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COMMERCIAL BREEDING WITH SEXED STALLION SEMEN: REALITY OR FICTION?

Servicio comercial con semen sexado de Potro: ¿Realidad o ficción?

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INTRODUCTION

Selecting or determining the gender of the offspring prior to conception or to birth has been a goal of scientists, farmers and lay people. Depending on the species in question each group will have different reasons for performing this procedure. Dairy farmers, for example, will obviously find an economic benefit of producing female calves, while beef producers would prefer males. Therefore, there is an easy and objective way to determine the difference in economic value between the desired and non-desired gender in the cattle industry. In addition, some deer species males are highly sought because of their antlers and female piglets are preferred because of castration issues. However in horses the difference between the desired and non- desired gender becomes a much more subjective issue.

IS THERE A REAL COMMERCIAL DEMAND?

One breed of horse where the difference in price of the female is higher than the male is the Polo horse. It is believed that female polo ponies are more desirable because they are more trainable, they learn to play quicker, and they have a residual value after their sport carreer is finished. However, the Polo Association and Polo breeders in general still do not have an objective way of measuring this difference. In addition objective comparisons would be difficult to perform since well over 80% of the horses playing at a high level are females and many of the male horses are never trained. In other breeds, selection of a desired gender is even more subjective and is determined by impressions of individuals owning specific mares or stallions. Some believe that fillies from a particular stallion are better while others believe the opposite. A niche market for sexed semen, however, would be for replacement breeding animals for some owners. Some might want a specific colt out of a particular mare to produce a stallion prospect, while others prefer a female as a prospective brood mare replacement. It is clear though, that horse breeders when having the opportunity to choose a specific gender would do so as an added bonus, but if the choice is a foal of any gender or no foal, clearly the option would be the first one as the residual economic value of the non-desired offspring is still much higher than in a dairy cow.

THE TECNNIQUE

Differences in size, weight, density, swimming speed, electrical surface charges, surface macromolecular proteins, differential effects of pH, or the effects of atmospheric pressure on sperm type using sperm sedimentation, electrophoresis, sephadex filtration, gradient centrifugation migration through albumin gradients, convection counter-streaming, electrical charge differences, immuno-bead binding and polymerase chain reaction (PCR) techniques have been used to try to separate X and Y sperm. This has resulted in the approval of more than 100 patents claiming to have technology to significantly alter the sex ratio. However most procedures are only anecdotal and have not withstood the rigorous scientific challenge over time.

It is possible to have deviation of sex ratios without separating the sperm carrying the X or Y chromosome. In cattle, old cows and embryos produced from in vitro fertilization (IVF) result in about 53-54% males, GARNER 1 and 2 On the other hand cattle management procedures can have slight impact on the number of male and female offspring. Others have suggested that the timing of AI can alter the sex ratio in cattle [3].

It is a common belief amongst individuals wanting a specific gender that breeding closer to ovulation would result in skewing the gender of the offspring. In a recent observation by Sanchez et al (unpublished) mares were inseminated with cooled or frozen semen before and after ovulation. The sex of the offspring from resulting 433 live births were recorded and compared to insemination timing. Clearly those mares bred post ovulation should have a higher number of males if the premise that Y sperm "swim faster" but last shorter than X sperm. Of the 433 births 214/433 (49.4%) were males an 219/433 (50.6%) females. Furthermore pre-ovulation cooled semen inseminations (n=244) resulted in equal number of males and females while cooled semen post-ovulation breedings (n=42) resulted in 45.2% males and 54.8% females. Frozen semen pre-ovulation breedings

(n=17) resulted in 8 and p males and females respectively (47 vs 53%) while frozen semen post-ovulation (n=130) resulted in equal number of males and females. The results from this observation clearly dismiss the concept of changing the sex ration based on proximity to ovulation breedings.

In Garner et al. [21] published a repeatable method that was capable to accurately determine the difference in DNA in male or female sperm from different species. In the process of making the sperm permeable to the DNA fluorescent dye, 4-6-diamindino-2-phenylindole (DAPI) the sperm, washed with DMSO, fixed with ethanol and their membranes digested with proteases rendering the sample unusable for insemination. Except for a few species the difference in DNA between X and Y bearing sperm is between 2 and 4%, with the stallion being around 3.4-3.7%

The only reproducible way of separating X and Y bearing sperm based on DNA content from an ejaculate or a semen sample is by the use high-speed flow cytometry technique developed by Johnson *et al.* 1989). This technique results in purities of over 90% with born live offspring consistent with the purity of the sperm inseminated. The current process to prepare stallion sperm for sorting includes an initial centrifugation, staining with the permeable DNA dye Hoechst 33342 , (Johnson *et al.* 1987a), a second counter staining. Sperm are then passed through a flow cytometer coupled with a forward fluorescence detector and a bevelled sample injection needle in order to orient sperm and detect DNA content. Johnson and Pinkel 1986). Three populations of sperm result from the sorting process: X and Y bearing sperm and a third population of unreadable and dead sperm. Once sperm are sorted then the a sample is reanalyzed to confirm the purity of the desired sperm population.

As in cattle, significant stallion to stallion variation for the sortability of a semen sample. Clulow e*t al. (*2009), using the MoFlo SX® reported that while some stallions are are excellent candidates for sex-sorting others are not. In their experiments, the percentage of dead spermatozoa or region R5 was a good predictor of sortability for stallions. However in recent clinical trials the variability between stallions can be significantly reduced by processing the initial semen sample through the sialinated coated colloid Equipure ®. Furthermore the efficiency (cells analyzed/second) of the sorting process for some stallions can be improved as much as 40%.

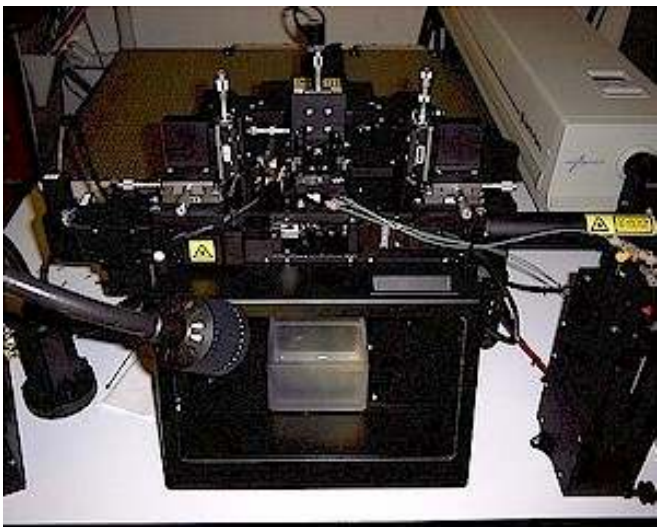
The fact that the current technology requires single sperm identification limits the number of sperm sorted per second. Although sperm numbers analyzed per second have significantly increased from 1-2 million in the early 1990's to the current 70-80 million (de Graaf personal communication) the process remains inefficient in the production of sperm for standard AI doses for different species. Of the total sperm analyzed approximately 25% are X bearing sperm and 15-20 are Y bearing sperm. Therefore conventional dose numbers of 250-500 million for frozen or fresh sperm in stallions are not attainable at the moment with this technology. Therefore to maximize the efficiency of machine time and maximum production of doses, the use of sex-sorted semen requires a significant reduction of sperm per dose. Furthermore since sperm are passed through the beveled needle by forced pressure (30 psi) reaching speeds of over 80 kms/hr, at a very high dilution rate it is possible that loosely attached proteins from the seminal plasma could be stripped off. Although Maxwell *et al. (*2011) reported that this is not the case for ovine sperm, this has not been tested for the stallion.

FERTILITY OF SEX-SORTED STALLION SEMEN

Fertility results with fresh sex-sorted sperm have been marginal to disappointing in most trials conducted since the early 2000's. Inseminating fresh sex-sorted semen pregnancy rates ranged between 10 and 40% after inseminating between 5 and 25 million million sex-sorted spermatozoa by endoscopic or rectaly guided insemination (Buchanan *et al.*, 2000, Lindsay *et al.,* 2002a, b, Clulow *et al.*, 2008). In the study by Lindsay et al 2002a mares inseminated with 5 million fresh unsorted semen from the same ejaculates resulted in similar results than those of the fresh sex-sorted inseminations. On the other hand, undiluted semen inseminated at a dose of 500 million spermatozoa yielded satisfactory conception rates (Clulow *et al.,* 2007) suggesting that the high dilution maybe directly responsible for the lower fertility rates reported in the trials using sex-sorted semen. This is in agreement with observations by Jasko et al who reported on reduced pregnancy rates when fresh semen was diluted at concentration of less than 5 million sperm per ml. Experiments conducted by Morris et al 2002, where acceptable fertility rate was achieved with as low as 100-250 uls containing 1 million sperm deposited hysteoscopically would suggest that perhaps the volume of fluid in which sperm are suspended needs to be taken into consideration. In the last 2 years independent work performed by Morris, Samper and Lascombes inseminating between 13 and 40 million and using 15 different stallions have resulted in pregnancy rates ranging from 40 to 60% using either rectally guided (Lascombes F and Samper J.) or hysteroscopic insemination techniques (Morris L). The better pregnancy rates are perhaps due to an increase in the, preparation of the semen prior to sorting, the sorting efficiency and excellent reproductive management. However, recently Pena *et al.,* (2012 in press), using the YoPro-1 fluorescent marker showed that sex sorting induces an increase in YoPro-1 positive cells. Yo-Pro-1 positive cells are indicative of slight alterations on membrane permeability characteristic of early stages of apoptosis (Ortega-Ferrusola *et al.*, 2008).

Unfortunately fertility of sex-sorted frozen-thawed stallion semen has not produced satisfactory results. In studies or trials by Lindsay (2001); Clulow (2009); Panarace (2009) and Morris (2009 and 2010) pregnancy rates have ranged beween 0 and 16.6% when inseminating between 5 and 20 million sperm by hysteroscopic insemination or rectally guided. Even though sperm motility, viability and morphology of sex sorted frozen-thawed sperm is similar to that of non-sorted frozen-thawed semen the results are very dissimilar. Possible explanations for this include mechanical effects such as the laser intensity, the high pressure and/or voltage that the sperm are exposed to, or the high centrifugation forces needed to re-concentrate the highly diluted sex-sorted semen sample. In addition to the mechanical effects, chemical or biochemical stressors could be responsible for reduction in the sex-sorted sperm fertilizing potential. Production of oxigen radicals during the prolonged incubation (45-60 min) for the Hoechst 33342 uptake of the dye by the sperm, some degree of sperm chromatin damage, pH changes, surface protein alterations, as well as cryodamage could account for some of the reduced fertility. In addition, the process of sex-sorting involves the suspension of sperm cells in what is known as the sheath fluid. This sheath fluid, although isosmotic with semen derives most of its ionic strength from a number salts. During the freezing process sperm are resuspended in a

freezing extender containing not just a cryoprotectant but also a myriad of high molecular weight sugars that probably causes a solution effect type of shock. This is in contrast to the bull, where the sheath fluid and the freezing extender have a similar composition and pregnancy rates are acceptable for commercial use.



SEX SORTED STALLION SPERM FOR ASSISTED REPRODUCTIVE TECHNOLOGIES

Since in-vitro fertilization is not a routine assisted reproductive technique in the horse, sex-sorted sperm has been used in a limited capacity for procedures such as intra-cytoplasmic sperm injection (ICSI). Unfortunately, the results have been very poor. In a small experiment, Galli *et al.,* (2009) reported a low cleavage rate, after oocyte sperm injection. In 2010 using a small number of stallions and oocytes sperm injections performed at two different locations in the United States produced no embryos. The use of sex-semen for ICSI procedures would be a highly sought process. However, one can only speculate as to why the eggs do not cleave or produce embryos. Lack of sperm decondensation, abnormal male pronucleus formation, lack of oocyte activation could be, among others, reasons for the lack of success during ICSI procedures.

COMMERCIAL USE OF SEX-SORTED STALLION SEMEN

In an effort to increase quality of sex-sorted semen several investigators have looked at factors such as cushion centrifugation before and after sorting (Knop *et al.,* 2005) as well as pre-selection of a more homogenous sperm population using a colloid (Heer 2007) as well as different freezing rates (Buss 2006; Clulow *et al.,* 2007). However all these investigations are isolated and are not part of a cohesive program long term research program. In order to implement the use of sex-sorted stallion semen commercially, the company holding the patents for this technology i.e. Sexing Technologies, Navasota,TX, must establish a methodical well controlled research program aimed at standardizing the processing procedure. This would include investigating what kind of damage if any, and its location within the sperm. Once this optimal processing protocol is established then short term (cooled) and long term (frozen) preservation should be investigated in-vitro. Fertility trials can be conducted once these variables have been sorted. In order to have a significant impact on the industry, sex-sorted semen must be able to be shipped to a sorting facility and shipped back to a mare either as cooled or frozen semen. Today, extended semen 1 or 2 conventional breeding doses can be sent to a central location for sorting, but the mares should be close to sorting facility for insemination. Full impact on the industry will not be able to be achieved until questions such as: a) What are the effects of sorting on membrane and sperm function, b) What are the ideal methods to preserve fresh cooled or to freeze sex-sorted semen, c) One can establish protocols to sort previously frozen conventional semen (reverse sorting) for use in ICSI programs.

In order to achieve this a dedicated person, with a dedicated sorter is necessary. However, extrapolating the techniques and the business model from the bovine, because it works, to the equine industry will set this technology back several years the same it did with the equine artificial insemination in the sixties.

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