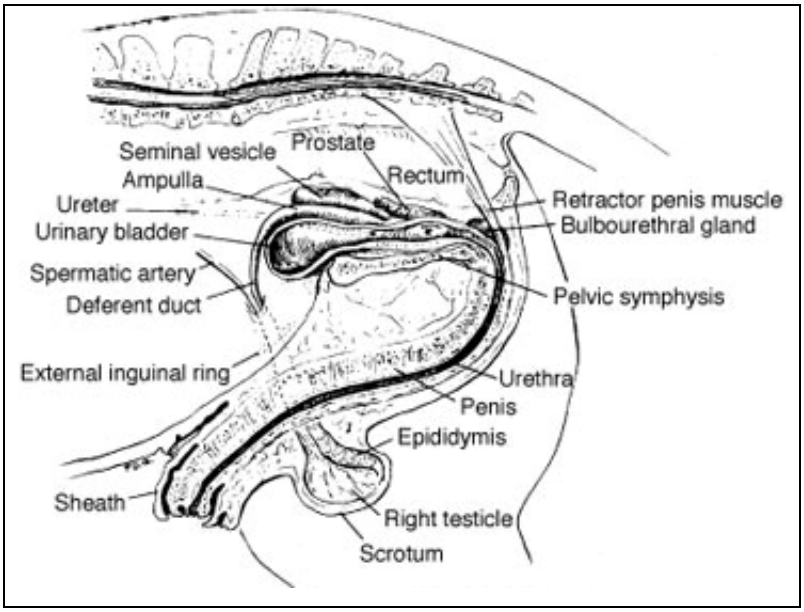
Different methods of harvesting epididymal spermatozoa have been described and cannulation or surgical removal of the epididymides is required (Bruemmer 2006). Epididymal sperm collection via epididymal tail aspiration from anesthetised and standing stallions has been successful (Bruemmer 2006) but sperm numbers harvested were low. Surgical removal of the epididymides is more efficient to obtain greater sperm numbers and one of two methods can be used: the float-up method or the retrograde flush technique. For both methods, the cauda epididymis and vas deferens have to be isolated from the testes and the rest of the epididymis. In the float-up method, the cauda epididymis and proximal ductus deferens are incised in 10-15 locations and suspended in approximately 5 ml semen extender, and sperm can float into the extender for 10 minutes (Cary *et al.* 2004). For the retrograde flush technique the vas deferens is catheterised and the spermatozoa are flushed in a retrograde fashion from the vas deferens through the cauda epididymis into a sterile test tube (Cary *et al.* 2004). Reported sperm numbers recovered per epididymis by the float-up method are 2.6 x 10^9 to 6.1 x 10^9 (James *et al.* 2002) and 4.9 x 10^9 (Cary *et al.* 2004); and 3.96 x 10^9 (Jimenez 1987), 4.5 x 10^9 (Cary *et al.* 2004) and 15-20 x 10^9 (Bruemmer 2006) for the retrograde flush technique.

4.4 Equine seminal plasma

Seminal plasma is secreted by the testes, epididymides and accessory sex glands (ampullae, prostate, vesicular and bulbourethral glands) (McKinnon and Voss 1992). Figure 1 demonstrates the anatomical location of the accessory sex glands in the stallion. Seminal

plasma is a transport medium for sperm cells, acts as a buffer, supplies nutrients and sustains the initial motility of spermatozoa. The sequence of release of ampullar, prostatic, vesicular gland and bulbourethral gland secretions during ejaculation in stallions has been studied by transrectal ultrasonography (Weber and Woods 1993) and by using biophysical parameters as markers for sequence of accessory sex gland secretions (Magistrini *et al.* 2000). These studies showed that ampullar, bulbourethral and prostatic secretions began prior to ejaculation, with fluid emission from the prostate continuing during the first urethral contractions and seminal vesicle fluid release after prostatic fluid emission stopped, forming the last ejaculatory fraction.

Figure 1
Figure 1 shows the anatomical location of the accessory sex glands (seminal vesicle, ampulla, prostate, bulbourethral gland) of a stallion.



Drawn by P.D. Garrett, DVM, University of Missouri, USA

4.4.1 Equine seminal plasma proteins

There are three major groups of equine seminal plasma proteins: proteins carrying two or four fibronectin type II modules (Fn-2 type proteins), cystein-rich secretory proteins (CRISP) and spermadhesins (Töpfer-Petersen *et al.* 2005). Eight stallion seminal plasma proteins (HSP-1 to HSP-8) with low molecular mass (14-30 kDa) have been characterised. All these proteins (except HSP-4) are peripherally bound to the sperm surface at the time of ejaculation. The major proteins in stallion seminal plasma, accounting for 70-80% of the total protein, are HSP-1 and HSP-2, belonging to the Fn-2 type proteins (Calvete *et al.* 1994).

Seminal Fn-2 proteins are the most abundant proteins in many species, including the stallion (Töpfer-Petersen *et al.* 2005). The majority of small proteins with only two Fn-modules (e.g. HSP-1 and HSP-2) are secreted in the ampulla, whereas the large proteins are produced mainly in the epididymis. The HSP-3 protein is probably a member of the CRISP family. CRISP-3 is primarily formed in the ampullae (Töpfer-Petersen *et al.* 2005) and a connection between the CRISP-3 gene polymorphism and stallion fertility has been presumed (Hamman *et al.* 2007).

The HSP-7 protein seems similar to the porcine spermadhesin AWN, and it is possibly involved in sperm-zona pellucida interaction (Töpfer-Petersen *et al.* 2005).

A protein profile for equine seminal plasma showed 14 different bands (Brandon *et al.* 1999; Frazer and Bucci 1996) of which four were significantly correlated with breeding scores (Brandon *et al.* 1999). Nevertheless, total protein concentration in equine seminal plasma was found not to be related to fertility (Barrier-Battut *et al.* 2005) but another study showed that a specific protein (protein number 19) was only detectable in ejaculates from highly fertile stallions whereas the amount of another (protein number 17) was greater in ejaculates of lesser fertility stallions (Jobim *et al.* 2005).

4.4.2 Enzymes in equine seminal plasma

There is a variety of enzymes present in seminal plasma. Alkalanine phosphatase (AP), acid phosphatase (ACP), lactate dehydrogenase (LDH), aspartate amino transferase (AST), γ-glutamyl transferase (GGT), glucosidases [β-gluronidase (BG), β-galactosidase, N-acetyl-β-D-glucosaminidase (NAD)] have been identified in stallion seminal plasma (Kareskoski and Katila 2008; Pesch *et al.* 2006). LDH concentrations for example were found to be strongly correlated with semen parameters. Semen volume was negatively correlated to LDH concentration while sperm concentration, live: dead-ratio and pathomorphology showed a positive correlation (Pesch *et al.* 2006). Glucosidases appear to play a role in sperm maturation as well as the acrosome reaction. But even though glucuronidases are important for sperm maturation and fertilisation, a gene over-expression of these enzymes seems to impair sperm function in infertile men (Corrales *et al.* 2002). It has also been found that the lipocalin-type prostaglandin D2 synthase and the angiotensin-I-converting enzyme in seminal plasma were strongly positive correlated with fertility in stallions (Barrier-Battut *et al.* 2005).

4.4.3 Electrolytes and trace elements in seminal plasma

A variety of different electrolytes and trace elements are present in seminal plasma. Concentrations of Ca, Mg, Zn, Cu, Fn, P, Cl in equine seminal plasma have been described (Barrier-Battut *et al.* 2002; Pesch *et al.* 2006) and the amounts vary between seminal plasma fractions (pre-sperm, sperm-rich, post-sperm) as well as between individual stallions (Kareskoski and Katila 2008). Barrier- Battut et al. (2002, 2005) found a variation in the

concentrations of Ca, Mg and Cu, but not Zn, between stallions but the differences did not explain the variation in post-thaw motility parameters.

In humans, infertility has been associated with abnormal levels of Ca (Pandy *et al.*1983), Mg (Pandy *et al.*1983), Zn (Chia *et al.* 2000) and Cu (Huang *et al.* 2000) in seminal plasma. Zinc plays a major role in sperm motility, exerts protective, antioxidant-like activity, and may act to stabilise sperm membranes. Intracellular Calcium is a necessary factor in the regulation of contractile function in sperm cells and motility activation of spermatozoa is related to the absorption of Calcium. It has been described that seminal plasma contains a factor which prevents or delays the uptake of Calcium into sperm mitochondria and that this factor acts upon the surface membranes of sperm (Babcock 1979).

4.5 Effect of seminal plasma on spermatozoa

Heparin-binding proteins, produced by the accessory sex glands, are bound to the surface of sperm cells. This process increases the permatozoa's ability to respond to capacitation-inducing glycosaminoglycans, such as heparin (Nass *et al.* 1990). It has been shown that the addition of seminal plasma to bovine epididymal sperm increased the susceptibility of spermatozoa to zona pellucida proteins (Flormann and First 1988). These components enable sperm cells to undergo the acrosome reaction. Seminal plasma treated epididymal sperm only undergo acrosome reactions when they are incubated under capacitation conditions. Furthermore the minerals contained in seminal plasma , which constitute the ionic environment, influence sperm function (Hamamah *et al.* 1998). Seminal plasma also has detrimental effects on spermatozoal longevity (Baas *et al.* 1983). Both the inhibitory and stimulating effect is present in stallions. One factor that may influence the longevity of spermatozoa, sperm motility as well as chromatin quality is the proportion of seminal

plasma. Previous studies have demonstrated that higher levels of seminal plasma reduced the longevity of spermatozoa (Brinsko 2000; Varner 1987) while others have not seen a difference (Jasko 1992b). A high proportion (>20%) of seminal plasma is detrimental to spermatozoal motility during long-term cold-storage (Pruitt 1993). Jasko *et al.* (1992b) recommended that 5-20 % of seminal plasma should be included in semen extender. According to Palmer (1984) 0 and 10 % of seminal plasma were superior to 20 and 50 % in semen extender for spermatozoal survival. Spermatozoa of some stallions may be more sensitive than others to the presence of seminal plasma. In these stallions, seminal plasma depresses duration of motility and reduces fertility (Brinsko 1992). Aurich *et al.* (1996) showed that the addition of seminal plasma from stallions with high post-thaw motility to semen from stallions with low post-thaw motility significantly improved membrane integrity and progressive motility. In stallions with good post-thaw semen quality, the addition of seminal plasma from horses with low post-thaw semen quality decreased progressive motility. It was therefore concluded that composition of seminal plasma is one factor that determines suitability of individual stallions for semen cryopreservation (Aurich *et al.* 1996).

Braun *et al.* (1994b) showed that the addition of seminal plasma (25 %) to spermatozoa flushed from the cauda epididymis stimulated spermatozoal motility immediately after mixing the sperm suspension with seminal plasma. Furthermore Braun *et al.* (1994b) described improved spermatozoal motility in stallions after addition of seminal plasma only before freezing but an adverse effect after freezing. On the other hand, Magistrini *et al.* (1988) described a reduction of freezability of stallion spermatozoa after mixing them with accessory sex gland secretions. It was also shown that post-thaw motility in centrifuged sperm-rich fractions was superior to both non-centrifuged sperm-rich fractions and centrifuged whole ejaculates, but that sperm plasma membrane integrity did not differ post-thaw between ejaculatory fractions (Sieme *et al.* 2004).

4.6 Effects of seminal plasma on spermatozoal motility of equine epididymal spermatozoa

The effects of seminal plasma on pre-freeze and post-thaw spermatozoal motility of epididymal spermatozoa have been investigated but results were contradictory. Most papers report a beneficial effect of seminal plasma on freshly harvested epididymal spermatozoa (Braun *et al.* 1994b; Stout *et al.* 1999; Tiplady *et al.* 2002) but reports on the effect of seminal plasma on post-thaw quality variables are not as clear. While Stout *et al.* (1999) found a beneficial effect of seminal plasma on post-thaw motility, others (Bruemmer *et al.* 2002; Cary *et al.* 2004; Morris *et al.* 2002; Papa *et al.* 2008; Tiplady *et al.* 2002) could not demonstrate a difference. Contrastingly, Volkmann *et al.* (2002) reported a negative effect of seminal plasma on post-thaw motility of stallion epididymal spermatozoa. The influence of seminal plasma is not only controversial for equine epididymal spermatozoa. Martinez-Pastor *et al.* (2006) reported a highly positive influence of seminal plasma on the freezability of Iberian red deer epididymal spermatozoa. Similarly, seminal plasma not only increased post-thaw motility and viability but clearly improved the fertilising ability of frozen-thawed canine epididymal spermatozoa (Hori *et al.* 2005). Herold *et al*. (2004) demonstrated that seminal plasma was detrimental to the post-thaw motility of African buffalo epididymal spermatozoa. This finding was supported by Harshan *et al.* (2006) who showed that buffalo seminal plasma heparin binding protein had a deleterious effect on cryopreserved buffalo cauda epididymal spermatozoa of this species.

To compare existing publications with regards to post-thaw spermatozoal motility and exposure or no exposure of equine epididymal spermatozoa to seminal plasma is difficult as experimental designs differ significantly which may have an influence on the outcome and explain the contradictory findings. The timing of addition and volume of seminal plasma

vary greatly between reported studies. Epididymal spermatozoa were just exposed to seminal plasma during collection (Braun *et al.* 1994b; Stout *et al.* 1999; Tiplady *et al.* 2002), after initial exposure to semen extender (Braun *et al.* 1994a; Cary *et al.* 2004; Papa *et al.* 2008) or together with (as a component of) freezing extender (Bruemmer *et al.* 2002). Major differences are also present for the harvesting of the testes and epididymides. Some studies used epididymides obtained after slaughter at an abattoir (James *et al.* 2002; Magistrini *et al.* 1988), while others preferred castrations under general anesthesia (Braun *et al.* 1994a; Morris *et al.* 2002), as opposed to standing castrations, because there are concerns regarding possible effects of a local anaesthetic on subsequent spermatozoal viability (Morris *et al.* 2002). Other reports did not specify the castration method (Braun *et al.* 1994b; Bruemmer *et al.* 2002; Tiplady *et al.* 2002).

4.7 Effects of seminal plasma on fertility of equine epididymal spermatozoa

To date, only few papers have been published on the fertility of stallion epididymal spermatozoa (Barker and Gandier 1957; Heise *et al.* 2010; Melo *et al.* 2008; Morris *et al.* 2002; Papa *et al.* 2008).

It has been demonstrated that stallion epididymal spermatozoa are fertile and pregnancies as well as live foals have been produced by conventional artificial insemination (AI) (Barker and Gandier 1957; Heise *et al.* 2010; Melo *et al.* 2008; Morris *et al.* 2002; Papa *et al.* 2008), hysteroscopic insemination (Morris *et al.* 2002) and ICSI (Rosati *et al.* 2004). Even though the first pregnancy using frozen-thawed equine spermatozoa was achieved with epididymal spermatozoa harvested after castration (Barker and Gandier 1957), pregnancy rates after conventional AI with fresh and frozen-thawed epididymal spermatozoa have been low

(Morris *et al.* 2002). Higher pregnancy rates than previously shown (Morris *et al.* 2002) using frozen-thawed epididymal spermatozoa have only been reported recently (Heise *et al.*, 2010; Melo *et al.* 2008; Papa *et al.* 2008). It is known that seminal plasma is not absolutely neccessary for fertilisation after AI (Barker and Gandier 1957; Melo *et al.* 2008; Morris *et al.* 2002; Papa *et al.* 2008). Furthermore, the addition of seminal plasma to epididymal spermatozoa has been shown to have no (Morris *et al.* 2002) or a beneficial effect on pregnancy rates (Heise *et al.* 2010).

4.8 Stallion sperm morphology

Evaluation of sperm morphology is part of the breeding soundness examination of stallions. Sperm morphology greatly impacts fertility in all species, and it would not be expected to be any different in horses (Brito 2007) even though the evaluation of fertility in horses has limitations due to experimental designs (e.g., reduced numbers of stallions or mares, use of different fertility endpoints) which makes interpretation of results difficult and partly questionable (Amann 2005). Some studies reported decreased fertility due to stallion sperm abnormalities (Bielanski 1975; Jasko *et al.* 1990) whereas others found no relationship between morphology and fertility of fresh stallion sperm (Bielanski and Kaczmarski 1975; Voss *et al.* 1981).

The examination for morphological sperm defects helps to identify causes of reduced fertility, stallions that can be expected to show potential fertility related problems and cases where measures can be taken to maximise stallion fertility (Brito 2007; Kenney *et al.* 1983). "An examination of sperm morphology alone can never justify the statement that the potential fertility of an ejaculate is high, but it is reasonable to state that potential fertility is low when a high proportion of spermatozoa have abnormalities"(Dott 1975).

Sperm morphology can be evaluated by examining wet mount preparations of unstained samples fixed in buffered formol saline (Jasko 1992a) or buffered glutaraldehyde solution; or by examining stained semen smears. Several staining methods have been used for this purpose (e.g. India ink, William's, Karras, Spermac, Diff-Quick, eosine-aniline blue, eosine-nigrosin) (Brito 2007). One of the most commonly used stains for evaluation of sperm morphology is eosin-nigrosin. It is a supravital stain that does not penetrate cells with intact membranes. Therefore, unstained spermatozoa have intact membranes (live) and those staining red have disrupted membranes (dead). Nigrosin provides a purple background that allows visualisation of unstained spermatozoa. The advantage of using eosin-nigrosin stain is that it facilitates morphologic evaluation as well as determination of the live-dead ratio at the same time (see Figure 2). It is generally accepted that at least 100 sperm cells should be evaluated and classified per sample. It is also preferred to describe all the defects on a single sperm cell to get a better overall picture of the condition and to enhance the ability to determine breeding soundness (Card 2005; Veeramachaneni *et al.* 2006). Classification systems used for bulls have been adapted by some authors for stallions (Barth and Oko 1989; Jasko 1992a) and several other classification systems for stallions have been reported where the nomenclature for specific defects varies widely (Bielanski *et al.* 1982; Dowsett *et al.* 1984; Jasko 1992a). These systems classify sperm defects according to 1) their origin in primary (sperm defects occurring during spermatogenesis), secondary (defects occurring during the maturation and storage period) and tertiary defects (occurring after ejaculation; these defects include iatrogenic defects) or 2) in major and minor defects depending on their impact on fertility or 3) in compensable versus non-compensable defects depending on the ability of the normal sperm to compensate for the abnormal ones or not. The current problem regarding the use of these classification systems is that a considerable body of data describing the effects of specific defects on fertility should be available for appropriate conclusions, which is not the case for stallions (Brito

2007). The present Society for Theriogenology forms for stallion breeding soundness examination have the following categories listed in the differential spermiogram: normal sperm, abnormal acrosomal regions/ heads, detached heads, proximal droplets, distal droplets, abnormal midpieces, and bent/ coiled tails. These categories are then further classified in specific sub-categories. There is a wide variation in sperm morphology among breeding stallions (Brito 2007; Dowsett and Knott 1996; Dowsett *et al.* 1984; Jasko *et al.* 1990; Love *et al.* 2000), but in general, the average stallion has approximately 50% morphologically normal sperm (Brito 2007; Card 2005). At this stage, there are no publications available describing stallion epididymal spermatozoal morphology.

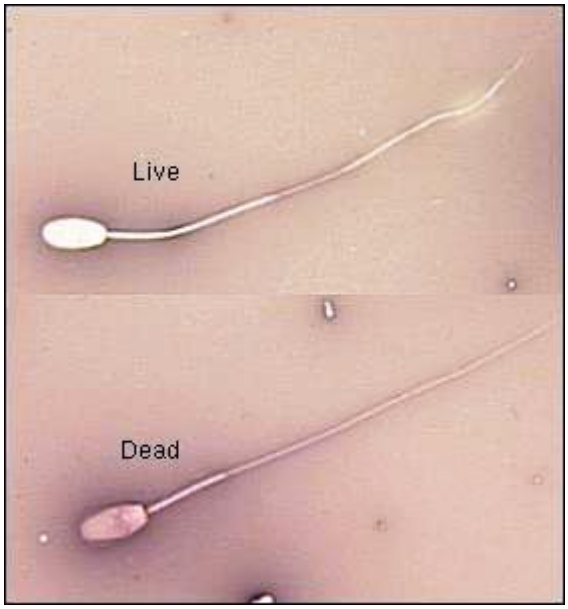


Figure 2
A live (intact membrane) and a dead (damaged membrane) stallion spermatozoa on a semen smear stained with eosin-nigrosin.

Chapter 2

5 Materials and Methods

5.1 Animals and Experimental design

Four stallions were used. Stallion A was a Warmblood (5 years old), stallion B and C were Boerperds (4 years old) and stallion D was a Welsh (4 years old). Stallions were housed in stables and fed concentrates and hay three times daily. Water was freely available to all horses.

Stallions used in this study were selected on results of breeding soundness examinations including semen evaluations for fresh and frozen-thawed (≥30%) progressive spermatozoal motility, spermatozoal concentration (≥ 120 x 10^6/ ml) and total spermatozoal numbers (≥ 5 x 10^9/ ejaculate).

Six sperm categories of each stallion were evaluated for motility, morphology and viability. These categories were fresh ejaculated spermatozoa (Fr-E), fresh epididymal spermatozoa that had been exposed to seminal plasma (Fr-SP+), fresh epididymal spermatozoa that had never been exposed to seminal plasma (Fr-SP-), frozen-thawed ejaculated spermatozoa (Cr-E), frozen-thawed epididymal spermatozoa that had been exposed to seminal plasma prior to freezing (Cr-SP+) and frozen-thawed epididymal spermatozoa that had never been exposed to seminal plasma (Cr-SP-).

Data for motility, morphology and viability of fresh and frozen-thawed epididymal spermatozoa that had and had not been exposed to seminal plasma was compared directly. Data for motility, morphology and viability of fresh and frozen-thawed epididymal spermatozoa that had and had not been exposed to seminal plasma was also compared to semen parameters of fresh and frozen-thawed ejaculated spermatozoa.

Fresh semen of all stallions was collected and evaluated for spermatozoal motility, morphology, viability and concentration every second to third day for approximately four weeks. A part of each ejaculate was frozen and stored. Castrations to harvest the epididymides were performed three to four weeks after the last semen collection. The epididymal semen was evaluated in the same way as the ejaculated semen. Spermatozoal concentration was determined for epididymal sperm categories to establish total sperm numbers recovered per epididymis.

5.2 Semen Collection of Ejaculated Spermatozoa

Semen of all stallions was collected on a teaser mare in oestrus using a Hannover Model artificial vagina.

5.3 Harvesting of Epididymal Spermatozoa

Castrations were performed three to four weeks after the last semen collection and both testes were recovered via open castration under general anaesthesia. The cauda epididymis and the vas deferens were separated from each testis. Connective tissue was removed from the cauda epididymis. The epididymal duct was cut at the site in the tail of the epididymis where the tubular diameter became distinctly narrower. A blunted 18G four cm hypodermic needle connected to a 20 ml syringe filled with flushing medium was inserted into the open end of the vas deferens. Spermatozoa were then flushed in a retrograde direction from the vas deferens through the cauda epididymis into a sterile 50 ml plastic tube.

For each stallion one epididymal tail was flushed with 20 ml seminal plasma and the other with 20 ml freezing medium. Left and right epididymides were randomly allocated to the different treatment groups.

Each epididymal semen sample was divided into two parts. One part was used for the evaluation of spermatozoal motility and the preparation of eosin-nigrosin smears for evaluation of sperm morphology of fresh epididymal spermatozoa. The other part was cryopreserved. The aliquot of epididymal spermatozoa flushed with seminal plasma and destined for cryopreservation was diluted with centrifugation medium to a concentration of 100 x 10^6 spermatozoa/ml and further treated like ejaculated semen (see semen freezing). Epididymal semen flushed with freezing medium was further diluted with freezing medium to a final concentration of 500 x 10^6 spermatozoa/ml and then cryopreserved as described below without centrifugation. Figure 3 shows the retrograde flushing technique of epididymal spermatozoa.

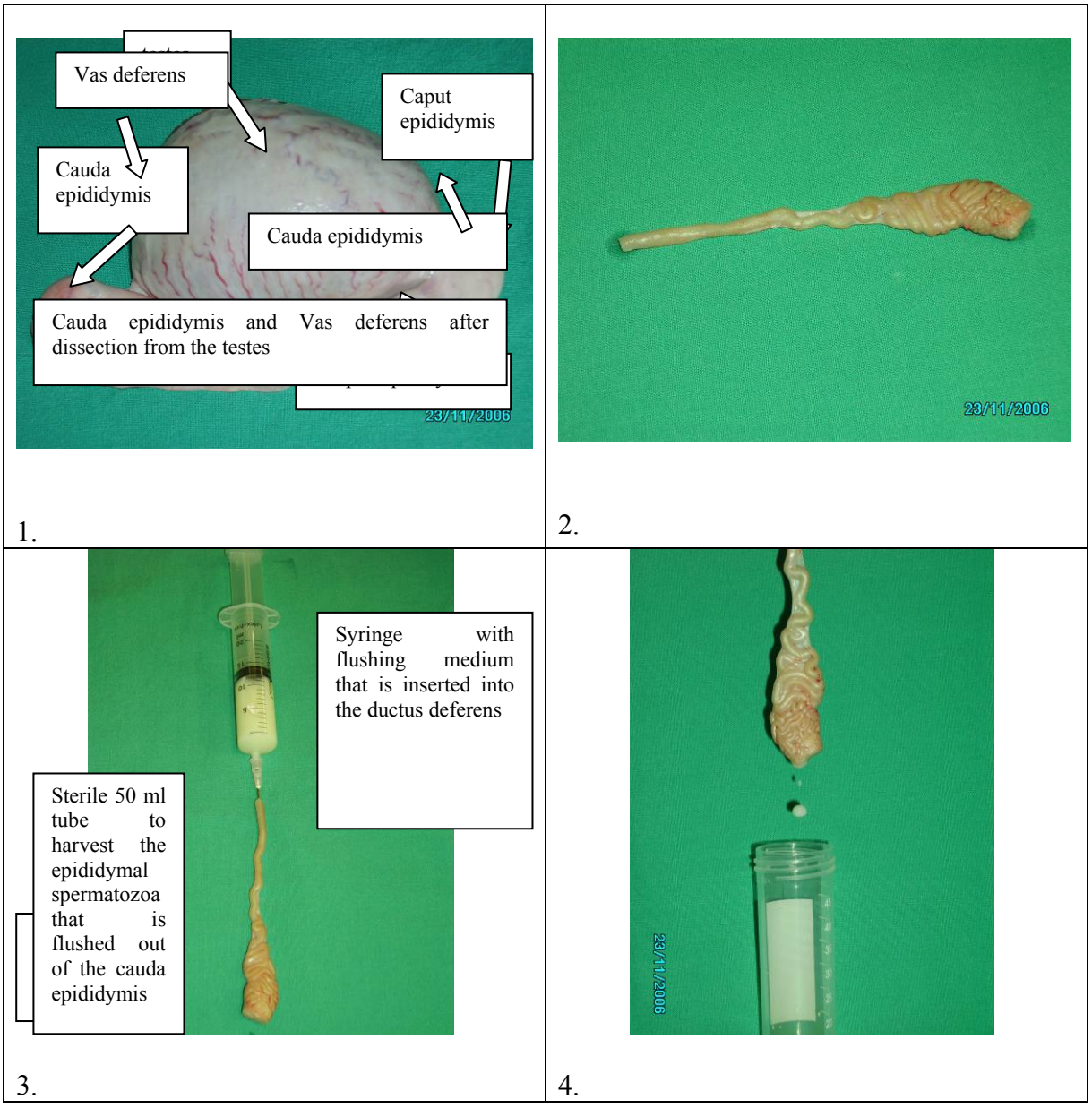


Figure 3
Retrograde flushing technique to harvest stallion epididymal spermatozoa.

5.4 Harvesting of Seminal Plasma

To harvest seminal plasma from the stallions, semen was collected and the sperm rich fraction was separated from the gel fraction using a sterile gauze filter and then centrifuged at 600 g for 15 min. The supernatant was placed into a sterile 50 ml plastic tube and the centrifugation was repeated twice more. Seminal plasma of all four stallions was pooled and frozen at -18ºC in aliquots of 50 ml. Aliquots of seminal plasma were thawed in a water bath at 37 °C before use.

5.5 Evaluation of Spermatozoal Motility and Concentration

An aliquot of semen (0.5 ml for fresh semen evaluation; 50 µl for frozen-thawed semen) was transferred into a 4 ml plastic tube, diluted with 2 ml skim milk extender (see semen extender: centrifugation medium) and kept in a 37°C water bath. Evaluation took place immediately. A drop of diluted semen was placed on a pre-warmed glass slide and covered with a 22 x 22 mm cover slip. The percentage of motile spermatozoa was determined by eyeball assessment at x 200 magnification using a phase contrast microscope with a heated microscope stage (37°C). Percentages of progressively motile, aberrantly motile and immotile spermatozoa were recorded; sperm concentration was determined by using a hemacytometer.

5.6 Evaluation of Spermatozoal Morphology and Viability

Recording of spermatozoal morphology and viability was performed by evaluating eosin-nigrosin stained semen smears.

The eosin-nigrosin stain was prepared by adding 2 g eosin and 5 g nigrosin to 100 ml of a water based buffer with a pH of less than 6.8 and an osmolality of 293. After adding eosin and nigrosin the solution was sonicated and filtered. The eosin-nigrosin stain had a pH of 8.49 and an osmolality of 420.

The staining procedure was performed by placing a drop of eosin-nigrosin stain on a prewarmed coverslip, adding a drop of semen to the stain and mixing it immediately. A drop of the mixture was transferred to a clean slide to make a thin smear using the back end of a spreader slide. The smears were dried on a heating stage and mounted with the rapid mounting medium Entellan® (Merck KgaA, Darmstadt, Germany).

A phase-contrast microscope at 1000 x magnification and oil immersion was used for the evaluation; 500 spermatozoa per smear of each sample (Fr-E, Fr-SP+, Fr-SP-, Cr-E, Cr-SP+, Cr-SP-) were evaluated. Data was captured using the data capture sheet and method described for bull semen morphology by Nöthling and Irons (2008) (Figure 4).

Identification of smear **Jason Warmblood frozen-thawed epid sperm+SP 20050125**		Stain or fixative or evaluation method **Eosin nigrosin**	
Number of nuclear defects (types 1-12) in excess of the number of spermatozoa affected by them II	1	Teratoid	
	2	Double	2
	3	Macrocephalic oo	2
	4	Microcephalic oo	
	5	Rolled/ crested head oo I	3
	6	Pyriform	
	7	Tapered (narrow) head	
	8	Diadem (nuclear crater)	
	9	Narrow base	
	10	Abnormal base	
	11	Other abnormal head shapes	
	12	Abnormal loose heads	
Spermatozoa with nuclear defects (Types 1-12) as well as acrosomal or tail defects (Types 13-28) oo I			3
Number of acrosomal or tail defects (Types 13-28) in excess of the number of spermatozoa affected by them ooo IIIII	13	Knobbed acrosome IIII oo II oooo II	14
	14	Stump tail	
	15	Pseudodroplet	
	16	Segmental aplasia or malpositioned mitochondria	
	17	Cork skrew	
	18	Dag oooo	4
	19	Other midpiece defects ooooo III ooo IIIIIIIII ooooooo II o IIII oooooo IIIIIIII	50
	20	Coilded principle piece	
	21	Proximal droplet III oooo IIIII oooo	16
	22	Distal midpiece reflex oooo IIIII oooooo IIIII	22
	23	Normally shaped loose heads oooooooooooooooooooooooooooooooooooo	36
	24	Fractured flagellum	
	25	Distal droplet IIII ooooooo IIIIIIIIII oooo IIIIIII ooooooooo IIIIIIIIIIIIIIIIII II IIIIII ooooooooooooo IIIIIIIIIIIIIIIIIIII IIIIIIIIIIIIIIIIIIIII oooooooo ooo	142
	26	Altered acrosome IIIIIIII oooo	12
	27	Bent midpiece oo	2
	28	Bent principle piece	
Spermatozoa with one or more acrosome or tail defects (numbers 13-28)			
Morphologically normal spermatozoa IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII Oooooooooo oooooooooo oooooooooo oooooooooo oooooooooo oooooooooo oooooooooo oooooooooo			208
Normal viable spermatozoa			80
Viable spermatozoa			212

Figure 4
Data capture sheet used to record sperm morphology and viability (Nöthling and Irons 2008)

o = marks dead spermatozoa I = marks live spermatozoa

5.7 Semen freezing

Immediately after collection the sperm rich fraction was separated from the gel fraction using a sterile gauze filter. Gel-free semen was evaluated for volume, concentration and percentage of progressively motile spermatozoa. Semen was diluted in skim milk centrifugation medium to a concentration of 100 x 10^6 spermatozoa/ml and centrifuged for 10 min at 600 g. After removal of the supernatant, sperm pellets were re-suspended in skim milk freezing extender to a final concentration of 500 x 10^6 spermatozoa/ml. Semen was packaged in 0.5 ml straws (0.5 cc semen straws, Minitüb, Tiefenbach, Germany) and equilibrated for 60 min at 4°C (straws placed on a rack with a distance of 10 mm between straws). Straws on the rack were then placed 3.5cm above liquid nitrogen for 20 min before being plunged into liquid nitrogen. Straws were thawed in a 37°C water bath for 30 s. The processing of epididymal spermatozoa for freezing is described under 5.3 (Harvesting of Epididymal Spematozoa).

5.8 Semen Extender

Semen extenders used were skim milk based and a modification of INRA 82 extender (Palmer 1984). The centrifugation medium contained glucose (25g), lactose (1.5g), raffinose (1.5g), sodium citrate (dehydrate) (0.25g), potassium citrate (0.41g), HEPES (4.76g), UHT sterilised skim milk (500ml), deionised water (500ml), Penicillin G (50 000IU), Gentamycin sulfate (5mg). The freezing medium contained the same ingredients but also egg yolk (40ml) and glycerol (25ml). For the preparation of egg yolk, 40 ml egg yolk were mixed with 40 ml deionised water and centrifuged at 15000 g for 10 min. Forty ml of the liquid component was harvested and used in the extender.

5.9 Statistical Analysis

The effects of flushing medium and of freezing on percentage progressively motile spermatozoa were estimated using Tukey-Kramer Multiple-Comparison Test.

Multiple logistic regression models were used to estimate the effect of flushing medium and freezing on morphological sperm defects. The main predictors of interact were flushing medium (skim milk extender or seminal plasma) and semen treatment (fresh or frozen). The clustering of observations within stallions was accounted for by including stallions as a fixed effect. Data were analysed in Stata 10.1 (StataCorp, College Station, TX, USA).

Effect of semen extender and treatment on occurrence of sperm defects was described according to the data capture sheet used (Figure 4): overall sperm defects (nuclear as well as acrosome and tail defects, Figure 4: number 1-28), nuclear defects (Figure 4: number 1-12), knobbed acrosomes (Figure 4: number 13), midpiece reflexes (Figure 4: number 22), distal droplets (Figure 4: number 25) and damaged/degenerated acrosomes (Figure 4: number 26). Only specific defects that constituted more than 2% of all defects were taken into consideration for evaluation.

Separate models were used for each of the following outcomes: overall sperm defects, nuclear defects, knobbed acrosomes, midpiece reflexes, distal droplets and damaged/degenerate acrosomes.

Comparisons between the 6 sperm categories (Fr-E, Fr-SP+, Fr-SP-, Cr-E, Cr-SP+, Cr-SP-) regarding sperm defects were done by Fisher's Exact test.

Fisher's Exact test was also used to test viability (live/ dead ratio) for morphologically normal sperm.

A paired t-test was used to compare the decrease in sperm motility between fresh and frozen-thawed ejaculated and epididymal spermatozoa.

Chapter 3

6 Results

6.1 Number of Spermatozoa Recovered from the Epididymides

Number of spermatozoa recovered from the epididymides varied between 8 x 10^9 and 28 x 10^9 spermatozoa per epididymis (stallion A, 22 x 10^9 and 22 x 10^9; stallion B, 19 x 10^9 and 28 x 10^9; stallion C, 8 x 10^9 and 8 x 10^9; stallion D, 18 x 10^9 and 17 x 10^9).

6.2 Effect of Seminal Plasma on Spermatozoal Motility

The effects of seminal plasma on spermatozoal motility are summarised in table 1.

For all stallions, Fr-SP+ contained more progressively motile spermatozoa than Fr-SP- ($P<0.05$) and was similar to Fr-E. However, after freezing and thawing, this difference in progressive motility between epididymal spermatozoa exposed and not exposed to seminal plasma was no longer present, even though it still appeared to persist in stallion D. For all stallions, post-thaw progressive motilities of epididymal spermatozoa that had been exposed to seminal plasma (Cr-SP+) and that had never been exposed to seminal plasma (Cr-SP-) were both significantly lower ($P<0.05$) than that of frozen-thawed ejaculated spermatozoa (Cr-E).

Table 1
Effect of seminal plasma on spermatozoal motility
Different letters in a row show significant (P≤0.05) differences between sperm categories for spermatozoal motility.

		Fr-E	Fr-SP+	Fr-SP-	Cr-E	Cr-SP+	Cr-SP-
Stallion A	**PMS (%)**	**67^{a}**	**75^{a}**	**30^{b}**	**50^{a}**	**10^{c}**	**10^{c}**
	AMS (%)	18^{a}	10^{a}	10^{a}	15^{a}	20^{a}	10^{a}
	IMS (%)	15^{a}	15^{a}	60^{c}	35^{a}	70^{c}	80^{c}
Stallion B	**PMS (%)**	**66^{a}**	**80^{a}**	**10^{c}**	**35^{b}**	**10^{c}**	**10^{c}**
	AMS (%)	19^{a}	10^{a}	10^{a}	20^{a}	20^{a}	10^{a}
	IMS (%)	15^{a}	10^{a}	80^{c}	45^{b}	70^{c}	80^{c}
Stallion C	**PMS (%)**	**77^{a}**	**80^{a}**	**75^{a}**	**40^{b}**	**10^{c}**	**10^{c}**
	AMS (%)	12^{b}	5^{c}	5^{c}	20^{b}	20^{b}	35^{a}
	IMS (%)	11^{a}	15^{a}	20^{a}	40^{b}	70^{c}	55^{b}
Stallion D	**PMS (%)**	**71^{a}**	**80^{a}**	**40^{b}**	**50^{b}**	**30^{c}**	**5^{d}**
	AMS (%)	14^{a}	10^{a}	10^{a}	5^{b}	20^{a}	5^{b}
	IMS (%)	15^{a}	10^{a}	50^{b}	45^{b}	50^{b}	90^{c}

Stallion A: Warmblood
Stallion B: Boerperd
Stallion C: Boerperd
Stallion D: Welsh

PMS (%) Progressively motile spermatozoa
AMS (%) Aberrantly motile spermatozoa
IMS (%) Immotile spermatozoa

Fr-E	fresh ejaculate
Fr-SP+	fresh epididymal spermatozoa that had been exposed to seminal plasma
Fr-SP-	fresh epididymal spermatozoa that had never been exposed to seminal plasma
Cr-E	frozen-thawed ejaculated spermatozoa
Cr-SP+	frozen-thawed epididymal spermatozoa that had been exposed to seminal plasma prior to freezing
Cr-SP-	frozen-thawed epididymal spermatozoa that had never been exposed to seminal plasma

6.3 Effect of Seminal Plasma on Spermatozoal Morphology

Table 2 shows the percentage of morphological sperm defects per sperm category combined for all stallions, calculated on the total number of sperm cells (n=2000) evaluated per category.

Sperm morphology of all stallions was within normal limits for fresh ejaculated spermatozoa as described by Palmer (1984).

Looking at all defects in the individual multiple logistic regression models, flushing medium and freezing had a significant influence on the occurrence of overall sperm defects (nuclear as well as acrosomal and tail defects), midpiece reflexes, distal droplets and damaged/degenerate acrosomes.

Results are listed in tables 3, 6, 7 and 8.

Flushing medium and freezing did not have a significant influence on the occurrence of nuclear defects and knobbed acrosomes as shown in tables 4 and 5.

Table 2
Percentage of morphological sperm defects for all sperm categories (Fr-E, Fr-SP+, Fr-SP-, Cr-E, Cr-SP+, Cr-SP-) combined for all stallions, calculated on the total number of sperm cells (n=2000) evaluated per category. Different letters in a row show significant (P≤0.05) differences between sperm categories for a specific defect

	Sperm category					
(%)	**Fr-E**	**Fr-SP+**	**Fr-SP-**	**Cr-E**	**Cr-SP+**	**Cr-SP-**
Normal sperm	68.15^{a}	34.1^{d}	35.42^{d}	63.30^{b}	49.00^{c}	29.90^{e}
Main groups of defects						
Sperm with defects (nuclear as well as acrosome and tail defects, Figure 5: 1-28)	31.85^{e}	65.90^{b}	64.58^{b}	36.70^{d}	51.00^{c}	70.10^{a}
Sperm with nuclear defects (Figure 5, 1-12)	2.95^{ab}	3.55^{a}	2.20^{b}	2.85^{ab}	3.15^{b}	3.45^{ab}
Sub-groups of defects						
Knobbed acrosome (Figure 5, 13)	2.60^{a}	1.55^{b}	1.35^{bc}	2.25^{ac}	2.25^{bc}	1.65^{ac}
Midpiece reflex (Figure 5, 22)	7.90^{c}	3.75^{d}	10.75^{b}	5.00^{b}	2.15^{e}	20.50^{a}
Distal cytoplasmic droplet (Figure 5, 25)	9.10^{e}	46.25^{a}	38.30^{b}	7.65^{e}	25.65^{d}	31.25^{c}
Sperm with damaged and/or degenerated acrosomes (Figure 5, 26)	4.20^{c}	2.30^{d}	2.30^{d}	7.70^{b}	9.20^{a}	5.80^{b}

Fr-E	fresh ejaculate
Fr-SP+	fresh epididymal spermatozoa that had been exposed to seminal plasma
Fr-SP-	fresh epididymal spermatozoa that had never been exposed to seminal plasma
Cr-E	frozen-thawed ejaculated spermatozoa
Cr-SP+	frozen-thawed epididymal spermatozoa that had been exposed to seminal plasma prior to freezing
Cr-SP-	frozen-thawed epididymal spermatozoa that had never been exposed to seminal plasma

Table 3
Effect of flushing medium, freezing and animal on occurrence of **sperm defects (nuclear as well as acrosomal and tail defects, Figure 4: 1-28)** in stallions: results of a multiple logistic regression model

Variable	Level	Odds ratio	95% C.I.	P
Extender	**Fresh**			
	Epidid-SP- vs. Ejac	4.27	3.73, 4.89	<0.001
	Epidid-SP+ vs. Ejac	4.54	3.96, 5.20	<0.001
	Epidid+SP vs. Epidid-SP	1.06	0.93, 1.22	0.369
	Frozen			
	Epidid-SP- vs. Ejac	4.46	3.89, 5.11	<0.001
	Epidid-SP+ vs. Ejac	1.86	1.63, 2.12	<0.001
	Epidid-SP+ vs. Epidid-SP-	0.42	0.37, 0.48	<0.001
Freezing	Ejaculated: Frozen-thawed vs. fresh	1.25	1.10, 1.43	0.001
	Epidid-SP-: Frozen-thawed vs. fresh	1.31	1.14, 1.50	<0.001
	Epidid-SP+: Frozen-thawed vs. fresh	0.51	0.45, 0.59	<0.001
Animal (4 levels)		–	–	<0.001
Extender*Freezing interaction		–	–	<0.001

Table 4
Effect of flushing medium, freezing and animal on occurrence of **nuclear defects (Figure 4: 1-12)** in stallion semen: results of a multiple logistic regression model

Variable	Level	Odds ratio	95% C.I.	P
Extender	Epidid-SP- vs. Ejac	0.97	0.75, 1.27	0.840
	Epidid-SP+ vs. Ejac	1.16	0.90, 1.50	0.247
	Epidid-SP+ vs. Epidid-SP	1.19	0.92, 1.54	0.174
Freezing	Frozen-thawed vs. Fresh	1.25	1.10, 1.43	0.001
Animal (4 levels)		–	–	<0.001

Table 5
Effect of flushing medium, freezing and animal on occurrence of **knobbed acrosomes (Figure 4: 13)** in stallion semen: results of a multiple logistic regression model

Variable	Level	Odds ratio	95% C.I.	P
Extender	Epidid-SP- vs. Ejac	0.61	0.44, 0.85	0.003
	Epidid-SP+ vs. Ejac	0.78	0.57, 1.06	0.107
	Epidid-SP+ vs. Epidid-SP-	1.27	0.90, 1.79	0.167
Freezing	Frozen-thawed vs. Fresh	1.12	0.86, 1.45	0.389
Animal (4 levels)		–	–	<0.001

Table 6
Effect of flushing medium, freezing and animal on occurrence of **midpiece reflexes (Figure 4: 22)** in stallions: results of a multiple logistic regression model

Variable	Level	Odds ratio	95% C.I.	P
Extender	**Fresh**			
	Epidid-SP- vs. Ejac	1.43	1.15, 1.79	0.001
	Epidid-SP+ vs. Ejac	0.44	0.33, 0.59	<0.001
	Epidid-SP+ vs. Epidid-SP-	0.31	0.23, 0.41	<0.001
	Frozen			
	Epidid-SP- vs. Ejac	5.49	4.34, 6.95	<0.001
	Epidid-SP+ vs. Ejac	0.41	0.28, 0.59	<0.001
	Epidid-SP+ vs. Epidid-SP-	0.07	0.05, 0.10	<0.001
Freezing	Ejaculated: Frozen-thawed vs. fresh	0.60	0.46, 0.78	<0.001
	Epidid-SP-: Frozen-thawed vs. fresh	2.30	1.91, 2.78	<0.001
	Epidid-SP+: Frozen-thawed vs. fresh	0.56	0.38, 0.82	0.003
Animal (4 levels)		–	–	<0.001
Extender*Freezing interaction		–	–	<0.001

Table 7
Effect of flushing medium, freezing and animal on occurrence of **distal droplets (Figure 4: 25)** in stallions: results of a multiple logistic regression model

Variable	Level	Odds ratio	95% C.I.	P
Extender	**Fresh**			
	Epidid-SP- vs. Ejac	6.32	5.29, 7.55	<0.001
	Epidid-SP+ vs. Ejac	8.81	7.38, 10.52	<0.001
	Epidid-SP+ vs. Epidid-SP-	1.39	1.23, 1.58	<0.001
	Frozen			
	Epidid-SP- vs. Ejac	5.57	4.60, 6.74	<0.001
	Epidid-SP+ vs. Ejac	4.21	3.47, 5.11	<0.001
	Epidid-SP+ vs. Epidid-SP-	0.76	0.66, 0.87	<0.001
Freezing	Ejaculated: Frozen-thawed vs. fresh	0.83	0.66, 1.03	0.097
	Epidid-SP-: Frozen-thawed vs. fresh	0.73	0.64, 0.83	<0.001
	Epidid-SP+: Frozen-thawed vs. fresh	0.40	0.35, 0.45	<0.001
Animal (4 levels)		–	–	<0.001
Extender*Freezing interaction		–	–	<0.001

Table 8
Effect of flushing medium, freezing and animal on occurrence of **damaged/degenerated acrosomes (Figure 4: 26)** in stallions: results of a multiple logistic regression model

Variable	Level	Odds ratio	95% C.I.	P
Extender	**Fresh**			
	Epidid-SP- vs. Ejac	0.52	0.36, 0.76	0.001
	Epidid-SP+ vs. Ejac	0.52	0.36, 0.76	0.001
	Epidid-SP+ vs. Epidid-SP-	1.00	0.66, 1.52	1.000
	Frozen			
	Epidid-SP- vs. Ejac	0.72	0.56, 0.93	0.013
	Epidid-SP+ vs. Ejac	1.20	0.98, 1.57	0.074
	Epidid-SP+ vs. Epidid-SP-	1.72	1.34, 2.21	<0.001
Freezing	Ejaculated: Frozen-thawed vs. fresh	1.99	1.50, 2.64	<0.001
	Epidid-SP-: Frozen-thawed vs. fresh	2.73	1.92, 3.90	<0.001
	Epidid-SP+: Frozen-thawed vs. fresh	4.70	3.36, 6.59	<0.001
Animal (4 levels)		–	–	<0.001
Extender*Freezing interaction		–	–	<0.001

6.4 Effect of Seminal Plasma on Spermatozoal Viability

Table 9
Percentage of morphologically normal spermatozoa per sperm category that are viable (i.e. with intact cell membranes). Different letters in a row show significant (P≤0.05) differences between sperm categories for viability.

	Sperm categories					
	Fr-E	**Fr-SP+**	**Fr-SP-**	**Cr-E**	**Cr-SP+**	**Cr-SP-**
Live morphologically Normal spermatozoa (%)	84[b]	91[a]	91[a]	64[c]	60[c]	60[c]

Fr-E	fresh ejaculate
Fr-SP+	fresh epididymal spermatozoa that had been exposed to seminal plasma
Fr-SP-	fresh epididymal spermatozoa that had never been exposed to seminal plasma
Cr-E	frozen-thawed ejaculated spermatozoa
Cr-SP+	frozen-thawed epididymal spermatozoa that had been exposed to seminal plasma prior to freezing
Cr-SP-	frozen-thawed epididymal spermatozoa that had never been exposed to seminal plasma

Chapter 4

7 Discussion

7.1 Effect of Seminal Plasma on Motility of Equine Epididymal Spermatozoa

Progressive motility of fresh epididymal spermatozoa that had been exposed to seminal plasma was higher than that of fresh epididymal spermatozoa that had not been exposed to seminal plasma. These findings agree with those of Braun *et al.* (1994b) and Stout *et al.* (1999) who found that seminal plasma significantly increased initial spermatozoal motility, which supports the conclusion that seminal plasma induces motility in live, but quiescent, spermatozoa (Stout *et al.* 1999). However, the post-thaw progressive motility of epididymal spermatozoa that had been exposed to seminal plasma prior to freezing and that had not been exposed was similar and poor in three of the four stallions. A difference in post-thaw motilities for Cr-SP+ and Cr-SP- only persisted in one stallion. The combined progressive motilities for Cr-SP+ and Cr-SP- for all four stallions were similar.

While some earlier studies (Bruemmer *et al.* 2002; Cary *et al.* 2004; Morris *et al.* 2002; Papa *et al.* 2008; Tiplady *et al.* 2002) also failed to find a beneficial effect of seminal plasma on post-thaw spermatozoal motility, others did detect differences due to the addition of seminal plasma to epididymal spermatozoa prior to freezing (Stout *et al.* 1999; Volkmann *et al.* 2001). Whereas Stout *et al.* (1999) showed a positive influence of seminal plasma on post-thaw motility, Volkmann *et al.* (2001) reported a negative influence of seminal plasma on cryopreservation of stallion epididymal spermatozoa. In the latter study

(Volkmann *et al.* 2001) post-thaw progressive motility of epididymal spermatozoa not exposed to seminal plasma was not only better than that of epididymal spermatozoa that had been exposed to seminal plasma, but also better than that of frozen-thawed ejaculated spermatozoa. The current study showed a significantly higher progressive motility in frozen-thawed ejaculated spermatozoa than in Cr-SP+ and Cr-SP- which contradicts the hypothesis that epididymal spermatozoa are less susceptible to cold shock and therefore more resistant to freezing and thawing than ejaculated spermatozoa (Johnson *et al.* 1980). Similar findings regarding poor post-thaw motilities for epididymal spermatozoa have been published previously (Bruemmer *et al.* 2002; Jimenez 1987; Tiplady *et al.* 2002).

Reports describing the influence of seminal plasma on post-thaw epididymal spermatozoal parameters are not only contradictory in horses. A positive influence of seminal plasma on post-thaw motility has been reported for bull and ram (Graham 1994), red deer (Martínez-Pastor *et al.* 2006) and dog epididymal spermatozoa (Hori *et al.* 2005; Nöthling *et al.* 2007). In dogs prostatic fluid did not only improve freezability but also post-thaw longevity (Nöthling *et al.* 2007) which is supported by Hori *et al*. (2005). Negative and detrimental effects of seminal plasma on post-thaw semen quality have been described for African (Herold *et al.* 2004) and water buffalo (Harshan *et al.* 2006). Buffalo seminal plasma heparin binding protein has been found to exert negative effects on buffalo frozen-thawed epididymal spermatozoa (Harshan *et al.* 2006). These negative effects seen as heparin binding protein mediated cryo-injury could be reduced through the use of an egg yolk containing extender (Singh *et al.* 2007).

A difference in post-thaw spermatozoal motility between Cr-SP+ and Cr-SP- was evident in one stallion, with higher post-thaw motility for Cr-SP+. As the same pool of seminal plasma was used to flush the epididymides for all four stallions, the composition of seminal plasma will not have influenced the post-thaw progressive motility of epididymal spermatozoa in this study. Instead, we suspect that stallion difference was responsible for differences in the

freezability of epididymal spermatozoa, just as it is for ejaculated spermatozoa (Magistrini *et al.* 1988; Vidament *et al.* 1997). In an extensive French study, 19% and 30% of stallions were considered to have unfreezable and poorly freezable semen, respectively (Vidament *et al.* 1997).

All four stallions chosen for the project produced post-thaw progressive motilities of at least 30% during a test freezing. Therefore, progressive motility of fresh or frozen-thawed ejaculated spermatozoa is not necessarily a good predictor for post-thaw epididymal spermatozoal motility in the same stallion. Magistrini *et al.* (1988) have however reported a strong correlation between motility of frozen-thawed ejaculated and epididymal spermatozoa of 19 stallions.

Looking at the motility results from a different angle, it can also be seen that the decrease in sperm motility between fresh and frozen-thawed spermatozoa differs for ejaculated spermatozoa and epididymal spermatozoa, even though the diffenence was only statistically significant ($p<0.05$) for ejaculated spermatozoa and epididymal spermatozoa that had been exposed to seminal plasma. For ejaculated spermatozoa, the decrease in progressive motility between fresh and frozen-thawed spermatozoa was 25%, 30%, 47% and 48% for stallions A, D, B and C, respectively. These decreases in sperm motility after cryopreservation are in agreement with findings for human spermatozoa of fertile men where sperm cryopreservation was reported to result in a 48% decrease in the average progressive motility (Donnelly *et al.* 2001). For epididymal spermatozoa that had been exposed to seminal plasma, the decrease in progressive motility between fresh and frozen-thawed spermatozoa was 63%, 87%, 88% and 88% for stallions D, A, B and C, respectively, which is a more pronounced decrease than for the ejaculated spermatozoa of the same stallions.

For epididymal spermatozoa that had not been exposed to seminal plasma, the decrease in progressive motility between fresh and frozen-thawed spermatozoa was 0%, 67%, 87% and

88% for stallions B, A, D and C, respectively. Here it is interesting to notice that for stallion B who showed a very low initial motility (Fr-SP-, table 1) the decrease in motility after cryopreservation was 0%.

These findings furthermore contradict the hypothesis that epididymal spermatozoa are less susceptible to cold shock and therefore more resistant to freezing and thawing than ejaculated spermatozoa (Johnson *et al.* 1980).

A positive relationship exists between post-thaw motility and fertility of frozen-thawed semen (Vidament 2005). Improving post-thaw motility of epididymal spermatozoa could improve its fertility as shown recently (Melo *et al.* 2008; Papa *et al.* 2008). For ejaculated spermatozoa, seminal plasma of stallions with good post-thaw spermatozoal motility improves post-thaw motility of stallions with poor post-thaw motility (Aurich *et al.* 1996). Therefore, seminal plasma of stallions with excellent post-thaw motility of ejaculated spermatozoa should be collected and stored for use when epididymal spermatozoa need to be harvested in an emergency situation. Furthermore, the post-thaw addition of seminal plasma may be benefial for post-thaw epididymal spermatozoa motility as well as for longevity, as it has been shown in dogs (Nöthling *et al.* 2007).

7.2 Effect of Seminal Plasma on Morphology of Equine Epididymal Spermatozoa

7.2.1 Effect of Seminal Plasma on Occurance of Overall Sperm Defects

Evaluating the different logistic regression models separately, it can be seen that fresh epididymal sperm (SP- as well as SP+) had similar numbers of overall sperm defects (table 4). Comparing fresh ejaculated spermatozoa with fresh epididymal sperm (SP- as well as SP+) shows that fresh ejaculated spermatozoa have significantly lower numbers of defects for this category. Freezing significantly increased the number of overall defects (nuclear as well as acrosome and tail defects) for Cr-SP- and Cr-E but significantly decreased numbers of defects for Cr-SP+. Frozen-thawed ejaculated spermatozoa still had significantly lower numbers for this category than Cr-SP- or Cr-SP+. The increase in overall defects (nuclear as well as acrosome and tail defects) after freezing for Cr-SP- and Cr-E is expected as membrane and other cryoinjuries to spermatozoa are always encountered due to the freezing process. The decreased overall defects for Cr-SP+ are somewhat suprising but it can be seen that this is due to the fact that the number of distal droplets for Cr-SP+ is dramatically decreased after freezing as compared to Fr-SP+. This explains the general decrease of defects as distal droplets constitute the major portion of tail defects for Fr-SP+.

7.2.2 Effect of Seminal Plasma on Occurance of Distal Droplets

The occurance of distal droplets was significantly higher for fresh and frozen-thawed epididymal spermatozoa (SP+ and SP-) than for fresh and frozen-thawed ejaculated spermatozoa. Interestlingly, before freezing Fr-SP+ showed more distal droplets than Fr-SP- whereas after freezing Cr-SP+ had less distal droplets than Cr-SP-. This indicates that seminal plasma influences the shedding of distal droplets during the cryopreservation process but that it is not absolutely necessary for the process.

It has been shown previously that seminal vesicular fluid in bulls has a droplet removing effect (Bialy and Smith 1958) and a haemolytic factor, phospholipid-binding protein (PBP), in bull vesicular gland fluid has been shown to support droplet removal (Matousek and Kysilka 1980). In our study, even though the exposure time to seminal plasma was comparable for ejaculated as well as epididymal spermatozoa that had been exposed to seminal plasma, ejaculated spermatozoa had significantly less distal droplets than epididymal spermatozoa that had been exposed to seminal plasma. This was occurent for fresh and frozen-thawed spermatozoa. The reason for that remains somewhat unclear but one possible explanation could be that ejaculated spermatozoa have spent a longer time period in the epididymis than epididymal spermatozoa that were flushed from the epididymis. Looking at all four stallions used in this experiment average total spermatozoal numbers in the ejaculates were 6 x 10^9 per collection and 16-47 x 10^9 for epididymal sperm recovery per stallion. Assuming that around 54 x 10^9 spermatozoa are stored in the cauda epididymides as part of the spermatozoal reserve, 2 x 10^9 spermatozoa are stored in the ampullae of the deferent ducts and 2 x 10^9 in the remainder of the deferent ducts (McKinnon and Voss 1992) it can be seen that the latter two fractions constitute a major part of the ejaculated sperm numbers whereas the sperm numbers recovered from the epididymides via flushing where much higher. Only a minor portion of ejaculated

spermatozoa originates from the cauda epididymis. To harvest epididymal spermatozoa we flushed further proximal in the epididymal tail, closer to the epididymal body, than where ejaculated spermatozoa would have been released from during a normal ejaculation. Therefore, ejaculated spermatozoa would have spent a prolonged time period in the epididymides moving from the tail into the ductus deferens and would have been exposed longer to epididymal secretions which are thought to play a role in the droplet shedding process (Barth and Oko 1989).

Another explanation could be that for ejaculated spermatozoa the seminal plasma was excreted during ejaculation from only the specific stallion, while pooled seminal plasma was used for the flushing of the epididymal spermatozoa. This could possibly indicate that stallion-specific factors in seminal plasma help shedding of "own" distal cytoplasmic droplets. Pooling of seminal plasma might have decreased these factors through dilution for the individual stallions and therefore decreased the number of distal droplets that were shed.

Freezing decreased the numbers of distal droplets for ejaculated spermatozoa, epididymal spermatozoa that had been exposed to seminal plasma (SP+) and epididymal spermatozoa that had never been exposed to seminal plasma (SP-).

Cytoplasmic droplets are formed during spermiogenesis, as the spermatid changes from a round to an elongated cell and the cytoplasm is drawn from the head (nuclear) region towards the tail region (Barth and Oko 1989). First, cytoplasmic droplets are called residual bodies which develop at spermiation into a droplet of dense cytoplasm in the neck region of the spermatozoon, the so-called proximal droplet. All spermatozoa show proximal droplets as they enter the caput epididymis. During sperm maturation in the caput and corpus epididymis, migration of the proximal cytoplasmic droplet from the proximal neck location to the distal portion of the midpiece follows. The stimulus for this migration is thought to be associated with the onset of motility (Barth and Oko 1989). Many spermatozoa stored in the cauda epididymis have distal cytoplasmic droplets, but the number of spermatozoa with

droplets in ejaculated semen is low. Our results confirm that the sperm maturation processes in the epididymis regarding cytoplasmic droplets were normal for all four stallions but that possibly the time that the milieu in the cauda epididymis has to influence spermatozoa was insufficient for complete shedding of all distal cytoplasmic droplets in epididymal spermatozoa. As distal cytoplasmic droplets do not have a special significance or a negative impact on fertility, it can be concluded that even though the number of epididymal spermatozoa with distal cytoplasmic droplets was high, it should not impair the use of epididymal spermatozoa for artificial insemination as it was shown in one study recently (Heise *et al.* 2010).

7.2.3 Effect of Seminal Plasma on Occurance of Midpiece Reflexes

Evaluation of midpiece reflexes showed that for fresh as well as for frozen-thawed epididymal spermatozoa SP+ had significantly less midpiece reflexes than Fr-SP- or Fr-E. At the same time, fresh and frozen-thawed epididymal spermatozoa SP- had significantly more midpiece reflexes than ejaculated spermatozoa. Freezing significantly decreased the numbers of midpiece reflexes for epididymal spermatozoa that had been exposed to seminal plasma and for ejaculated spermatozoa, but increased these numbers for epididymal spermatozoa that had never been exposed to seminal plasma.

Midpiece reflexes are common sperm defects in stallions (Brito 2007) and develop in the corpus and cauda epididymis (Barth and Oko 1989). It has been hypothesised that the susceptibility to bending in that area may be due to an increased sensitivity to ions that is aquired in the epididymis (Barth and Oko 1989; Swanson and Boyd 1961). It was also shown that frequent ejaculations decreased the numbers of midpiece reflexes in some bulls indicating that the incidence of midpiece reflexes is related to the length of time

spermatozoa spent in the epididymis (Swanson and Boyd 1961). However, this does not explain our results where epididymal spermatozoa that have been exposed to seminal plasma (fresh and frozen) have significantly less midpiece reflexes than epididymal spermatozoa that have not been exposed to seminal plasma (fresh and frozen). The castration and removal of both epididymides was performed exactly at the same time and the spermatozoa recovered from the epididymes have been exactly the same time in the epididymis prior to the procedure. It does indicate though that seminal plasma exerts a positive effect on epididymal spermatozoa preventing midpiece reflex formation. In bulls, midpiece reflexes can be induced *in vitro* by rapid cooling of semen, exposure of semen to hypotonic solutions, treatment of bulls with estradiol and scrotal insulation (Barth and Oko 1989). This could possibly explain why Fr-SP- had significantly more midpiece reflexes than Fr-SP+ as the different flushing media (freezing extender vs. seminal plasma) created different ionic environments for the epididymal spermaztozoa at the time of flushing.

In some cases the underlying cause for midpiece reflexes has been thought to be a weakness in the midpiece caused by abnormal development in spermiogenesis (Barth and Oko 1989). This cause for midpiece reflexes can be excluded in our case as all sperm categories (Fr-E, Fr-SP+ and Fr-SP-) should have had the same incidence of midpiece reflexes if it was due to spermiogenesis.

Ejaculated spermatozoa and epididymal spermatozoa that had been exposed to seminal plasma both showed significantly less midpiece reflexes after freezing. A possible explanation for that could be that the freezing process helped to shed the cytoplasmic droplets trapped in the bend of midpiece reflexes and consequently helped to straighten the midpieces. Such a bending of sperm tails followed by resuming of a straight conformation has been thought to be possible previously (Dickey 1965) but was not supported by others (Barth and Oko 1989). This could possibly also explain why the percentage of midpiece reflexes increased after freezing for epididymal spermatozoa that had never been exposed to

seminal plasma as it might indicate a protective effect of seminal plasma on midpieces during cryopreservation. Furthermore, epididymal spermatozoa that had never been exposed to seminal plasma did not experience the effect of seminal plasma on distal droplet shedding which might predispose them further to midpiece reflex formation during cryopreservation due to a higher percentage of retained distal droplets.

Spermatozoa with midpiece reflexes display a reverse swimming motion in a circular pattern and are therefore not able to fertilize an oocyte as they do not reach the site of fertilization. Midpiece reflexes have been classified as minor defects with no or little effect on fertility (Barth and Oko 1989) and midpiece reflexes have been found in up to 25% of spermatozoa from normal fertile bulls. It can probably be assumed for our experiment that the effect of the observed midpiece reflexes on fertility would be low.

7.2.4 Effect of Seminal Plasma on Occurance of Damaged/ Degenerated Acrosomes

The occurance of damaged/degenerated acrosomes was similar for Fr-SP- and Fr-SP+. Fresh ejaculated spermatozoa had significantly more damaged/degenerate acrosomes than Fr-SP- and Fr-SP+. Freezing significantly increased the incidence of damaged/ degenerate acrosomes for ejaculated as well as epididymal spermatozoa (SP+ and SP-). After freezing, Cr-SP+ had significantly more damaged/degenerate acrosomes than Cr-SP- whereas the number of spermatozoa with damaged/ degenerate acrosomes was similar for Cr-SP- and Cr-E.

These findings show that the percentage of damaged/ degenerate acrosomes is significantly increased for frozen-thawed spermatozoa (ejaculated and epididymal spermatozoa) which is most likely due to cryoinjury during the freezing process. Our findings also show that the

exposure of epididymal spermatozoa to seminal plasma prior to freezing increases the chances for cryoinjury regarding the acrosome. Even though the percentage of spermatozoa with damaged/degenerate acrosomes increased after freezing and thawing, the values were still within normal limits expected for fertile stallions as described by Palmer (1984) and should therefore not have a major impact on fertility.

7.2.5 Effect of Seminal Plasma on Occurance of Nuclear Defects and Knobbed Acrosomes

The occurance of nuclear defects and knobbed acrosomes in epididymal spermatozoa was not influenced by flushing medium or freezing. This finding was to be expected as nuclear defects in general and knobbed acrosomes specifically have been shown to be developed during spermiogenesis (Barth and Oko 1989) and should not be affected by sperm maturation, exposure to different flushing media or cryopreservation.

7.3 Effect of Seminal Plasma on Viability of Equine Epididymal Spermatozoa

There was no difference in viability for morphologically normal fresh epididymal spermatozoa that had been exposed to seminal plasma and fresh epididymal spermatozoa that had never been exposed to seminal plasma. Furthermore, the percentage of viable morphologically normal spermatozoa is higher for epididymal spermatozoa (Fr-SP+ and Fr-SP-) than for fresh ejaculated spermatozoa. After freezing however, there is no difference in percentage viable morphologically normal spermatozoa for epididymal spermatozoa (Cr-SP+ and Cr-SP-) and ejaculated spermatozoa. This shows that seminal plasma does not influence the viability of morphologically normal epididymal spermatozoa (fresh and frozen-thawed) and that the viability of morphologically normal frozen-thawed epididymal spermatozoa (Cr-SP+ and Cr-SP-) is similar to frozen-thawed ejaculated spermatozoa. Viability was evaluated as spermatozoal membrane integrity using the supra-vital stain eosin-nigrosin. An intact spermatozoal membrane is necessary for capacitation to take place in order to ensure the acrosome reaction and fertilisation. Heparin-binding proteins, produced by the accessory sex glands, are bound to the surface of sperm cells and increase the sperm cell's ability to respond to capacitation-inducing glycosaminoglycans, such as heparin (Nass 1990). These components enable sperm cells to undergo the acrosome reaction.

It has also been shown that the addition of seminal plasma to bovine epididymal sperm increased the susceptibility of spermatozoa to zona pellucida proteins (Flormann 1988). Even though these processes are necessary it has been shown that prolonged exposure of spermatozoa to seminal plasma affects sperm longevity (Babcock 1979). This could possibly explain why fresh ejaculated spermatozoa showed less live spermatozoa than fresh

epididymal spermatozoa (SP+ and SP-). As mentioned earlier, ejaculated spermatozoa would have spent a prolonged time period in the epididymides as compared to epididymal spermatozoa and would have been exposed longer to epididymal secretions which constitute a part of the seminal plasma and which could have decreased longevity (Baas *et al.* 1983; Babcock *et al.* 1979) and increased the percentage of dead spermatozoa. The time factor could possibly also explain why there is no difference in viability of fresh epididymal spermatozoa that had been exposed to seminal plasma and that had never been exposed to seminal plasma as the exposure time was possibly too short to have exerted a negative effect on viability for epididymal spermatozoa that had been exposed to seminal plasma.

After freezing however, there was no difference in viability of ejaculated spermatozoa and epididymal spermatozoa (SP+ and SP-). Freezing injuries to spermatozoa are mainly caused by plasma membrane disruption due to thermal, mechanical, chemical and osmotic stresses (Amann and Pickett 1987; Parks and Graham 1992). These stresses compromise sperm membranes due to reordering of membrane lipids, thus disturbing the lipid-lipid and lipid-protein associations that are required for normal membrane function (Parks and Graham 1992).

Cryopreservation also induces changes in the plasma membrane of "premature capacitation" that are probably due to lipid loss from the plasma membrane during freezing and thawing as well as loss of decapacitation factors that are removed with the seminal plasma due to centrifugation (Watson 1995). Capacitated sperm have a destabilised plasma membrane, which is sensitive to even small environmental stress. The fragility of plasma membranes is shown by the fact that they will undergo spontaneous acrosome reaction as a result of simply cooling them from 38 to 30 °C (Gadella *et al.* 2001; Gadella 2001). This explains the decreased viability for frozen-thawed ejaculated as well as epididymal spermatozoa (SP+ and SP-).

Our results agree with previous findings (Moore *et al.* 2005) which showed that seminal plasma did not affect post-thaw viability of ejaculated spermatozoa. It is possible that no difference in post-thaw viability was seen for ejaculated and epididymal spermatozoa (SP+ and SP-) due to the pre-freeze centrifugation process which removed most of the seminal plasma from ejaculated as well as epididymal sperm (SP+).

7.4 Epididymal sperm numbers

Expected sperm numbers that can be harvested from both cauda epididymides of a stallion should be about 54 x 10^9 sperm or approximately 61% of the sperm in the excurrent duct system (Amann *et al.* 1979). Epididymal sperm numbers recovered in this experiment were close to these expected numbers for three of the four stallions and much higher than in previously published reports (Bruemmer *et al.* 2002; Cary *et al.* 2004; James *et al.* 2002) but quite consistent with the numbers provided by Bruemmer (2006). Our results support the use of retrograde flushing to harvest epididymal spermatozoa as opposed to float-up methods.

8 Conclusions

The authors realise that the outcome regarding the occurance of certain spermatozoal defects might have been accentuated due to the fact that only four stallions were used for this study. Nevertheless, our study guides the way to a possible larger scale trial investigating the effects of seminal plasma on post-thaw parameters of stallion epididymal spermatozoa.

This study confirms findings of previous reports that seminal plasma stimulates initial motility of fresh epididymal stallion spermatozoa while this difference in progressive motility is no longer present post-thaw.

Our results indicate that progressive motility of fresh or frozen-thawed ejaculated stallion spermatozoa is not always a good indicator for post-thaw progressive motility of epididymal spermatozoa.

We also show that seminal plasma has a positive influence on the incidence of overall sperm defects, midpiece reflexes and distal cytoplasmic droplets in frozen-thawed stallion epididymal spermatozoa while the occurance of midpiece reflexes is likely to be linked to distal cytoplasmic droplets.

Furthermore, we could show that seminal plasma does not have an influence on viability of fresh and frozen-thawed morphologically normal epididymal spermatozoa.

In conclusion, based on the results of this study and taking recent findings concerning fertility of epididymal spermatozoa (Heise *et al.* 2010) into consideration, we recommend the retrograde flushing technique using seminal plasma as flushing medium to harvest and freeze stallion epididymal spermatozoa.

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