CANINE SEMEN COLLECTION AND ASSESSMENT

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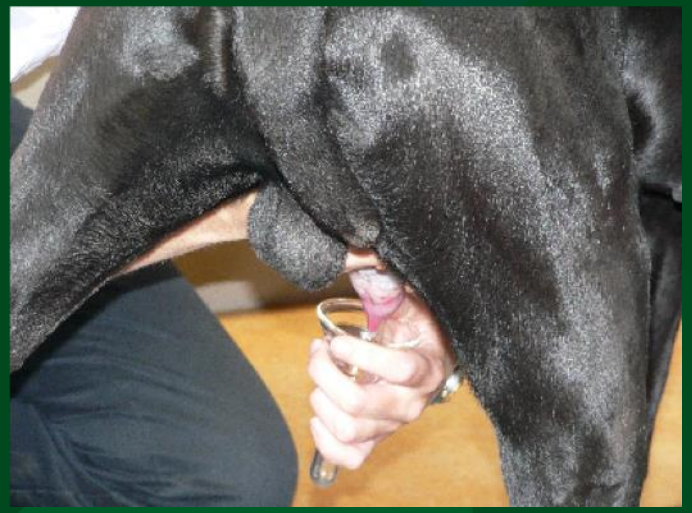

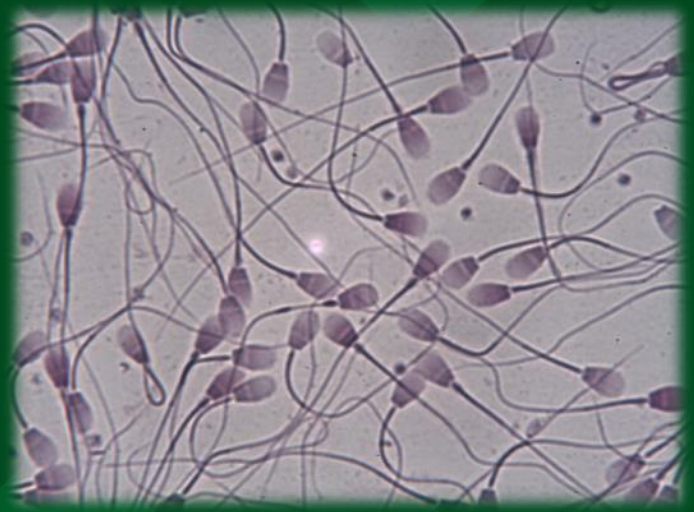

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OVERVIEW 'OF THE BOY'

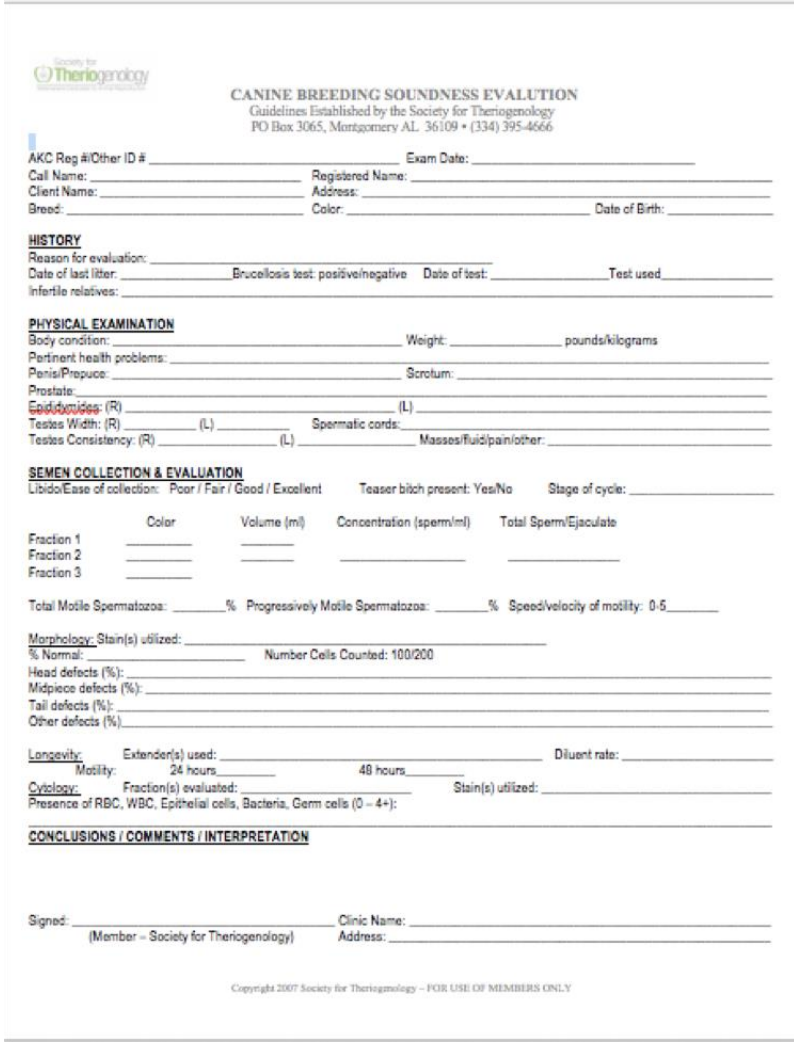
1. Basic breeding soundness examination
2. Semen collection
3. Semen assessment: basic and advanced techniques
4. Processing and preparing semen for chilled shipment
5. Freezing and storing frozen semen
6. Semen thawing
7. Equipment, supplies and space requirements for semen collection, analysis, handling , freezing and storage

Breeding Soundness Examination

A canine BSE consists of performing:

1. Physical examination (BCS, structural soundness)
2. Examination of scrotum, testicles, epididymis by palpation; scrotal Circumference measurement, visual inspection of penis
3. Semen Evaluation

Aim: to detect problems/issues that may reduce fertility, cause infertility or make the dog unsuitable for breeding



CANINE BREEDING SOUNDNESS EVALUTION

Guidelines Established by the Society for Theriogenology
PO Box 3065, Montgomery AL 36109 • (334) 395-4666

AKC Reg #/Other ID # _____ Exam Date: _____
Call Name: _____ Registered Name: _____
Client Name: _____ Address: _____
Breed: _____ Color: _____ Date of Birth: _____

HISTORY
Reason for evaluation: _____
Date of last litter: _____ Brucellosis test: positive/negative Date of test: _____ Test used_____
Infertile relatives: _____

PHYSICAL EXAMINATION
Body condition: _____ Weight: _____ pounds/kilograms
Pertinent health problems: _____
Penis/Prepuce: _____ Scrotum: _____
Prostate: _____
Epididymides: (R) _____ (L) _____
Testes Width: (R) _____ (L) _____ Spermatic cords: _____
Testes Consistency: (R) _____ (L) _____ Masses/fluid/pain/other: _____

SEMEN COLLECTION & EVALUATION
Libido/Ease of collection: Poor / Fair / Good / Excellent Teaser bitch present: Yes/No Stage of cycle: _____

	Color	Volume (ml)	Concentration (sperm/ml)	Total Sperm/Ejaculate
Fraction 1				
Fraction 2				
Fraction 3				

Total Motile Spermatozoa: _____% Progressively Motile Spermatozoa: _____% Speed/velocity of motility: 0-5_____

Morphology: Stain(s) utilized: _____
% Normal: _____ Number Cells Counted: 100/200
Head defects (%): _____
Midpiece defects (%): _____
Tail defects (%): _____
Other defects (%)_____

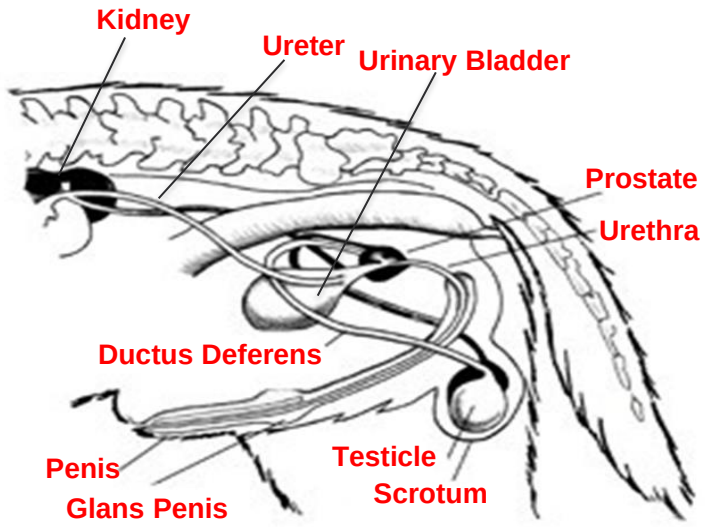
Longevity: Extender(s) used: _____ Diluent rate: _____
Motility: 24 hours_____ 48 hours_____
Cytology: Fraction(s) evaluated: _____ Stain(s) utilized: _____
Presence of RBC, WBC, Epithelial cells, Bacteria, Germ cells (0 – 4+):

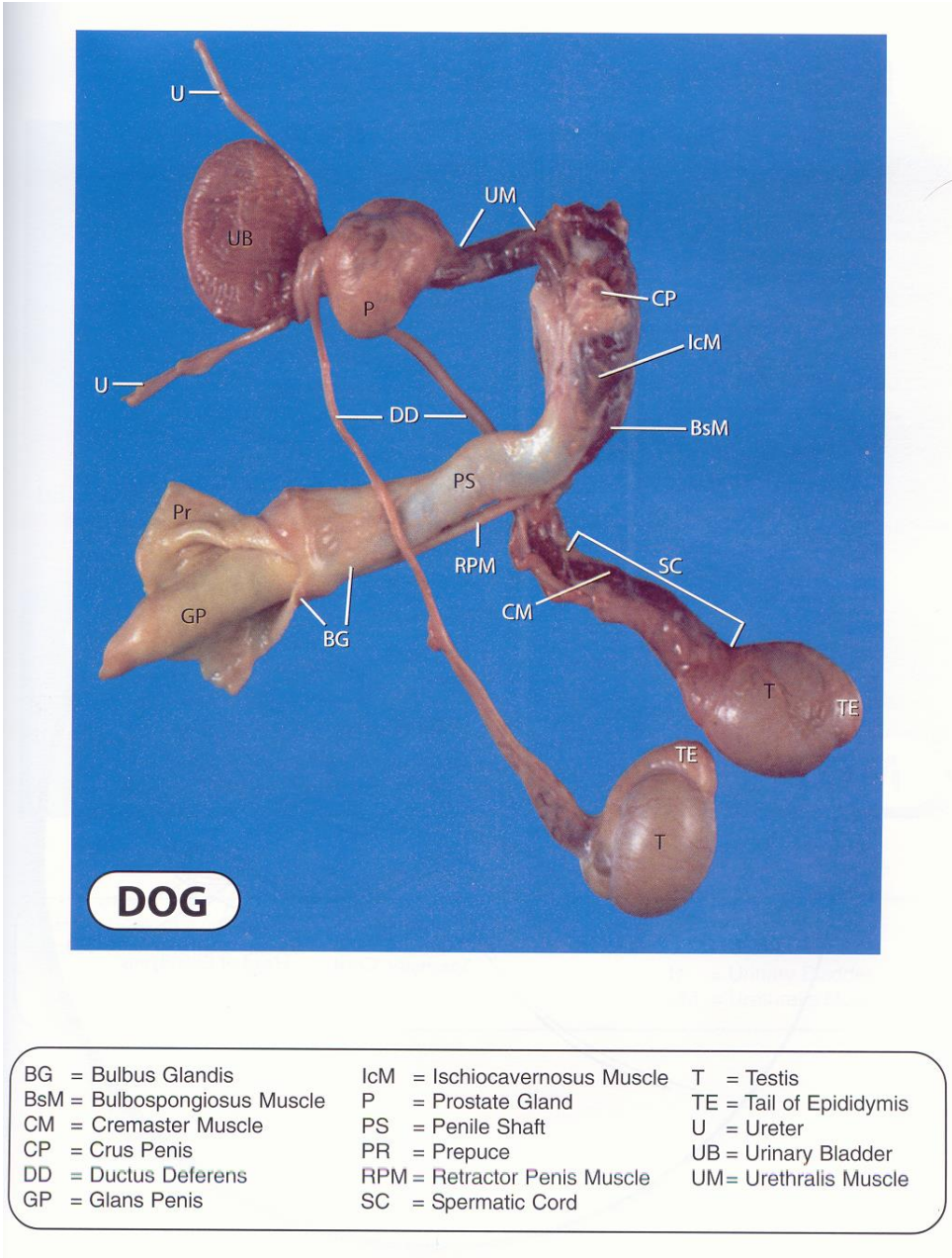
CONCLUSIONS / COMMENTS / INTERPRETATION

Signed: _____ Clinic Name: _____
(Member – Society for Theriogenology) Address: _____

Anatomy-Dog

- Note testes are oriented horizontally and the epididymis is located dorsally
- Large prostate gland (only accessory sex gland)
- Dogs have an *os penis*





Senger, P. L. (2012). Pathways to pregnancy and parturition. ed. *Current Conceptions, Redmond, OR*.

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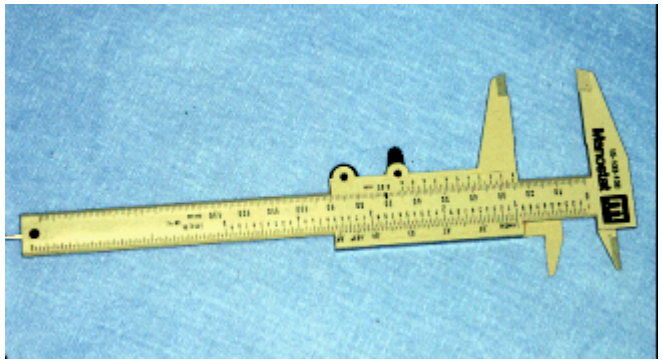
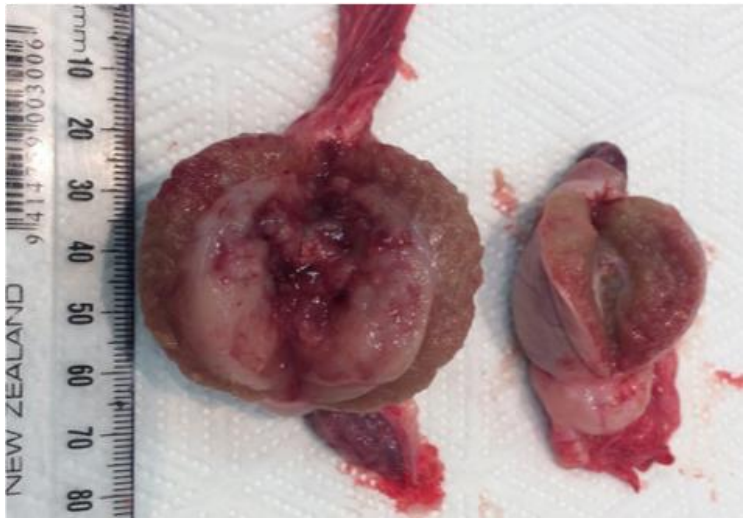
REPRODUCTIVE EXAMINATION: SCROTAL CONTENTS



The normal size of the testes can be estimated using the equation:
log Scrotal Width = 0.324(log Body Weight) + 1.249



Testicular Asymmetry

Ref: Sertoli cell tumor, Ontiveros, Hanlon and Hollinshead. Clinical Theriogenology, 2018

Do not select any cryptorchid dog for your breeding program

https://urbananimalveterinary.com/event/cryptorchidism-retained-testicles-in-dogs/

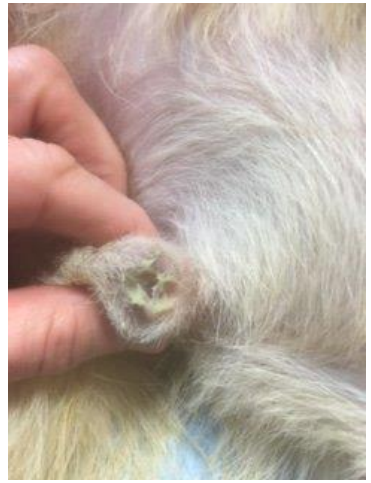
Aim: On palpation to detect:

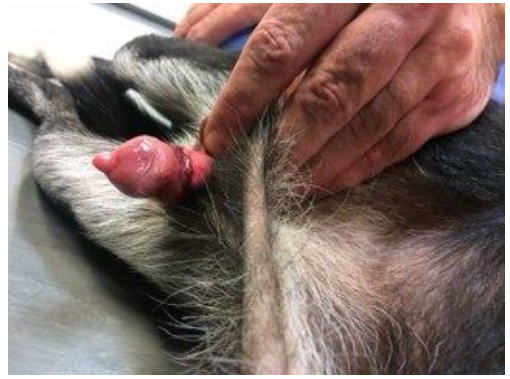
- Testicles and Epididymi (tail, head , body): i)Symmetry, ii) Consistency ii) Size: SC iv) Masses/lumps v) presence of two descended testicles by 6 weeks (cryptorchidism)
- Scrotum: skin lesions (hot spots) that will interfere with thermoregulation
- Spermatic Cord: thickness

REPRODUCTIVE EXAMINATION: PENIS

Check for abnormalities to the penis and prepuce:

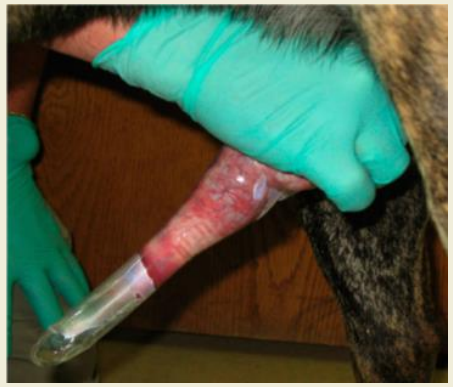
- Blood
- Trauma
- Tumors/masses
- Paraphimosis
- Short Sheath
- Phimosis
- Short Penis
- Stricture
- Priapism - Seen with Spinal Injury
- Os Penis -Fractures, Congenital Abnormalities





B. canis - The Brucella canis status of the male should be ascertained before breeding or freezing semen. The rapid slide agglutination test has many false positives and a true positive titer may wane over time.

SEMEN COLLECTION



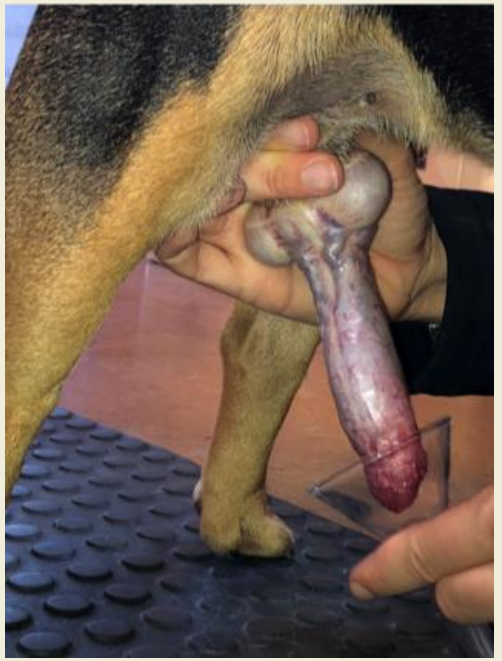
Equipment:

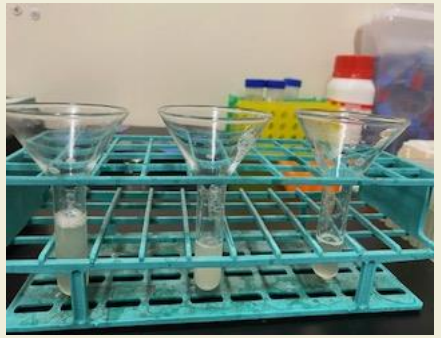
- Open hand technique=

  Glass bovine collection vials

  Plastic funnel with a tube attached

- Collection Cone (latex or PVP) with or without a test tube (disposable): twist technique to fractionate
- Pre-warm vials/vessels (and collect in a warm space)





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Fractionate the ejaculate

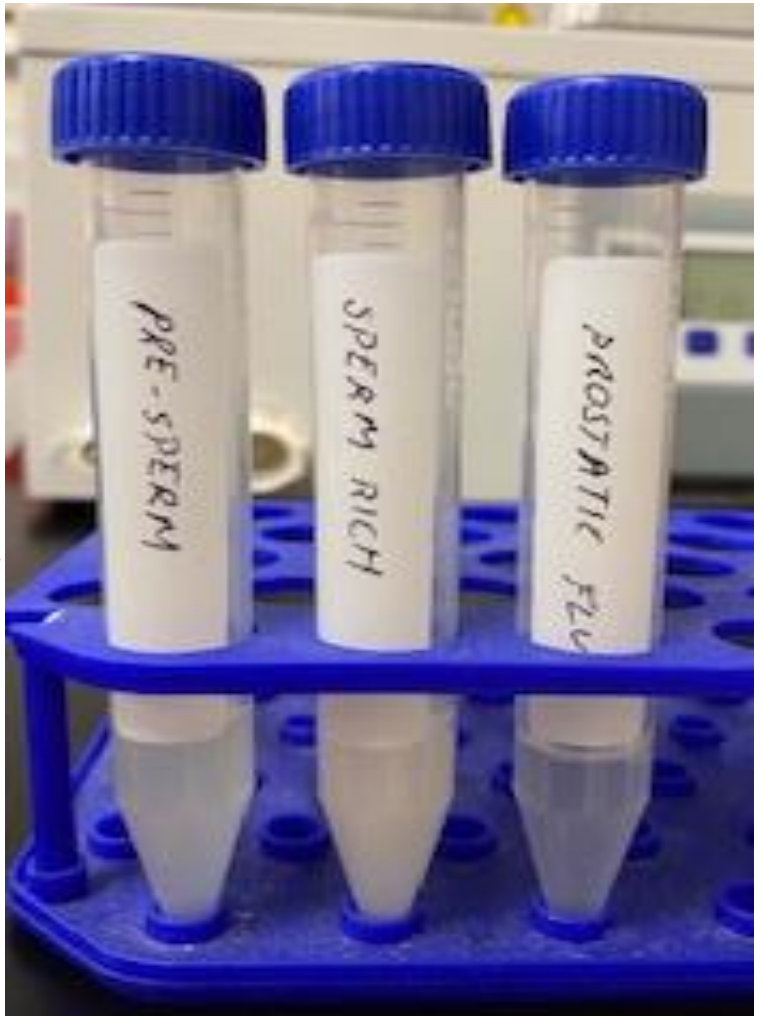
Important to collect separate 3 ‘fractions’ of the dog ejaculate into 3 separate vessels (especially when freezing semen):

1st Fraction: Pre-Sperm: CLEAR Seminal and prostatic fluid (during thrusting): spray/spurt over the floor

2nd Fraction: Sperm Rich: **WHITE** drips 0.5-2 ml (no thrusting):<1-2 min

Switch collection vial when fluid goes from white to clear

3rd Fraction: Prostatic Fluid: CLEAR drips 20-80 ml but only collect 1-2ml (mimic the tie after mating): switch when goes from white to clear



- *Canine sperm don’t like prostatic fluid (fractionation important)*
- *Don’t throw out the 3rd fraction: very effective and easy way to examine the prostate*

Importance of a teaser bitch

- Increased likelihood of obtaining an ejaculate
- If he is a novice or timid dog: ‘play’ increases likelihood of collecting an ejaculate (organized, monitored play sessions prior to collection for ‘training’)
- Increase likelihood of getting a representative/ complete ejaculate : important when freezing semen.

- More likely to get an ejaculate that will freeze well = higher post thaw motility
- More likely to get an ejaculate that has more sperm= more straws= more doses per freeze

Many owners say ‘*my dog collects well without a teaser*’ which is very handy especially when a sample needed for shipment BUT important to understand the benefits of a teaser, particularly when spending the money on shipping or freezing and storing semen for future generations.

Teaser= bitch a standing heat/estrous

If you have to perform a semen collection with out a teaser bitch:

- Use rags or swabs collected from the vulval discharge of a bitch in standing heat i.e on the day of mating/AI: store them in the freezer

- Collection mat with lots of smells (don't clean it!)

If you have a novice or timid dog with low libido:

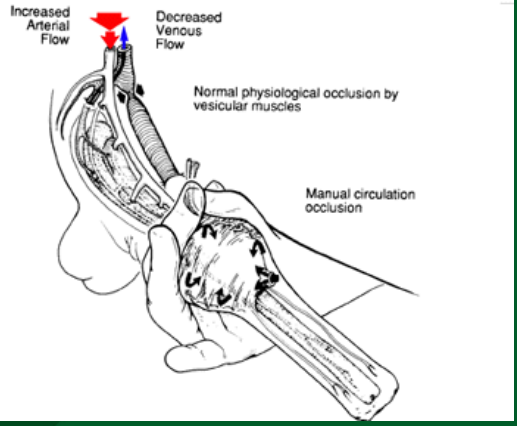
- Sexual 'play' and presence of a bitch they know, even if not in heat can help especially if combined with estrual swabs: the visual as well as olfactory stimui increases chances of being successful

- Sometimes not having the owner in the room during collection will allow the dog to exhibit sexual behavior which usually has negative consequences/re-enforcement

COLLECTION SITE/SPACE

- Use the same ‘collection mat’ and do not wash or clean it: smells and odors of previous bitches in heat will be a positive stimuli
- Make sure the room is quiet and calm: no loud noises or distractions + no other dogs around (not next to the kennels!)
- Use the same room/space every time: consistency so the dogs learn they are ‘allowed’ to behave in a way that is often discouraged
- Fear, anxiety and pain will inhibit a complete erection and ejaculation= collect nothing or a ‘partial ejaculate’
- Need a warm room at a constant temperature (21ºC) without any wind or air currents to not damage sperm viability
- Significant and sudden temperature changes detrimentally affect semen: consistent careful handling of semen from collection to end use is key: *You don’t want to cook it or don’t cold shock it!*

SEMEN COLLECTION PROCEDURE



- **Stimulation of erection:**
  - Vigorous massage?
  - Pressure at base of penis (especially if has an erection)
- **Stimulation of ejaculation**:
  - Firm constant/pulsing (not back and forth massage) pressure behind the bulbous glandis to help stimulate ejaculation

Importance of getting prepuce back over bulbous glandis???

- **Allow dog to 'tie':**
  - If he lifts his back leg to 'tie' and turn then help place his leg over your collection arm
  - Gently pull the penis caudally away from the dog if the prepuce is behind the bulbourethral glands: rotate penis 180º

- **Mounting**

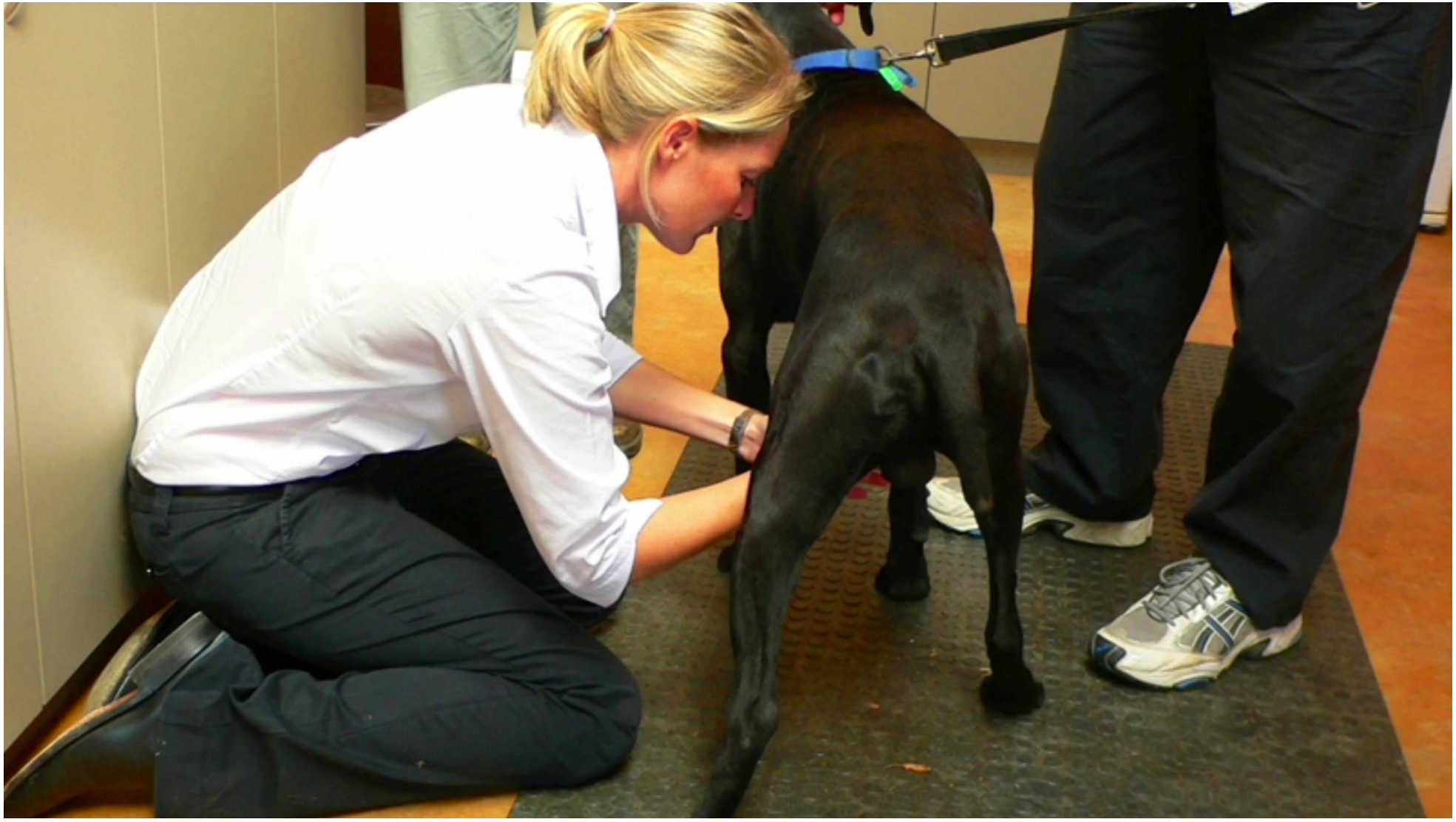
If the teaser bitch allows mounting this can facilitate collecting a complete ejaculate: deviate his penis to the side

- **Detumescence and retraction:**

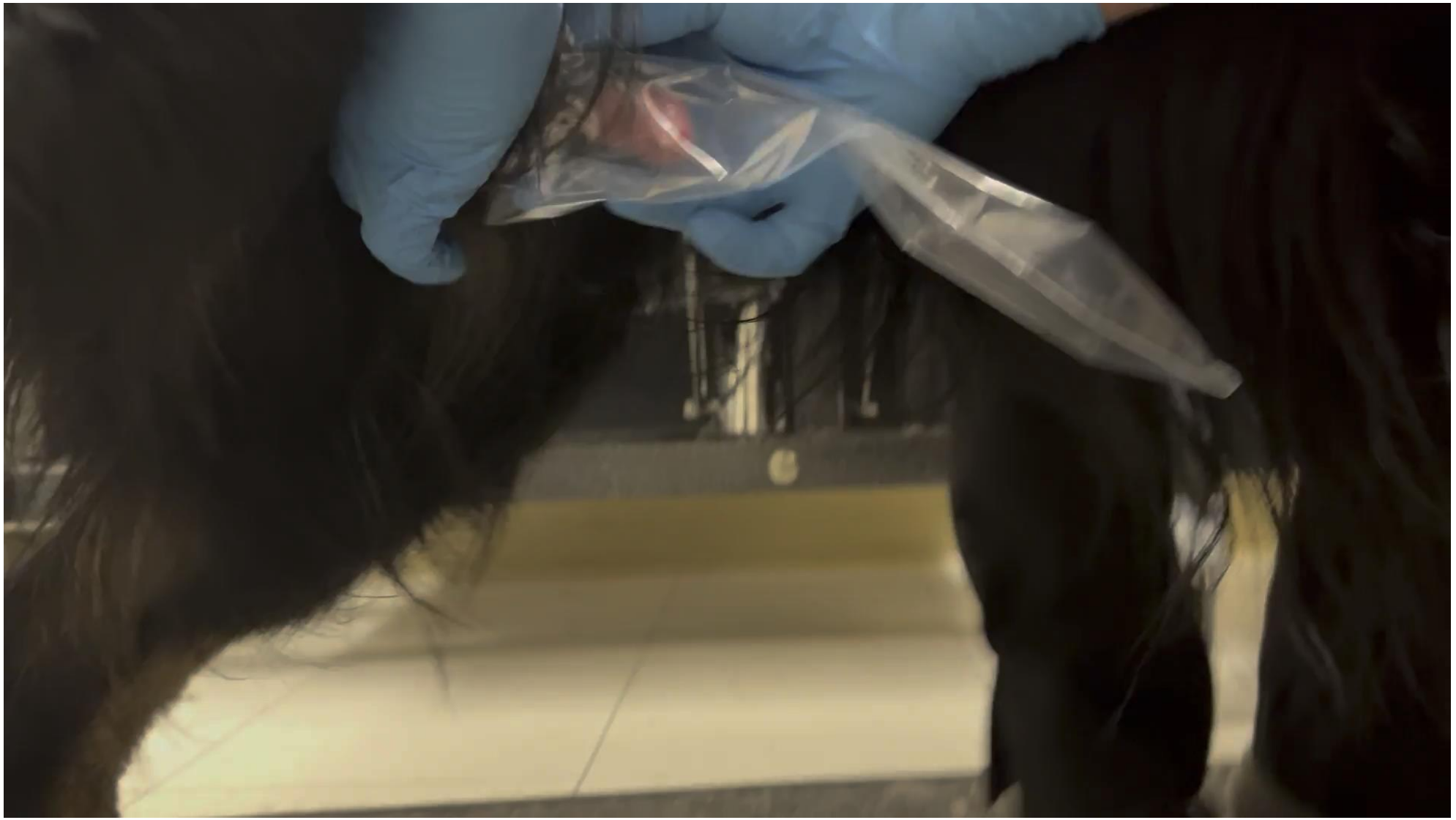
Prevention of rolling in of prepucial skin and/or surrounding hairs getting trapped between the penis and prepuce opening=PAIN

  - Lubrication
  - Jogging
  - Take care with assistance

SEMEN COLLECTION: OPEN HAND

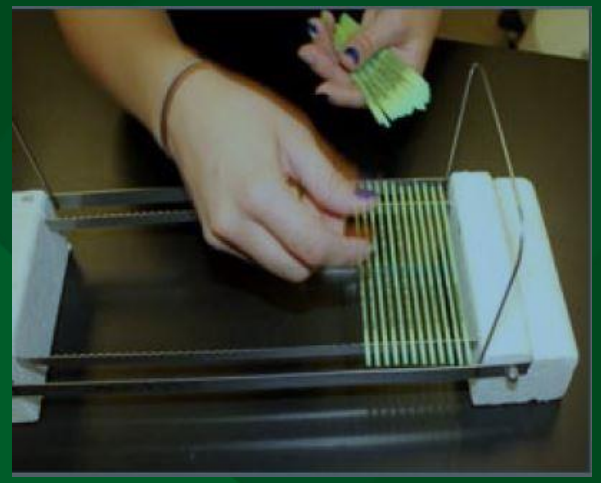


SEMEN COLLECTION: SLEEVE



SEMEN COLLECTION AND ASSESSMENT FOR:

1. Fresh: room temp (21ºC): for immediate AI or infertility work up
2. Chilled: 5 º C chilled-shipment : last 24-32h (box)
3. Frozen: -196 º C (liquid N): stored indefinitely



BASIC SET UP FOR SEMEN HANDLING

Semen lab space is similar to a basic kitchen

Key aspects to consider when setting up a basic semen processing room to ensure sperm viability is not negatively affected:

- Warm and consistent temperature (No wind and drafts)
- Access to water, electricity and bench space (= small kitchen)
- No light exposure
- Clean, sterile, dry equipment and semen vessels

BASIC SEMEN ASSESSMENT

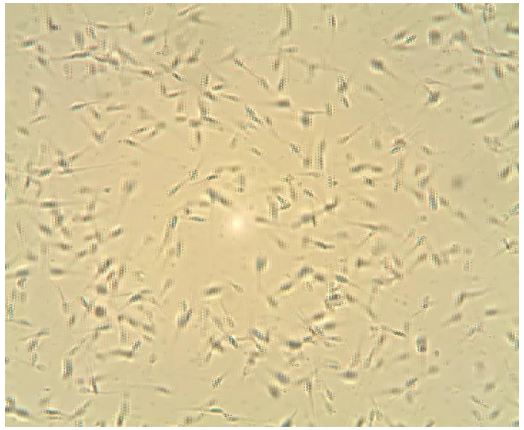
HOW TO PERFORM THE DIFFERENT ASSESSMENTS

↓

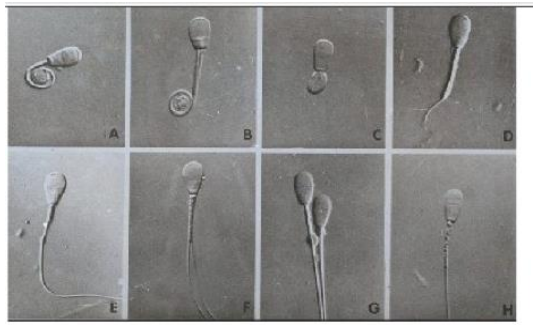
WHAT YOU NEED

- Routine methods of assessment of semen quality are relatively simple:
  - Volume
  - Motility (% motile)
  - Concentration
  - Morphology
- *No one measurement is correlated to fertility or can predict with absolute certainty the fertility of a dog **BUT** can be suggestive of potential breeding success **AND** the more tests you perform, the greater the predictability*
- High variation between assessors: especially motility: QC as a group weekly/monthly (even via zoom): having your microscope connected to a camera and monitor advantageous

Concentration



Subjective Motility



Morphology

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Older dog ejaculate: 3rd fraction blood tinged= Benign Prostatic Hyperplasia (BPH)

Each fraction is analysed for :

- **Volume**- Sp rich: 0.5-2ml ; PF: up to 80ml! (breed, BW dependent)
- **Colour**- Sp Rich white; Healthy PF-clear; BPH- blood tinged
- **pH**- Healthy PF is acidic, BPH (less healthy) is more alkaline
- **Density**-wave motion presence only in very concentrated ejaculates

What do you need for gross semen assessment:

- *sterile, non spermicidal test tubes*
- *pipettor to move semen and measure volume of the ejaculate*

CANINE SEMEN EVALUATION

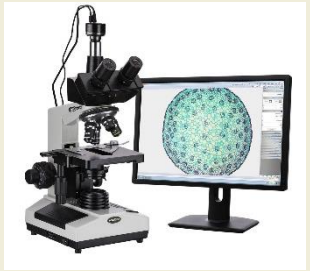
1. GROSS ASSESSMENT

2. MOTILITY

METHOD FOR STANDARD SUBJECTIVE ASSESSMENT

Basic light microscope with X100, X400and X1000 objectives and built in warm stage

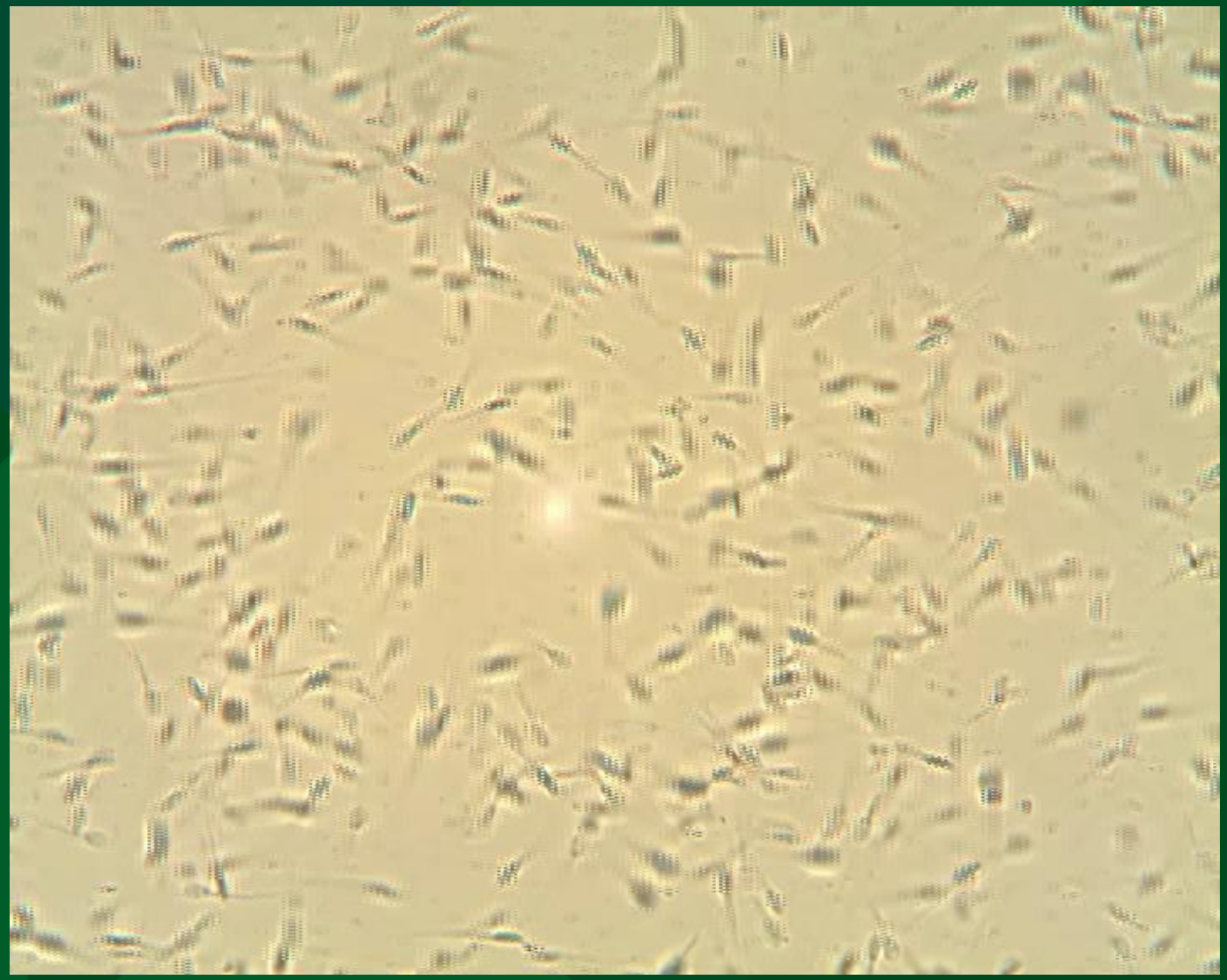
Warm stages



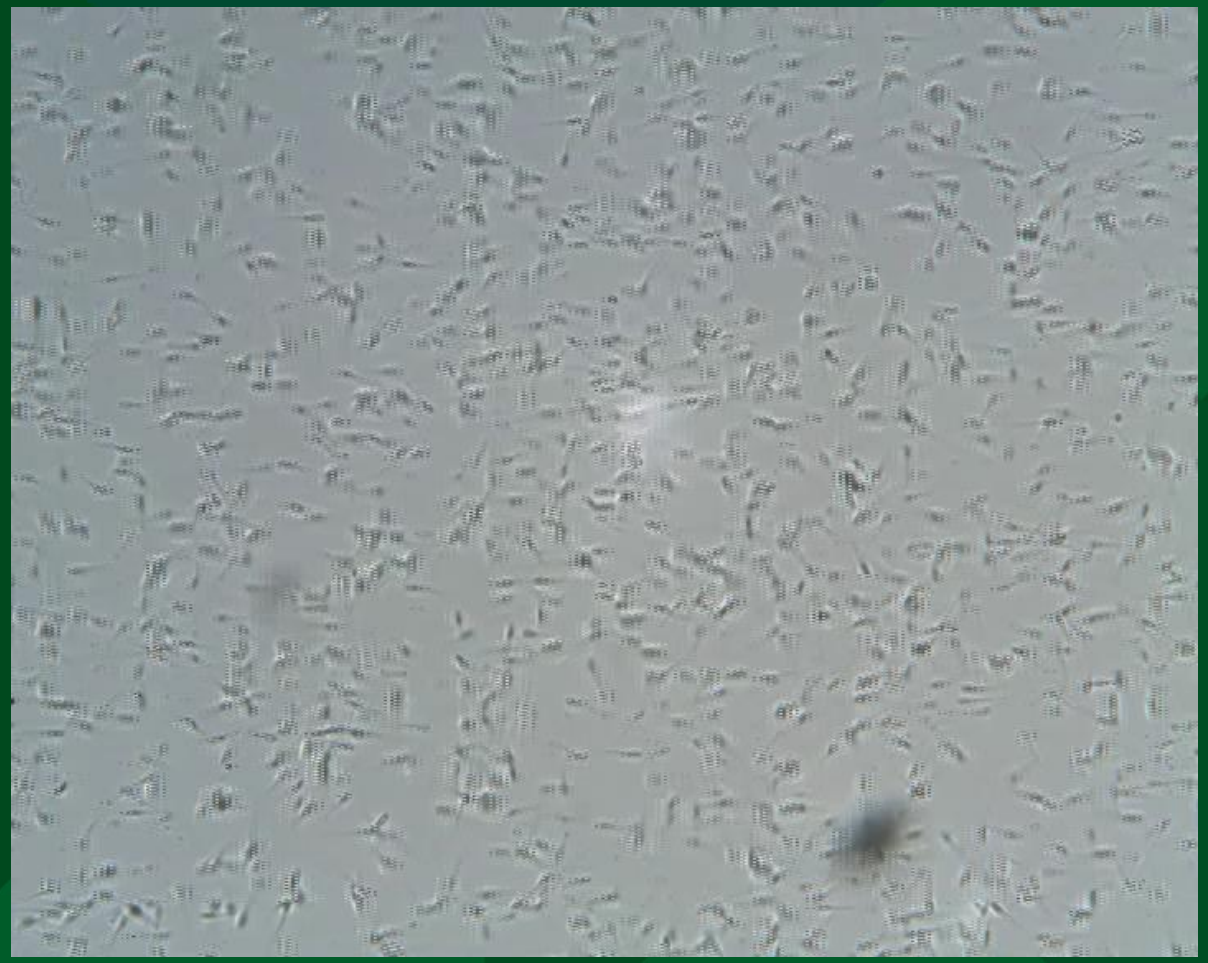
Optional: camera head and monitor

- Must assess with with a warm stage @ 37°C (built in or placed on top of microscope stage)
- Place 10 µl of sperm suspension on pre-warmed (37°C) slide; cover with coverslip (22mm x 22mm)->
- Observe sperm monolayer at 100-200X
- Observe at least 200 sperm from 4 different fields in mid-slide (not at edges)
- Assess motility in 5% increments
- First assess 'raw' semen then dilute apx 1:4 with extender and assess (can see individual sperm)

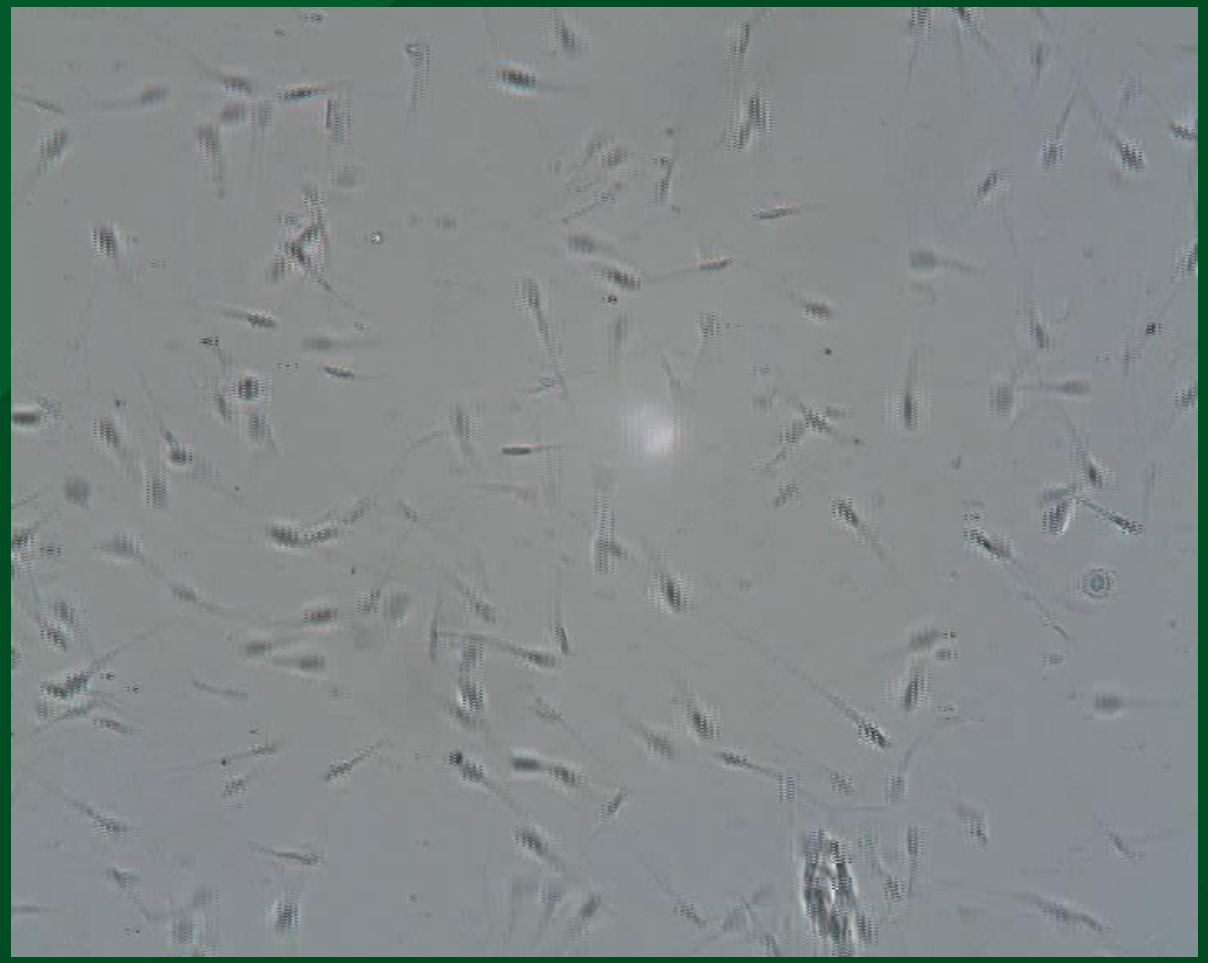
Fresh collected high quality canine semen > 90% PMS; FPM: 5/5



Post-thaw: Examples of high quality frozen-thawed canine semen (using Uppsala extenders and a 2 step freezing technique)

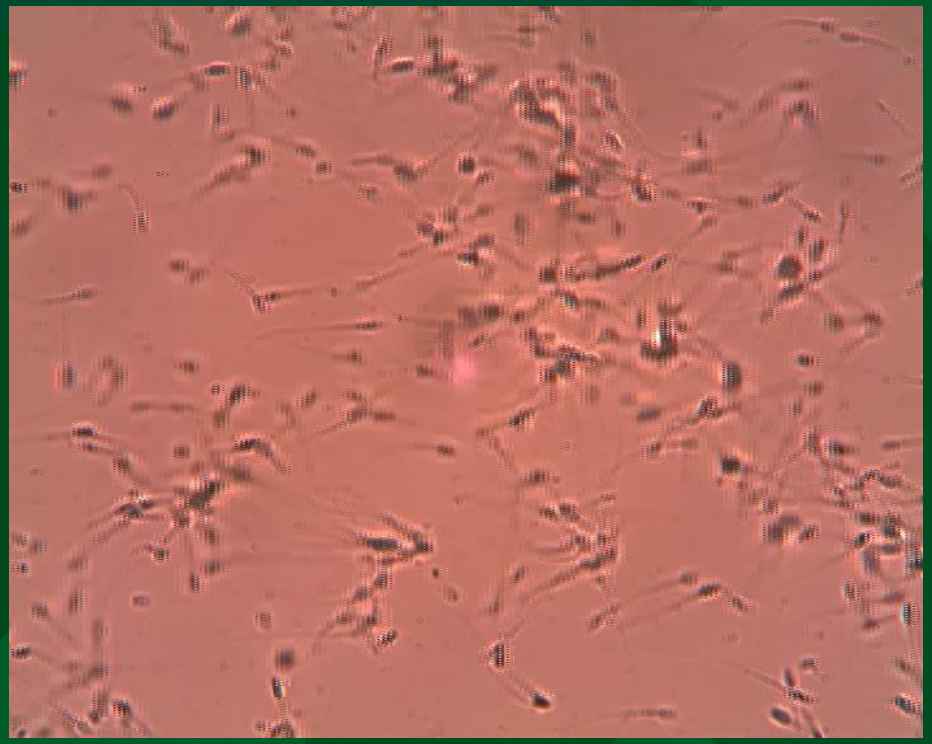


>75% PMS; FPM: 5/5 (200X)

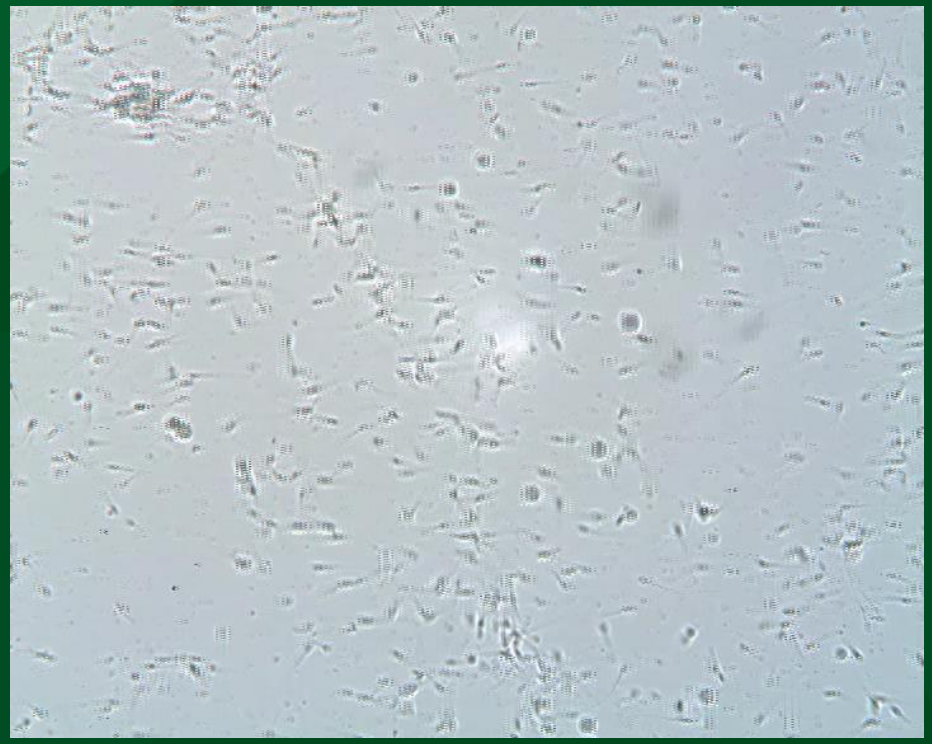


>75% PMS; FPM: 4.5-5/5 (400X)

Post thaw: Examples of frozen-thawed poor quality canine semen (imported semen)

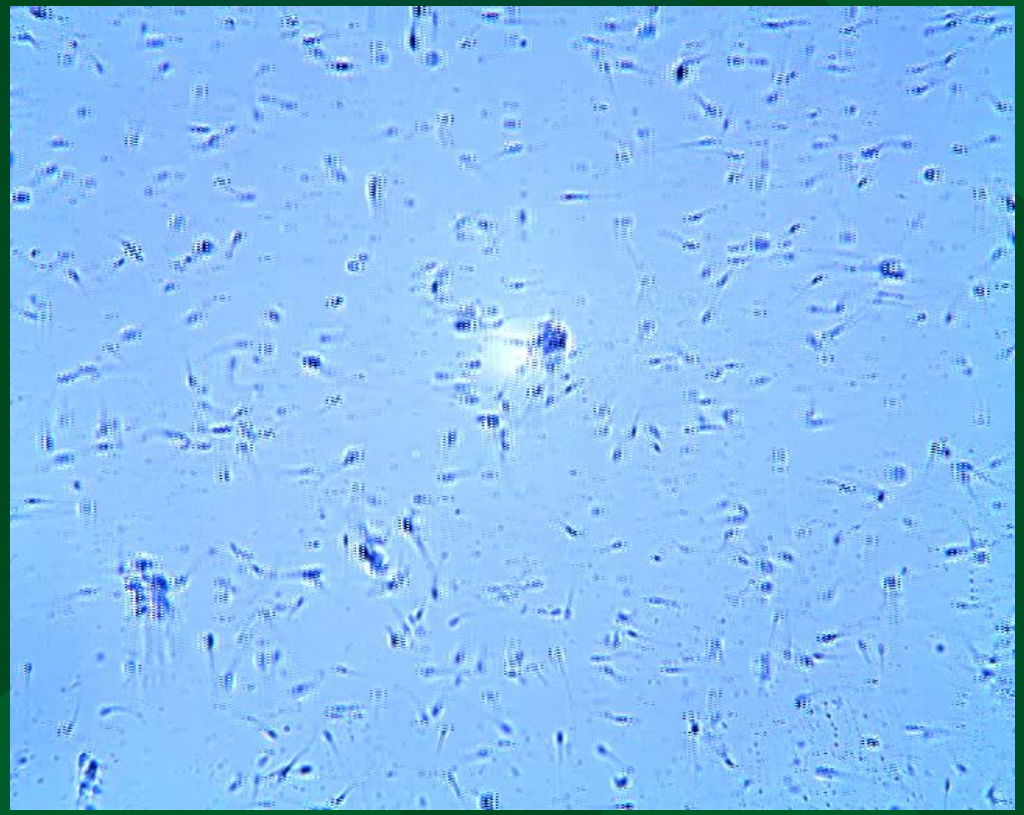


<5% PMS; twitching : FPM 0/5

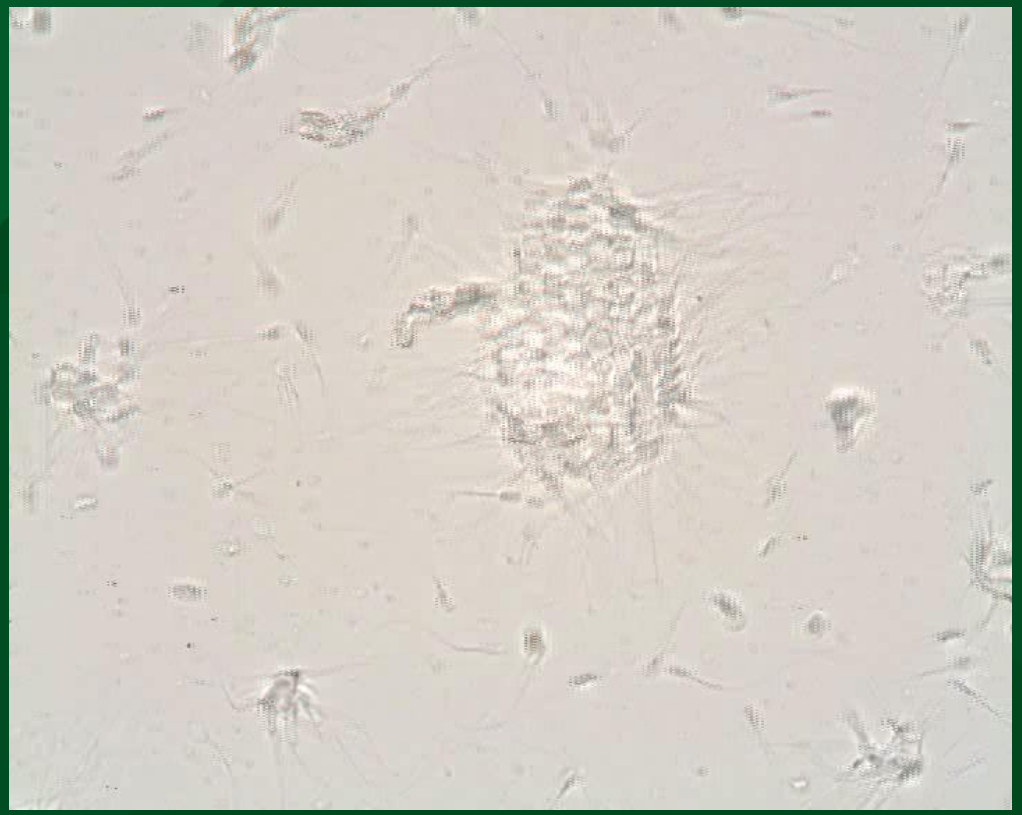


45-50% TMS; FPM 3.5/5,
Line agglutination= variable fields-need to look at more fields to get overall % motility

Post-thaw: Examples of sperm agglutination: sperm head stick together or to cellular debris forming clumps or lines: common after thawing and incubation

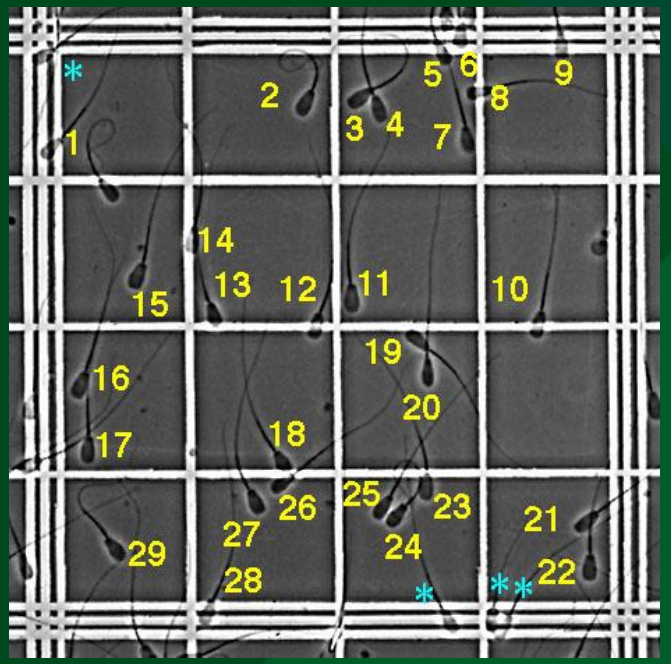


>80%, FPM: 5/5 but agglutinated



Sperm trying to fertilise a squamous cell

3. CONCENTRATION: SPERM COUNT



- **Haemocytometer-manual count**
  - Most accurate: gold standard
  - Requires accurate dilution of sperm in saline to make immotile
  - Cost effective
- **Spectrophotometer/** Densimeter
  - Evaluates percent light transmission
  - Can only use with raw semen (not diluted)
  - Requires standardisation and calibration for canine sperm.
- **CASA** (Sperm analyser)
  - Generally inaccurate, though precise
- **Nuclear Counter**
  - -uses fluorescent dye that stains the sperm nucleus
  - - Raw and extended semen
  - - Gold standard but $$$

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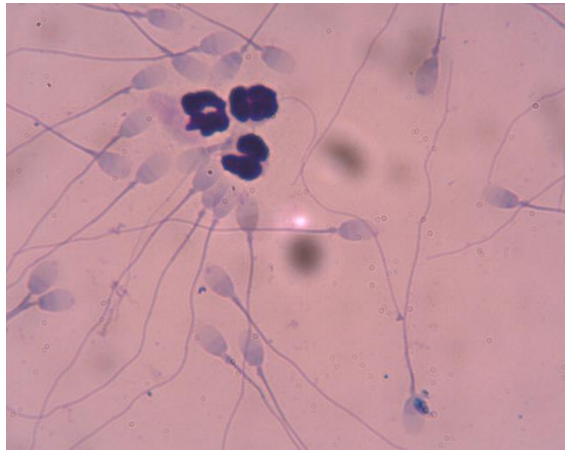
MORPHOLOGY

Eosin nigrosin stained sperm: red sperm are non viable



Diff Quik stain: sperm and inflammatory cells (neutrophils)

Dry smear stains

-Eosin-Nigrosin: live-dead stain and morphology(SFT recommended)

-Diff-Quik cytology: Morphology and cytology

Wet mount preparations

- Immobilise sperm in 3% NaCl or 0.5% formalin

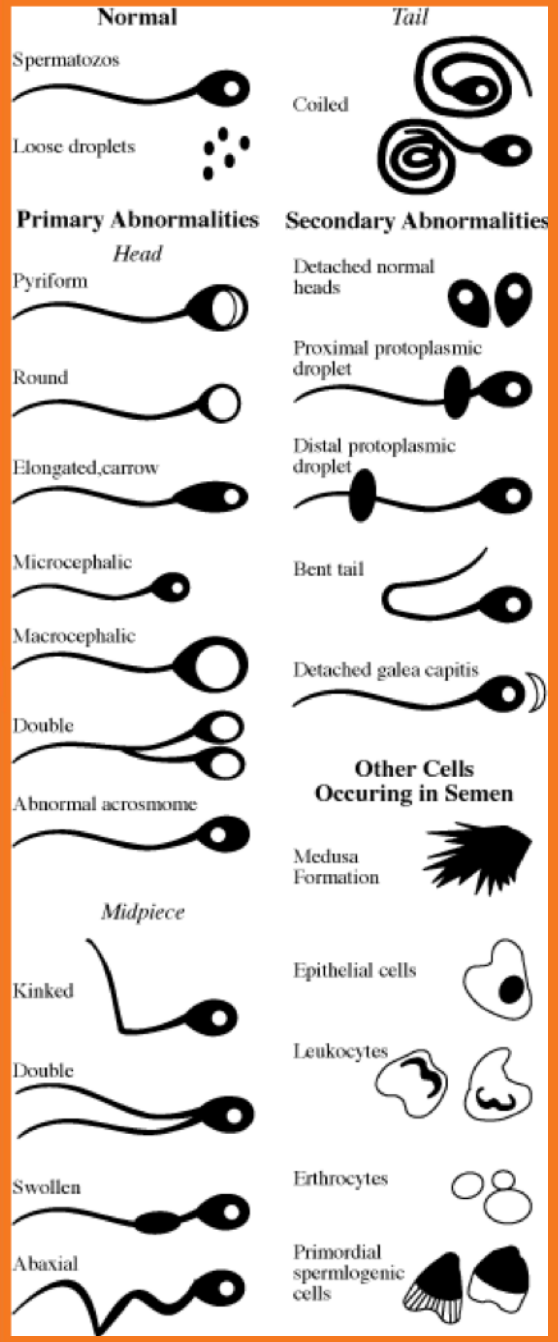
Observe under 1000X, oil immersion

- Count 200 sperm and classify accordingly: normal/abnormal (head, tail, neck defects) using a sperm counter
  - Use bright field or phase-contrast microscopy or DIC (gold standard for morphology)

Sperm counter

MORPHOLOGY

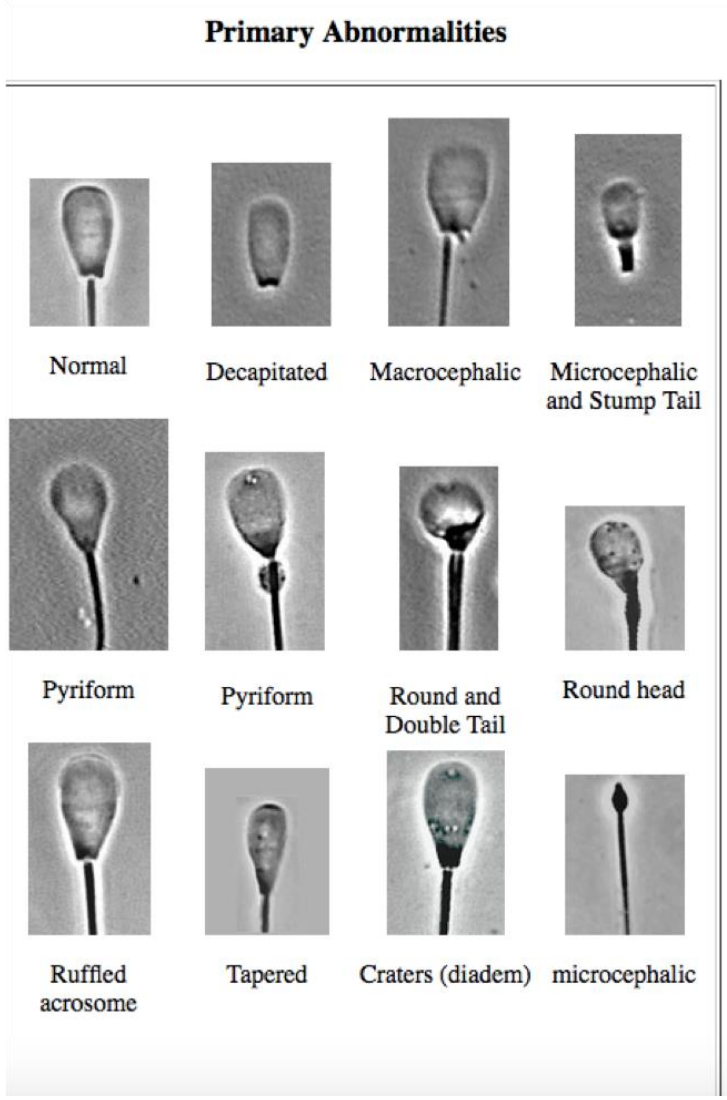
Classification systems:



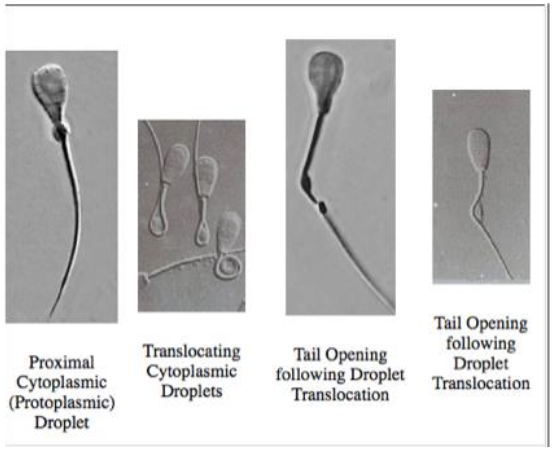
- **ANATOMIC SITE:**
  - Head, midpiece or tail- some sperm may have multiple sites affected
- **ORIGIN OF DEFECT:**
  - **Primary**- defect originates in testis during spermatogenesis
  - **Secondary**- defect originates within epididymi during maturation
- **PERCEIVED ADVERSE EFFECTS ON FERTILITY:**
  - **Major** –defects @with proven infertility* : non-compensable (@head)
  - **Minor**- structural defects but not @ with decreased fertility: compensable (@tail)
- **THE INTERACTION BETWEEN SEMEN QUALITY AND QUANTITY AND ITS EFFECT ON FERTILITY:**
  - **Compensable-** increasing sperm numbers may increase chance of pregnancy
  - **Non compensable-** increasing sperm numbers will not improve pregnancy rate eg DNA damaged sperm @ with embryonic loss

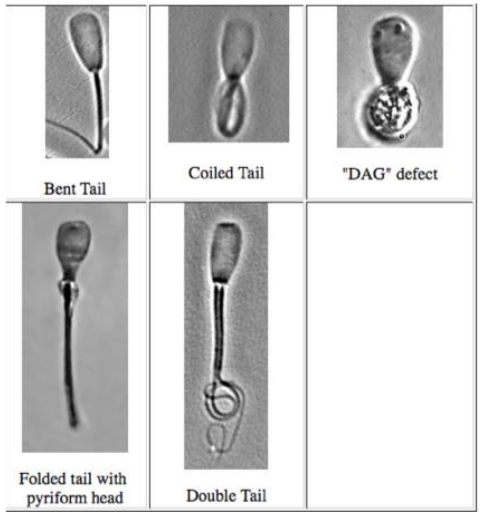
*Jasko, Lein and Foote Determination of the relationship between sperm morphologic classifications and fertility in stallions.1990 JAVMA 197, 3, 389-394

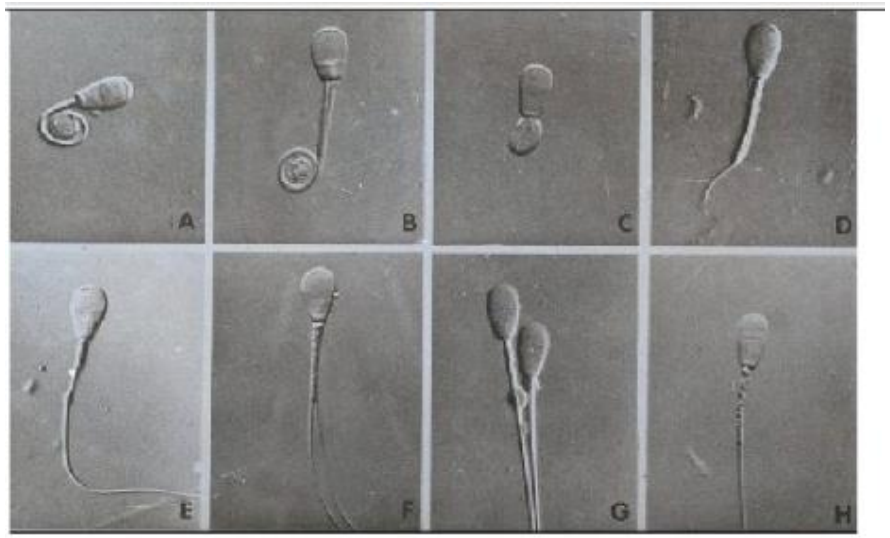
ORIGIN OF THE DEFECT CLASSIFICATION



Secondary Abnormalities





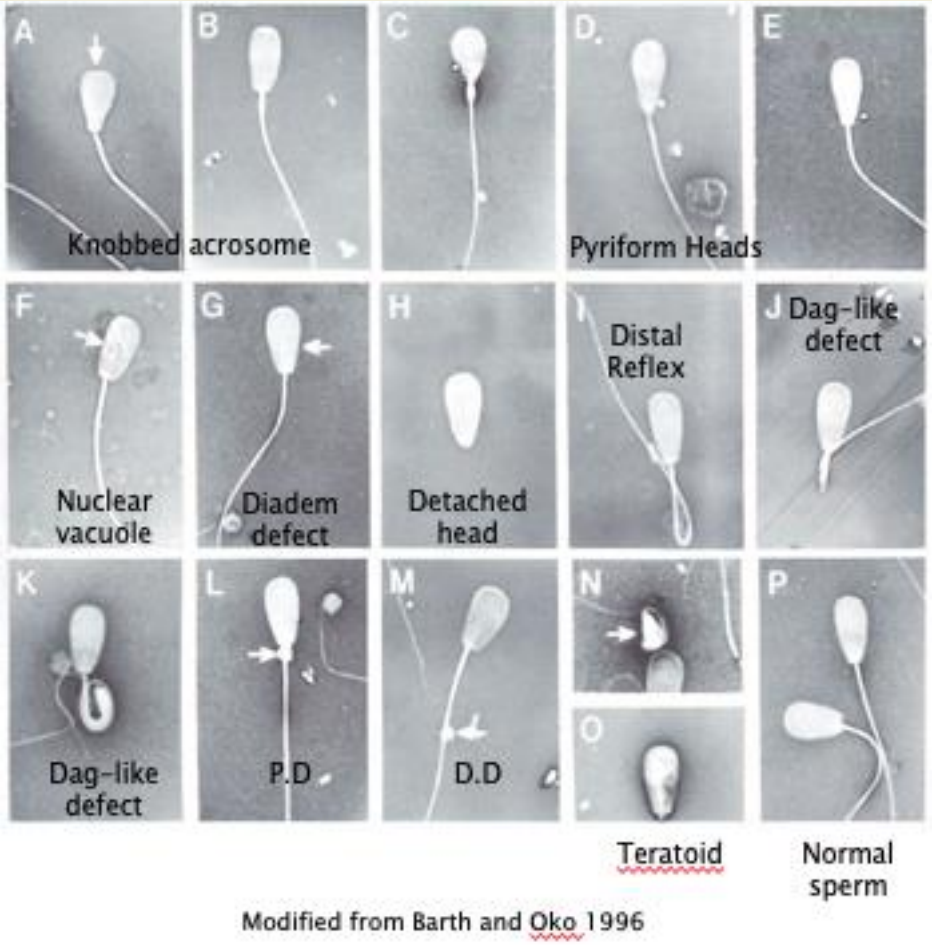


A. Coiled Tail with Droplet
B. Coiled Double Tail
C. "DAG" defect
D. Folded tail
E. Filamentous
F. Double Tail
G. Corkscrew Midpiece with Droplet
H. Corkscrew Midpiece

http://www.ansci.wisc.edu/jjp1/ansci_repro/lab/procedures/sperm/bovine_abnormals.html

PERCEIVED EFFECTS ON FERTILITY CLASSIFICATION:

Examples of major and minor defects in bull spermatozoa



Modified from Barth and Oko 1996

Sperm Abnormality	Pathogenesis	Effect on Fertility
Knobbed Acrosome	Abnormal pathogenesis due to disturbances in testis heat regulation e.g. systemic illness, toxicity, nutritional deficiencies, fat deposition around scrotum. Heritable.	Bull with 83-99% of spermatozoa affected was used for natural mating (single sire) – calving rate of cows mated was 9%. Sperm do not attach to zona pellucida.
Pyriform Head	Abnormal spermiogenesis due to disturbances of either heat regulation in testis of endocrine control of testicular function. Possible genetic predisposition.	Reduced fertility in superovulated heifers inseminated with semen containing a high proportion on affected cells.
Nuclear vacuoles (pouches, craters, diadem defect)	Unknown, possible stress induced. Diadem also thought to be associated with feeding high concentrate rations.	Bull with 80% diadems had a history of low fertility but produced apparently good quality semen (e.g normal post thaw motility). However, fertilization rate was reduced by 54%
Detached Heads	Testicular hypoplasia. Testicular degeneration, inflammation of ampullae, and epididymis. Appears to be a genetic predisposition.	Bull suffering from laminitis and toxaemia had 91% sperm cells affected. Four to 6 months later incidence decreased to 33%. Can be associated with sperm accumulation and cell death.
Distal Midpiece Reflex	Thermoregulation disturbances of the testis. Low testosterone levels. Can be induced when spermatozoa exposed to hypotonic solutions or cold shock. Genetic disposition.	High incidence may result in reduced fertility. Usually transient.
Dag-like Defect	Disturbance of spermiogenesis. Genetic disposition.	High incidence results in significant impairment of fertility.
Proximal droplet	Disturbance in later stages of spermiogenesis and in function of the caput epididymides. Immaturity of testicular function in young bulls.	Marked reduction in in vitro fertilization rate following use of semen containing >30% proximal droplets.
Teratoid (undeveloped)	Severe disturbance in spermatogenesis and spermiogenesis. Tubule degeneration and fibriosis.	High incidence results in significant impairment of fertility.

Also see: http://www.vivo.colostate.edu/hbooks/pathphys/reprod/semeneval/morph.html

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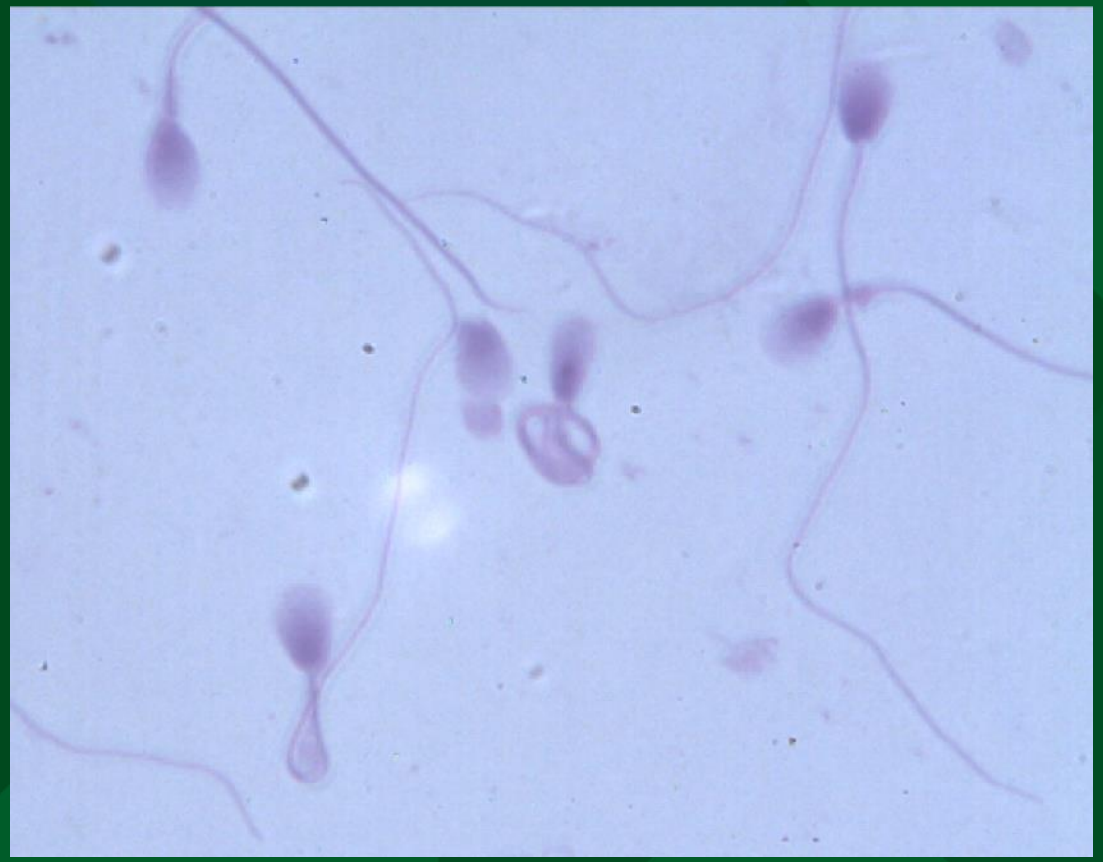
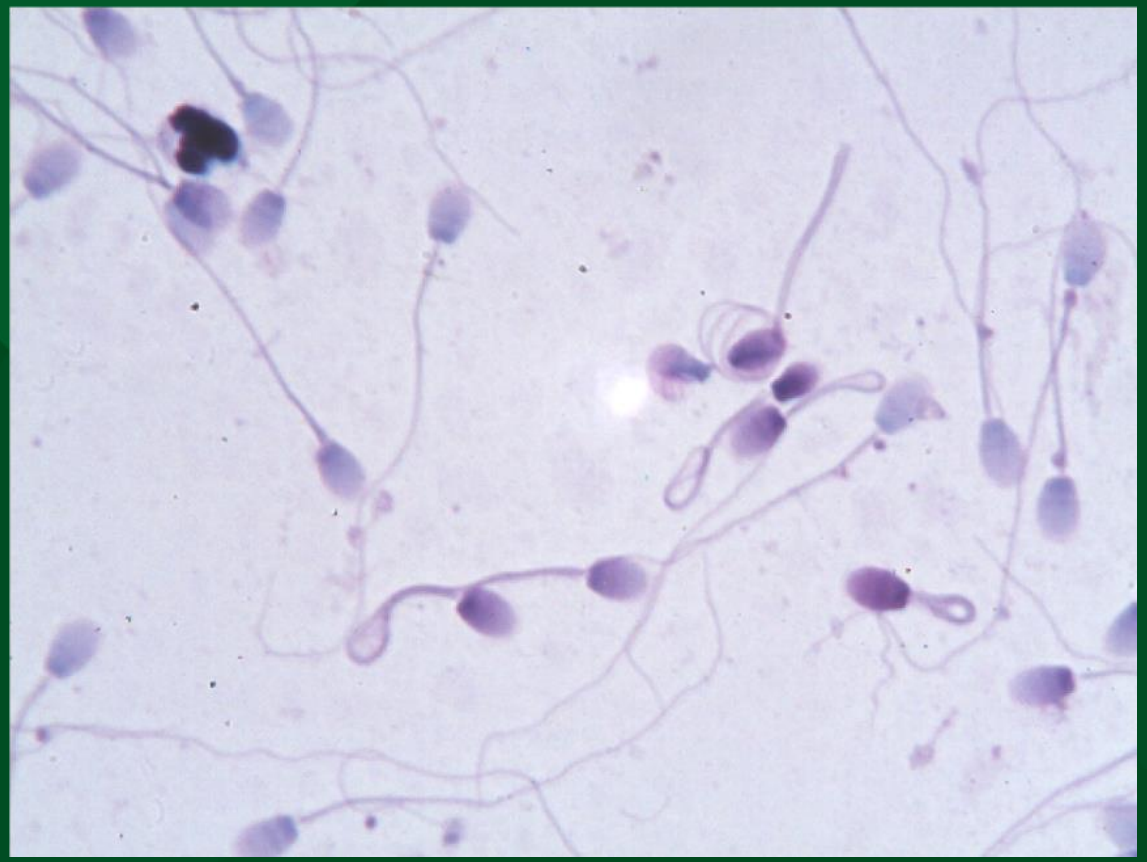
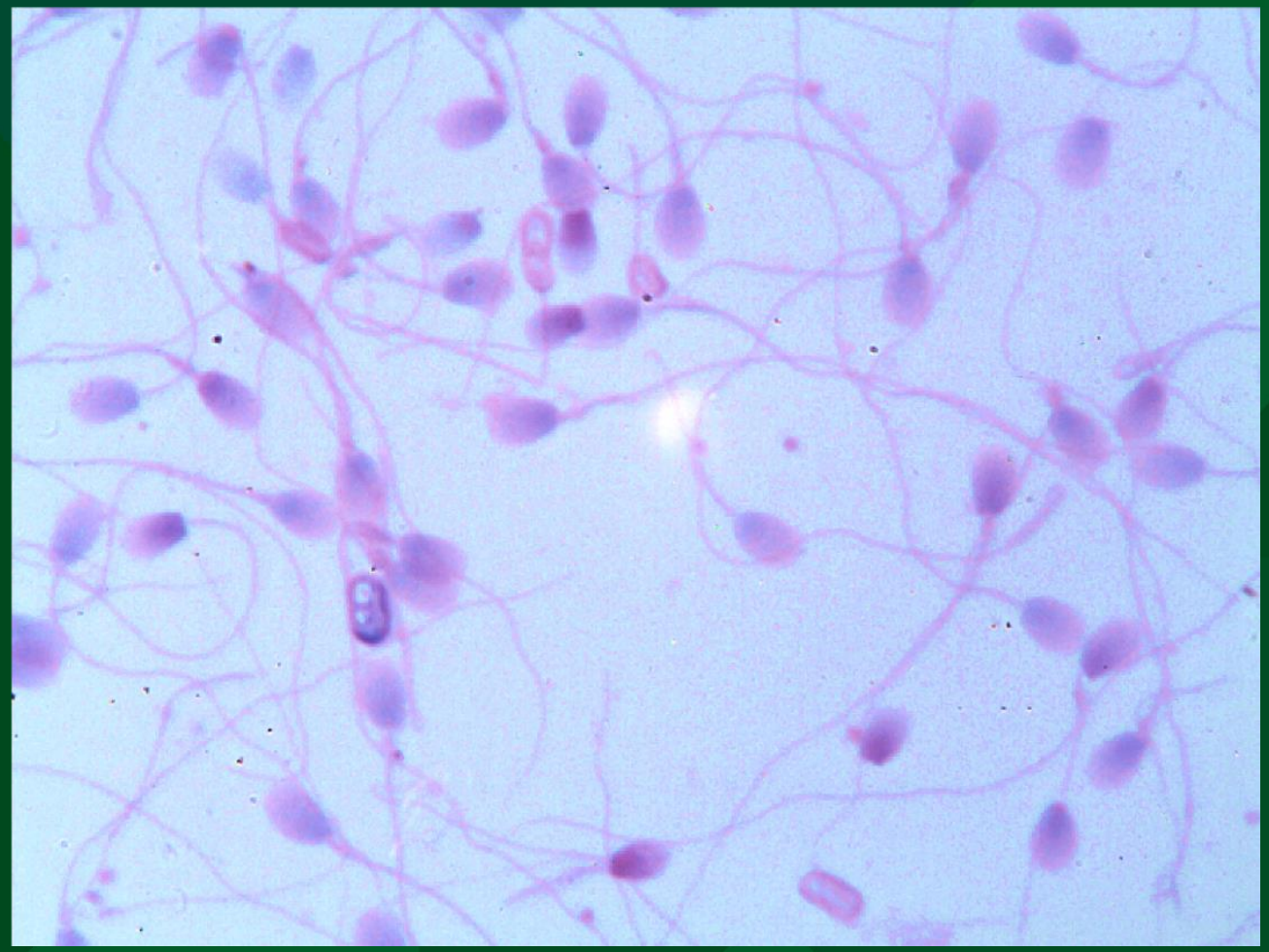
EXAMPLES OF CANINE SPERM MORPHOLOGY ABNORMALITIES





Mixture of defects: Coiled tails, dag, proximal droplets, bent midpiece reflex, macrocephalic heads, no inflammatory cells (X1000 oil): Old dog with history of declining fertility; testicular degeneration (soft small testicles)

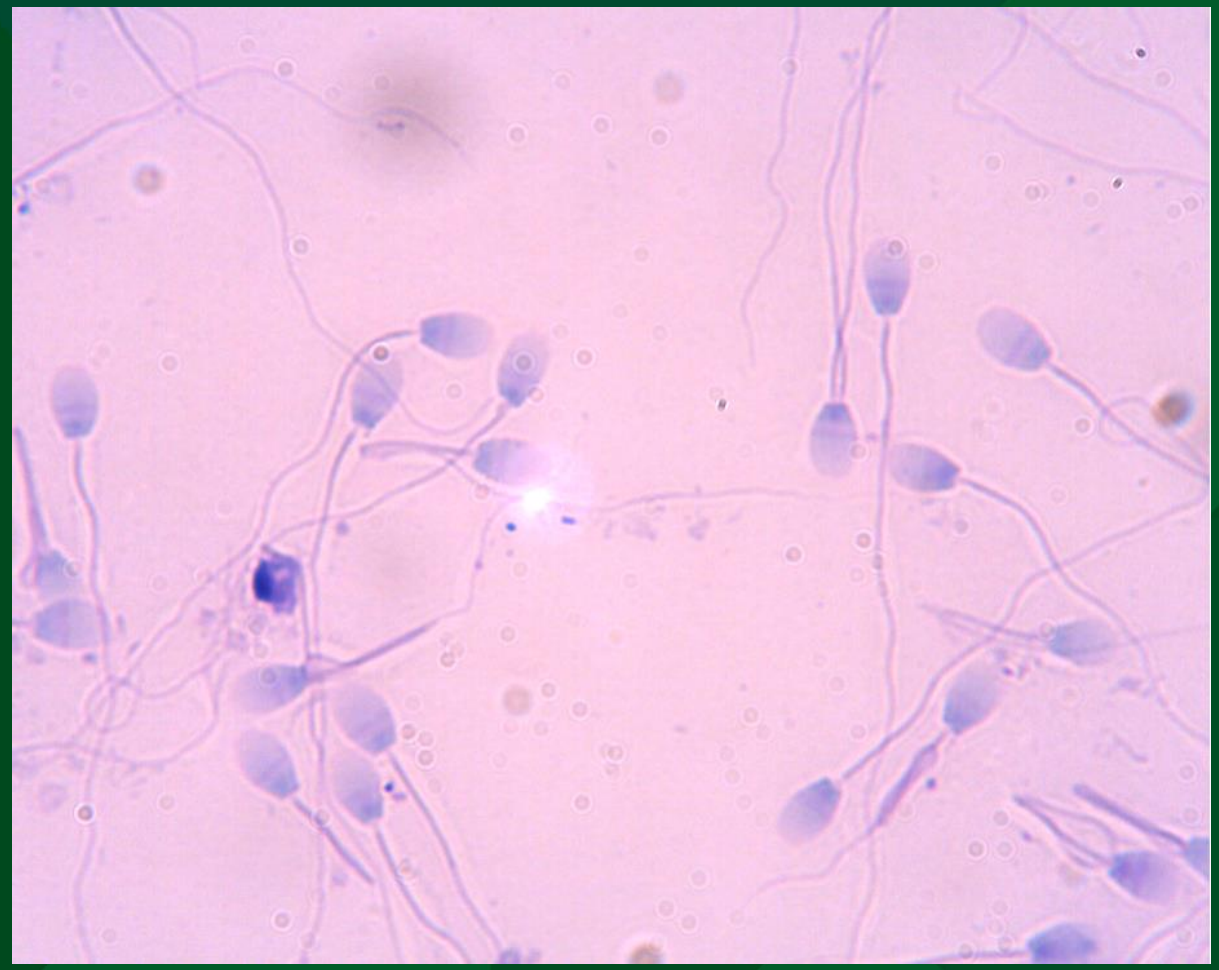


Mixture of detached heads and coiled tails (X1000 oil)

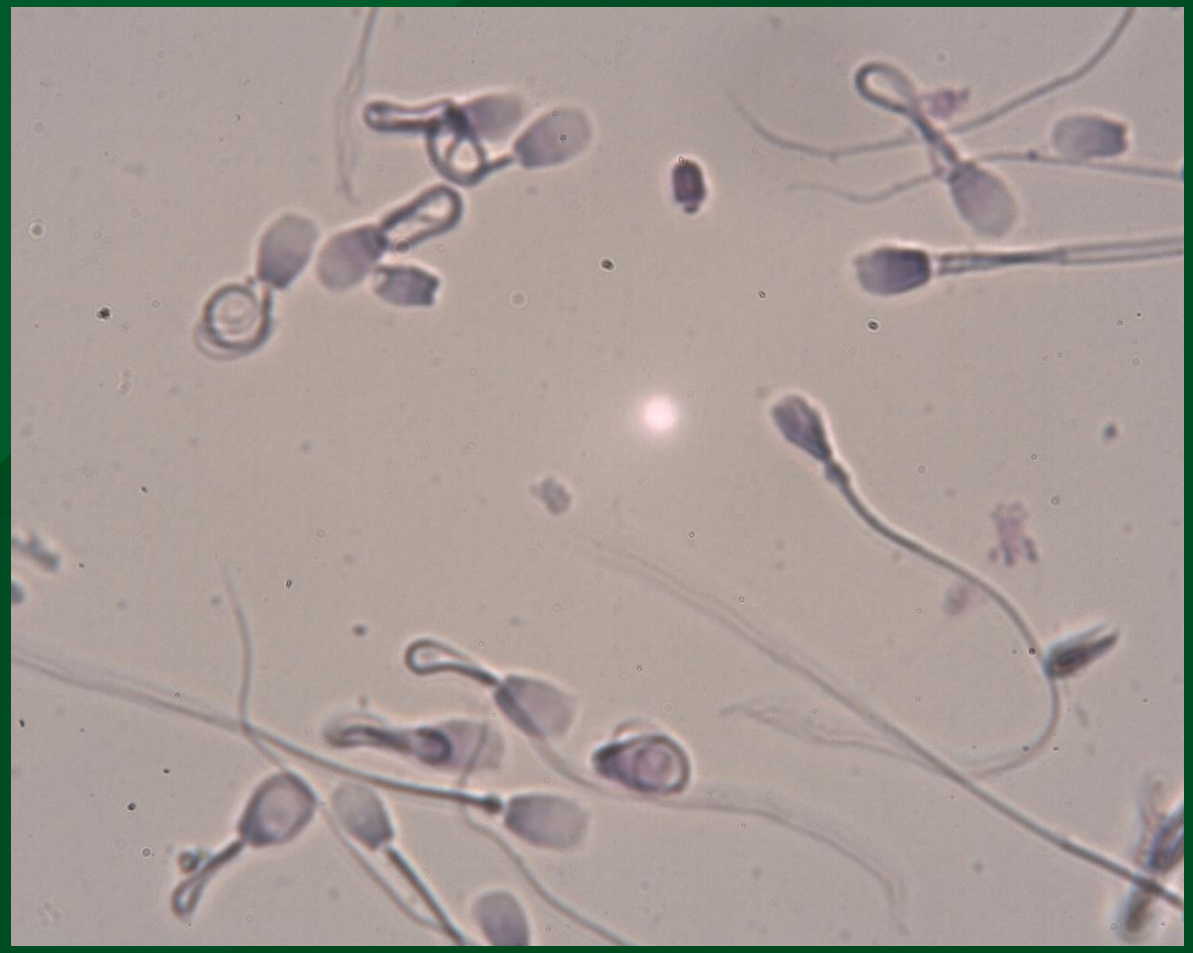
Dag defect

Coiled tail

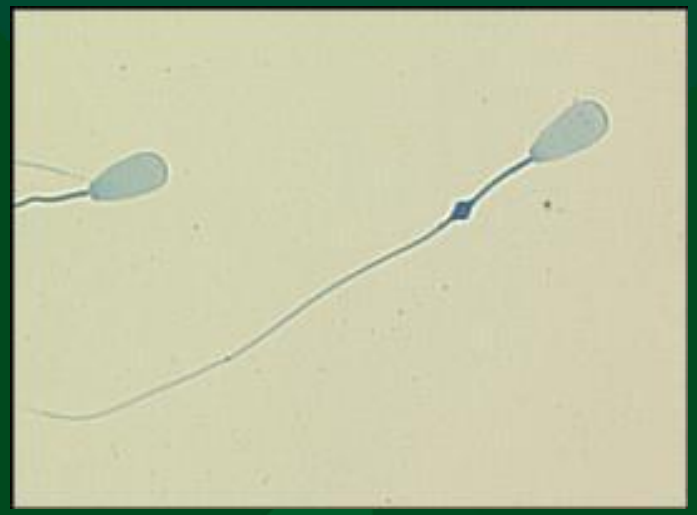
Loose/detached head



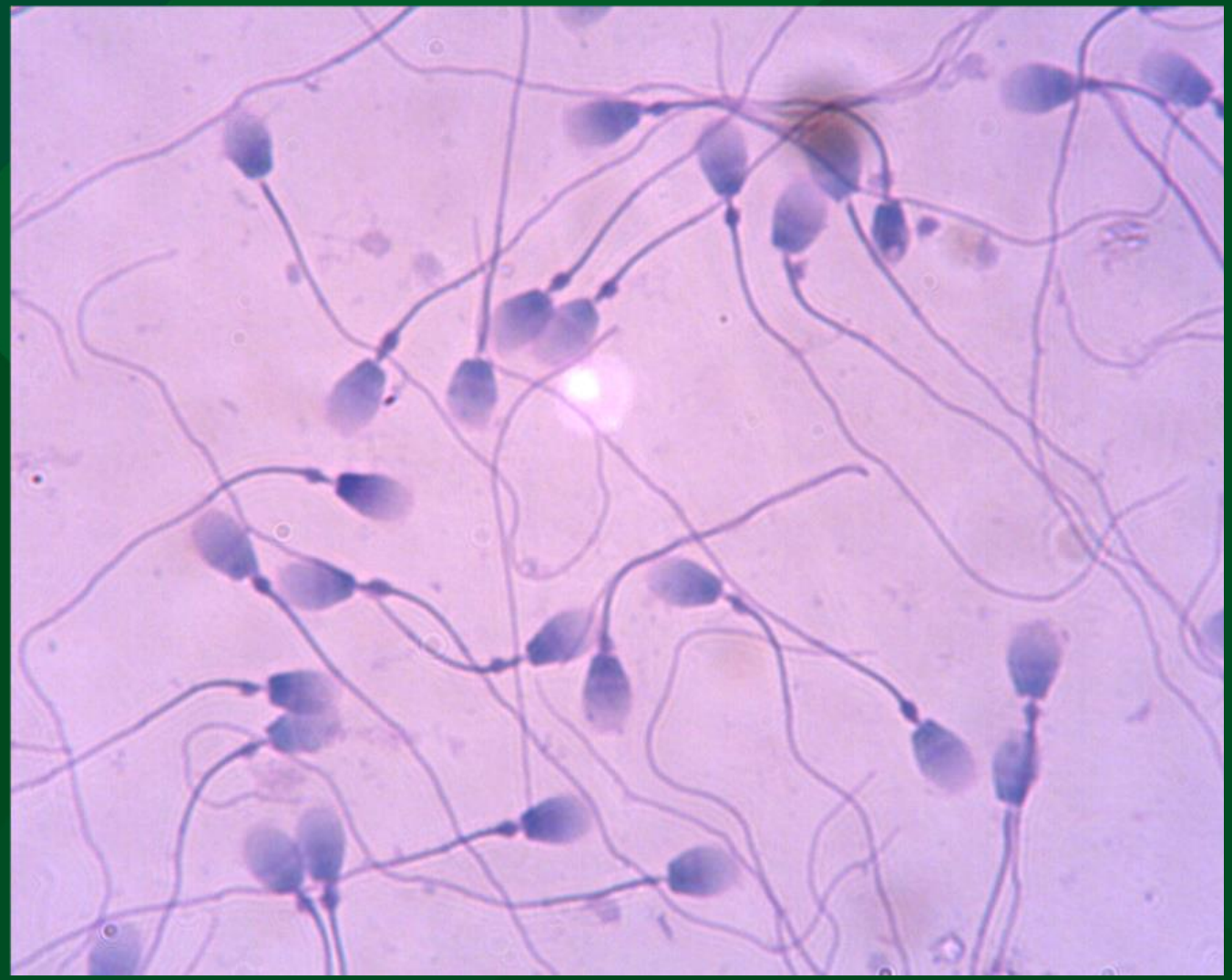
Double Tail and Bent midpieces



Bent midpiece reflex
(double tail, PD, coiled tail, DH)

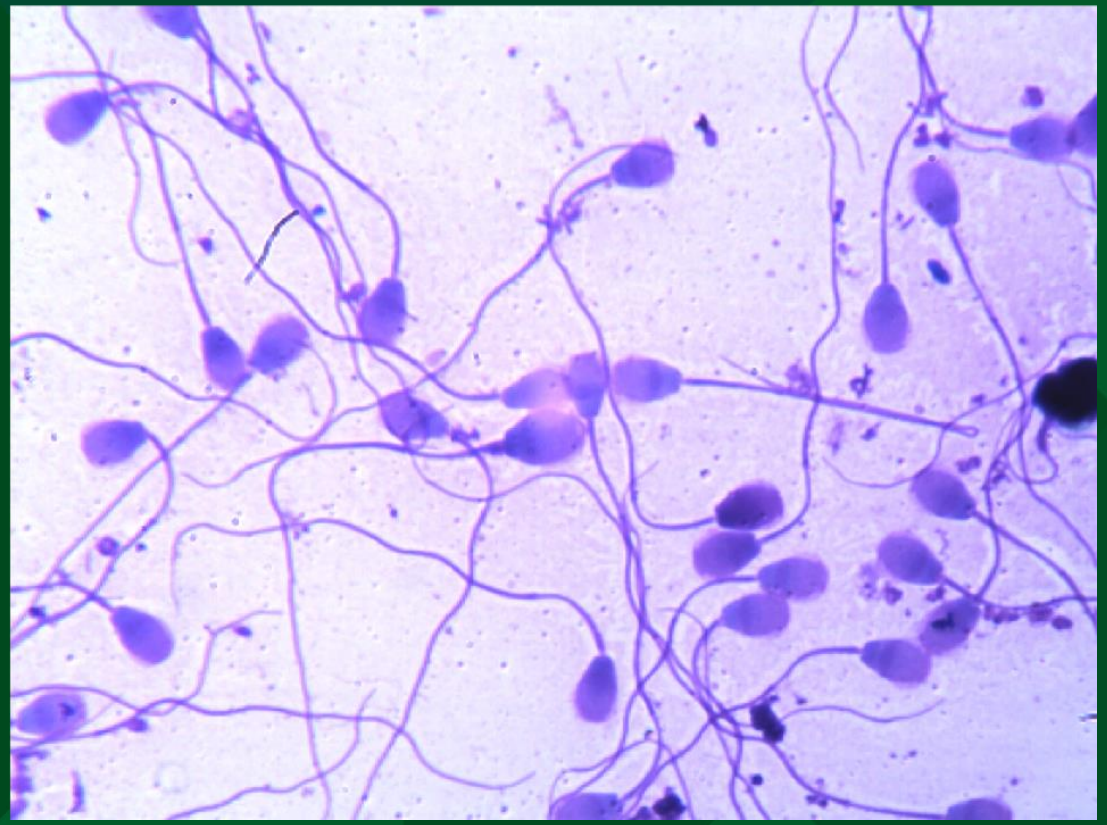


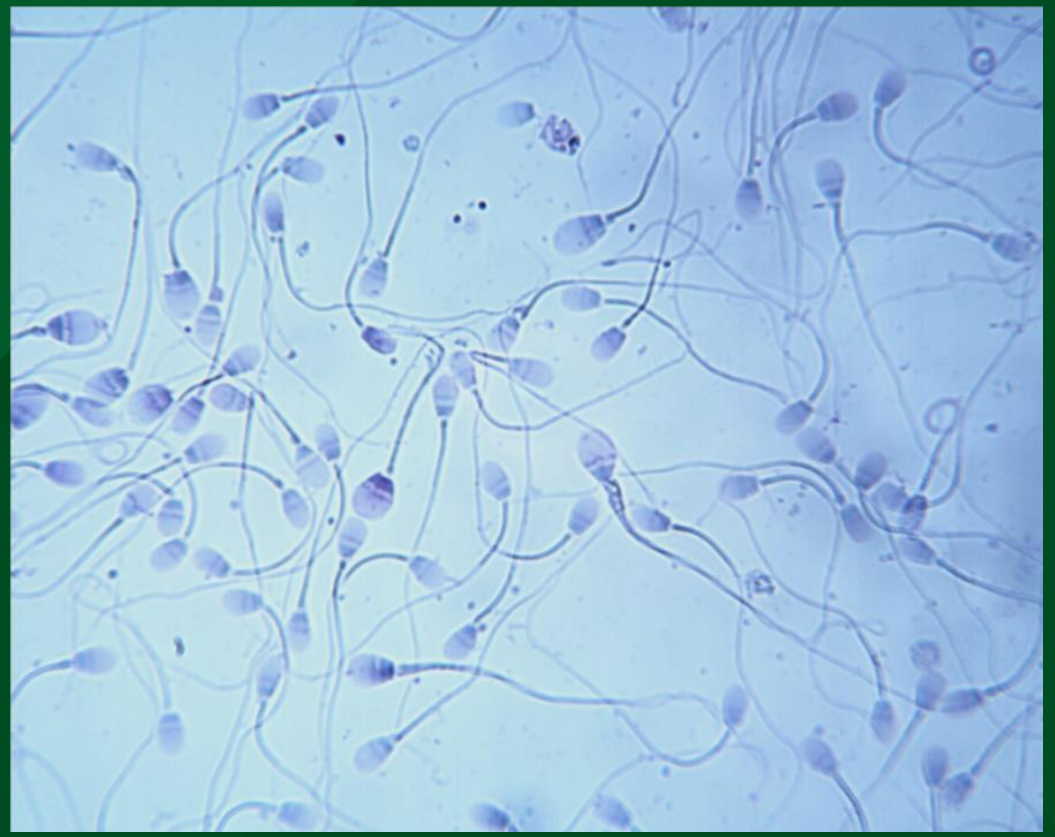
Distal droplet: Minor defect (compensable)



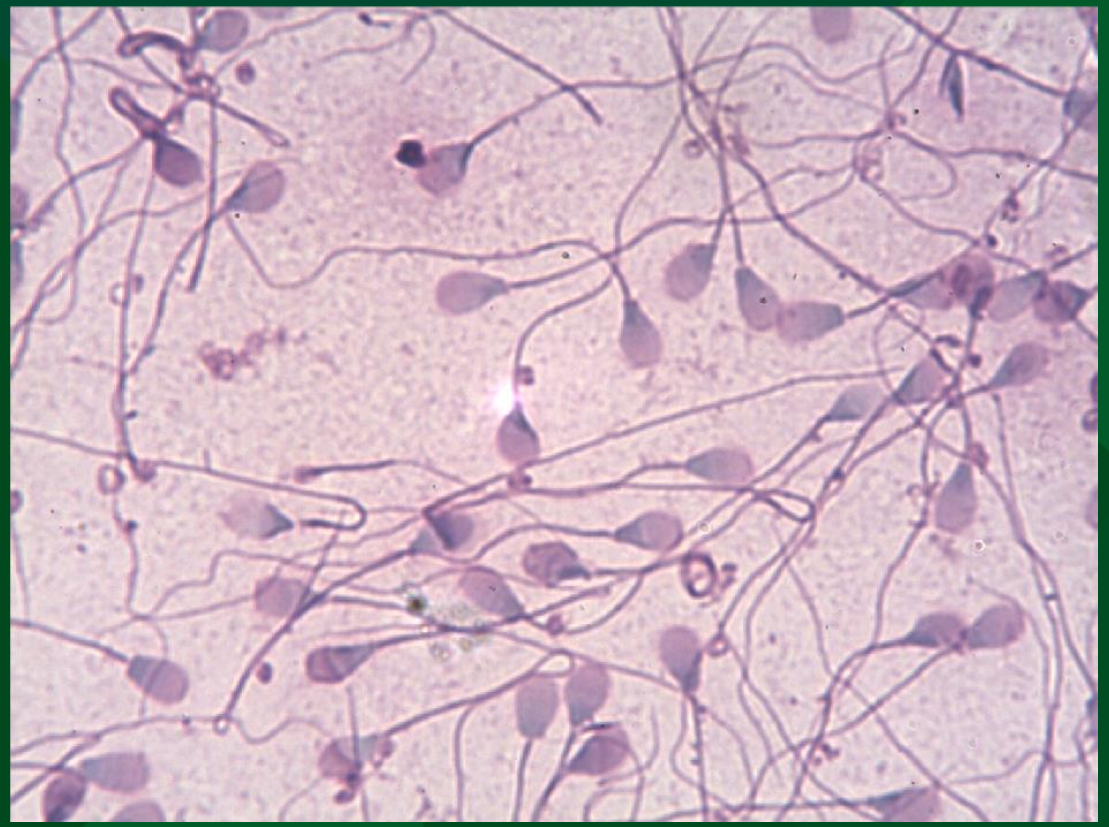
Proximal Droplets : >90% : familial defect that resulted in infertility in this young dog : Major defect (non compensable)

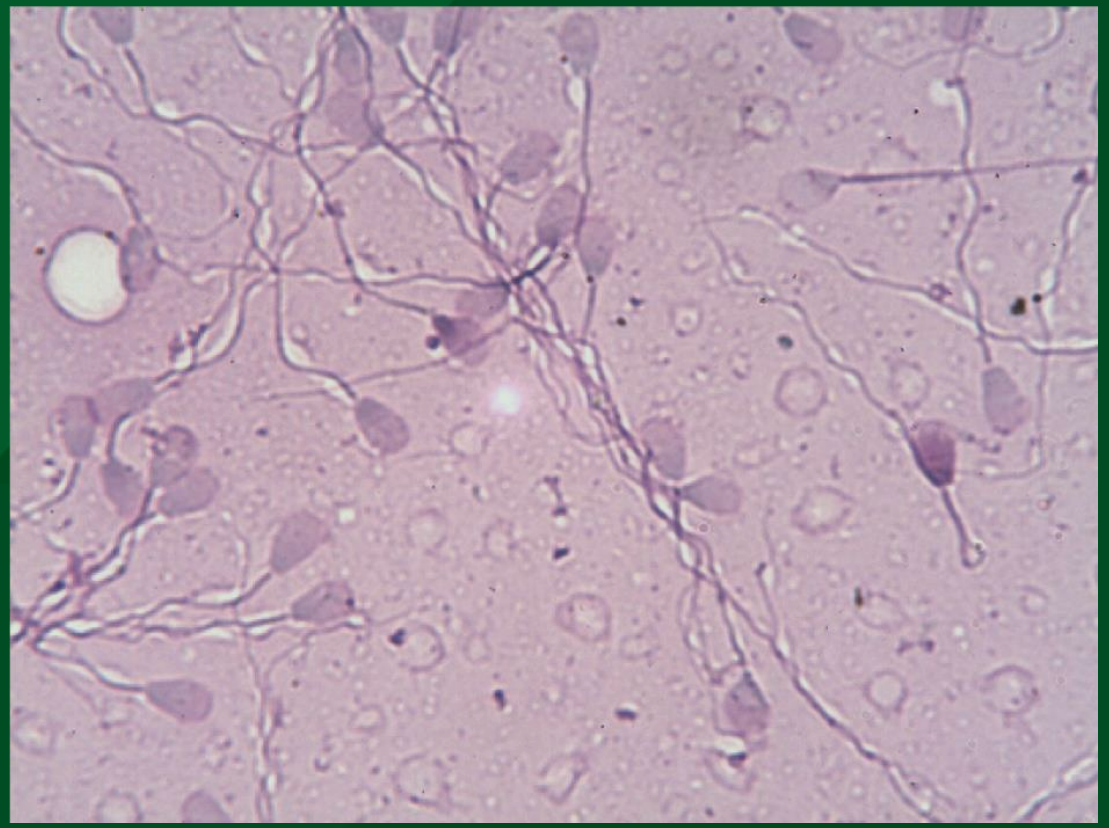
(X1000 oil)





Macrocephalic heads (X1000 oil): major defect->infertility

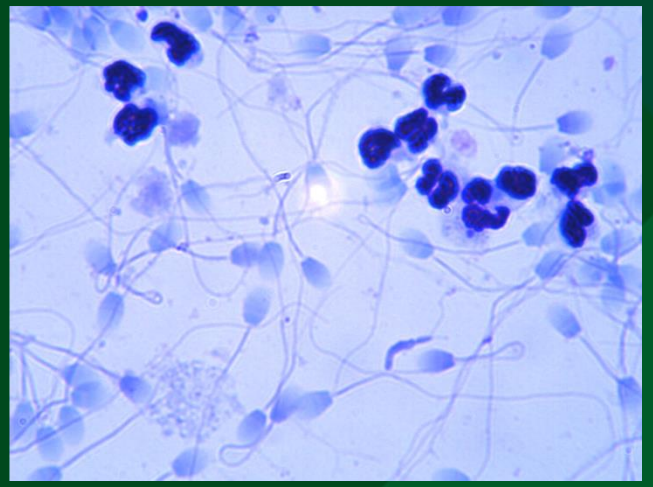




Mixed head defects – mostly pear or pyriform and tapered heads

(familial defect @ with infertility): brothers all infertile (X1000 oil)

CYTOLOGICAL ASSESSMENT



- Important for detection of non -sperm cells (i.e inflammatory cells, germ cells, squamous cells, etc) in either sperm rich or PF fractions
- Presence of inflammatory cells in the sperm rich fractions can be indicative of infection in the testicles (orchitis)+/or epididymi (epididymitis)
- But could also be contaminants (urinary tract)
- Collection (+/- cytospin) of PF fraction= assist in diagnosis of prostatic disease : BPH, prostatitis
- **Advantage of Diff Quick stain for morphology assessments**

Cytological Evaluation at morphology assessment: Inflammatory cells (>5 per HPF) in sperm rich fraction: Source?? Cause??

CANINE BSE:

RECOMMENDATIONS

Morphology:

- No > 20% head defects
- No >25% acrosome and tail defects
- Overall > 70% normal sperm

Motility:

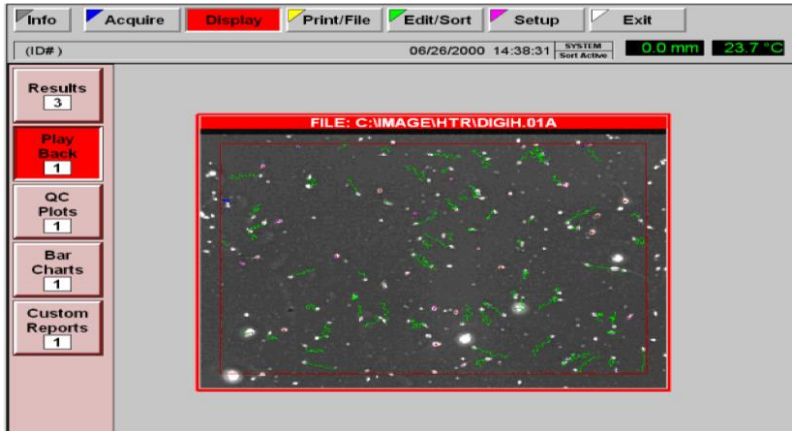
- > 75% (raw)

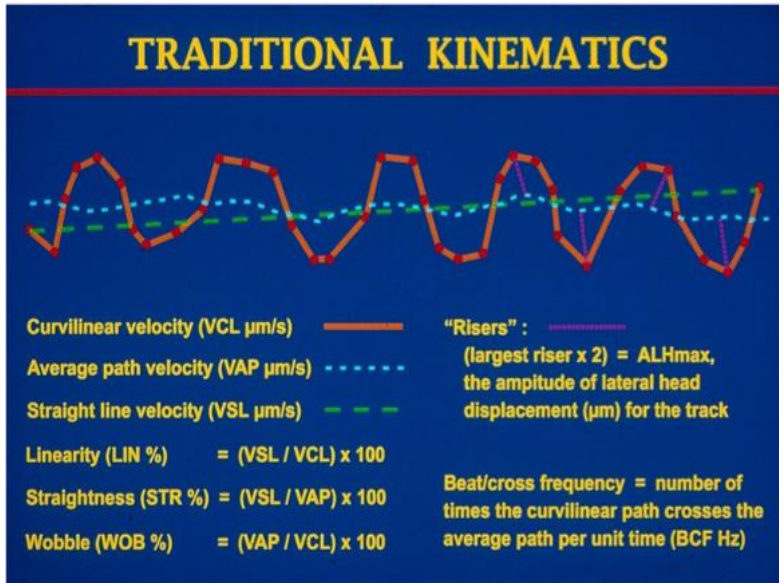
(all young dogs should have high quality semen= >85-90% motility)

- Percent Normal sperm is very important
- Abnormal sperm can indicate:
  - Institute potential management to correct
  - Many cases cannot be corrected (testicular degeneration)

ADDITIONAL SPERM FUNCTION TESTING AND EVALUATION

COMPUTER ASSISTED SPERM ANALYSIS (CASA)





- Objective measurement of sperm motility, kinematics (and concentration)
  - The speed and pattern of movement is as important as the proportion of sperm which are viable
- Concentration: inaccurate as counts contaminants in the sample as sperm

See demonstration of CASA on: https://www.youtube.com/watch?v=G_0o4X8zzqc

ASSESSING FLUORESCENT SPERM STAINS

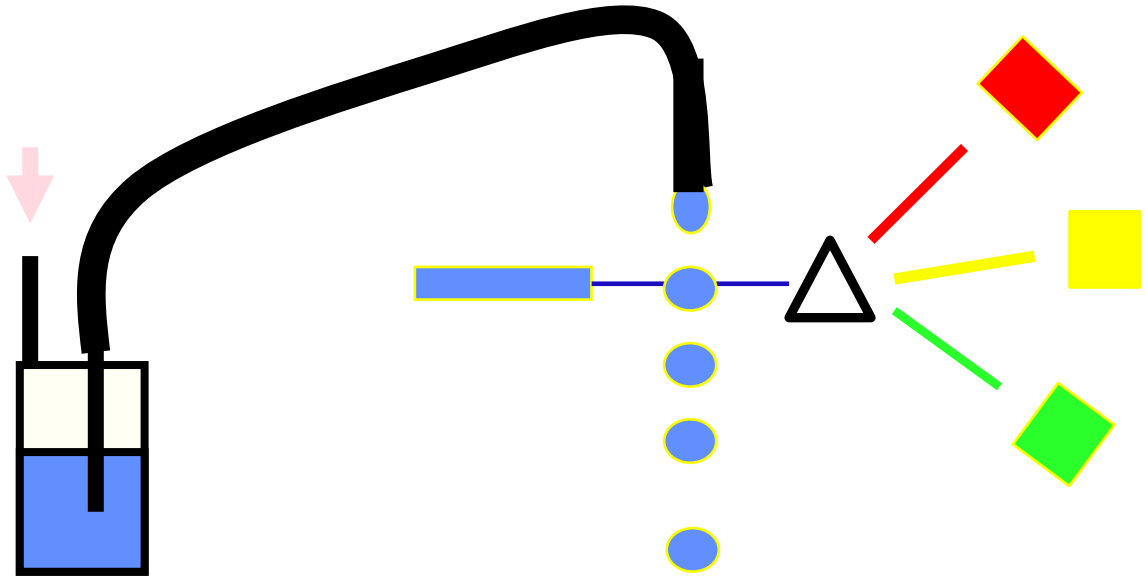
- Fluorescent microscopy

  - Manual=Slow (count ~200 sperm) and subjective

- Flow cytometry

  - Evaluate >10,000 sperm in ~10 seconds and objective

- -Evaluate multiple functional stains simultaneously



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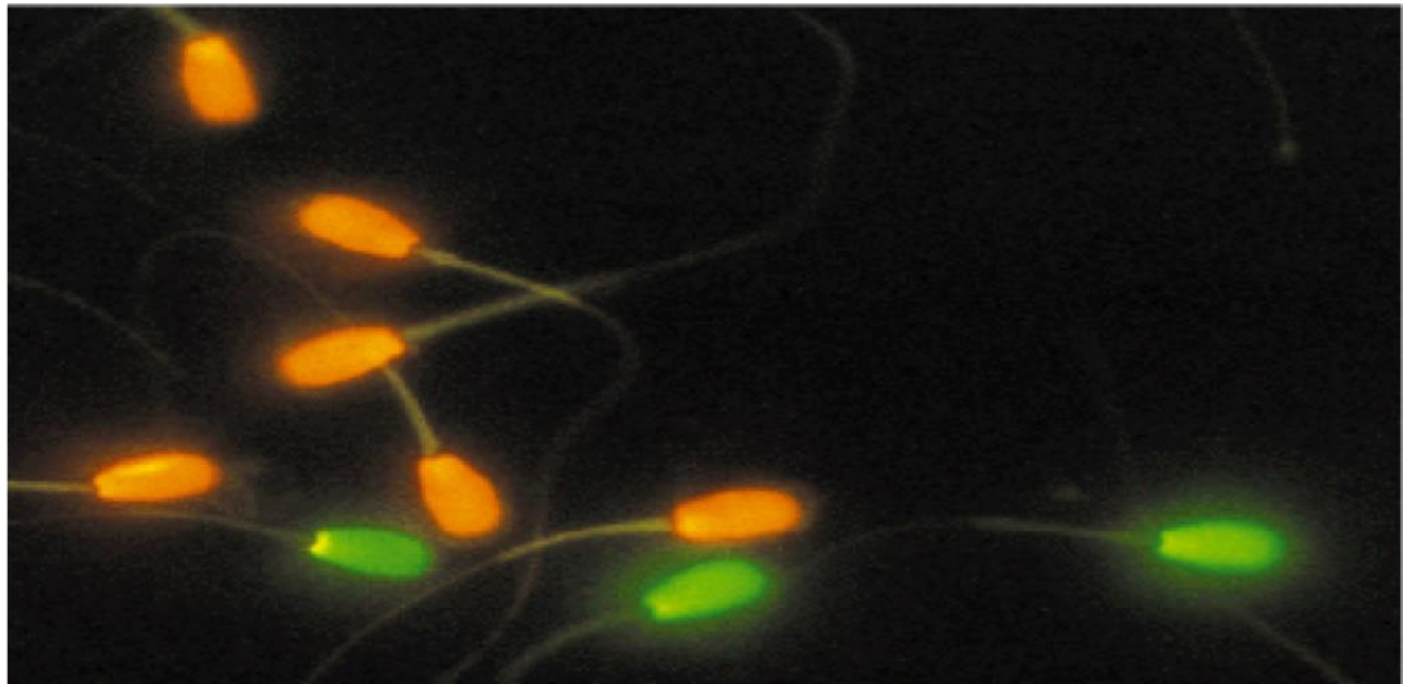
Flow cytometry and some common sperm assays used to determine various sperm characteristics and function:

Viability: PI/SYBR-14 (Live/dead)

- Propidium Iodide (PI) only labels nucelic acid in membrane compromised sperm= red
- SYBR-14 binds nucleic acid in live sperm= green



Red heads= non viable

Green heads= viable

SPERM FUNCTION:

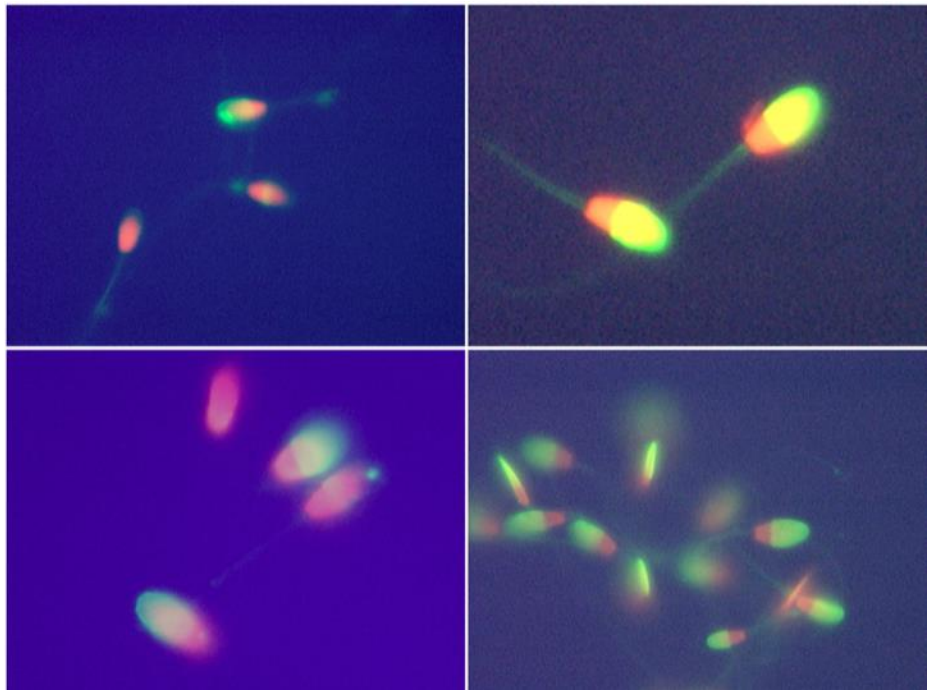
Viability

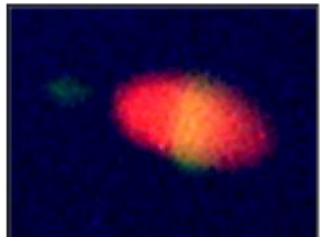
SPERM FUNCTION:

Acrosome Integrity

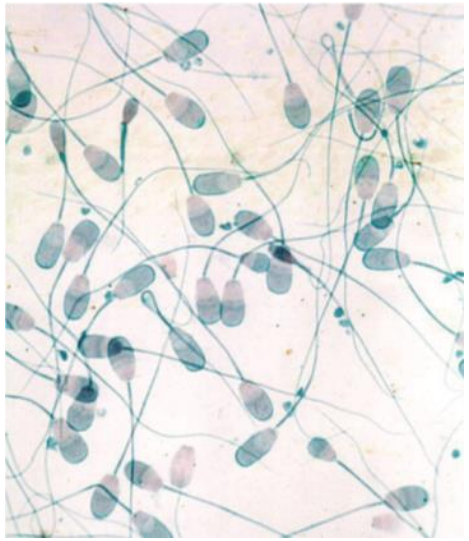
Acrosome stains:

- Napthol yellow/erythrosine B
- Spermac (light microscopy)
- FITC-PNA (fluorescent stain)





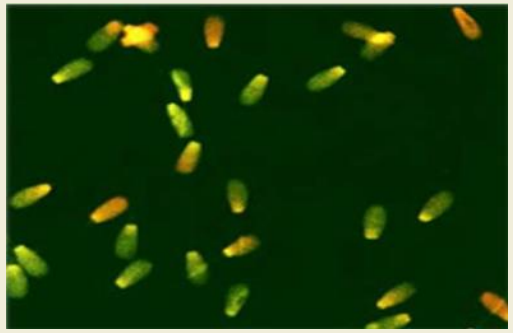
Fluorescein isothiocyanate-peanut agglutinin is a lectin which binds exclusively to outer acrosomal region of sperm
- Green heads – acrosome intact
- Equatorial staining – acrosome reacted

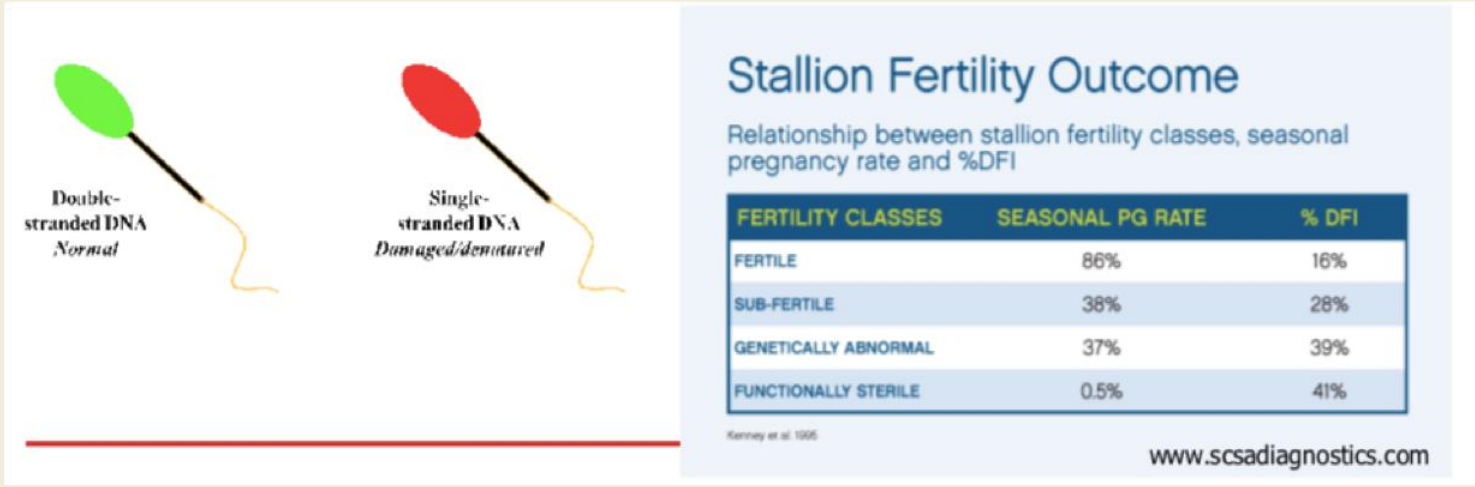


Spermac stained sperm: acrosomes clearly visible (courtesy Minitube®)

SPERM FUNCTION: **DNA Integrity**

- DNA and chromatin intactness stains:
  - Many techniques (FISH, SCD, Comet, TUNEL) and stains (Aniline and toluidine blue, Hoechst, Fuelgen, Chromocyin A3)
  - Most commonly used is **Sperm Chromatin Structure Assay (SCSA)** using Acridine Orange
- AO intercalates between parallel bases of double-stranded DNA as a monomer (green) whereas it binds to single-stranded DNA (or RNA) as an aggregate (red/orange).





Stallion Fertility Outcome

Relationship between stallion fertility classes, seasonal pregnancy rate and %DFI

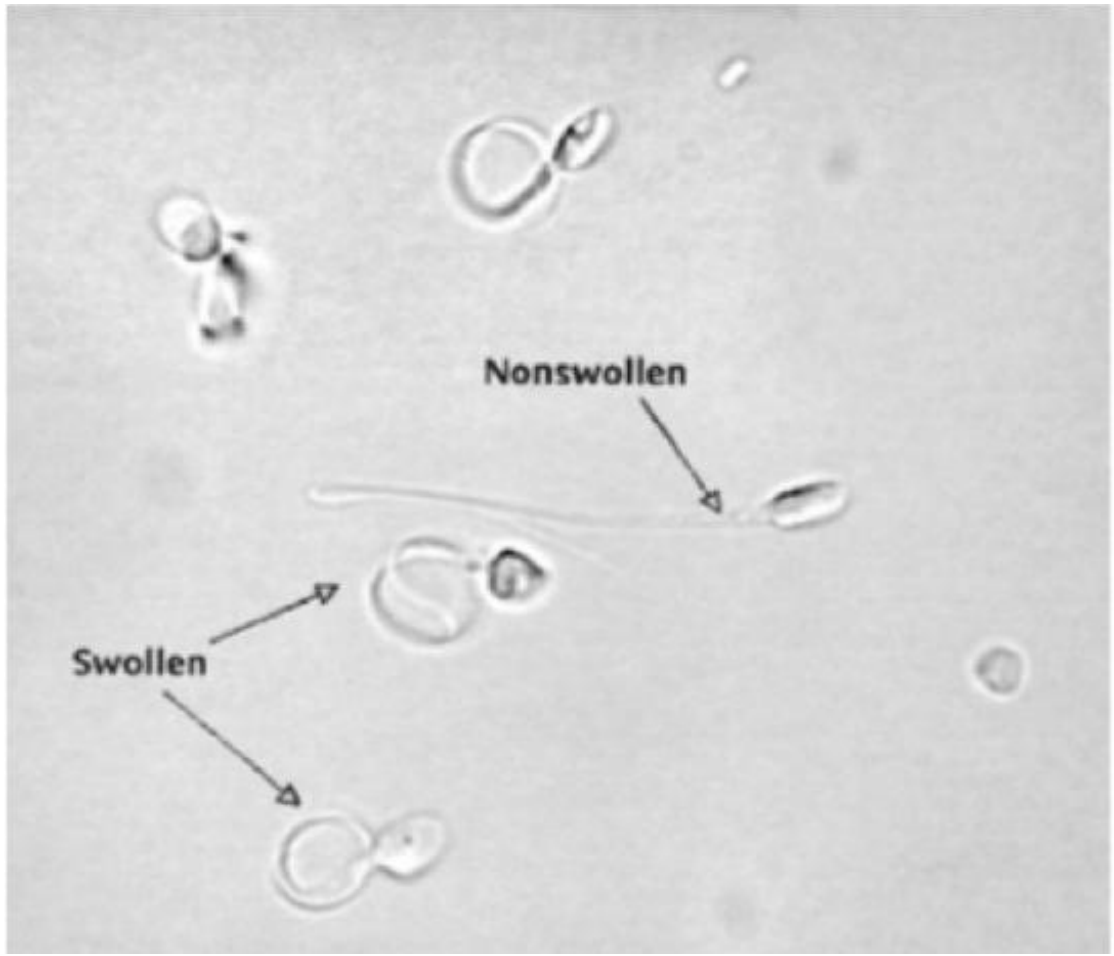
FERTILITY CLASSES	SEASONAL PG RATE	% DFI
FERTILE	86%	16%
SUB-FERTILE	38%	28%
GENETICALLY ABNORMAL	37%	39%
FUNCTIONALLY STERILE	0.5%	41%

Kenney et al. 1995

www.scsadiagnostics.com

SPERM VIABILITY

Hypo-osmotic swelling test (host)-membrane integrity



The hyperosmotic swelling test (HOS) relies on the physiological phenomenon that membrane-intact sperm will swell when placed into a moderately hypoosmotic environment, whereas membrane-damaged sperm do not. The induced swelling produces a characteristic coiling of the flagellum inside the swollen membrane which is easily observed under phase-contrast microscopy.

Validated HOST Method for Dog Semen:
5ul semen in 0.2ml distilled water
(0mOsm) at 37°C for 5 min
Ref: Santos et al EVSSAR 2018

CHILLED CANINE SEMEN: SHIPMENT

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BENEFITS:

- Overcome situations here the bitch and dog cannot geographically be at the same location
- Good semen quality and increased numbers of sperm in the AI dose: better pregnancy rates and litter size compared to frozen-thawed semen
- Costs: Shipment in a styrofoam box cheaper (and non hazardous) than a dry shipper or liquid N tank
- Can last 3-4 d at 5°C (fertility after storage for 10 d reported)
- Can use with vaginal AI- economical, technically simple, minimal equipment, non invasive insemination technique

CHILLED SEMEN

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WHAT IS INVOLVED IN SHIPPING CHILLED SEMEN?

COLLECTION-> ASSESSMENT

PREPARATION: SLOW DILUTION OF ENTIRE EJACULATE IN WARM CANINE EXTENDER AT APX 1:4 (SEMEN: EXTENDER) OR TO 100-150 x10^6 SPERM/ML

(extenders contain protectants against cooling damage i.e milk, EY and buffers and sugars but **no glycerol** or equex paste)

Place extended semen in a sterile test tube with minimal air or syringe (no air) with cap and in semen whirlpak bag in case leakage occurs.

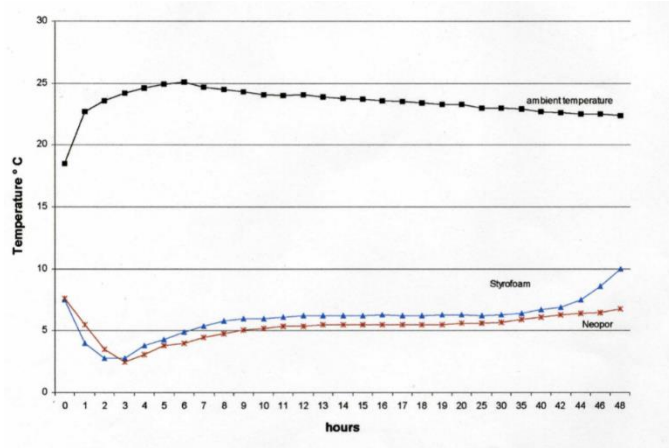
COOL IN SPECIALLY DESIGNED SHIPPING BOXES OR AN EQUITAINER*

(cool semen at a specific rate to 5°C during transport and hold at 5° C for over 24-36 h)

- Too dilute = dilution shock
- Too concentrated = by products reduce viability

Minitube® chilled shipment box

**If shipping during time of year when extreme heat or if delays a possibility and equitainer may be more reliable but less economical:*



Cooling curves for canine semen in chilled shipment boxes (Minitube® Data)

* I.C.N. Cunhaa, H. Henning, C. Urhausen, M. Beyerbache, A.R. Günzel-Apel 2014 Anim. Reprod. Sci.147

WHAT IS INVOLVED IN SHIPPING CHILLED SEMEN?
CONTINUED

SHIP OVERNIGHT-MOST BOXES WILL HOLD AT 5ºC FOR UP TO 32H

↓

INSEMINATE THE BITCH THE FOLLOWING DAY

(Management of bitch oestrous cycle important for timing of insemination in co-ordination with FEDEX!)

CONSIDERATIONS: ***One vs Two inseminations when chilled semen can last 4 days in the bitch ??***

- Age of the dog
- Age of the bitch
- Timing

If there is a delay in doing the AI : if the semen is placed in fridge on arrival-can last up to 3-5 d depending on individual male and extender type ***BUT*** *best to time to inseminate as close to the time of collection as possible* ***AND*** *the semen is always better in the bitch than in the box!*

TIPS FOR SHIPPING CANINE SEMEN:

IMPORTANCE OF PAPERWORK!

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Colorado State University

CHILLED CANINE SEMEN SHIPMENT Date:

Stud Dog Details:	
Owners Name:	
Address:	
Phone Number:	

Animal Kennel Name:	
Animal Call Name:	
Registration Number:	
Date of Birth:	
Breed and Colour:	

Semen Assessment:	
Raw Ejaculate (sperm rich fraction only):	Volume:
	Progressive Motility:
	Concentration:
Extended ejaculate:	Diluent Used:
	Dilution Rate:
	Sperm Concentration (10^6/ml):
	Progressive Motility:
Total Volume of AI dose shipped	
Number of Doses shipped	
Total number of motile sperm per dose	
Route of Insemination (vaginal vs I.U)	

Shipping Address:	

Bitch I.D for Insemination:	
Kennel and Call Name:	
Date of Birth:	
Owner of Bitch Contact Details:	

Semen Preparation and Assessments carried out by:

Dr Fiona Hollinshead
BVSc (Hons), PhD, Diplomate ACT
Registered Specialist in Canine Reproduction

Dr Mary Ontiveros
DVM
Theriogenology Resident

Veterinarians
Fiona Hollinshead BVSc (Hons), PhD, MANZCVS DipIACT
Dave Hanlon BVMS (Hons), PhD, MANZCVS, DipIACT
Mary Ontiveros DVM

Veterinary Nurse - Administrator
Vicki Knox DVN (dist)

Semen Storage & Distribution Manager
Hillary Schifferer BS
P 07 888 8193

Always include a basic semen report and information on the dog semen was collected from

Label all tubes, bags with the dog's complete name and date of collection

FREEZING CANINE SEMEN

FROZEN SEMEN

BENEFITS:

- Preservation of valuable genetics forever
- Allows for breeding when the male is dead or no longer fertile
- Insurance against loss of these genetics
- International semen exchange: efficient method for injection of new genetics for greater genetic gain
- Economic advantage and reduced risk in importing a tank of semen from many stud dogs' vs importing a single live dog (all the genetics and fertility placed in 1 dog= limitations and risk)

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WHAT IS INVOLVED IN FREEZING DOG SEMEN?

1. Overview: Freezing technique → Set up: what you need for semen freezing

Semen collection-> Initial assessment

↓

Dilution with extender with EY and no glycerol

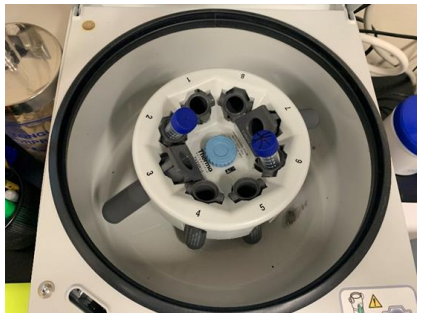
↓

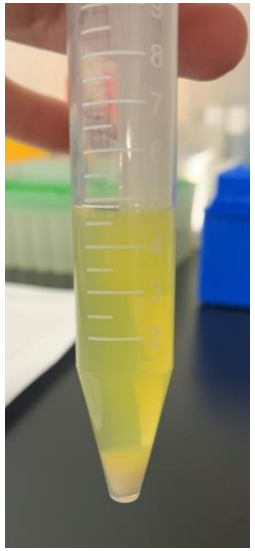
Centrifugation: remove prostatic fluid

↓

Dilution of semen pellet in pre-warmed extender containing antibiotics, sugars, buffers, cryoprotectants (10-20% egg yolk, 5-8% glycerol v/v)to a final concentration 200 (1- step) or 400 (2-step) x 10^6 sperm/ml

↓





2. Overview: Freezing technique

Cool semen in a 200ml beaker of water (2-step)

or in straws (1-step) from room temp to 4º C over

1.5-2 h in a cool room or fridge (ideally upright)

Packaging: Fill pre-labelled, pre-cooled straws

at 4ºC (2-step) and seal straws with colored

PVA or use an ultrasonic straw sealer

Set up: what you need for semen freezing







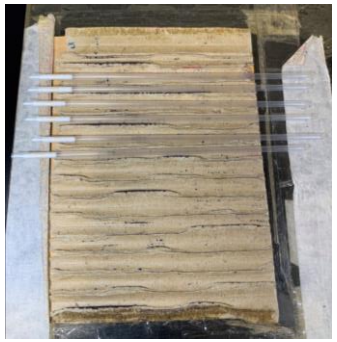

IMPORTANT NOTE: **LABELING STRAWS**

Automated straw labeler : $

Each straw requires the minimum following information:

1. Identity In house number: location
2. Dog's Registered Kennel Name
3. Breed: AKC abbreviation to be used
4. Owners last name (semen vs dog)
5. **Microchip #** (check dog with scanner)
6. Date

Hand written straws- most common for canine semen as few straws per ejaculate



The more information on the straws the more likely they will be eligible for export to any country in the future. Aim is to meet all current import requirements on the day of freezing.

Example:

CSU 101 Gale Winds Dreamin in Color HENDERSON Dach 985113002116596 08.21.21

3. Overview: Freezing technique

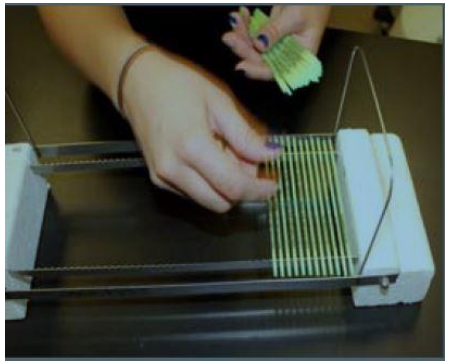
Set up: what you need for semen freezing

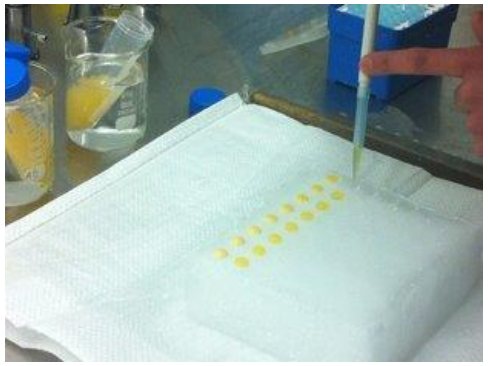
Place straws 4 cm above liquid N in vapor for 10 min using a 'floating rack and freezing box' filled with liquid N or a programmable freezing machine

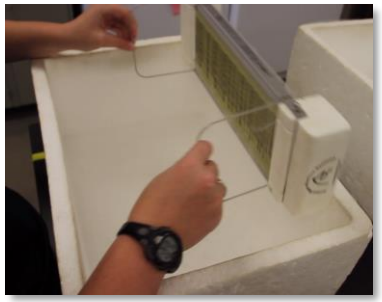
(**OR** in 50-200 ul pellets on dry ice for 10 min)

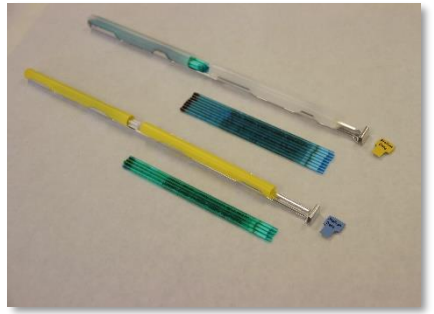
Plunge straws or pellets into liquid N (-196° C)

(Note: pellets need to be loaded into labelled vials and then the vial are placed on a labelled cane)





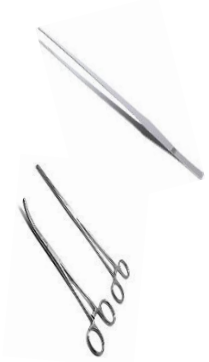


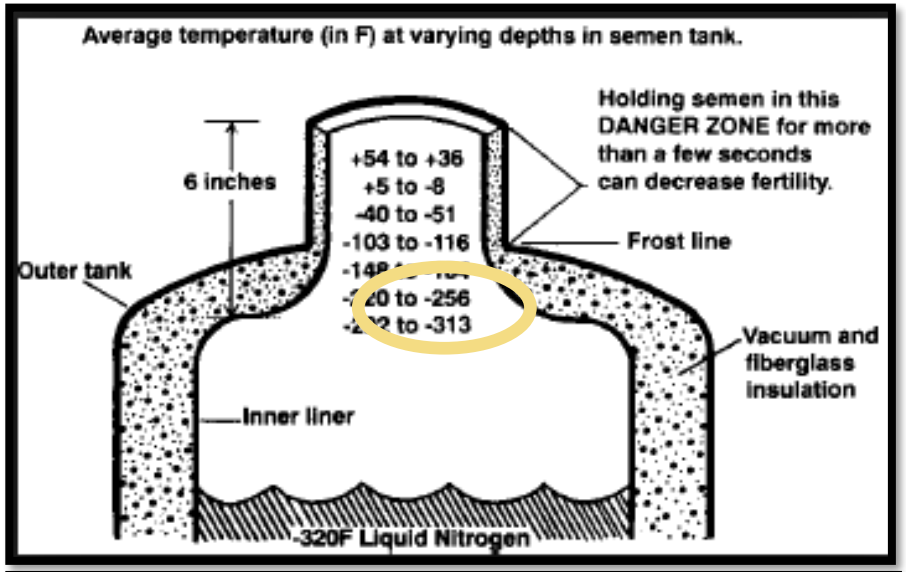


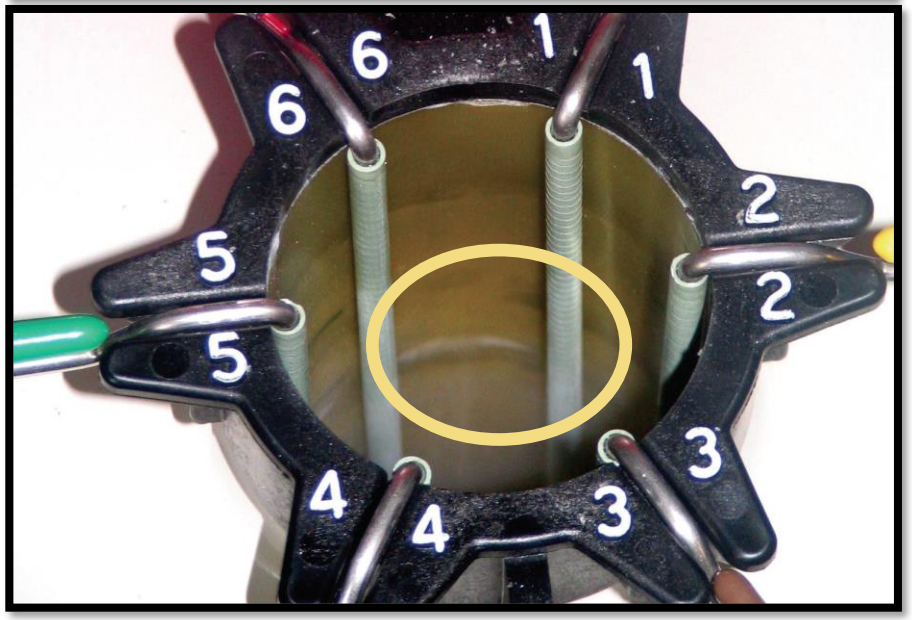
STRAW HANDLING AND LOADING GOBLETS AND CANES

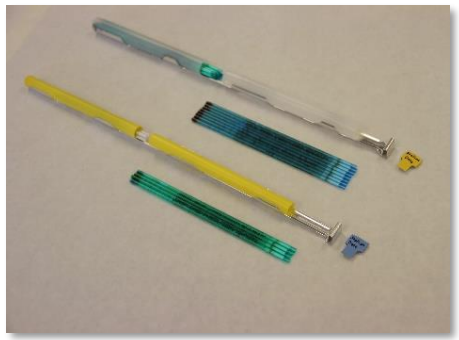
- Straws are fragile
- Move straws into goblets
- Place goblets on labelled canes
- Always use precooled tongs
- Transfer as quickly as possible into tank
  - Minimal air exposure (2-3 sec)
- Frost Line ~4" down the neck

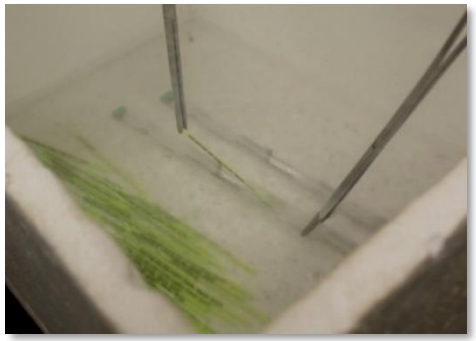
Store frozen semen indefinitely in maintained long term liquid nitrogen tanks









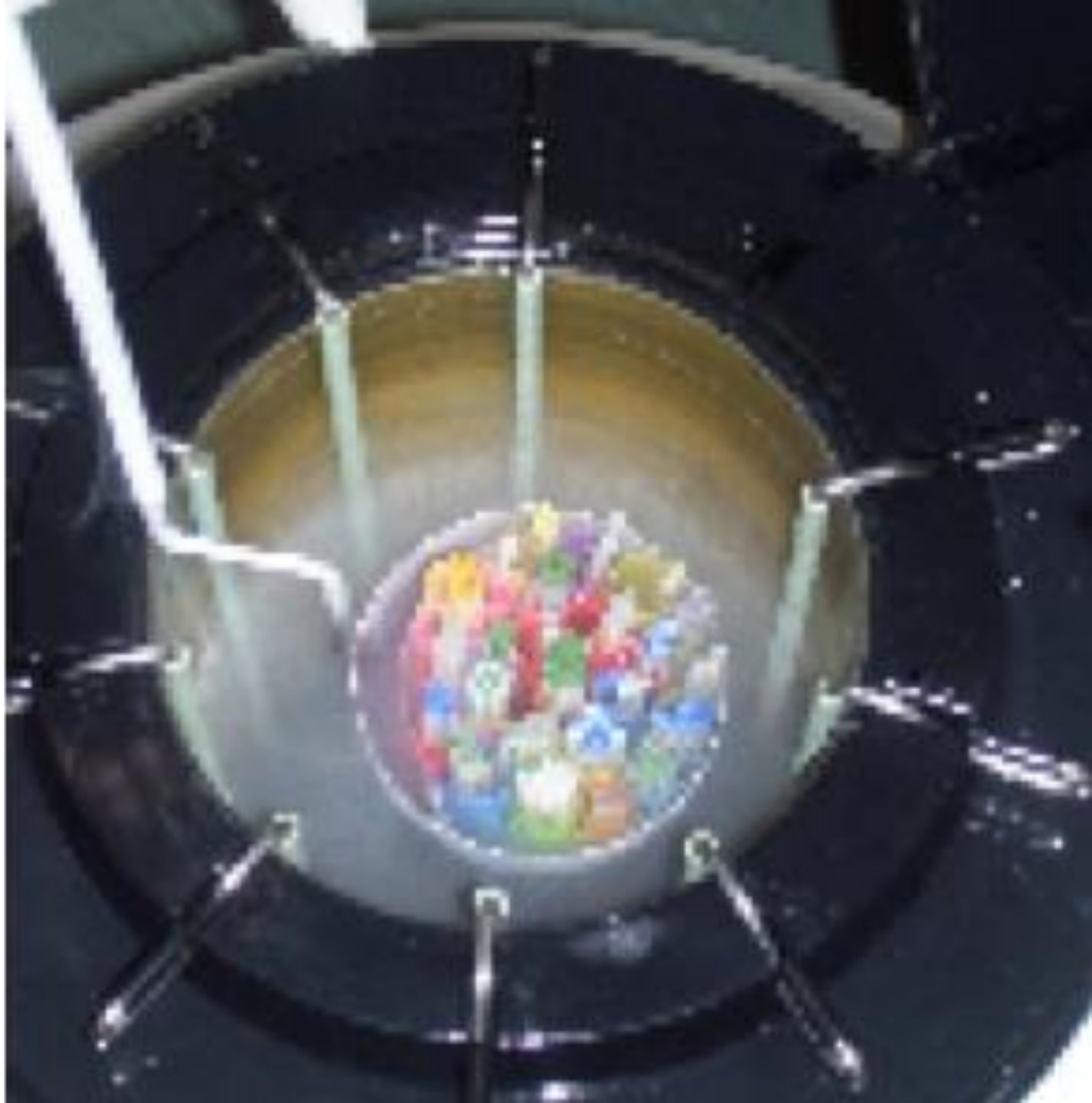


IMPORTANT NOTE:

IDENTIFICATION OF STRAWS

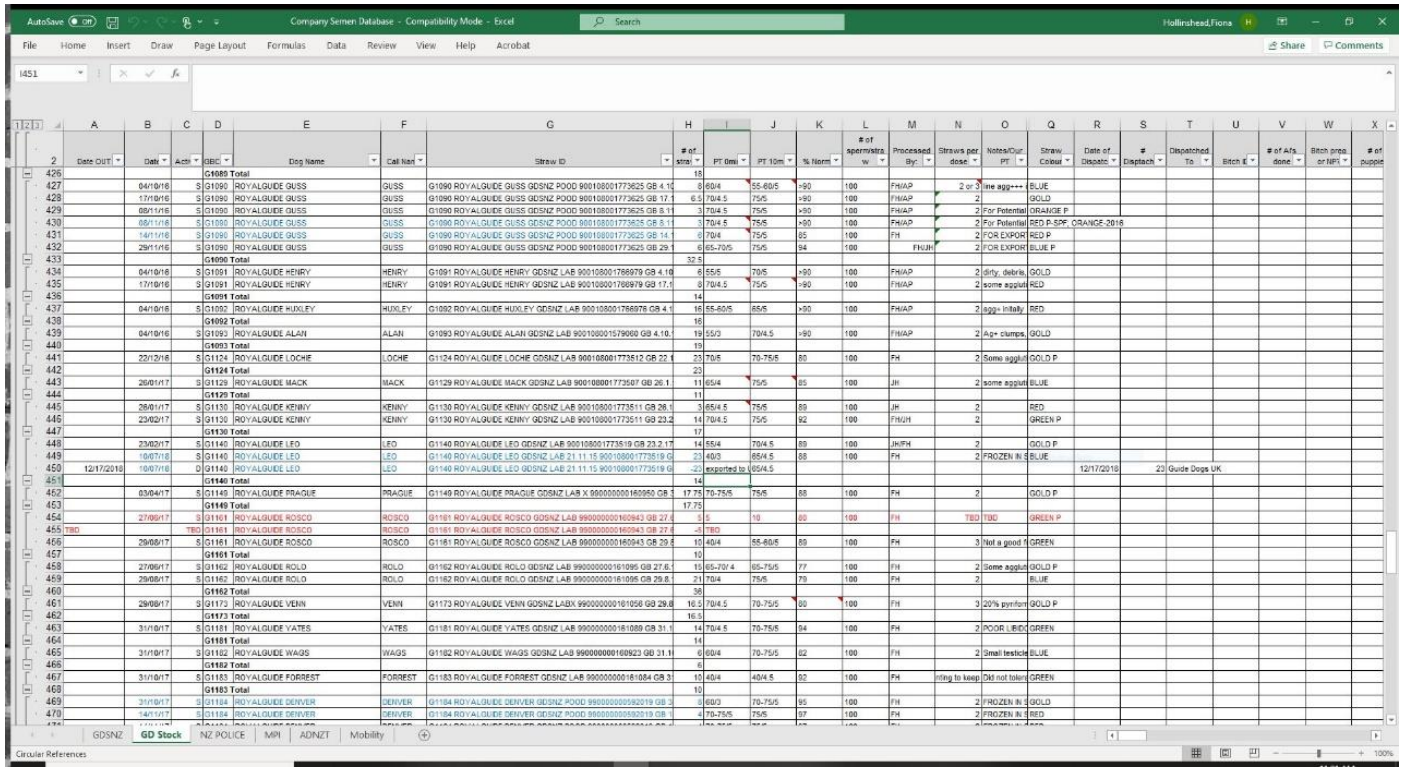
Importance of different colors of PVA plug and goblets for ID of batches of semen in a **large l**ong term storage tank:

Facilitate ID of straws from a specific ejaculate/ individual dog amongst 10,000 straws and semen from 100’s of dogs



IMPORTANT NOTE: **RECORD KEEPING**

- Electronic bookkeeping
  - Must keep up to date
- Paper records
  - Filed
- Remember to keep track of what is used
  - Shipped out
  - Used in breeding program
  - What comes in



Screenshot of a typical excel spreadsheet for semen storage documentation

ESSENTIAL PART OF STORING SEMEN !!

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THAWING CANINE SEMEN

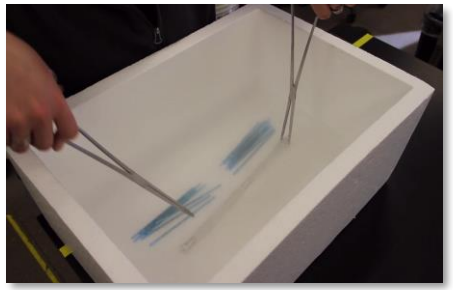
Thawing

i) Remove straws from liquid N using pre-cooled tongs and ii)plunge into 37ºC waterbath for 30 sec

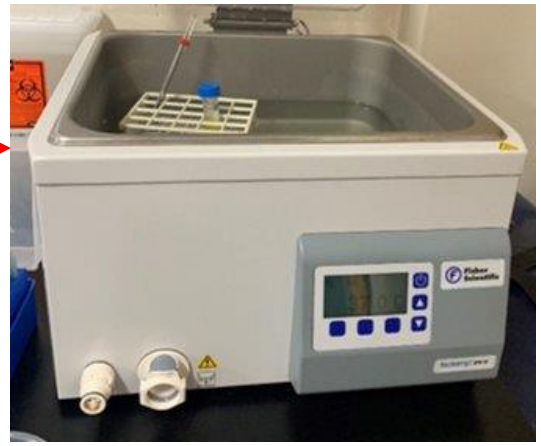
iii) Wipe straws dry then cut off PVA end then cotton plug end of straw while holding over a warm test tube: contents will drain into the tube

iv) Slowly add pre-warmed (37ºC) thawing extender (no glycerol) to thawed semen with pipettor and assess motility at 0 and 10 min at 37ºC

Set up: what you need for semen freezing



i)



ii)

iv)

iii)

Water kills sperm! : Care when thawing

GOALS OF FREEZING CANINE SEMEN

- Remove prostatic fluid by dilution and centrifugation
- Concentrate sperm by centrifugation
- Re-extend sperm with a freezing extender
  - Dilute to 200 million per ml =100 million per straw (0.5 ml)

    Insemination dose= > 100 million motile sperm so long as post thaw >50% and >70% morphologically normal sperm = 2 straws per AI dose/1 breeding unit
- **Goal:** post-thaw motility of **≥ 50% (average)**
- **Aim: >70-75%** ☺**:** this is possible as (young) dog sperm freezes better than many other species= increase our expectations in a canine BSE
- **Poor: <30% :** This is associated with decreased pregnancy rate and litter size*

*Hollinshead and Hanlon Theriogenology 2017,

IMPORTANCE OF STORING ONLY GOOD QUALITY FROZEN SEMEN

1. It is not the extender, pellets vs straws that play a role in freezing semen well BUT the techniques and attention to detail that facilitates successful freezing

2. Don't store/ keep poor quality semen: the long term economic and genetic costs to your program is significant i.e storage costs, costs in timing of AI and inseminating with frozen semen-often several times-> all leading to no offspring= loss of a small breeding program and genetically valuable lines in larger programs

3. We can't do IVF, ICSI in canids yet- only have AI so no indication to keep poor quality semen

4. Poor quality semen can eventually be sold or traded with others unknowingly and exported all over the world: this will damage your reputation and the ability to share genetics

Take Home Message: ***Only keep high quality semen: >50-70% to endure the future of a successful breeding program***

Limitations of frozen semen : reduced fertility

- Damage to sperm during cooling, freezing and thawing processes reduces sperm viability and longevity i.e survives only 6-24h in reproductive tract
- This limitation is compounded further by the use of **low numbers** of sperm per AI dose i.e 1/10th ejaculate (100 million motile sperm)
- Significant variation between males (and even ejaculates)

BUT despite these limitations, with canine semen, we still achieve very good pregnancy results when:

- Good breeding management (ovulation timing)
- Deposit as close to the site of fertilisation as possible (Intrauterine AI)
- Optimal bitch selection –young (<4 y)
- Use high quality frozen semen (collected from young dogs and >65% pt)
- Potentially by increasing sperm numbers by using two doses and performing two inseminations

THE KEY TO SUCCESSFUL FREEZING OF CANINE SEMEN: SOME CONSIDERATIONS

At what age to collect and freeze canine semen?

1. Freeze canine semen from dogs when they are **YOUNG** i.e 2 yrs age !!

2. Freeze before 4 yrs age when start to develop prostatic and testicular issues which affect semen quality and quantity

3. Freeze semen before a dog 'proven ' in the show ring, field trials, performance testing by using pedigree information and genetic tests – becoming much more available and possible to do this with the IWD registry

4. Can freeze after all health testing complete OR bank at least one ejaculate while young as a safegaurd and discard if subsequent health tests not positive

YES ☺

NO !!

SUMMARY: CHILLED VS FROZEN SEMEN

PROS AND CONS

Chilled semen

- Increased numbers of sperm (whole ejaculate) in the AI dose
- Greater longevity and viability (only cooled not frozen-thawed): lasts 3-4 days
- Can do either vaginal or intrauterine AI= low cost and anyone can do a vaginal AI
- Public holidays and shipment impediments
- High pregnancy rate and litter size

Frozen semen

- Low number of sperm in the AI dose (1/10th ejaculate)
- Reduced longevity and viability due to cryopreservation process : lasts12-24 h
- Must place semen into the uterus= cost, equipment, skill
- Need equipment, liquid nitrogen and training to freeze, handle and thaw frozen semen
- No reliance on Fedex: long term storage
- No reliance on presence of the dog (geographical, age, deceased)
- Lower and variable PR and LS

EQUIPMENT, SUPPLIES AND SET UP INFORMATION

BASIC SPACE REQUIREMENTS

1) Benchtop space for processing and assessing semen

2) Space for performing inseminations

3) Space for performing semen collections

Space for performing inseminations on a hydraulic grooming table

Semen lab similar to a basic kitchen-benchtop required for equipment

4) Space for storage of long -term semen tanks

- Split semen batches and have two storage locations if you can for safety precaution: fire, flood, natural disaster to protect against loss of genetic bank
  - Multiple tanks
  - Geographically separate facilities

- Need a fireproof, alarmed, ventilated, security controlled room/s with concrete floor but tanks kept on a roller base (prevent corrosion)

Location 1

Location 2

CANINE SEMEN EXTENDERS

Commercially available extenders or can make them up in house or some pharmacies will make them up with published recipes.

- Fresh semen assessment
- Chilled semen shipment
- Artificial Insemination
- Freezing: 1 step or 2-step

- Skim-milk based
- Egg-yolk based

minitube

Animal Reproduction Systems, Inc.

UPPSALA CANINE SEMEN EXTENDERS

Available online at www.sciencedirect.com
SCIENCE DIRECT
Theriogenology
Theriogenology 65 (2006) 584–593
www.journals.elsevierhealth.com/periodicals/the

Freezing of stored, chilled dog spermatozoa

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Received 2 March 2005; received in revised form 3 June 2005; accepted 6 June 2005

Table 1
The extenders used for semen chilling, freezing and thawing

	EYT/1	EYT/2	UE-2/1	UE-2/2	UE-2 thaw medium
Tris	3.025 g	Ditto	Ditto	Ditto	Ditto
Citric acid	1.7 g	Ditto	Ditto	Ditto	Ditto
Fructose	1.25 g	Ditto	Ditto	Ditto	Ditto
Streptomycin	0.1 g	Ditto	Ditto	Ditto	Ditto
Aqua dest.	To 80 ml	To 69 ml	To 77 ml	To 72 ml	To 100 ml
Bensyl penicillin	0.06 g[a]	Ditto	Ditto	Ditto	Ditto
Glycerol	None	10 ml	3 ml	7 ml	None
Equex STM paste[a]	None	1 ml	None	1 ml	None
Egg yolk	20 ml	Ditto	Ditto	Ditto	None
pH	6.78	6.70	6.72	6.74	6.76
Osmolarity	325 mOsm	1934 mOsm	865 mOsm	1495 mOsm	324 mOsm

[a] Nova Chemical Sales Inc., Scituate, MA, USA in 0.3 ml dest. H_2O.

**2-step freezing technique extenders*

SEMEN EVALUATION EQUIPMENT: SUMMARY

- Sterile test tubes
- Pipettors + tips
- Sperm count (pre & post extension)
  - Densimeter/spectrophotometer
  - Hemacytometer
  - NucleoCounter®
- Sperm motility evaluation
  - Microscope, phase contrast, 400x magnification
- Sperm Morphology

  Diff stain

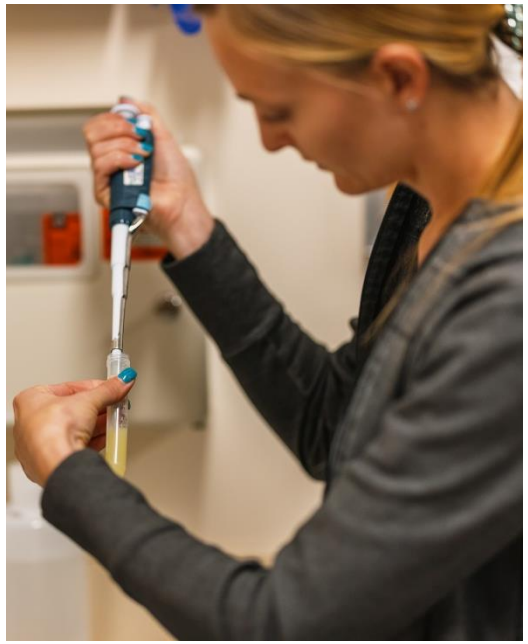
  Eosin + Nigrosin stain

  Microscope: X1000 oil

PIPETTORS

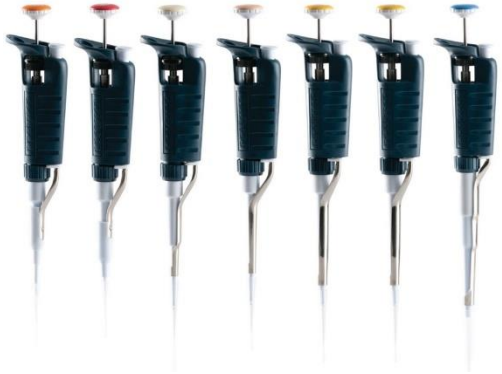
- For assessment of semen
- For diluting semen accurately
- For measuring semen volume accurately
- For transferring semen from collection vials to tubes
- Pipettors need to be calibrated regularly
- Ideal sizes for canine semen processing are *20 ul and 1ml* pipettors
- There are many different brands

Plastic pipettor for diluting semen with extender: not accurate



Calibrated pipettor for accurately measuring and slowly diluting semen

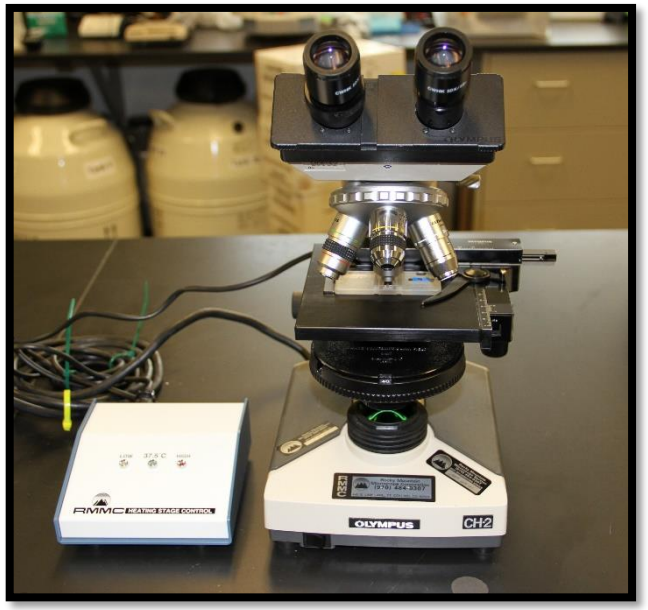
Pipette tips



Different sized pipettors

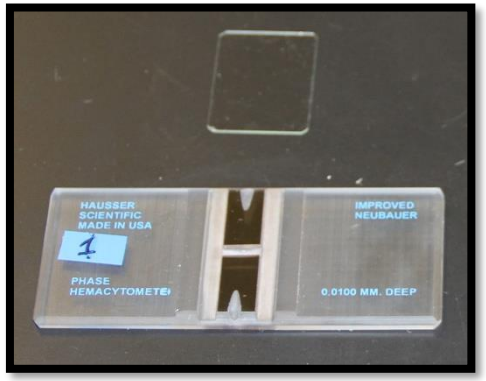
MICROSCOPE

- Compound microscope
  - Bright field vs phase contrast vs DIC
  - Filter, blue or green
  - Sperm are translucent
- Brands
  - Olympus, Nikon, Zeiss, others
  - Heated stage, important to maintain motility
- ± Camera with a monitor
  - CASA system, Computer Assisted Sperm Analysis
- Phase contrast, 100x, 200x, 400x, 1000x magnification and filter
  - Best option to visualize sperm



SPERM COUNT

- Spectrophotometer
  - Evaluates percent light transmission
  - Raw semen only
    - Not accurate for extended semen
- Hemacytometer- manual count
  - Raw and extended semen
  - Cost effective and godl standard
- NucleoCounter® SP-100™
  - Raw and extended semen
  - ChemoMetec A/S, Denmark
  - Gold standard but $

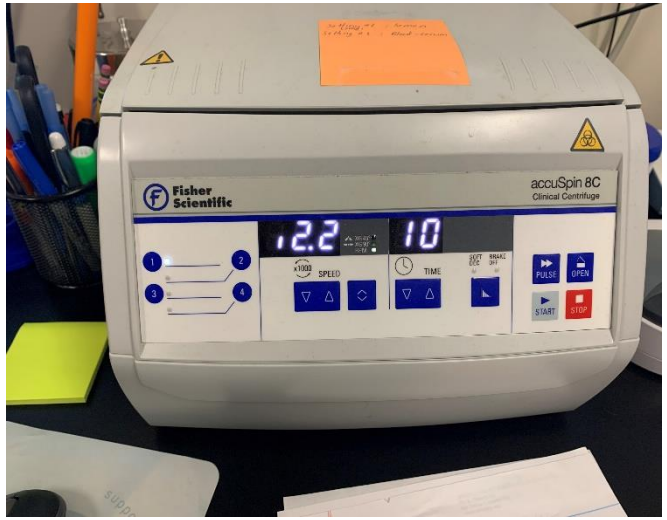


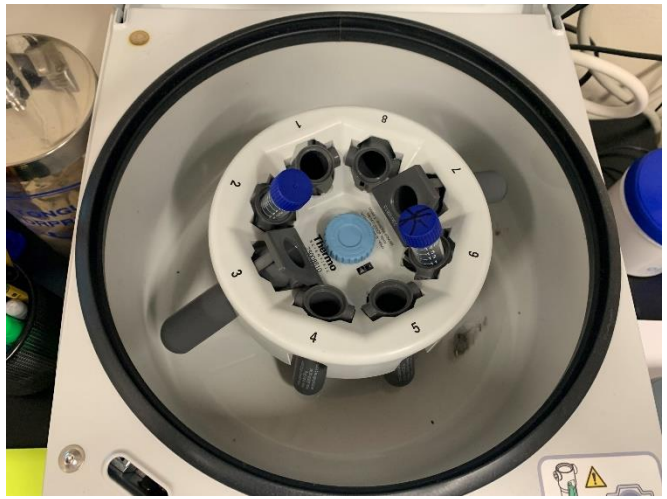
Hemacytometer

- Hemacytometer - ~$250 - $350
  - Neubauer/Improved Neubauer ruling w/cover slips
  - Count cells/sperm visually with microscope
  - Manual technique
  - Accurate lab technique, ~ 10 minutes
  - Can be used on raw and extended samples
  - Requires a diluted sample, Thrombo-TIC® or 1:100 with Formalin 10 solution
  - ~$4/sample for supplies

CENTRIFUGE

- Remove prostatic fluid
- Concentrate sperm
- Size – small benchtop
  - Rotor
    - Swinging buckets, 4 or 8 x 15 ml tubes
  - 1000 x g, fully loaded
- Investment





COOLING SEMEN

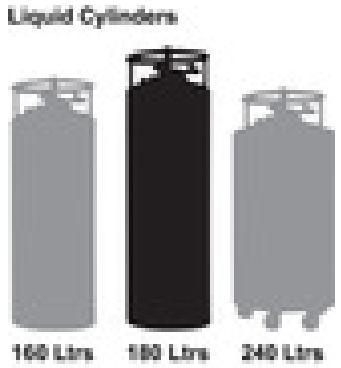
- Refrigerator: Limitations
- Upright chiller: supermarket
- Walk in cold room

BASIC EQUIPMENT FOR FREEZING: FLOATING RACK AND BOX

- Inexpensive
- Floating straw rack & box
- Rack floats straws ~4 cm from LN2

ADVANCED EQUIPMENT FOR FREEZING: ICECUBE™ MINITUBE

- Controlled rate cell freezer
  - Programmable
  - Compact, easy to operate
  - $$$$$
  - Need large dewar of LN2 at 22 PSI
    - Dewars will only last a few days, exchange frequently
    - LN2 supply company



Food Grade Grade Liquid Nitrogen, 180 Liter Cylinder, 22 PSI

Airgas Part #: NI FG180LT22

LIQUID NITROGEN TANKS

- Large Dewar/Cylinder: liquid N supply
  - Pressurized with safety valve
  - Bulk LN2: ~160 L or more
  - Need special hose and phase separator

- Dewar Tank in Semen Room
  - Storing excess/working LN2
  - Does not have canisters
  - Low liquid consumption

- Semen Storage Tank/s in storage space
  - Multiple sizes available
  - Different manufacturers
  - Variable liquid consumption
  - Variable configuration

DRY SHIPPERS: TRANSPORT FROZEN SEMEN

- SC- small capacity
- 4/3 – 4 liters/3 weeks at vapor
- v – vapor not liquid
- ~120 straws
- 1 canister
- Cost ~$1,400
  - *Some places will rent

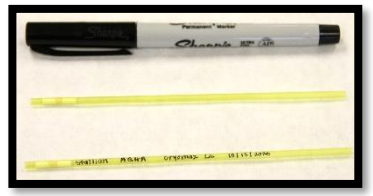
SUPPLIES: LIQUID NITROGEN AND HANDLING

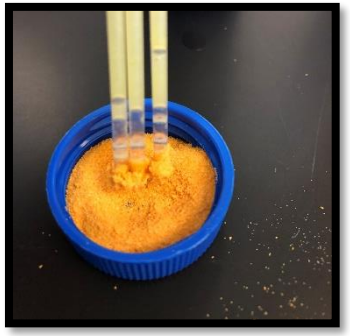
- Liquid Nitrogen (LN2) is a cryogenic liquid
- Hazardous material
- Use safety protection, goggles, gloves, forceps
- Necessary for freezing sperm
- Need to have a space with concrete floor, ventilation, alarms

- Evaporation is constant
- You need a continuous supply of LN2
- LN2 companies will deliver

SUPPLIES: 0.5 ML STRAWS

- Available from several companies
  - ARS, Minitube, IMV
  - Variety of colors optimal
- Labeling the straws
  - Custom printing by ARS, Minitube
  - Or hand write with small Sharpie®
- Sealing the straws
  - PVC powder, ‘crimp by machine’, heat seal or BB’s





TAKE HOME MESSAGE TO SET UP FOR FREEZING

- Specialized equipment is required to freeze canine sperm: not all $
- Space is important
- A reliable and continued supply of LN2 is needed
- The expendable supplies needed depends on the number of ejaculates to be frozen
  - Pre-planning is key
- Laboratory equipment should be serviced annually
- It is a commitment and investment to do it properly, regularly and most importantly, well!
- The size of your breeding program (and collaborators) will determine if economically feasible to do it yourself or use an external company

THANK YOU!

- CSU Small Animal Reproduction Team
- ARBL, CSU team
- NZ Working dog Breeding programs: Guide Dogs, NZ Police and MPI

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