SEMEN EVALUATION, HANDLING AND THAWING

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INTRODUCTION

Artificial insemination (AI) is becoming a very useful tool for breeding programmes in all farmed species as a means of exploiting top sires with desirable performance characteristics. In the dairy industry AI is well established and has an excellent track record. The Deer farming industry however, has only just "discovered" the benefits.

There are some restraints which make AI in Deer a very imperfect science. There is considerable pressure on people who process semen to "freeze anything that wriggles". Therefore there is pressure to process and freeze even when evaluation of semen shows characteristics which fall below acceptable levels in other species. It is very much a case of once something works at a reasonable level don't change the recipe. With diluted semen often valued at more than $1,000 per ml operators and owners are loathe to risk experimenting on different ways of handling it.

Stag semen is collected, almost universally, by electro-ejaculation under anaesthetic. It cannot be denied that this technique is time consuming and traumatic to the animal. The amount of semen available for use either for experimentation or in an AI programme is limited since frequent collection from individuals is not desirable.

Finally, experimentation to evaluate subtle changes in handling, freezing, thawing and AI techniques requires large numbers of animals to show statistical differences. It is difficult to find herd owners willing to risk the reproductive performance of a large group of hinds for an AI experiment.

Despite these drawbacks AI is still a viable technique. There will be many breeders who want to use AI with fresh semen. While this is a perfectly legitimate use of the technique, the fact that semen is generally collected by electro-ejaculation is a major drawback. It is difficult to achieve predictable, consistently good quality samples from an individual. When an expensive fresh AI programme is at stake the insurance policy of a backup of frozen semen is wise planning. The use of frozen semen in an AI programme avoids the problem of relying on a satisfactory semen collection "on demand".

Whichever method is used it is important that the semen quality has been thoroughly checked and that steps have been taken during handling so that quality is not compromised.

EVALUATION

There are two places where semen is likely to require evaluation. In the field, at the point of collection, some sort of gross evaluation is required even if only to set the operators mind at rest that the collection

contains live cells. Once the sample is taken to the processing laboratory evaluation becomes more critical.

i. Field evaluation

Primary evaluation in the field generally involves identifying contamination of the semen with accessory fluids. The two contaminants most likely to be encountered are urine and seminal vesicular fluid. Both are detrimental to the sample. Urine generally dilutes the semen and gives it a more yellowed "watery" appearance. Vesicular fluid on the other hand is a deeper yellow and has a consistency more akin to honey. A semen sample grossly contaminated with either fluid should be discarded. In an attempt to minimise the effects of accessory fluid contamination we currently use a series of collection vessels and collect samples in fractions related to the manual "cycling" of the electro-ejaculator.

Some idea should be gauged of the gross motile activity of the semen sample in the field. Generally a "good" sample will have a "thick, milky" to "creamy" appearance and some swirling should be visible just looking at the test-tube. If a low power microscope is available a small drop of semen on a warmed slide can be examined at about 40x magnification. Observation should be made of the edge of the drop. The gross motility of the sample is seen as wave patterns with larger and stronger waves indicating a higher proportion of live, motile cells.

ii. Laboratory evaluation

Motility

Once the sample comes into the laboratory it is important to make a more detailed assessment of the motility of the sperm. A further drop should be examined to recheck the gross motility of the sample. Wave action is generally measured on a scale of 0-5. This scale (table 1) has been used for many years for evaluating sheep semen, and can be adapted to other species.

To make a more accurate assessment of motility a drop of diluted semen should be placed on a warm microscope slide and a cover slip placed on it. The drop should not be too small as the weight of the coverslip may slow the sperm down. The slide can be examined under 200x magnification and an assessment made of the % of sperm which are progressively motile.

Volume

The simplest way to measure volume is to connect a tuberculin syringe to a pasteur pipette and draw the semen into the pipette. The volume of air displaced in the syringe will equal the volume of the sample. The sample is then transferred to a clean dry tube for dilution.

Density

The density of the ejaculate is the most important factor in determining the final number of doses available. Density can be measured by one of two methods.

a. Haemocytometer Counts

When the semen sample is collected a small volume (20μl) is diluted to 10 mls with a saline/stain mix and put into a haemocytometer cell by capillary action. After allowing the sample to settle the grids are located under the microscope and the number of sperm cells counted. Only complete sperm are counted. A simple mathematical formula based on the dilution rate and the volume of the counter gives the concentration of sperm per ml of sample.

b. Optical density

The number of sperm per millilitre of sample is assessed by using a known dilution rate of the semen and measuring the optical density of the subsequent solution. The reading is matched to a calibrated standard curve to give the sperm concentration in the sample. Although this is the routine technique we use for sheep semen I prefer to use haemocytometer counts for stag semen. The main logic for this is the likely contamination of the sample as mentioned initially. We use a Gallenkamp colorimeter to measure optical density. Yellow discolouration interferes with light penetration in this instrument thus giving false readings. Another disadvantage is that it may also be damaged during transport and is therefore restricted to use in the laboratory.

Following determination of the sample density the number of doses available is worked out by the following formula.

$$\frac{d \times v}{c} = n$$

where d = the concentration of the sample
v = the volume of the ejaculate
c = the concentration of sperm per dose
n = the number of doses available

The dilution factor is worked out by the formula

$$\frac{d \times v}{F} - v = D$$

F = the final concentration per ml (dose concentration x no doses per ml)
D = volume of diluent to add.

iii. Morphological evaluation

Once an animal has been collected from several times in a season there is little need to make a morphological evaluation of each sample. However, it is a good idea to examine a sample from each animal for morphological abnormalities prior to starting an extensive semen collection programme.

A small sample of semen is placed on a warm slide and mixed with a couple of drops of live-dead stain: (see below). Following a smear being made the slide is air-dried. An absolute minimum of 100 individual sperm are examined at 400x magnification with no more than 20 individuals counted per field to eliminate isolated groups of damaged cells biasing assessment. Abnormalities to be noted (in increasing order of importance) are: malformed acrosome, droplets, malformed tail, decapitated and dead sperm.

Live-Dead stain

Eosin (water soluble)	1.67g
Nigrosin (water soluble)	10.00g
Distilled water	100 ml

This mix should be filtered and can be stored for long periods in a refrigerator. It must be warmed to 37°C before use.

There are some important points to note

1. The slide must be clean and warm
2. The semen and stain should be at the same temperature
3. The smear should be thin
4. To avoid damaging sperm the smear should be pushed rather than pulled, using only one pass.

HANDLING SEMEN

Semen which is good quality at the time of collection can be damaged by several factors. When handling semen it is important to be aware of these and to avoid the possibility of killing the sperm.

i. Extending semen

The principal reason for handling semen prior to AI is to dilute the sample to a standard number of sperm per inseminate volume and in doing so extend the viability of the sperm by up to 12 hours prior to insemination. Most operators use extenders based on the NZ Dairy Board two part recipe (Fennessy et al 1987). As each laboratory may have its own refinements contact should be made with individual laboratories to establish exact protocols. Most extenders are based on a Sodium Citrate buffer plus egg yolk. Semen to be frozen-stored will also have a cryoprotectant added to the extender (generally glycerol).

Extenders should be kept at 30°C prior to adding to semen and should be added very slowly. Laboratories will supply the operator with a primary diluent to be added at the point of collection. In some cases this may require the addition of egg yolk before use. Eggs **must** be fresh and the **yolk only** used. Care must be taken to dry off all albumin and to avoid the yolk membrane. Careful, thorough mixing is also essential.

ii. Temperature

Rapid fluctuations and extremes of temperature must be avoided. It is important that collection vessels are warmed to about 37°C prior to use. Exposure to high temperatures will increase the metabolic rate and impair the life span of the sperm. A temperature which is too low will cause cold shock and kill sperm out-right. Some form of warming device for equipment is essential. This may vary from a sophisticated thermostatically controlled incubator to a simple wooden box heated with a low wattage light bulb.

iii. Contact with metal

Metal will kill sperm, therefore all vessels should be either glass or plastic.

iv. Contact with water

Although all buffers used are made up using distilled water the chemical composition is designed to maintain quite strict osmotic pressures. Direct contact with unbuffered water will kill sperm and must be avoided. All glassware must be dry.

v. pH

Buffer pH should be around neutral definitely not below 6.8 nor above 7.2.

vi. Contact with other agents

Disinfectants and antiseptics are harmful. During cleaning all glassware should be thoroughly rinsed with distilled water or cold boiled water prior to drying. Cleaners suitable for tissue culture equipment can be used if rinsed off thoroughly. For sterilization, glassware may be wrapped in tinfoil and heated at 100°C for 20 minutes. Alternatively an alcohol rinse may be used, provided the equipment is thoroughly dried before use.

Dust, bacteria, hair etc will all reduce viability. In common practice these impurities are difficult to avoid, however all care should be taken. The penis should be cleaned prior to collection.

vii. Sunlight

Most collection will be done in a yard situation where direct sunlight is not a problem. However, it should be remembered that direct sunlight is detrimental to semen and should be avoided.

TRANSPORT OF SEMEN

Usually semen will have to be transported from the point of collection to a processing laboratory. This is covered in a previous paper (G Bowen) however there may be one or two useful tips we can offer.

We transport semen (with part of the primary diluent added at 1:1) from the Invermay deer yards to the processing lab in a very simple water jacket. The tube of semen is carried suspended in water which was initially at 30°C. This is adequate for short distances. It is wise to also carry some of the diluent so that it is at the same temperature as the sample when added in the laboratory.

If semen is to be transported chilled, a thermos flask with 2 or 3 acetic acid "bombs" will maintain 15°C. Acetic acid bombs are made by putting approximately 40 ml of glacial acetic acid in a sealed container and freezing. Before use these should be run under a cold tap to start the thawing process. For transport, plenty of damp cotton wool or paper towels wrapped around the tubes help maintain the cool temperature.

Semen being transported and cooled in a car prior to freezing can be kept in a refrigerated chilly bin plugged into the car's cigarette lighter.

THAWING SEMEN

While the principle of freezing semen for storage is to lower the temperature relatively slowly to avoid damaging the cells, thawing should be rapid.

The widely accepted method for thawing semen is to remove the straw from the liquid nitrogen container, flick it to remove any nitrogen from the cavity in the plug end and immerse the straw in a water bath. Some operators recommend a temperature of approximately 70°C with immersion for 5-10 seconds. The procedure we prefer to use is to immerse the straw in 35-37°C water for 30 seconds since immersion time is less critical and delaying factors do not compromise the semen. The straw is then removed from the water bath and the bubble flicked to the sealed end of the straw. It is important to wipe the straw completely dry with tissue and then cut off the sealed end. If the insemination equipment will accept a complete straw, insert the straw in the instrument and use as quickly as possible. I prefer to hold the instrument at room temperature, unless of course the ambient temperature is very cold, when the equipment may be held in the assistant's jacket until required.

If the AI gear is not designed to accept a 0.25cc straw it may be necessary to empty the thawed semen into a clean dry test tube by holding its open end into the tube and snipping off the plunger end.

The water bath may be a laboratory model, a small dedicated straw thawer or a deep open necked thermos. If the latter is used, careful attention to a thermometer may be necessary with temperature loss in the flask being greater as the ambient temperature drops.

CONCLUSION

Successful semen processing depends on a careful, methodical approach to all phases from collection through processing to thawing. There are no shortcuts. Since failure to apply quality control at any step will compromise the whole job the operator must be prepared to discard all samples which do not meet standards negotiated with the stag owner prior to collection.

Table 1: Wave motion scoring of semen

SCORE:	WAVE MOTION
5.	**Very good motility.** Dense vigorous waves, changing direction very rapidly. 90% + alive
4.	**Good motility.** Vigorous movement less rapid direction changes. 70% - 85% alive.
3.	**Fair Motility.** Small or no wave motion. Individual sperm visible. 45% - 65% alive.
2.	**Poor motility.** No waves some individual sperm show movement. 20% - 40% alive.
1.	**Very poor motility.** Very few individuals show movement.
0.	**No motility.** All sperm dead.

References

Bowen G. Proceedings NZVA Deer Branch Course 1989 (In Press)

Fennessy P.F., Beatson N, Mackintosh C.G.. Proceedings NZVA Deer Branch Course No. 4 1987 p 33.