New methods to assess and optimise fertility in breeding stallions: H1047052. Final report

In December 2010 SSH granted funding for two years for a (modified) project involving researchers at the Swedish University of Agriculture (SLU) and Flyinge AB. The objectives were as follows: (i) to test whether the improvement in sperm quality observed in laboratory assays following Single Layer Centrifugation (SLC) is translated into improved pregnancy rates in normally fertile artificially inseminated mares; and (ii) to determine whether SLC and/or oocyte- or zona pellucida binding assays, or some other marker, could be used as an indicator of a stallion´s potential fertility when his semen is used for artificial insemination (AI). The third objective (to develop new extenders and protocols for cryopreserving stallion semen, or modifications to existing transport boxes for cooled semen doses, to enhance sperm survival) was refused.

Background

The equine industry in Sweden represents an important part of the national economy, having an annual turnover of approximately 45 billion SEK (Jordbruksverket, 2008); horse breeding, and the AI industry particularly, are significant sources of income for Sweden. The total number of mares bred by AI in Sweden in 2008 was 4978, of which 71% received cooled, transported semen doses (Elisabet Ernblad, personal communication to Anne-Marie Dalin). Over the last 15 years in Sweden, there has been a steady decline in foaling rates in warmblood mares following AI, concomitant with the increased use of transported, cooled semen doses for AI (Avelsförening för Svenska Warmblodiga Hästen, 2008).The reason for this apparent decrease in fertility has not been determined but may be due, in part, to lack of an effective, objective *in vitro* evaluation of semen quality – the "fertility potential" of a stallion (Colenbrander et al., 2003). Alternatively, it may represent an increase in the use of stallions whose semen quality is initially good but whose spermatozoa do not tolerate cooling (Aurich, 2008). Semen quality evaluation at the stud is usually confined to a subjective assessment of sperm motility immediately after semen collection (Malmgren et al., 1994), despite the fact that this parameter is not generally indicative of sperm fertilising ability (Watson, 1990). Therefore, it is difficult to make a meaningful retrospective analysis of the relationship between semen quality at the time of semen collection and pregnancy rates following AI. Moreover, although a subjective evaluation of sperm motility may be made on receipt of transported semen doses or at the time of insemination (up to 24 h after semen collection), no objective assessments of sperm quality are routinely made prior to AI with stored semen.

In studies using semen from Swedish warmblood stallions, a significant correlation was observed between pregnancy rate and the proportion of spermatozoa with normal morphology in the original ejaculate, as well as a significant negative correlation between pregnancy rate and sperm chromatin integrity (Morrell et al, 2008). Obviously, semen quality is not the only factor influencing pregnancy rate: there are many stages involved in the whole process, such as collection and preparation of semen doses, their transport, storage and handling at the receiving stud, quite apart from factors relating to the mare or the skill of the inseminator. However, one could anticipate that improving the quality of the sperm doses for AI will lead to an increase in pregnancy rate, if all other stages in the process are optimal, thus leading to a reversal in the downward trend in foaling rates.

Recently, a method has been developed at the Swedish University of Agricultural Sciences (SLU) whereby the most robust spermatozoa are selected from stallion ejaculates by centrifugation through a single layer of colloid (Single Layer Centrifugation, SLC). The SLC-method was shown to select the most motile spermatozoa, and those with normal morphology,

intact plasma membranes and good chromatin integrity from the rest of the ejaculate (reviewed by Morrell & Rodriguez-Martinez, 2009). The capacitation status of the spermatozoa is not affected by the centrifugation (Bergqvist et al. 2009), and they retain their motility, viability and chromatin integrity for longer than unselected spermatozoa (Johannisson et al, 2009; Morrell et al, 2009a). Furthermore, in a collaborative study with a Spanish group, SLC was shown to be highly effective in selecting robust stallion spermatozoa from frozen-thawed semen (Garcia Macias et al, 2009; Macias et al, 2009). SLC-selected spermatozoa function normally when injected into equine oocytes. The SLC-method has been scaled-up to allow large volumes of semen to be processed (Morrell et al, 2009b). A limited number of AIs have been carried out with SLC-selected sperm doses prepared from stallions with poor quality semen and the results are encouraging, in that SLC-selected sperm doses can generate pregnancies after AI (Morrell et al, 2010). However, the benefits of using SLC for stallions of "normal" fertility have not been investigated. The first objective of the present study, therefore, was to conduct an AI trial with mares inseminated with either SLC-selected sperm samples or control (unselected) sperm doses from normal stallions, to determine whether the improvement in sperm quality is reflected in an improvement in pregnancy rate. It would also be interesting to determine how long SLC-selected sperm samples retain their fertilizing capability.

The presence of seminal plasma (SP) in an equine insemination dose may be necessary for promoting uterine clearance prior to implantation. SLC removes seminal plasma from spermatozoa at the same time that the best spermatozoa are selected from the rest of the ejaculate. Therefore, the effect of adding back SP to SLC-selected sperm samples should be investigated.

The second main area of interest in this project concerned predicting which males potentially have good fertility. Many attempts have been made to identify predictive laboratory tests of sperm fertility, both to improve the quality of semen doses for AI and to identify potential breeding sires. The most widely-used method of assessing sperm quality at the semen collection station is by evaluating subjective motility, yet this parameter is poorly linked with subsequent fertility. Some authors have called for a battery of tests to be used in semen assessment, to arrive at an estimate of likely fertilising capability of the ejaculates (Rodriguez-Martinez 2007), but such analyses are not usually available on studs. Thus the most accurate evaluation of male fertility is still by means of a time-consuming AI trial (Colenbrander et al, 2003). There would be a considerable saving in time if a test could be developed for use at the point of semen collection that is a reliable indicator of the potential fertilising capability of the spermatozoa after AI under normal conditions, or even of the likely fertility of the individual stallion.

There are several candidates that could serve as potential indicators of sperm- or individual male fertility.

1) The yield of spermatozoa obtained after SLC, i.e. the number of spermatozoa passing through the colloid, expressed as a proportion (%) of the initial load. Retrospective analysis of our previous results showed that the yield could be related to sperm morphology and chromatin integrity in the original ejaculate (Morrell et al, unpublished data). Since these parameters are known to be related to pregnancy rate, it seems logical that the yield of spermatozoa after SLC may also be related to pregnancy rate and, therefore, indicative of a stallion´s potential fertility.

2) Hyaluronan binding assay (HBA). As epididymal spermatozoa mature, they develop the capability to be motile and also to fertilise. Both of these abilities have been linked to the formation of hyaluronic acid receptors on the sperm membrane and their ability to bind to hyaluronic acid. An assay is commercially available to test the ability of human spermatozoa to bind to glass slides coated with synthetic hyaluronan, which is indicative of fertility in IVF

(Huszar et al, 2003). However, it is not known whether the same hyaluronic acid binding sites found on human spermatozoa are present on animal spermatozoa, nor whether the results of the test are related to pregnancy rates following AI, since spermatozoa face many more challenges in the female reproductive tract than in IVF.
3) Activation of stallion spermatozoa exposed to zona-pellucida (ZP) proteins derived from equine oocytes. It may be possible to detect differences between stallions in the ability of their spermatozoa to activate when challenged with ZP-proteins and to correlate this parameter with *in vivo* pregnancy results after AI. Unfortunately it is not practical to develop an *in vitro* assay for stud use based on equine oocytes since such material is scarce in Sweden and the procedures would require a specialised laboratory and skilled personnel. However, if an assay based on the sperm response to challenge with ZP-proteins was correlated with fertility, it might be feasible to develop an assay for on-stud use based on synthesised proteins. Equine oocytes are available from Italy and a collaboration has been established with Professor Cesare Galli to send material to SLU for a preliminary study. It was intended to extract the ZP proteins by procedures already available at SLU and determine whether spermatozoa become activated when challenged, sperm activation being a pre-requisite for fertilisation.
4) Heterologous penetration assay. The ability of stallion spermatozoa to penetrate denuded cow oocytes will be investigated and correlated with pregnancy rate after AI using spermatozoa from the same ejaculates. Such penetration tests have been reported previously (Sinowatz et al, 2003) but a potential correlation with *in vivo* fertility has not been determined.

The third part of the project (cryopreservation) was removed at the request of SSH. An additional modification specified that the number of mares to be used per stallion in the fertility trial was to be increased.

Methods and Results

1) <u>Fertility trials</u>

a) Cooled sperm doses

Ten stallions with no known fertility problems were chosen for the trial. Ejaculates (10 per stallion) were collected and extended immediately 1:1 (v/v) in extender at 35°C. The ejaculate was split, with one part being used to prepare AI doses and the other half being processed by SLC as follows. The sperm concentration was adjusted to approximately 100 $x10^6$/mL (90-$120x10^6$/mL) and used for SLC with Androcoll-E to prepare doses for AI. Control and SLC-selected sperm doses were sent out to other studs in insulated boxes containing a cold pack and were used for AI approximately 24h after semen collection. The mares for AI were of normal fertility i.e. not suspected of being sub-fertile. They were checked for follicular development by ultrasound examination and inseminated at a suitable time relative to ovulation according to standard practice. Pregnancy diagnosis was made by ultrasound scanning at approximately 16 days post-ovulation.

The response from studs in Sweden was initially very encouraging with 9 studs expressing an interest in participating in the study, some with more than one stallion. However, only two studs were able to complete the number of mares required for each stallion. Since it is important to have sufficient numbers of mares for each treatment and each stallion, data sets with less than 6 mares per treatment have been removed from the statistical analysis. Colleagues in other countries were approached and were able find sufficient mares for further stallions. The results from one stallion were removed from the data set because the poor pregnancy rate achieved with the control inseminations indicated that there was a severe fertility problem with the stallion. Pregnancy data have not been received yet for two stallions or from another stallion that has been recruited to replace the one that was removed. However,

the data for the seven stallions so far show a highly significant improvement in pregnancy rate when SLC-selected sperm doses are used for AI (49/67 (73%) compared with 31/68 (46%) for controls; Chi squared value =10.6; P<0.01)
For 3 sets of donor mares for embryo production, where the same mares were used for control and SLC doses in different cycles, also produced a significant result in favour of SLC-selected sperm samples. The results were 11 and 19 embryos from 29 inseminations for control and SLC doses, respectively, which is significant (Chi squared = 4.4; P<0.05).

Table 1: Pregnancy rates in mares artificially inseminated with either SLC-selected sperm doses or control (untreated) sperm doses.

Stallion	Pregnancy rates	
	Control (uncentrifuged)	SLC
1	1/7	6/7
2	5/10	4/7
3	4/7	6/6
4	7/15	9/16
5	4/10	8/10
6	5/9	9/11
7	5/10	7/10
Total	31/68	49/67

Note: inseminations with semen doses and SLC-selected spermatozoa from another stallion were done but these were not included in the statistical analysis due to the poor quality of the original semen. Inseminations are currently being performed with semen from a further 3 stallions.

b) Increased storage time
SLC-selected sperm samples show better retention of motility and chromatin integrity than unselected sperm samples during prolonged cooled storage. However, it was not known whether this improved quality was associated with retention of fertilising capability. Therefore, SLC-sperm doses were prepared at Flyinge, cooled and sent to SLU for insemination at 48h, 72h or 96h after semen collection. The results (Table 2) indicated that fertilising capability was retained for at least 96h after semen collection in SLC-selected samples. Although it was not possible to include control inseminations due to the small number of mares available, the accepted useable life of cooled stallion semen doses is usually considered to be 24-36h. Therefore, achieving such good conception rates after 96h of storage is a very encouraging result.

Table 2: Pregnancy rates in mares artificially inseminated with SLC-selected sperm doses at 48h, 72h or 96h after semen collection.

Time in cooled storage	Conception rate
48h	4/5
72h	4/7
96h	3/4

c) SLC with stored semen

The purpose of this experiment was to determine if the quality of stored stallion semen doses could be enhanced by SLC using Androcoll-E with stored semen at 24h after semen collection. Three semen doses from each of fifteen stallions were transported overnight to the Swedish University of Agricultural Sciences (SLU) for processing 24h after semen collection. Sperm quality in the resulting SLC-selected samples was significantly improved compared to the uncentrifuged samples: mean progressive motility was increased by 8% on the day of processing ($P<0.001$) and by 13% after 24h cold storage ($P<0.001$), normal morphology was increased by 4% ($P<0.01$), whereas mean %DFI was decreased by 2% ($P<0.001$). The improvement in sperm quality was similar to that obtained by processing fresh samples by SLC with Androcoll-E, although sperm quality was not maintained for as long as with fresh samples. A mare was inseminated with one of the sperm doses at 72h after semen collection (48h after SLC) and an embryo was detected 16 days later by ultrasound examination. These results suggest that SLC can be used with stored semen as an additional option for improving sperm quality for artificial insemination.

d) Effect of season on sperm quality

There are anecdotal reports that equine fertility may decline towards the end of the breeding season. Previous studies have examined differences in sperm quality between the breeding season and non-breeding season but few studies have investigated the proportions of superoxide or peroxide containing spermatozoa at different times during the breeding season. The content of these reactive oxygen species (ROS) in stallion semen was measured at the beginning and end of the Swedish breeding season, using flow cytometric analysis of the fluorescence produced after staining with hydroethidium and dichlorodihydrofluorescein diacetate. Superoxide production by stallion spermatozoa was found to be higher at the start than at the end of the breeding season in Sweden (22±16% versus 9±6%, $P<0.05$), whereas sperm motility was lower (total motility 80±9% versus 90±6%, $P<0.01$; progressive motility 55±12% versus 60±8%, $P<0.05$, at the beginning and end of the breeding season respectively). The mean values of the other parameters of sperm quality measured did not differ with time within the breeding season although differences did occur for individual stallions. Since ROS are known to affect fertility, there may well be differences in stallion sperm fertility between the beginning and end of the breeding season.

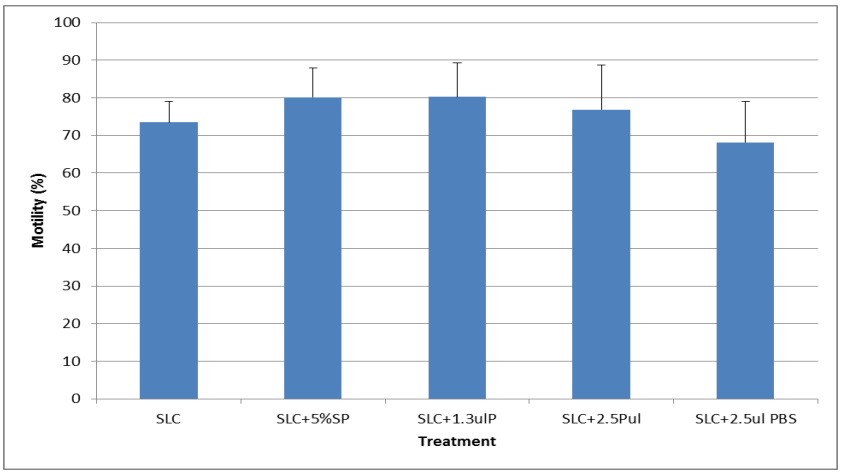
In addition, the effects of a SLC on ROS-content were investigated. SLC was found to select motile spermatozoa that contained less superoxide (16±14% versus 23±18%; $P<0.01$) and less peroxide (0.3±0.8 versus 1±2%; $P<0.01$) than uncentrifuged controls, although the spermatozoa were capable of producing ROS when stimulated with menadione. This reduced peroxide production may contribute to the enhanced sperm survival (retention of motility) seen in the SLC samples during storage.

e) The effect of seminal plasma and prostasomes on SLC-selected sperm samples

Sperm progressive motility ($P<0.01$) and velocity were increased by SP compared to SLC samples. However, after 24 h cold storage of treated samples, progressive motility was not different for the SP-treated groups compared to SLC, whereas chromatin damage (%DFI) was higher. In contrast, adding SP to untreated 24h-stored SLC samples did not affect progressive motility although it did increase the proportion of sperm velocity. Thus to summarize, 5-10% SP can be added back to SLC-selected samples if considered necessary for an optimal uterine response but it should be added immediately before insemination rather than before storage of the sperm dose, to benefit from the transient increase in sperm progressive motility and avoid increased chromatin damage.

In an attempt to identify the component of SP that activates the spermatozoa, prostasomes were prepared from stallion semen by co-workers at the Academic Hospital, Uppsala. When added to SLC-selected stallion spermatozoa in an amount corresponding to 5 or 10% SP, the same mean increase in sperm motility was observed as when SP was added (6.7% and 6.8% for SP and prostasomes, respectively), whereas the corresponding volume of phosphate buffered saline caused a 5.3% decrease in motility (Figure 1), suggesting that prostasomes may indeed be the component of SP responsible for activating sperm motility. Further studies will be made to determine the mechanism of action by which prostasomes operate and whether prostasomes from individual stallions differ in their effects on spermatozoa.

Figure 1: effect of adding seminal plasma or prostasomes to stallion spermatozoa from which all seminal plasma had been removed by Single Layer Centrifugation.



2. Predictors of fertility

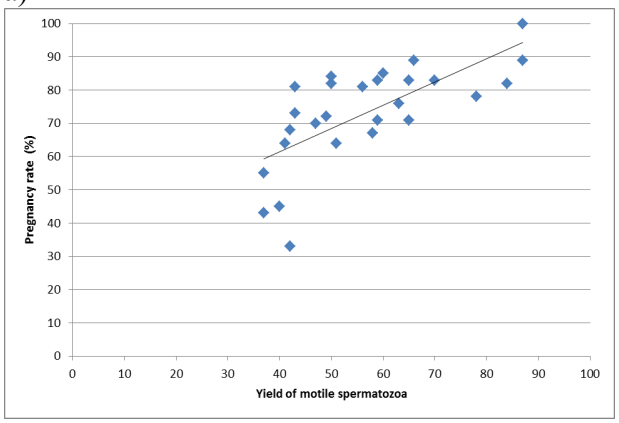
a) Yield after SLC. Our hypothesis is that one or more of the following assays: yield of spermatozoa recovered after SLC, HBA, response to ZP, and a heterologous sperm penetration assay (*in vitro* penetration of denuded bovine oocytes by stallion spermatozoa), could be used as diagnostic tools for predicting the likely fertilising capability of equine insemination doses. Aliquots of ejaculates from 10 stallions breeding stallions (4 ejaculates per stallion) were used for SLC under standard conditions, to establish whether the yield of spermatozoa recovered after SLC was correlated to certain parameters in the original ejaculate known to be associated with fertility. The remainder of the ejaculates was used to prepare AI doses and was sent to studs in the usual manner.
The yield of motile spermatozoa after SLC was found to be highly correlated with pregnancy rate ($r = 0.69$; $P<0.001$) (Figure 2). In this respect it is a similar indicator of potential fertility as viability ($r = 0.76$; $P<0.001$) or chromatin damage ($r = - 0.74$; $P<0.001$) and better than progressive motility ($r = 0.6$; $P<0.001$) or normal morphology ($r = 0.56$; $P<0.01$). The advantage of being able to use SLC to predict fertility rather than viability or chromatin integrity is that SLC does not require expensive equipment whereas viability and chromatin integrity require a flow cytometer, which is beyond the means of most studs.

b) **Zona pellucida binding proteins**. Equine oocytes were collected and sent from Prof. Galli´s laboratory in Cremona. Proteins were extracted from the zona pellucidae, which were then characterized and tested for their effects on spermatozoa. Sperm capacitation was elicited in both control and SLC samples, with no difference between groups. However, the extraction

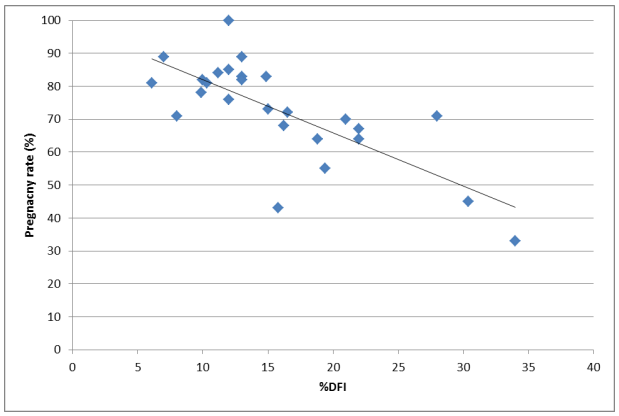
process was judged to yield too low a concentration of protein to be useful as a diagnostic test of sperm fertility.

Figure 2: Relationship between a) Pregnancy rate and yield of motile spermatozoa after SLC, (b) Pregnancy rate and %DFI, and (c) Pregnancy rate and normal morphology (c).

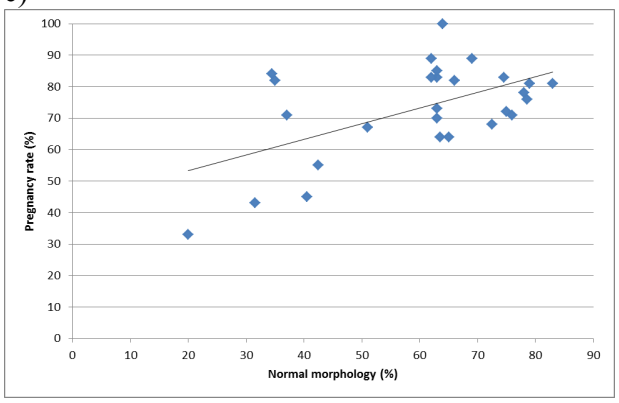
a)



b)



c)



c) **Heterologous zona binding assay.** The ability of stallion spermatozoa to bind to bovine oocytes was investigated, with the intention of correlating the binding ability with pregnancy rate after AI using spermatozoa from the same ejaculates. Unfortunately the method did not function well in our hands. We have since made contact with a researcher in Portugal who has been successful with this technique and is prepared to help us to set up this assay.

3. Removal of pathogens from stallion semen by SLC

This experiment was carried out at the Gluck Equine Research Center, Kentucky, where three stallions that are shedding equine arteritis virus are kept. The use of several modifications of Single Layer Centrifugation (SLC, SLC with an inner tube and double SLC) were evaluated for their ability to separate spermatozoa from virus in ejaculates from carrier stallions. The three types of SLC significantly reduced the virus titer in fresh semen at 0h and for stored semen at 24h ($P<0.001$) but did not completely eliminate the virus. Although virus titers were reduced in the SLC-samples, significant levels of infectivity still remained, especially in stallions shedding large amounts of virus. It remains to be determined whether SLC-processed spermatozoa from stallions shedding low virus titers retain sufficient EAV to cause infection in mares through artificial insemination.

The ability of SLC to remove bacteria was determined by adding loading doses of cultured bacteria to freshly collected ejaculates before processing by SLC. The resulting sperm pellet and controls (unprocessed semen) were cultured and the bacteria identified. In the first experiment, doses of *E. coli* $2x10^2$ to $2x10^7$ colony forming units were added to aliquots of pooled ejaculates from three stallions. For loading doses of > $5x10^4$cfu/mL i.e. added bacteria plus natural contaminants, more than 90% of the bacteria were removed. In the second experiment, *T. equi* or a mix of *E. coli, Klebsiella* and *Streptococcus zooepidemicus* were added to eight stallion ejaculates. Varying proportions of different bacteria were removed, ranging from 68% for naturally occurring *Corynebacterium* to >97% for added cultured *E.coli.* These results suggest that SLC can separate spermatozoa from bacteria in stallion ejaculates but does not remove all the bacteria. Whether the remaining bacteria would be present in sufficient numbers to affect sperm quality during storage or, in the case of *T. equi,* cause infection inseminated is not known. However, physical removal of the bacteria could be a useful alternative to adding antibiotics to semen extenders.

Relevance to the equine industry in Sweden

The equine breeding industry in Sweden has expressed considerable interest in being able to improve the quality of sperm doses for AI, both to increase pregnancy rates on studs throughout the country and to improve the reputation and competitiveness of the national semen industry globally. Previous results indicated that the sperm quality of poor quality ejaculates could be improved by SLC.The results from the present study show that the fertilizing ability of SLC-selected sperm samples from stallions of “normal” fertility is retained during storage for up to 96h, and the scaled-up method is suitable for use by stud personnel at any stud. The second question, identifying an indicator of potential fertility in AI, is also of considerable interest to the equine breeding industry. At present, the only way of determining the stallion´s current fertility is to use the semen for AI, which is a time-consuming and costly method. Sufficient AIs must be carried out to allow a proper evaluation to be made and to overcome extraneous factors, such as the ability of the mares to conceive, correct AI technique and timing of AI relative to ovulation, which might affect the results. There is also an environmental “cost” of transporting cooled AI doses around the country, only to discover that the sperm doses do not generate pregnancies after AI. Thus a reliable indicator of potential fertility could help to reduce the environmental impact of the equine breeding industry. There was a significant relationship between the yield of spermatozoa obtained after SLC and the pregnancy rate of the unselected ejaculate, indicating that SLC could be used as a diagnostic tool for predicting the fertility of a given stallion or ejaculate without needing an AI trial. The method is fast and can be used at the stud, whereas other potential diagnostic methods require either expensive equipment (CASA or a flow cytometer) or that the sample is submitted for morphological examination, which is time consuming and expensive.

Several countries have adopted strategies for preventing and controlling equine viral arteritis based on vaccination and restricting the breeding activities of carrier stallions. However, in some cases, carrier stallions are only identified after they have transmitted virus to a mare. Therefore, a mechanism for separating virus from spermatozoa in the semen of carrier stallions would facilitate control measures for preventing disease transmission. Although SLC significantly reduced the virus titer in sperm samples, some virus remained. Further research into the use of SLC to separate spermatozoa from virus is warranted. Similarly, the possibility of removing bacteria from semen doses by SLC instead of adding antibiotics to semen extenders is interesting and requires following up.

We would like to thank Stiftelsen för Häst Forskning for financing this project. The funding enabled us to present results on the usefulness of SLC for the equine breeding industry, both for the Swedish market and in other countries. We were able to continue our international collaborations in Spain, Italy, Portugal, the United Kingdom and the United States, and to develop new collaborations e.g. in Belgium, Germany, and with new groups in Spain and the United States. The re-arrangement of the stud business at Flyinge AB resulted in the loss of this stud as a collaboration partner but new collaborations have been established with other studs in Sweden.

Dissemination of results

a. Papers arising from the project

Morrell JM, Macias Garcia B, Pena FJ, Johannisson A. (2011) Processing stored stallion semen doses by Single Layer Centrifugation. Theriogenology 76, 1424-1432.
Morrell JM. Biomimetics in Action: Practical Applications of Single Layer Centrifugation for equine breeding. Veterinary Science and Technology 2:107 Doi:10.4172/2157-7579.1000107.
Morrell JM, Pihl, J, Dalin A-M, Johannisson A. (2012) Restoration of seminal plasma to stallion spermatozoa selected by colloid centrifugation increases sperm progressive motility but is detrimental to chromatin integrity. Theriogenology 78; 345-352.
González-Fernández L, Morrell JM, Peña FJ, Macías-García B. (2012) Osmotic shock induces structural damage on equine spermatozoa plasmalemma and mitochondria. Theriogenology 78, 415-22.
Macías García B, González Fernández L, Gallardo Bolaños JM, Peña FJ, Johannisson A, Morrell JM. (2012) Androcoll-E-Large selects a live subset of stallion sperm capable of producing ROS. Animal Reproduction Science 132, 74-82.
Morrell JM. (2012) Stallion sperm selection: past, present and future trends. Journal of Equine Veterinary Science 32, 436 – 440.
Lindahl J, Dalin A-M, Stuhtmann G, Morrell JM (2012) Stallion spermatozoa selected by Single Layer Centrifugation are capable of fertilization after storage for up to 96h at 6˚C prior to artificial insemination. Acta Veterinaria Scandinavica 54, 40.
Morrell JM, Timoney P, Klein C, Shuck K, Campos J, Troedsson M. (2012) Single Layer Centrifugation reduces equine arteritis virus titer in the semen of shedding stallions. Reproduction in Domestic Animals doi: 10.1111/rda.12133.
Balao da Silva CM, Ortega Ferrusola C, Morillo Rodriguez A, Gallardo Bolaños JM, Plaza Dávila M, Morrell JM, Rodriguez Martínez H, Tapia JA, Aparicio IM, Peña FJ. (2013) Sex sorting increases the permeability of the membrane of stallion spermatozoa. Animal Reproduction Science *In press*

Ronquist KG, Ek B, Ronquist G, Morrell JM, Carlsson L, Larsson A (2013) Biochemical characterization of stallion prostasomes and comparison to their human counterparts. Systems Biology in Reproductive Medicine. *In Press*
Morrell JM, Winblad C, Georgakas A, Stuhtmann G, Humblot P, Johannisson A. Reactive oxygen species in stallion semen can be affected by season and colloid centrifugation. Animal Reproduction Science *In press*
Ronquist GK, Ek B, Morrell JM, Stavreus-Evers A, Ström Holst B, Humblot P, Ronquist G, Larsson A. Prostasomes from four different species are able to produce extracellular adenosine triphosphate (ATP) Biochimica et Biophysica Acta *In press*

b. Conference presentations (ESDAR 2011, 2012; ICAR 2012; ISSR 2012).

Lindahl J, Dalin A-M, Morrell, JM (2011) Pregnancies in mares inseminated with spermatozoa selected by Single Layer Centrifugation and stored for 48h or 72h. Reprod Domest Anim. 46, Suppl. 3, P158.
JM Morrell, B Macias Garcia, F.J Peña, S Meurling, A Johannisson. Single Layer Centrifugation of cooled stallion semen with Androcoll-E improves sperm quality. Reprod Domest Anim. 46, Suppl. 3, P189.
Morrell, JM, Baule C, Wallgren M, Blomqvist G. (2011) Removal of Equine Arteritis Virus from stallion semen doses by Single Layer Centrifugation with Androcoll-E. Proc Annual meeting of British Equine Veterinary Association, Liverpool, UK.
Lindahl J, Dalin A-M, Morrell, JM (2011) Pregnancies in mares inseminated with spermatozoa selected by Single Layer Centrifugation and stored for 48h or 72h. Reprod Domest Anim. 46, Suppl. 3, P158.
Balao da Silva CM, Ortega Ferrusola C, Macías García B, Morillo Rodriguez A, Gallardo Bolaños JM, Aparicio IM, Tapia JA, Morrell JM, Rodriguez Martinez H, Peña FJ.
Effect of Hoescht 33342 on stallion spermatozoa incubated in KMT or modified INRA96-Tyrodes. Reprod Domest Anim. 46, Suppl. 3, P37.
JM Morrell C Winblad P Humblot, A Johannisson (2012) Influence of time within in the breeding season on ROS production and stallion sperm quality. Reproduction in Domestic Animals 47, Suppl 4, p585, #3008.
I. Oritz , J Dorado, JM Morrell, D Acha, L Ramírez, M Urbano, JJ Carrasco, V Gómez-Arrones, R Calero, M Hidalgo (2012) Androcoll-E Large improves kinematic parameters on donkey semen after 24h cool storage. Reproduction in Domestic Animals 47, Suppl. 3, 113-114.I Ortiz, J Dorado, JM Morrell, D Acha, L Ramírez, M Urbano, JJ Carrasco, V Gómez-Arrones, R Calero, M Hidalgo (2012) Sperm motility differences between donkey cooled sperm processed or not with Androcoll-E Large. JEVS 32, 504-505.
M Hoogewijs, S Piepers, J Govaere, C De Schauwer, A de Kruif, JM Morrell (2012) Sperm longevity following pre-freeze sperm selection. JEVS, 32, 489.
JM Morrell, C Winblad, A Johannisson (2012) Production of Reactive Oxygen Species is lower in stallion spermatozoa after Single Layer Centrifugation with Androcoll-E. JEVS 32, 500.
Morrell JM, Lundgren A, Humblot P, Johannisson A. (2012) Reactive oxygen species in stored stallion semen. Reprod Fertil Dev. 25, 153-4.

c. Presentations to Swedish Stud personnel
The preliminary results were presented during the Aveslchampionat at Flyinge in October 2011 in the form of posters and handouts.
Specialisation course for stud veterinarians SLU 2011, 2012.
Numerous personal visits to studs.