FORENSICS FOR THE BULL STUD AND SEMEN PROCESSING LAB: INTERPRETING TANK, STRAW AND SEMEN QUALITY CLUES

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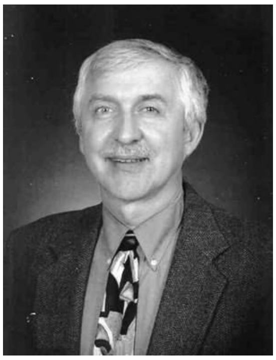

KAPROTH

Dr. Mike Kaproth completed his B.S. and M.S. degrees at the University of Minnesota, supervised by Dr. Ed Graham. Mike processed semen at Minnesota Valley Breeders Cooperative, and supervised semen processing at Eastern Artificial Insemination Cooperative. Following mergers which created Genex Cooperative, Mike accepted the responsibility of Associate Vice President for Laboratories and Research. In 2005, Mike completed a Ph.D. program under the direction of Dr. John Parks at Cornell University. Research interests include sperm metabolism and preservation, spermatogenesis and production of bovine spermatids from transplanted tissue.

Introduction

Semen is processed and distributed using methods and techniques developed over many years by professional artificial insemination staff and dedicated academic institutions and interested cattlemen. When processed semen leaves the AI center, good solid advice and procedures provide ample safeguards for maintaining semen quality in the field, until it gets into the cow.

However, at times, a tank of semen becomes “suspect” and is returned for evaluation and questions about the quality of the tank, straws and semen emerge, or unexpected outcomes and observations are presented to the lab manager as clues to provide assistance in advising on the use of these products.

Develop and Spread the Expertise?

At each facility, someone should know **why** specific activities, cautions and preparations exist in maintaining tank, straw and semen quality. With expansion, remote supervision and staff transitions, this is not ensured at every operation. What was the original justification for each procedure? Who designed it? What was this person’s knowledge base and reasoning for the procedure? Is this person still available to answer questions when deviations occur? What are some common deviations? How should we interpret these?

Why Should We Conduct Our Own Experiments in Tank, Straw and Semen Quality?

Dr. Ed Graham, a regular contributor to the NAAB Annual meeting and the NAAB Technical Conference from 1956 through 1986, had an impulse to design simple experiments to dispel myths, or find useful “rules of thumb”. In trying to instill this impulse in his students, Ed told this story: *At one time, lactation physiologists “observed” that in milking a cow, the last milk to emerge was highest in butterfat. This stimulated* *debates and notions about whether or not the high fat milk was the last milk ejected because it buoyantly rose in the cow. After hearing these protracted discussions, Dr. Graham turned his cows upside down and milked them. The "Cream" still came out last.* The lesson learned was that useful observations and simple tests aid in making proper interpretations.

Steps in Design of Experiments?

- What is it that you need to know? Desire for understanding.
- Observation. Discern the nature of the problem.
- Think about the investigation: What properties will provide answers?
- Hypothesis. Trick is that it aids in development of understanding.
- Reduce the dimensions of the problem.
- Choose variables related to the problem. Plan variables to also be measurable.
- Experiment. Simple tools will generally suffice.
- Measurement. Expect variability, therefore do replicates.
- Consider all responses. Unexpected responses may provide new insights.

Whose Problem Are We Solving?

When presented with a problem, we must exercise caution against a situation where a *reason* for the problem may be suggested, but the *problem* isn't solved. An example of this, was given by Cohen and Medley (2), using an example from a human infertility clinic. In this report from Guzick, 84% of *infertile* couples had at least one test outcome which was an abnormal, but important result (i.e. a reason for infertility) and was taken to explain why they were childless. However, nearly as many, 69%, of the *fertile* couples have a similar evaluation (abnormal, important result). The temptation is to align our field of *expertise* with the *apparent* problem. When this occurs, *our* problem is solved once a *reason* has been identified. The *real* problem however may not be solved.

Section 1: Equipment, Rationales for Straw, Tank and Semen Quality Experiments

Thermocouples

Each processing and distribution laboratory manager should have and routinely use modern thermocouple meters. An inexpensive useful meter is the Omega Model HH23, (Omega Engineering, Stamford, CT, USA). The proper thermocouple for a cryogenic lab would be the Type T, (Cu/CN) variety (8). When measuring seminal temperatures, the wire gauge affects the junction size and heat transfer from the environment into the straw. A 36-gauge wire (0.005 inch diameter, Omega 5TC-TT-T-36) and an exposed "bead" junction is preferred for both atmospheric and within-straw measurements, with the thermocouple being placed within the center of the straw without contacting its wall. Thermocouple gauge size is important. In the course of studies at Genex, when the gauge is too thick, heat can be conducted through the thermocouple into the material being studied. In one set of paired values, atmospheric readings for .010 inch and .005 inch thermocouples were -130°C and -104°C respectively.

Shipping Monitors

Useful shipment monitors include:

1. Tip N Tell™ (Decker Tape Products, Fairfield, NJ). Self-adhesive indicator will reveal whether a tank has had at least one episode where it has been tipped on its side during transit.

2. Drop N Tell™, Shockwatch™ (Arrow Packaging, San Jose, CA). These self adhesive indicators are available in different sensitivities to reveal if the shipment has been roughly handled.

3. Temperature monitor ampules (ABS Global). These ampules are used as a set of two indicators, which are frozen upside down at cryogenic temperatures. These are placed in the shipping tank. The first ampule, containing a blue liquid, melts at -100°C. This temperature is perhaps before semen has been damaged; however semen quality should still be checked. The second ampule, containing a red liquid, melts at -55°C. This indicates that the product has definitely passed into a dangerous atmospheric temperature.

Rules of Thumb for Sperm and Temperatures

How Cold is "Cold Enough" for Frozen Semen?

There is a range of published or understood temperatures which have been thought of as a limit for seminal temperatures.

- "Below the frost line". Since it is easy to see the frost line in the neck of the tank, this was a handy "rule of thumb", but also impractical: By measurement, we found this to vary from -40 to -120°C in different tank models. We would expect climatic conditions and tank liquid nitrogen levels to also affect the "frost line".
- -80 to -100°C. The zone identified as dangerous by Rapatz (11).

- -100°C. Cited as a critical temperature by Mitchell and Doak (7).
- Less than -100°C. Frozen sperm was recommended to be kept at temperatures less than -100°C in separate works by Pickett and coworkers (10) and Elliott (4).
- -130°C. Temperature suggested by Berndtson and coworkers as a maximum for holding frozen straws of semen (1).
- Less than -130°C. One variation in maintaining straws in the field is the use of single-decked cups of gobletted straws in inseminator field units with use of long tweezers, tweezer lights and cup lifters. This combination of procedures achieves storage and working conditions for straws of semen at less than -130°C.

Why Consider a Temperature Less Than -130°C?

In Elliott's work, using a photographic method for determining post thaw semen motility, ampules of semen stored at -196°C were determined to have greater post thaw motility than samples stored at -103°C and much greater than samples stored at -78°C (Figure 1).

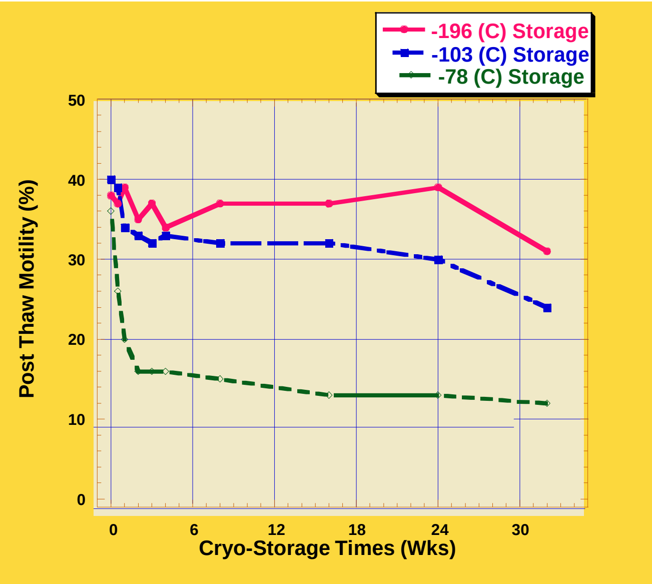

Figure 1. Effect of storage temperatures on post thaw motility. Adapted from Elliott (4).

Below -132 °C, all water is in a glass or crystalline state. During cryopreservation, particularly in faster freezing procedures, water freezes out as small crystals. Sperm are pushed into remaining regions of liquid water, but within regions of ever higher solutes. Then, as cooling proceeds, water in these regions of high solute concentration also eventually freezes. However, the last remaining water was a liquid at temperatures as low as -80°C. Sperm in these regions are very susceptible to damage from crystal growth, recrystalization.

Very importantly, storage at temperatures below -130°C, allows a safety cushion for seminal temperatures, in the case of mishandling, before critical temperatures for recrystalization are experienced.

Section 2: Simple Experiments in Forensics for Straw, Tank and Semen Quality

Straw Quality

International commerce and distribution of semen following long airline travel is common, and in recent years, has been facilitated by the replacement of liquid nitrogen shipping tanks (wet tanks) with nitrogen vapor tanks (dry shippers). It is both interesting and important to know whether a particular combination of nitrogen tank, straw packing method, and straw type is best suitable for airline transports of long duration.

From one tabulation of straw quality defects reported for a 20-month period (16,000 total straws) associated with international shipments, the following straw defect rates were found (Figure 2): straw breakage rates (shattered at the bottom rim of the straw), at 20 incidences/10,000; blown or moved plugs, at 25-28 at incidences per 10,000.

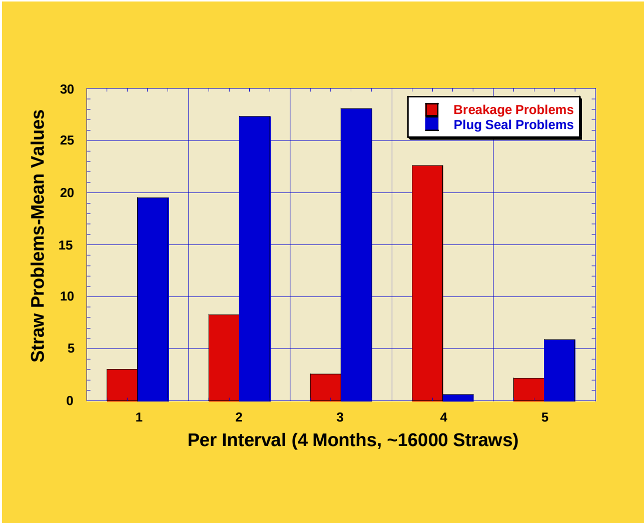

Figure 2. Rate of reported straw problems per 10,000 straws shipped by air.

Does Vibration Cause Additional Damage? Would Packing or Type of Tank Change This?

We could conjecture that without the cushioning effect of liquid nitrogen, straws may be excessively vibrated. Would this lead to damaged bottom rims of the straws? Would the wick-and-powder seal be loosened, permitting liquid nitrogen to migrate into the straw at the final destination, causing a moved or blown plug?

Would loosely-packed straws suffer more damage than tightly packed straws?

To test this, we conducted a shipping trial. Variables included tank style (wet tank, vapor tank); packing (in each goblet straws are either tightly packed, loosely packed); Shipping (4400 miles by air, central storage). To fully represent normal processing methods, 10,000 straws representing 20 batches and 4 processing days were prepared and split to 4 groups: (1) Vapor tank-packed loosely-4400 miles; (2) Vapor tank-packed tightly-4400 miles; (3) Wet tank-packed tightly-4400 miles; (4) Wet tank-packed tightly-Central storage. Following shipments all straws were stored under liquid nitrogen to allow for potential liquid nitrogen migration, then thawed and examined.

To better estimate the potential for a customer receiving straws which have plugs that blow out, we evaluated these test straws for a gradation of changes related to the plugs: Blown plugs > Moved plugs > Moved semen column (minimum change).

What is the Best Combination for Long-distance Shipments?

In all four groups, we generally found low levels of straw damage, within the typical range of reported defects. We found that vapor tanks may be superior to wet tanks in preventing blown plug problems (Figure 3). Wet tanks had more blown plugs and moved semen columns. We found that wet tanks did cushion the rear rim of the straw somewhat better than a vapor shipper (Figure 4). However, overall rates of straw rim breakage were very low (20/10,000). Surprising to us, packing straws loosely within goblets did not "cause" more bottom rim breakage in shipped straws (Figure 5). From these observations, we conclude that making long distance air shipments by vapor shippers are very satisfactory. Straws may be packed tightly or loosely without affecting breakage.

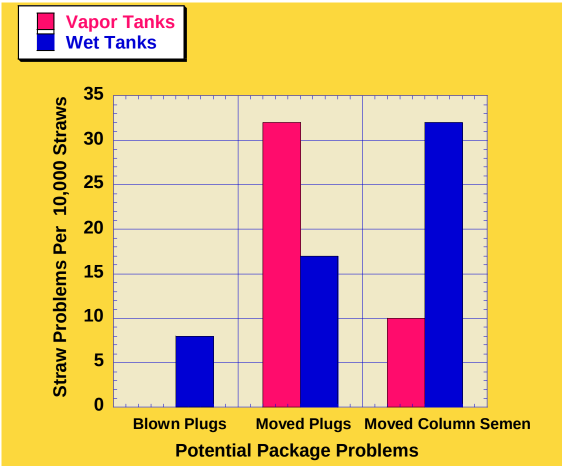

Figure 3. Effect of shipping method on potential straw problems. Vapor shippers do not cause additional potential plug problems.

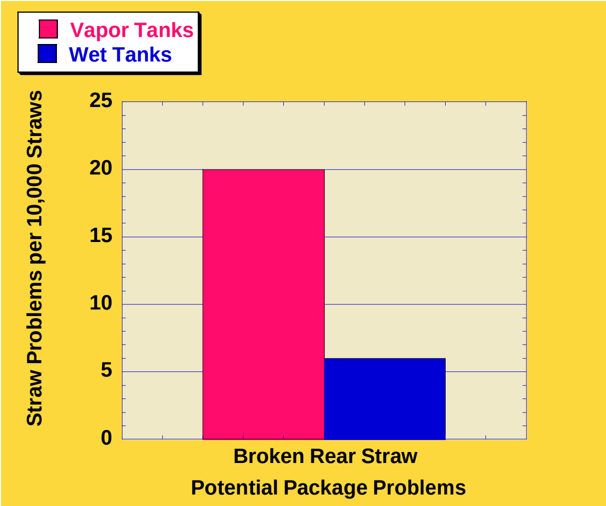

Figure 4. Effect of shipping method on potential straw problems. Numbers of straws with broken rims are low, but use of wet tanks did decrease broken rim incidence.

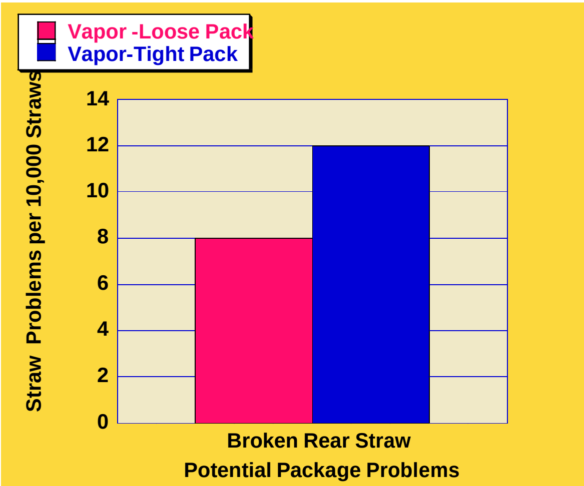

Figure 5. Effect of shipping method on potential straw problems. Loosely packing straws is accept-

breakage.

What We Learn From Monitoring Nitrogen Tank Liquid Levels (How Did It Fail Me?)

industry and reported this in Dairy Herd Management:

- Holding time can be reduced by one month

  to 48 canisters per day.
- Poor neck plug-can reduce holding time by 20-30%.
- Loss of insulation-tank can last one more day-or a year. It is impossible to tell.

- Not using the neck plug-can increase nitrogen usage rate eight times faster.

Why is the Tank Not Lasting as Long as It Should?

Did filling pattern change? If more than one person is filling the tank, or adding it at an irregular interval, interpreting the nitrogen usage rate is impossible. Consider this: A labeled 20 week tank that has been "topped off" twice a month for years may disappoint the tank owner when service patterns change and a tank is not replenished for a month.

Vapor Shipping Observations?

Vapor shippers are produced with ranges from 8 to 85 days. Within the specified holding time, semen quality is adequately maintained. Vapor shipper efficiency is reported by the manufacturer to be reduced by half or more when the vapor shipper is tipped on its side, and by 90% or more for shippers positioned upside down. Parks (9) observed no difference in post thaw semen quality over 20 days for straws held in either the top or lower goblet, in a vapor shipper stored in the upright position (Figure 6). For straws stored in a tipped over shipper, semen quality declined on the fifth day (Figure 7).

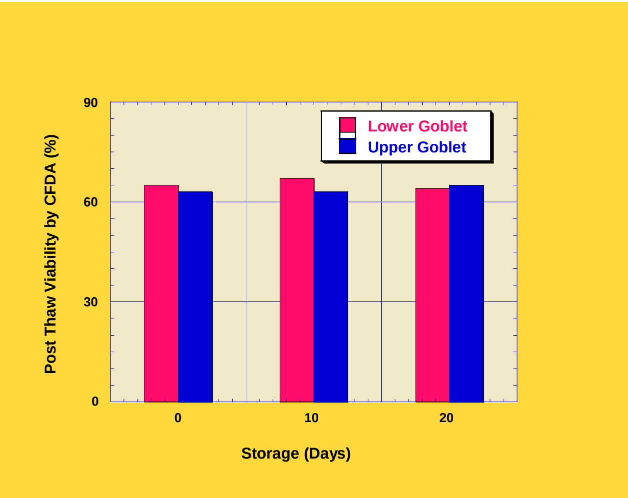

Figure 6. Effect of position and storage time in a vertical nitrogen vapor shipper on post thaw viability (CFDA staining). No difference in semen quality over 20 days storage. Adapted from Parks (9).

Reasons Why We Don't Really Want to Hold Straws Up in the Neck of the Nitrogen Tank.

A good inseminator will work with tweezers and can perform all the needed cane and straw manipulations with the straw never being raised closer than 12 cm below the tank opening. Above this height, the straw is going one-way only…to the cow.

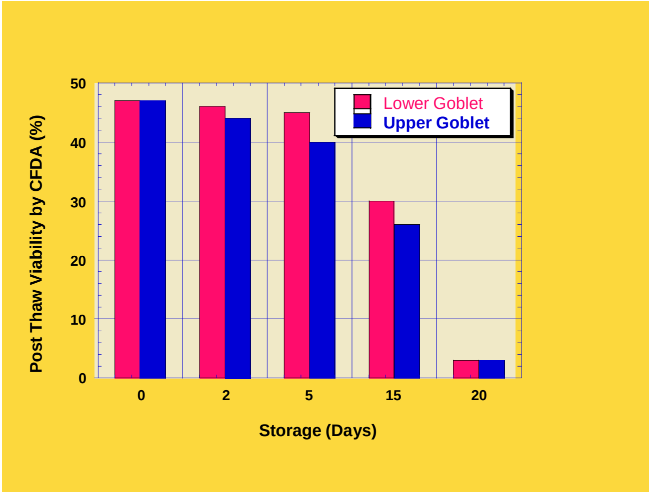

Figure 7. Effect of position and storage time in a tipped-over nitrogen vapor shipper on post thaw viability (CFDA staining). Decline in semen quality by day 5. Adapted from Parks (9).

Temperatures at the Neck of the Tank?

One can consider a depth of 12 cm, as a commonly taken "working" distance for an inseminator using tweezers to retrieve straws. At this depth, a safe working temperature should be in the range of -130°C. At progressively warmer temperatures, through -100°C, shorter working times should be utilized. In measurements at this depth, atmospheric temperatures were found to range from -160°C to -130°C (Figure 8) in a small field unit (Linde LR-21). The warmer temperature was associated with a lower nitrogen level. This tank is clearly suitable to work from.

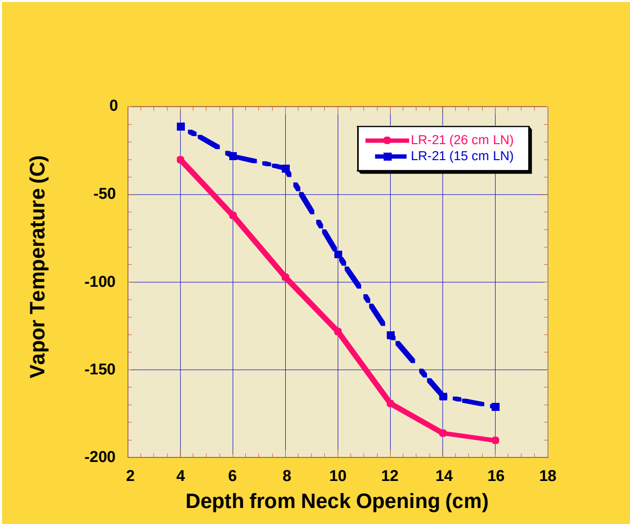

Figure 8. Relationship between depth from tank opening and vapor temperature in an LR-21 tank. Depicts relative temperatures in a conventional wet tank at high and low nitrogen levels.

What are the Temperatures in a Vapor Tank Compared to a Wet Tank?

One model of vapor shipper, the MVE Doble 34 has a wide mouth and a deep neck. It is designed to be used as a vapor shipper or a storage tank. In Figure 9, the relationship between depth from tank opening and vapor temperatures is depicted. The atmospheric temperature values were found to be similar whether the Doble is full of nitrogen, or are being used as a vapor shipper. At a depth of 18 cm, the atmosphere is -190°C or colder.

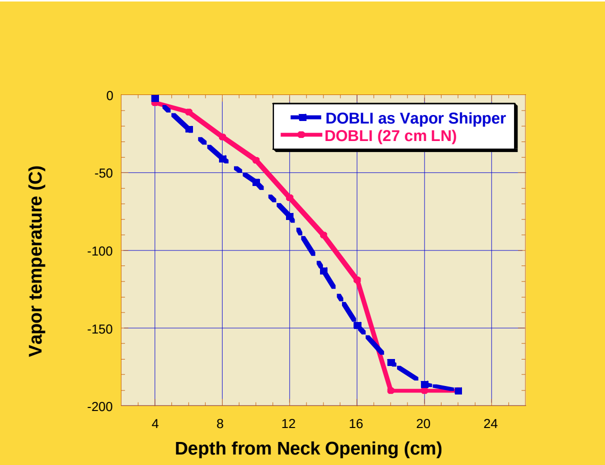

Figure 9. Relationship between depth from tank opening and vapor temperature in a Doble 34 tank . Depicts relative temperatures in a Doble tank when used as a conventional wet tank at a high nitrogen level, or as a vapor shipping tank.

However, at the depth of 12 cm, taken as the maximum working tweezers distance, the atmospheric temperature of this tank model was found to approx-imate -70°C (Figure 10), a fairly warm temperature.

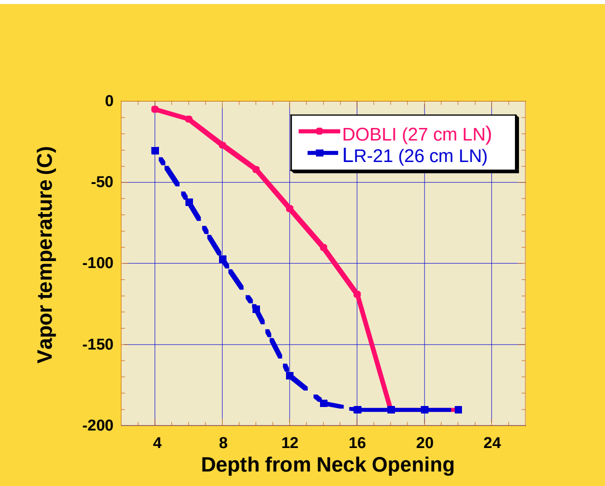

Figure 10. Different tank styles (Doble 34, LR-21) as examples where temperatures are distinctly different at the conventional working depth, 12 cm.

An inseminator working in this style of tank should be aware of this temperature and the consequent shortening of working time necessary to prevent the straws from dangerous warming. Other styles of vapor tanks from the same manufacturer, with less deep necks, were not as extreme in temperatures. This is pointed out to encourage that each cryostorage unit be measured and characterized by the user so that the inseminator can be given guidance in its proper use.

What is the Effect of Continued Activity in a Field Unit?

A common inseminator practice is to work from a canister for a short time, and then lower the canister for some 15-20 seconds to "cool down", then the canister is lifted to continue working. *Does this practice work?* This probably does not work as expected. Berndtson and coworkers (1) reported that lowering canisters for a full minute in a field unit which was relatively high in nitrogen did recool the seminal straw temperatures as expected, however, in a field unit which was low in liquid nitrogen, seminal temperatures only recooled by 30° to 40°C. The longer the session continued, the warmer the overall seminal temperatures became (Figure 11).

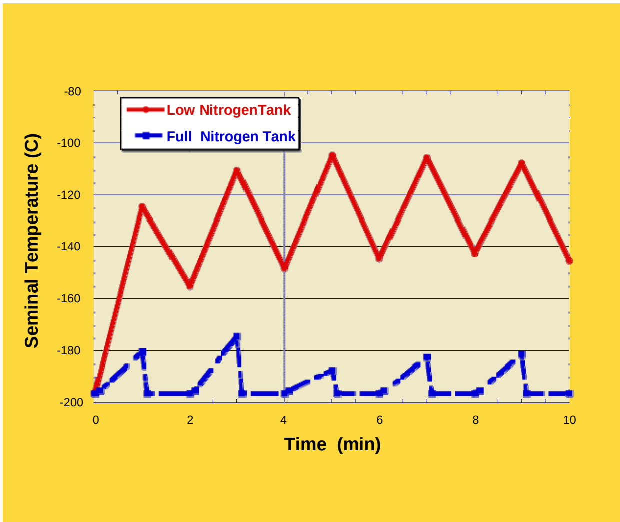

Figure 11. Effect of continued activity in a field unit. When a field unit is low on nitrogen, lowering the canister for a full minute only cools the straws slightly, 30-40°C. Adapted from Berndtson (1).

How to Recognize a "Warmed" Nitrogen Tank Problem, When It Isn't Warm Anymore?

Senger and coworkers (13) reported 5% of the nitrogen tanks went "dry" in his two year study of Washington State dairies (60 farms). We depend on use-ful clues to recognize when straws have been warmed, then refrozen. Generally, field clues include:

- Thawed straws and goblets first warm up, and then get wet.
- Wet straws and goblets turn to ice.
    - Hard to remove straws from goblets.
- Thawed straws, when quickly refrozen, show these clues:
    - Nonsymmetrical cylindrical shape, crooked.
    - Bulge or blister, 1-2 cm, at middle of the straw.
    - Radial cracks, middle of semen column.
    - Broken straws, middle of semen column.

Is Seeing a Foggy Vapor Trail Sufficient?

One practice, is the determination that there is “enough” nitrogen still in the tank at refill, if a foggy trail of vapor is visible when the neck plug is removed. However, by our measurements, a freshly empty field unit, allowed to warm to -50°C, still made foggy streams of vapor. Any straws still in a tank at that temperature would be compromised.

Completely Thawed Straws Will Soon Have Sperm Settling to the Bottom of the Straw.

In Figure 12, the rate of sperm settling is depicted for thawed straws of sperm extended in milk extender. Once straws have been thawed, settling rates were similar at room or cold room temperatures.

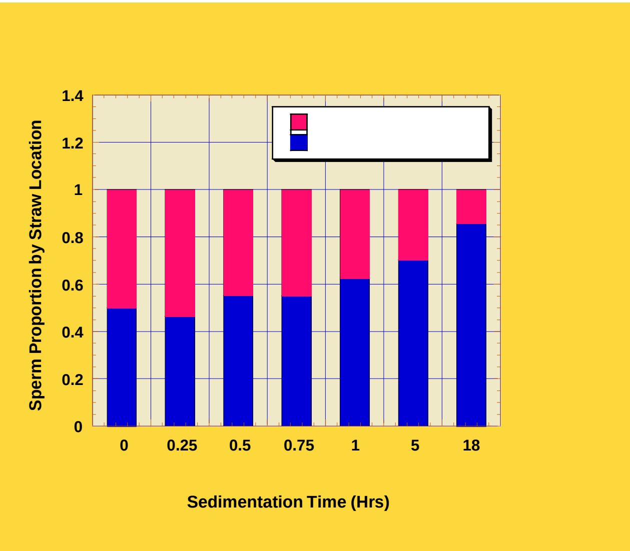

Figure 12. Rate of sperm sedimentation at cold room temperatures.

What About Sabotage? Can a Canister be Removed From a Tank for Mischief, and Then be Replaced Without Anyone Knowing?

In the course of normal field business, it is almost impossible to warm and then refreeze a canister of straws undetected due to adhering moisture on the straws and goblets, and the straw damage done with rapid refreezing. Whether wet or dry, completely thawed straws cannot be rapidly refrozen as the straws would have distinctive damage: breaks, bulges and blisters.

In one experiment, we found that canisters of cryopreserved caned-gobletted straws warmed to -2°C to -3°C, but in dry condition, could be slowly recooled to cryogenic temperatures without straw damage. In this trial the canes were placed in a freshly emptied nitrogen unit at a tank temperature of -140°C. Small amounts of nitrogen (6 oz) were added to the tank at 90 second intervals. After 10 additions of nitrogen, cryogenic temperatures were reached in the straws. Similar results were obtained with a warmer tank (5°C), but required 13 additions of nitrogen.

In further trials, canes of straws allowed to warm to room temperature and dried were found to be able to be refrozen without detection. In this instance, the canister of caned straws was placed in the canister location of a cold vapor shipper. This enabled a slow refreeze, allowing straws to maintain an undamaged appearance.

A Worst-Case Scenario? Vapor Shipper-to-Vapor Shipper Transfer.

Taken together, this suggests the need to be aware of an unlikely chain of events, where it is improbable, but still possible, to accidentally produce normal-looking straws filled with dead sperm. This would be a two-vapor-shipper system for distributing semen.

- A vapor tank shipment is prepared, ensuring tank and straws are pristine and dry.
- Shipment warms enroute or after arrival. Sperm may even be warm enough to settle to the bottom. Unnoticed by destination staff.
- Destination staff pulls canisters and transfer them intact into another vapor shipper for shipment to final destination.
- Second vapor shipper is cold enough to slowly refreeze straws.
- Straws reach final destination, are cold, look normal, but are infertile.

It appears needful that in a two-vapor-shipper distribution system, a positive temperature measurement should be made at the mid-point and endpoint of this system.

Semen Quality

Should We Expect to See Some Straws with Lowered Quality?

Declines in sperm quality are one expected outcome of mishandling (3). Lineweaver and coworkers measured semen quality in field stored (3-year) straws and noted overall decreases in post-thaw motility and acrosome quality (6).

Examination of Suspect Semen?

When requested to "check" field-returned straws, to tell if the tank had been warmed up, consider this:

- Get two canes of straws with full goblets from two different canisters.
- Check three straws from the top goblet. Three straws from bottom goblet.
- Checking straw integrity and sperm numbers in top vs. bottom stored straws.

Estimate the relative quality differences in top vs. bottom stored straws. Determine the absolute quality values. Use QC measures generally practiced in the production lab to assess sperm quality: motility, acrosomes. It is best if a lab only checks straws produced by that lab, and refers other straws back to the producing organization. Recognize that having *some* sperm with motility in the straw is not a good diagnosis…*Some* sperm are hard to kill off. With this in mind, when straws from a tank of suspect field semen are forwarded for examination, the finding of some level of motility is not necessarily evidence that the field semen is undamaged.

What Are Exposure Effects on Straws?

Berndtson and coworkers (1), performed a series of experiments to demonstrate the effects of exposing cryopreserved straws to ambient temperatures for differing time spans prior to refreezing, on resulting post thaw motility values. They compared the effect of exposure duration in 0.5 and 0.25 mL straws (Figure 13), pointing out the sensitivity of 0.25 mL straws to ambient conditions. In comparing single or gobletted straws (Figure 14), it was pointed out that gobletted straws did offer a measure of protection as compared to single straws. It was emphasized that this was not to be taken to mean that fertility is uncompromisingly maintained after exposures of these durations.

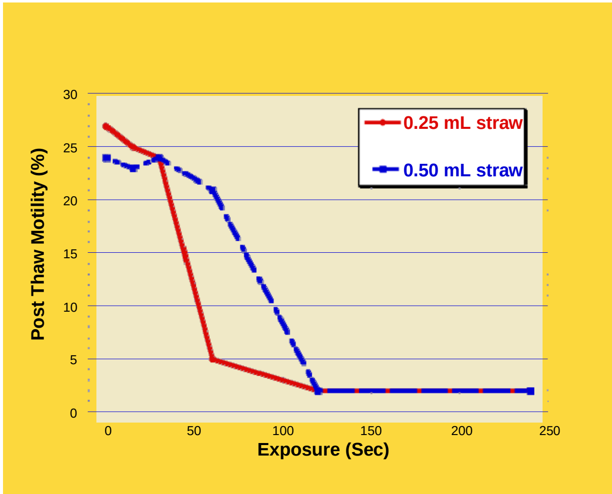

Figure 13. Effect of exposure on post thaw motility in single straws. Duration of exposure to ambient temperatures as indicated, followed by refreezing. Straws of 0.25 mL volume were more susceptible to damage. Adapted from Berndtson (1).

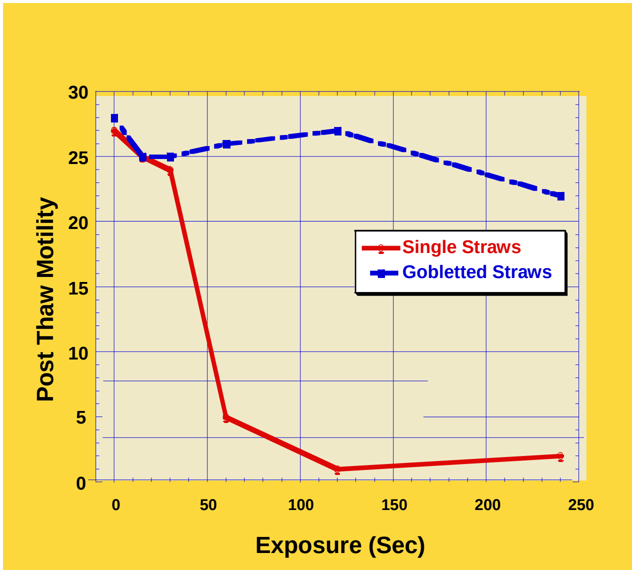

Figure 14. Effect of exposure on post thaw motility in single straws and straws in goblets. Duration of exposure to ambient temperatures as indicated, followed by refreezing. Goblets offer protection. Adapted from Berndtson (1).

Similar patterns of motility declines after exposure were recorded in our laboratory using CASA evaluations (Figure 15). Note persistence of motility, although at declined levels, at 60 and 75 sec intervals. Repeatedly warming and cooling straws after short durations (10 sec-5x, 15 sec-3x, 30 sec-2x) did not seem to damage straws as long as they completely re-cooled prior to their next exposure. However, 45 sec-2x and 60 sec-2x treatments did diminish motility values (Figure16).

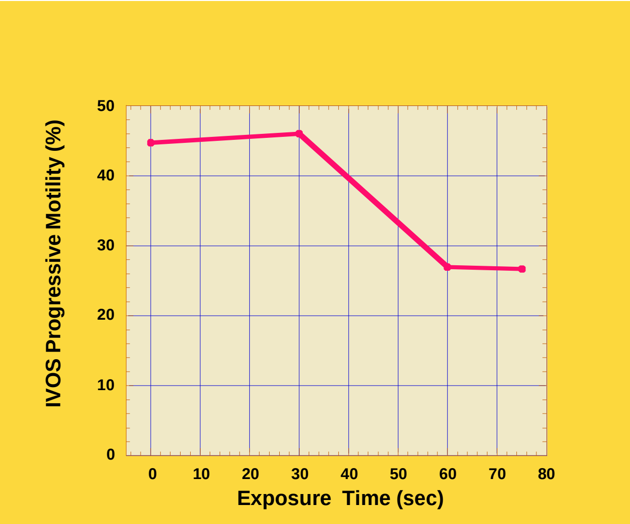

Figure 15. Effect of exposure on CASA (IVOS) post thaw motility in single straws. Duration of exposure to ambient temperatures as indicated, followed by refreezing.

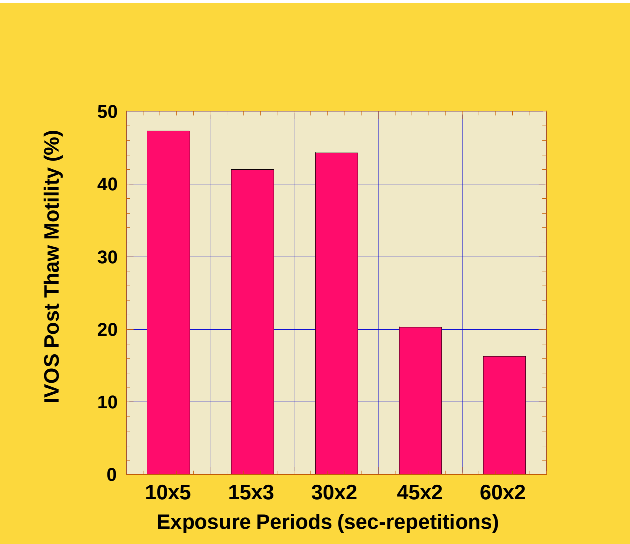

Figure 16. Effect of repeated short exposures on CASA (IVOS) post thaw motility in single straws. Duration of exposure, number of exposures to ambient temperatures as indicated, followed by re-freezing. Repeated short exposures did not cause motility decline.

How Fast Can Single Straws Warm Up?

Saacke (12) provided measurements of seminal warming in cryopreserved straws (Figure 17). From this it can be seen that it takes less than 10 sec for seminal temperatures to rise above -130°C. This is a good justification for minimizing exposures in the processing and distribution lab as well as the field.

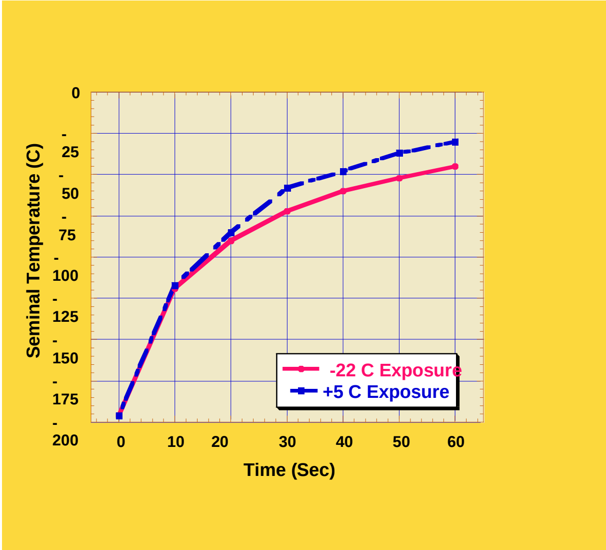

Figure 17. Effect of exposure at varying temperatures and times on resulting seminal temperature. Straws reach critical -130°C temperature in less than 10 sec. Adapted from Saacke (12).

The Lab Suction Exhaust Fan?

The lab "suction-exhaust fan" is a potential culprit for inadvertently damaging straws before they get to the field. The portable suction-exhaust fan is sometimes used to clear the atmosphere within a nitrogen work tank to allow for good visualization of the canes and straws in the tank. Obviously these suction-exhaust fans should only be used where straws are strictly kept below the liquid surface, but staff should also be aware that dangerous atmospherical temperatures can be introduced. Depending on where the exhaust fan inlet is placed, we have recorded temperatures as warm as -5°C to -10°C at a height of 2 cm above the liquid level and at some distance away from the inlet. Since the atmosphere is moving in this region, straws may begin to be damaged in a few seconds.

What Are the Alternatives?

Measurements should be made by staff to determine whether and under what conditions a suction-exhaust fan is appropriate. Relocating the exhaust fan inlet to the highest possible location at the work tank may offer some margin of safety as less room air is introduced at the level of the nitrogen surface. Saacke (12) pointed out that removing water vapor with a suction-exhaust fan was dangerous, and suggested that nitrogen vapor piped in from the stand tank may be a better alternative. In our experience, moving vapor created by forcing a low of nitrogen vapor (~20°C) through liquid nitrogen results in the boiling of liquid nitrogen with production of a steady upward flow of cold nitrogen vapor.

Conclusion

When processed semen leaves the AI center, we have ample and effective routine safeguards for "typical conditions". We however, have to use our wits to understand inevitable unexpected outcomes.

Acknowledgements

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