Cryobiology and Reproduction

Basic principles, recent advances

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Centre for Genetic Resources
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Animal Breeding &
Genomics Centre

In this presentation:

- Briefly about CGN
- Explain some aspects of fundamental cryobiology

  (and how this helps to understand and improve methods)
- Examples of developed methods

CGN (Centre for Genetic Resources, The Netherlands)

- Government funded organisation

- Plant Genetic Resources
- Forest Genetic Resources
- **Animal Genetic Resources (AnGR)**

→www.cgn.wur.nl

Species in CGN Gene Bank

CGN semen collections (2016)

Species	# of breeds	# males per breed	# Doses
Cattle	18	1 – 5,223	239,793
Sheep	10	8 – 71	31,154
Goat	5	5 – 33	6,590
Horse	9	1 – 41	3,307
Pig	28	1 – 56	20,464
Chicken	31	1 – 20	18,828
Duck	3	14 – 34	1,588
Dog	5	1 – 8	410
Rabbit	8	3-12	1,957

Fundamental Cryobiology

Fundamental Cryobiology

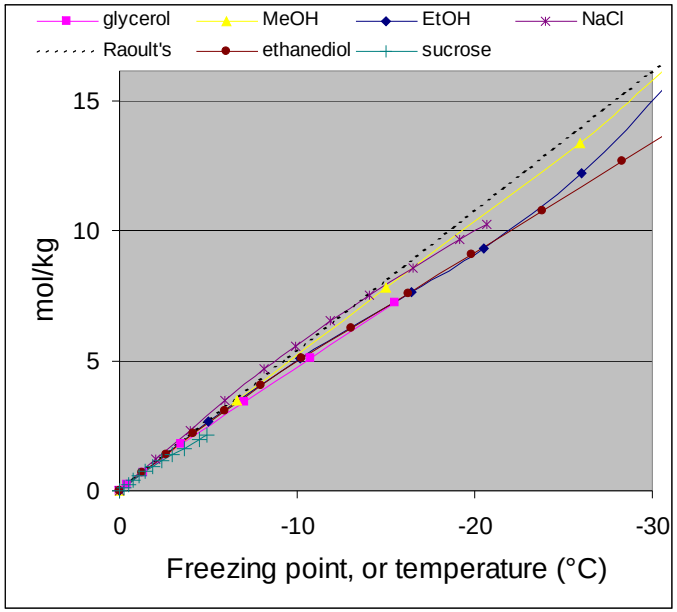
How to survive cryopreservation?

Much about water (and solutes)

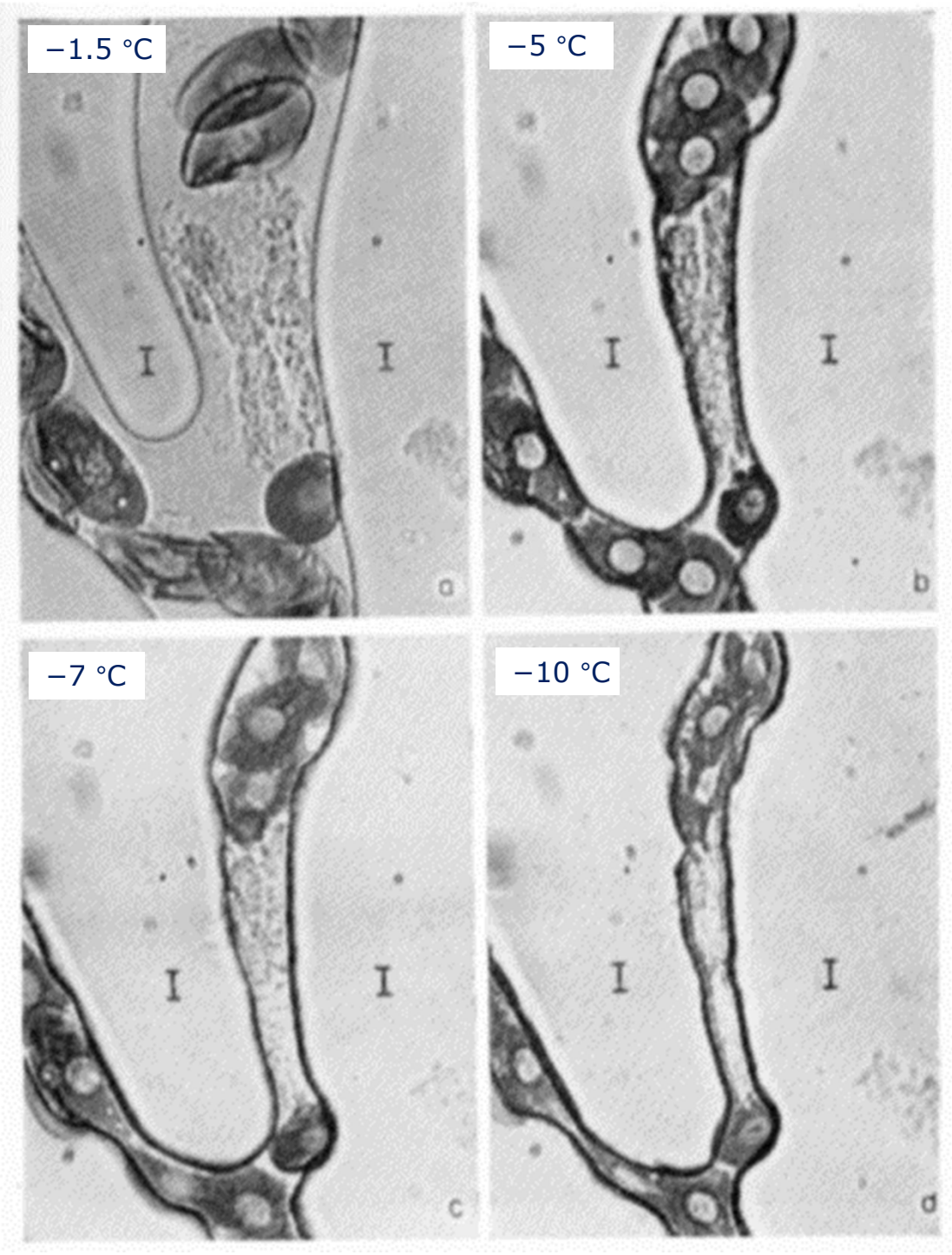
- Prevent intracellular ice
- Therefore, remove water
- Without excessive dehydration
- Without excessive shrinking and swelling

Slow Freezing

Erythrocytes
Rapatz & Luyet, 1960



Woelders and Chaveiro 2004



Slow Freezing

Water freezes (extracellularly) as pure ice

An unfrozen fraction remains that contains all solutes

- The volume of unfrozen fraction ↓
- Water content ↓
- Solute (salt) concentration ↑
- Osmotic pressure ↑
- Viscosity ↑

} While IIF is prevented!

At some point of temperature and concentration

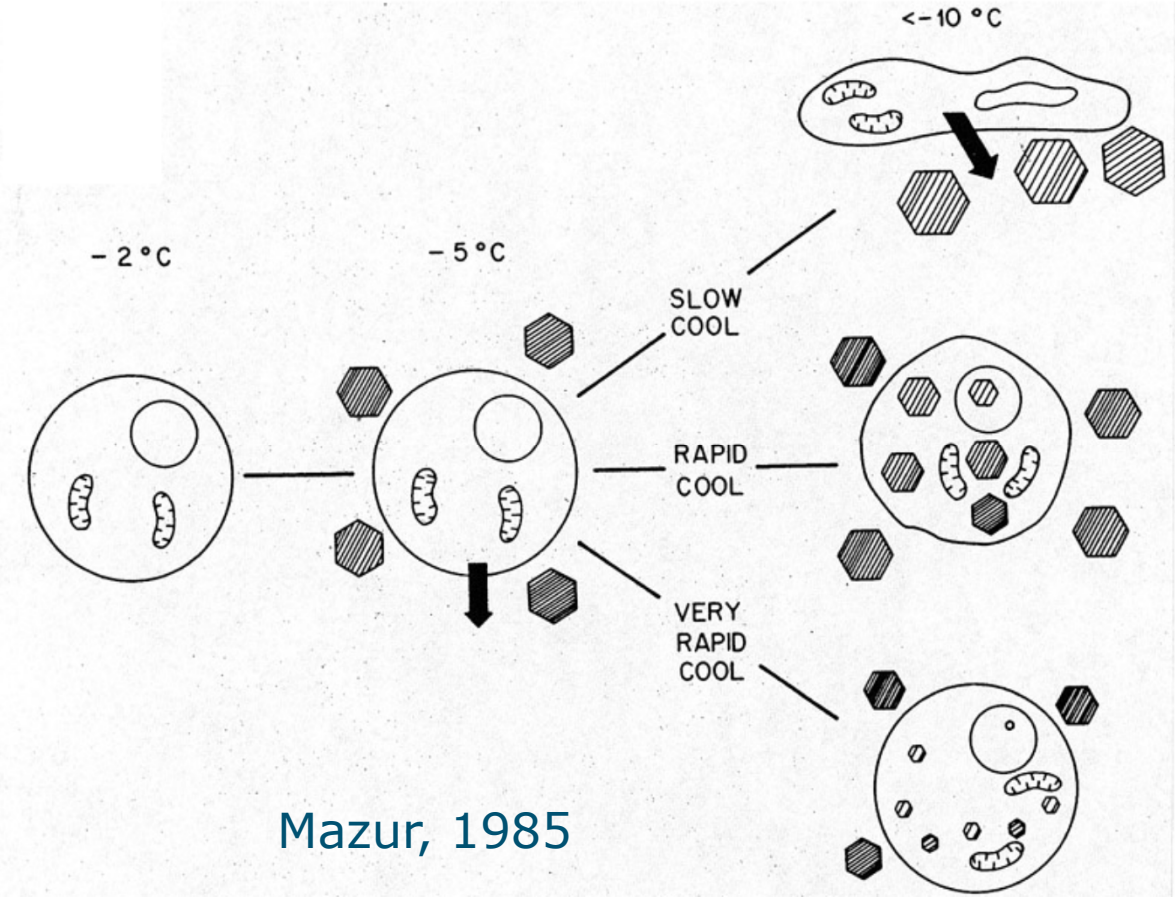
➔Glass transition

Glass transition

Glass transition means that a liquid becomes solid in an amorphous state. The lateral mobility of molecules becomes practically zero.

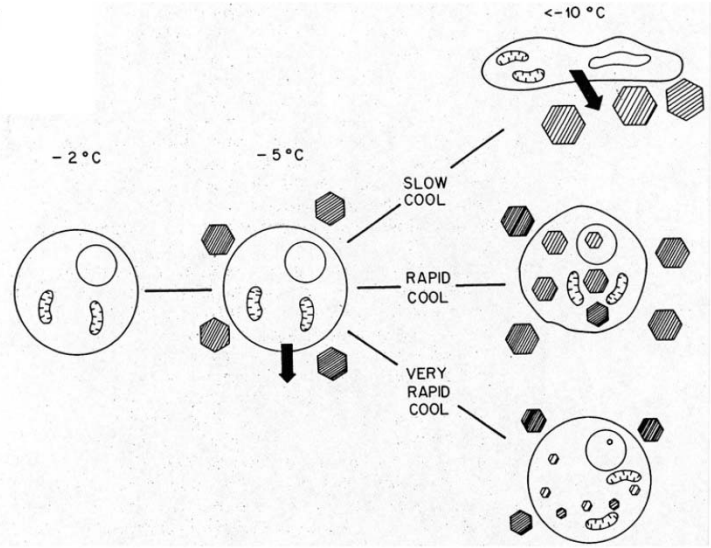
That is why a glass is stable: Molecules have lost the ability of lateral movement. No significant biological or chemical changes will take place.

Slow Freezing



Mazur, 1985

Slow Freezing



So, do cells survive slow freezing?

NO

Polge et al. (1949) thought that they had to draw out water before freezing by using a high sugar concentration.

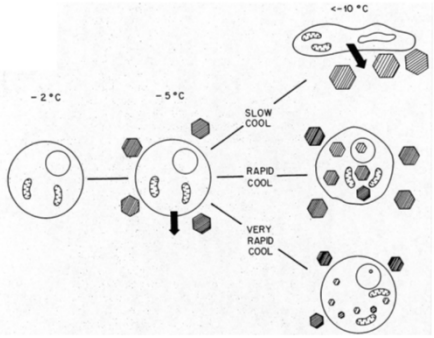
Do cells survive this?

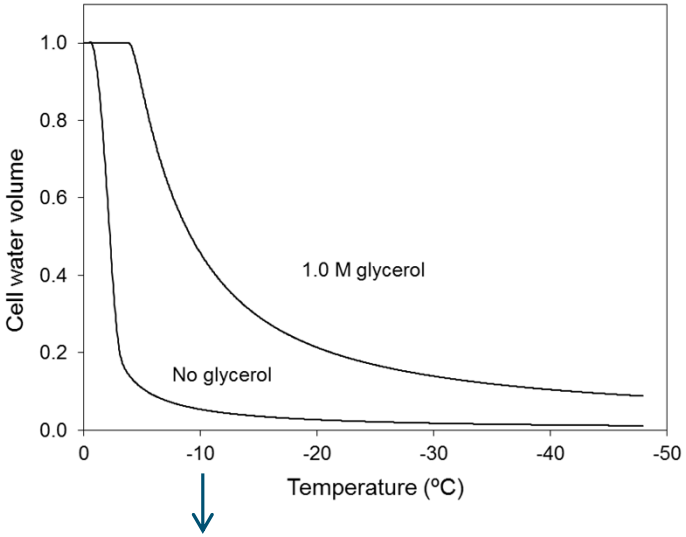
NO

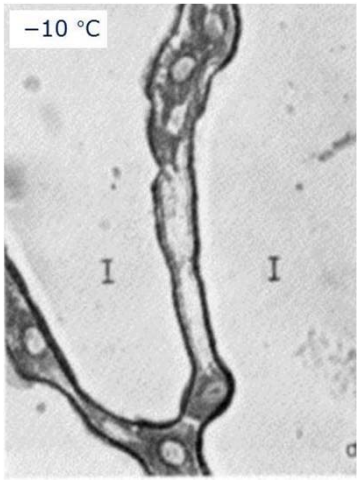
But they were lucky....

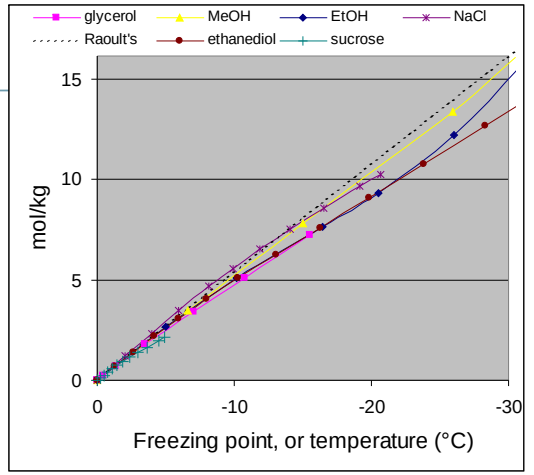
Serendipitous 'invention' of glycerol as CPA

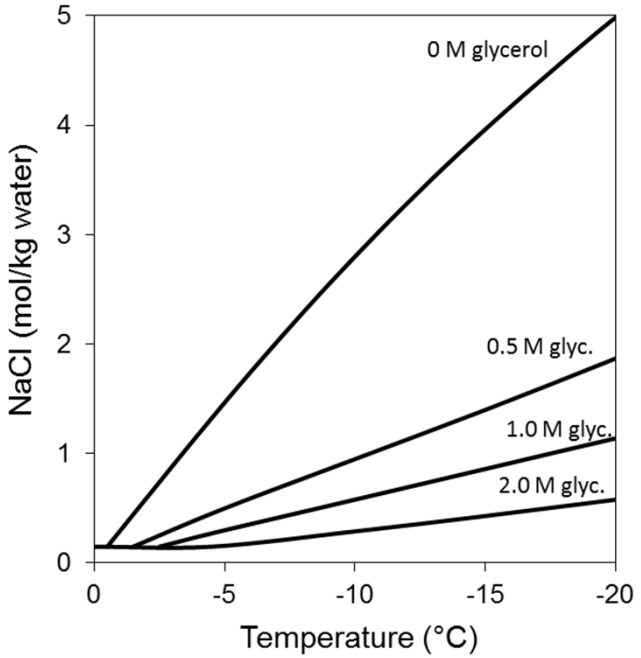
Cryoprotectants











Mazur & Rigopoulos, 1983

Cryoprotectants

Examples of such compounds:

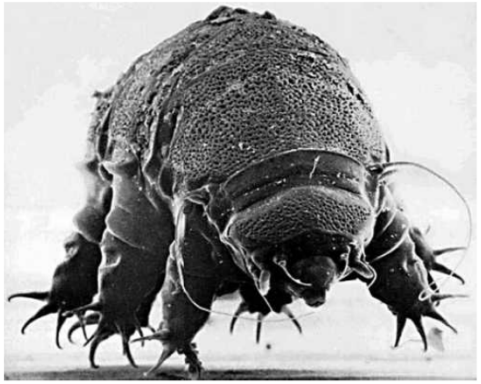
- propane triol (= glycerol)
- propane diol (= propylene glycol (PG))
- ethane diol (= ethylene glycol (EG))
- butane diol
- ethanol
- methanol
- dimethyl sulfoxide (DMSO)
- dimethyl acetamide (DMA)
- methyl acetamide (MA)
- methyl formamide (MF)
- etc.

Cryoprotectants from nature

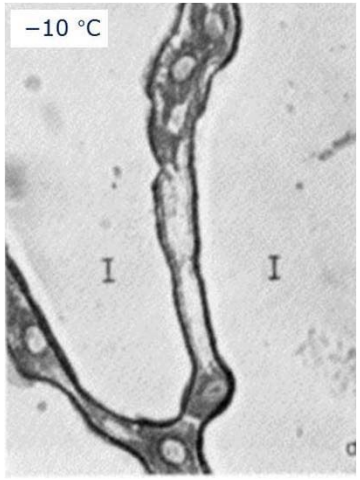
How to get the cryoprotectant inside the cells?

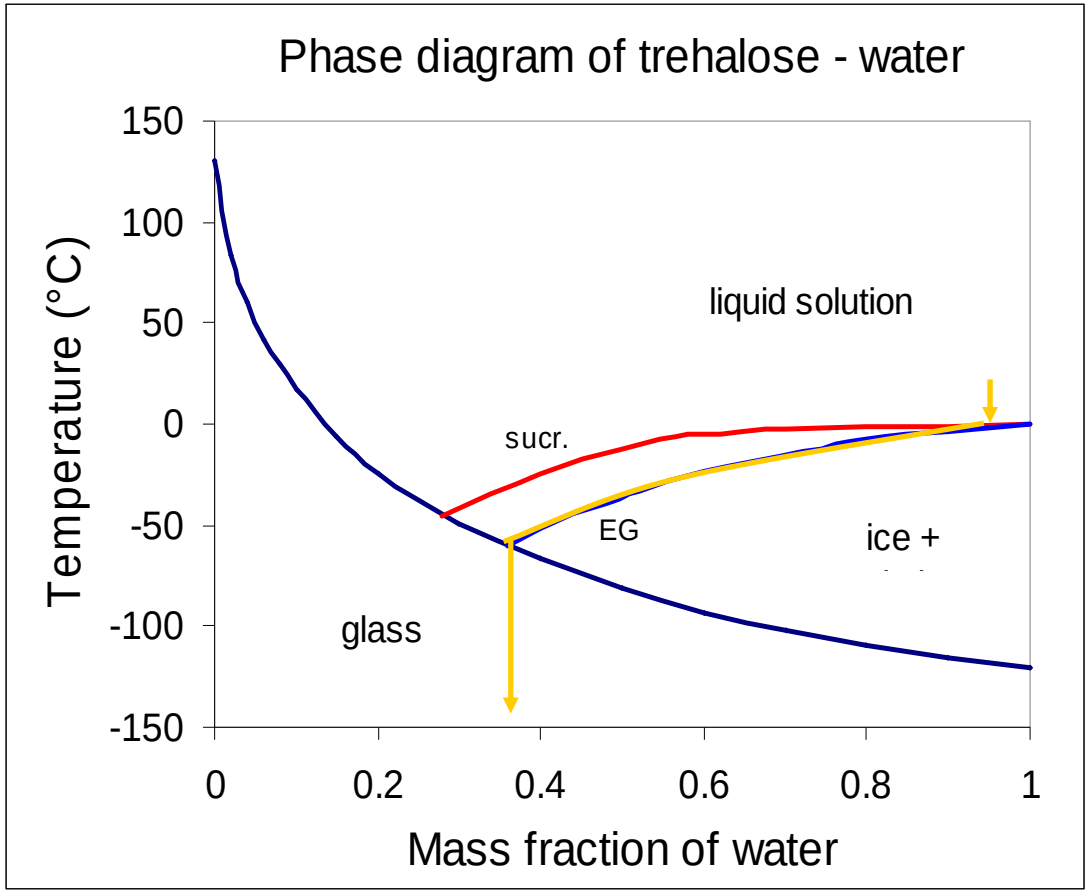
1. We use membrane permeable CPAs, like glycerol

2. Nature has another trick:
   Cold hardy plants and animals can produce high intracellular concentrations of sugars

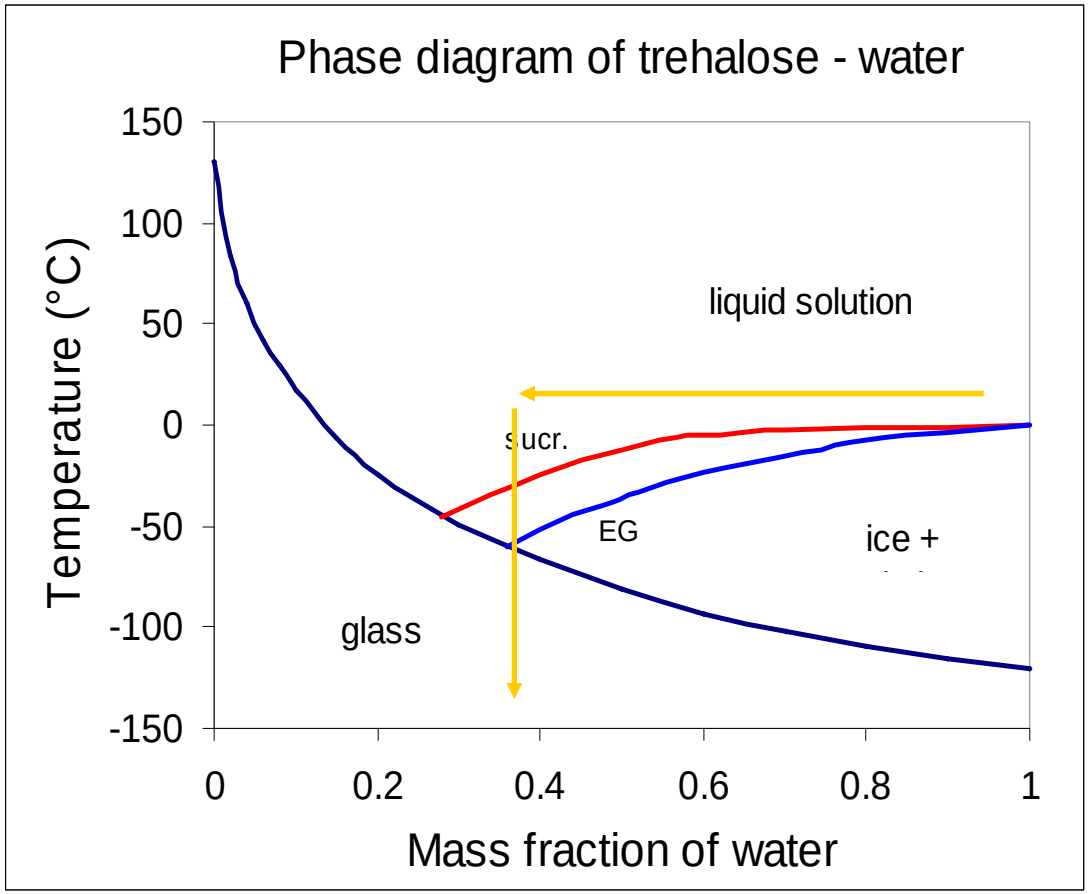


Slow Freezing → Vitrification



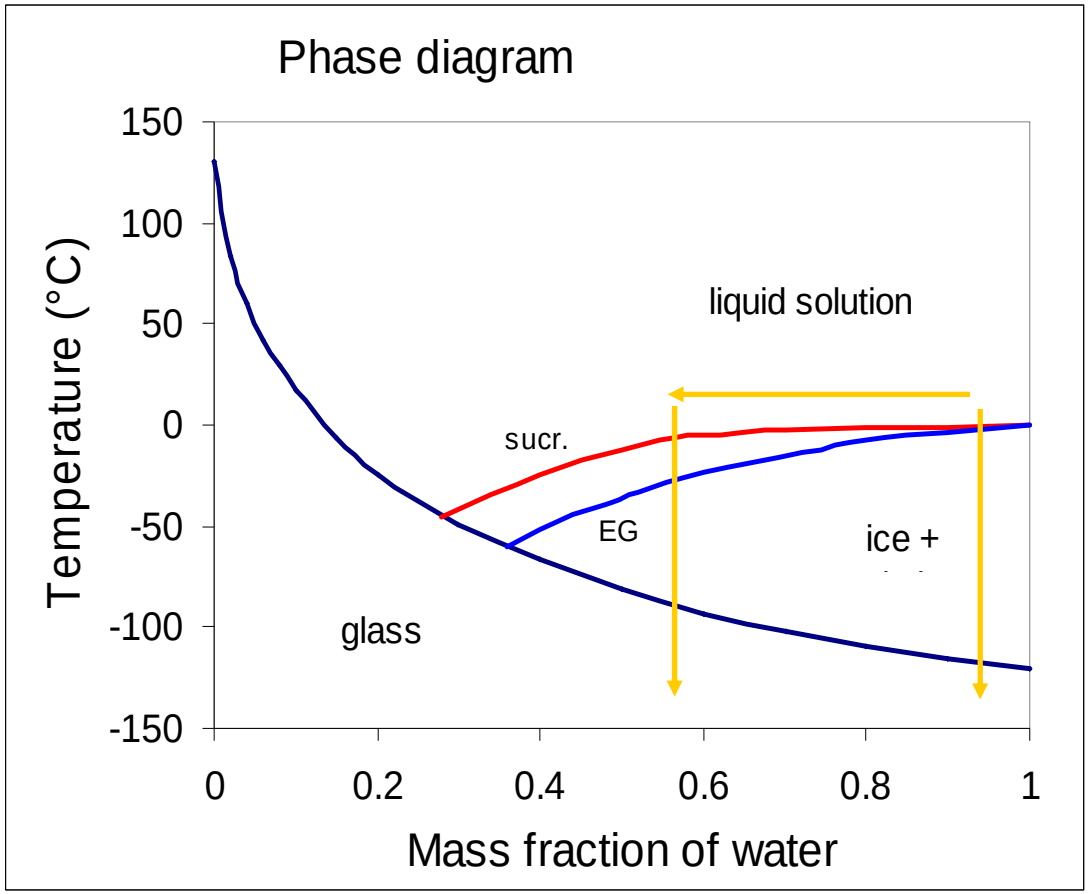


Vitrification



Vitrification, with very high cooling rates

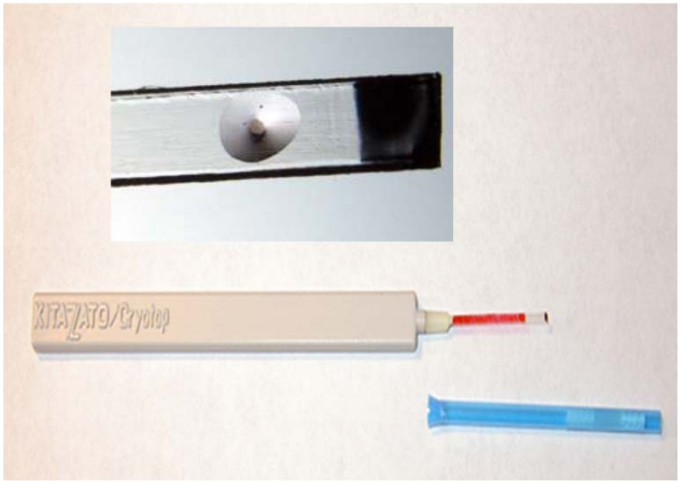
- Vitrification with lower CPA concentrations
- Outrun Spindle depolymerization
- and other hypothermia induced changes
- Semen vitrified without any CPA (Isachenko et al., 2003)

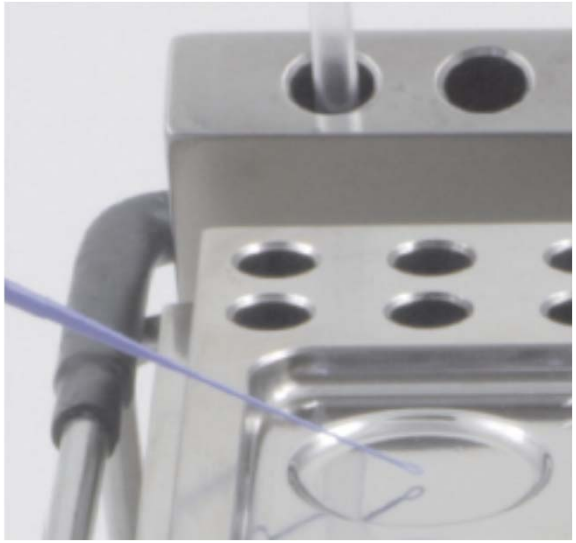


Vitrification

Very high cooling and thawing rate by:

- Minimal volume (o.p.s., grid, cryoloop, cryotop, QMC etc.
- Solid surface vitrification(cryologic CVM)
- N_2 at freezing point versus at boiling point (Vitmaster)

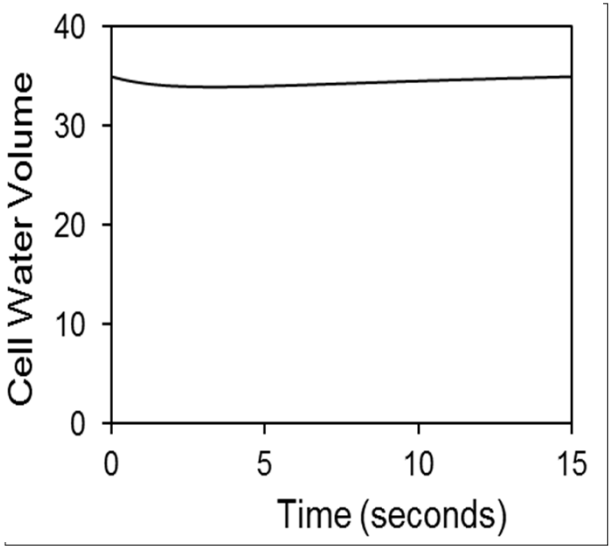




Volume changes due to adding glycerol

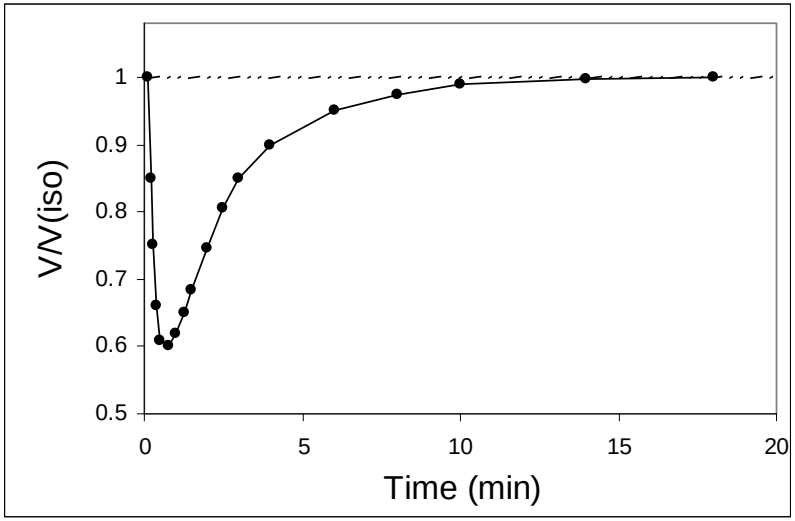
Semen

- Equilibration of CPA is very rapid (10 seconds)
- Volume changes are not very large → little chance of damage

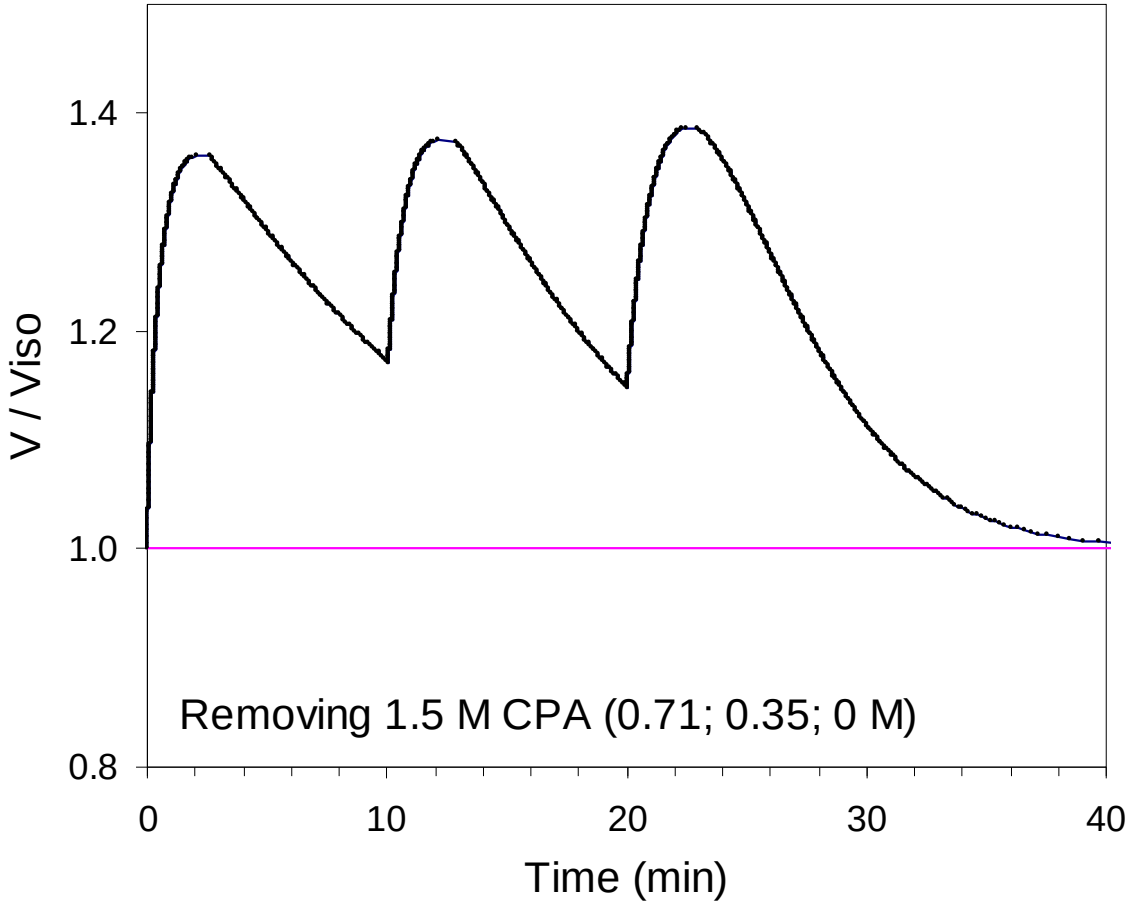


Embryos

- Equilibration of CPA may take 5-15 minutes
- Volume changes are very large → large chance of damage



Removal of CPA (embryos, oocytes)



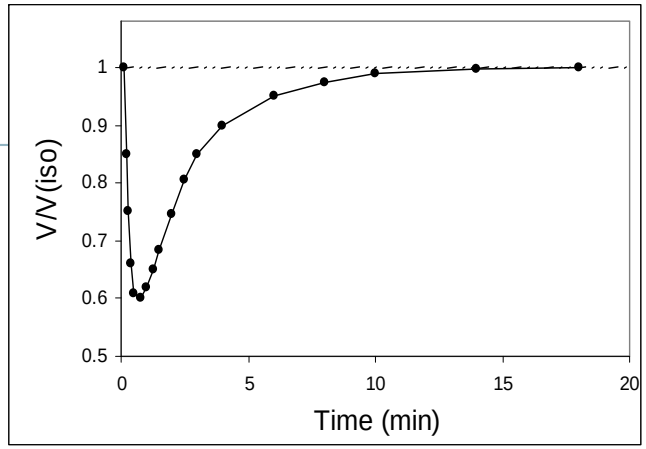
Improving cryo-methods and cryomedia

- Measurements and simulations help to understand what actually happens
  - During freezing or during vitrification
  - During adding and removal of cryoprotectants.

✓ What happens with cell volume

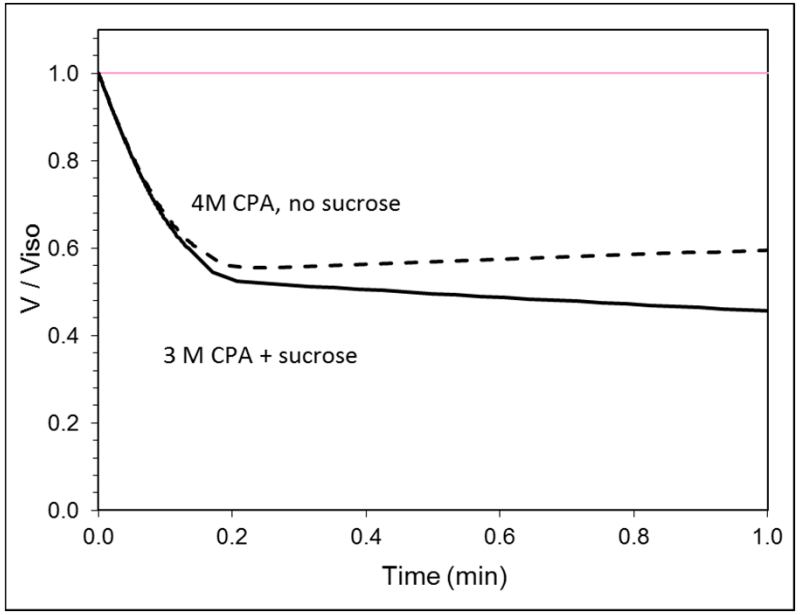
✓ What happens with concentrations of cryoprotectants and other solutes.

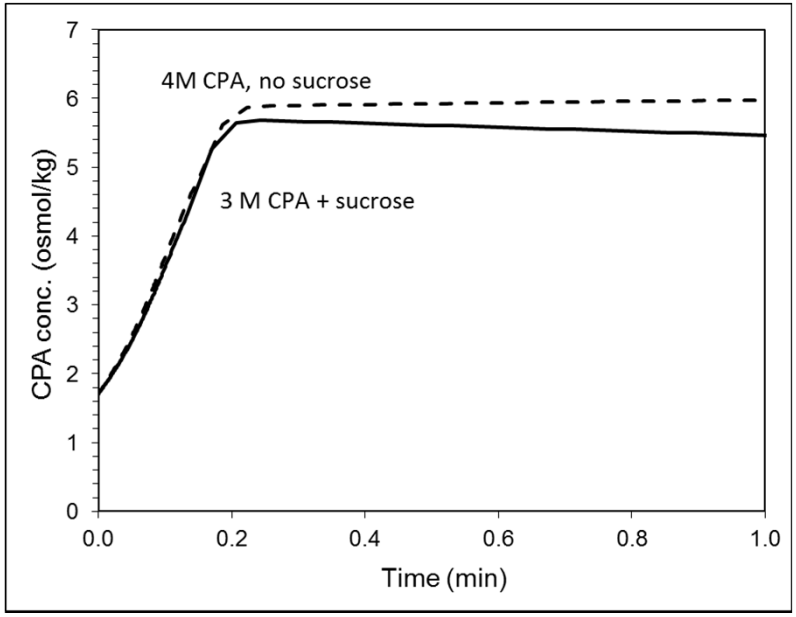
Vitrification



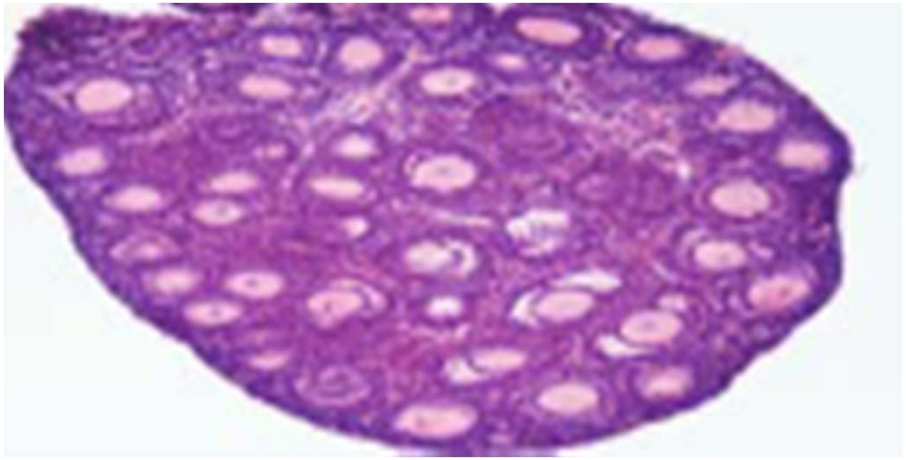
- ✓ What happens with cell volume?
- ✓ What happens with concentrations of cryoprotectants and other solutes?

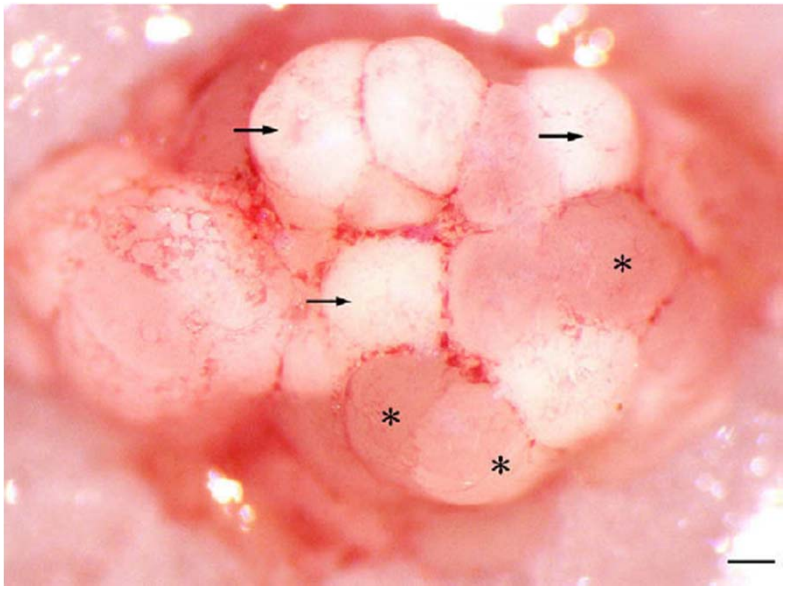
Do we need membrane permeant CPAs?





Vitrification of ovaries (and embryos)





Huang et al. 2010

- Larger structures. Penetration of CPAs is probably slow
- No logic.... But vitrified as good as fresh! (in birds, mice)

If CPAs do not penetrate anyhow, why use EG and DMSO in 2nd step?
In ovaries, perhaps even water does not equilibrate?
What does that mean?

Methods for gene banking

Cryobiology and reproduction

Methods for specific species; specific germplasm

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Freezing of epididymal ram semen

Epididymal ram semen

- Heath sheep rams often too 'wild' to train for semen collection → Alternative: epididymal sperm

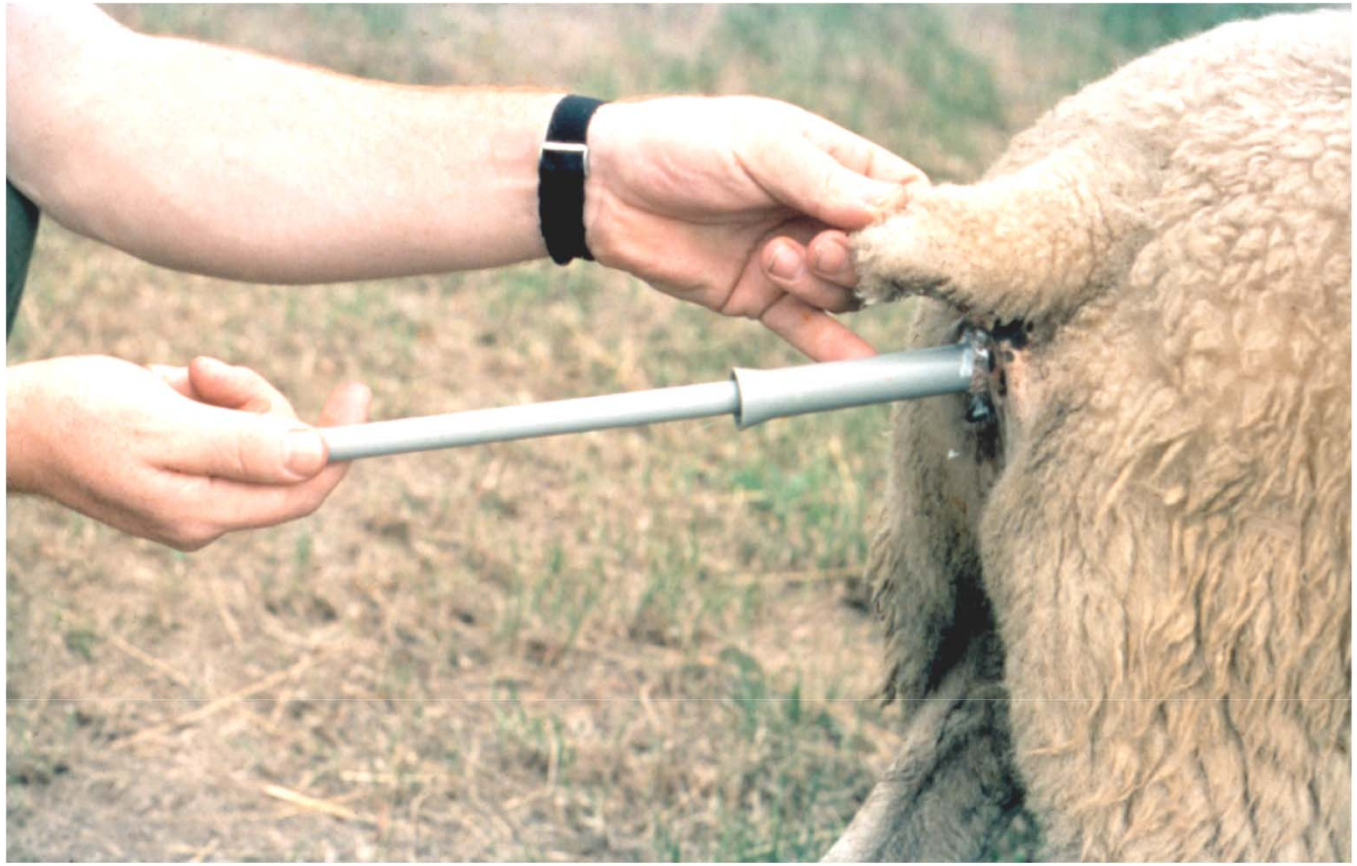
- We use a rapid method for semi-quantitative collection from the caudae epididymidis.
- Rams are culled anyway. We collect testes from slaughterhouse. → Very cost-efficient

- More than **20 billion** sperm **per ram**.
  = >100 doses of 0.2 billion sperm/dose.

Semen from Veluwe rams

	% motile sperm	% live sperm
Ejaculated semen	42 ± 4.5	48.8 ± 2.1
Epididymal semen	60 ± 0	62.3 ± 5.6

Synchronised Swifter ewes



Lambs born

Results

	Ejaculated semen		Epididymal semen	
	pregnant	lambs/ewe	pregnant	lambs/ewe
Cervical AI	0/11*		4/10	2.0
Laparoscopic AI	6/10	2.3	7/10	3.1

* Cervical AI with ejaculated semen scored 12-30 % in later experiments

Other species

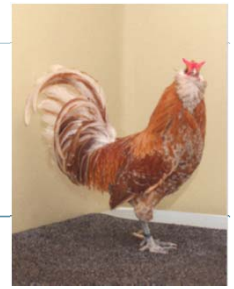
Goat

- We developed a freezing medium for buck semen.
  - Alleviates deleterious effect of lipase in buck seminal plasma
  - Good post-thaw sperm survival
  - Used now by Dutch goat AI

Horse

- We developed a freezing medium for buck semen
  - Improved motility and live sperm during cooled storage
  - Improved post-thaw sperm quality
  - Used by CGN gene bank

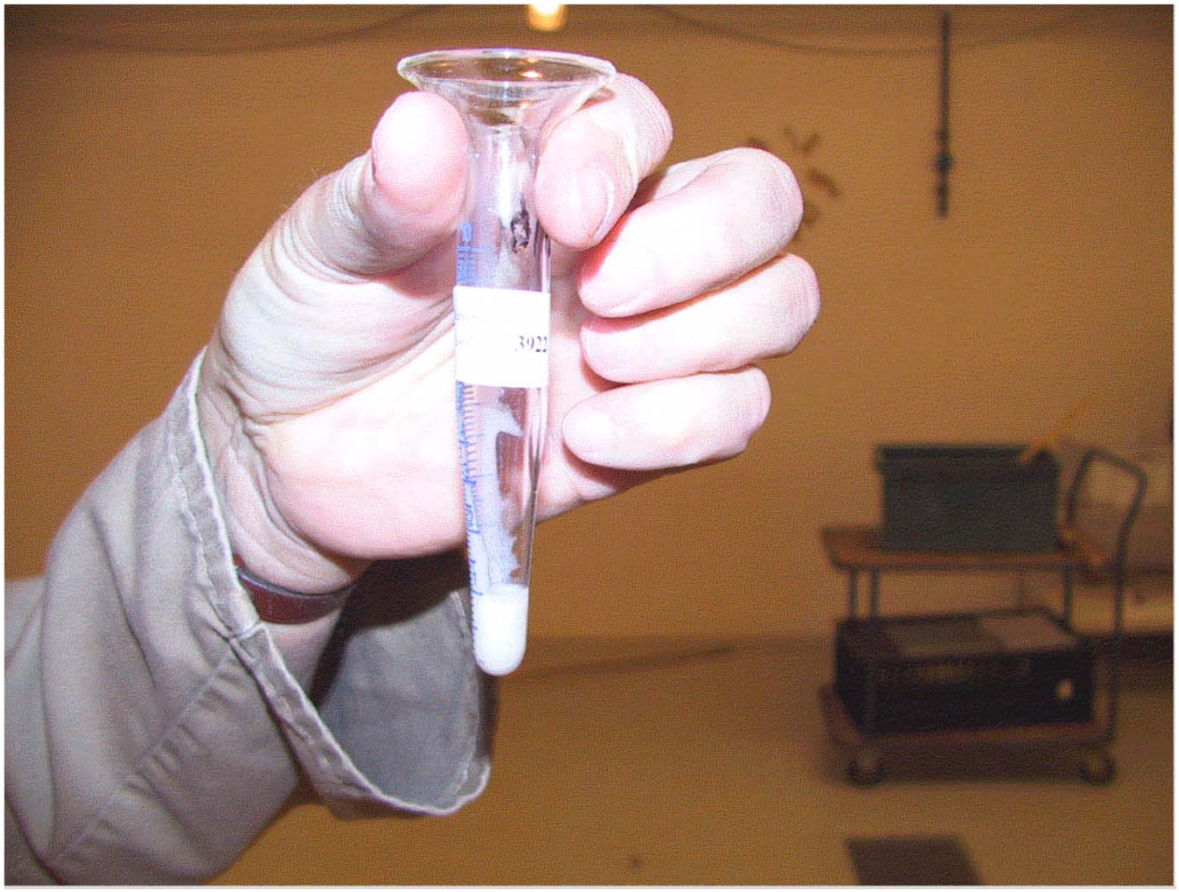
Birds



- Gene banking of birds for:
  - Wild life conservation
  - Poultry rare breeds
  - Breeding lines (breeding companies); Research lines
- Ova or embryos are not a possibility because of the large yolks
- Possibilities include semen, ovaries, and PGCs

Collection of semen

Collection of semen



Freezing poultry semen

"The enigma of Glycerol"

- Glycerol is generally seen as the better cryprotectant

  Hammerstedt en Graham 1992; Tselutin et al 1999; Phillips et al. 1996

- But glycerol acts as a contraceptive.

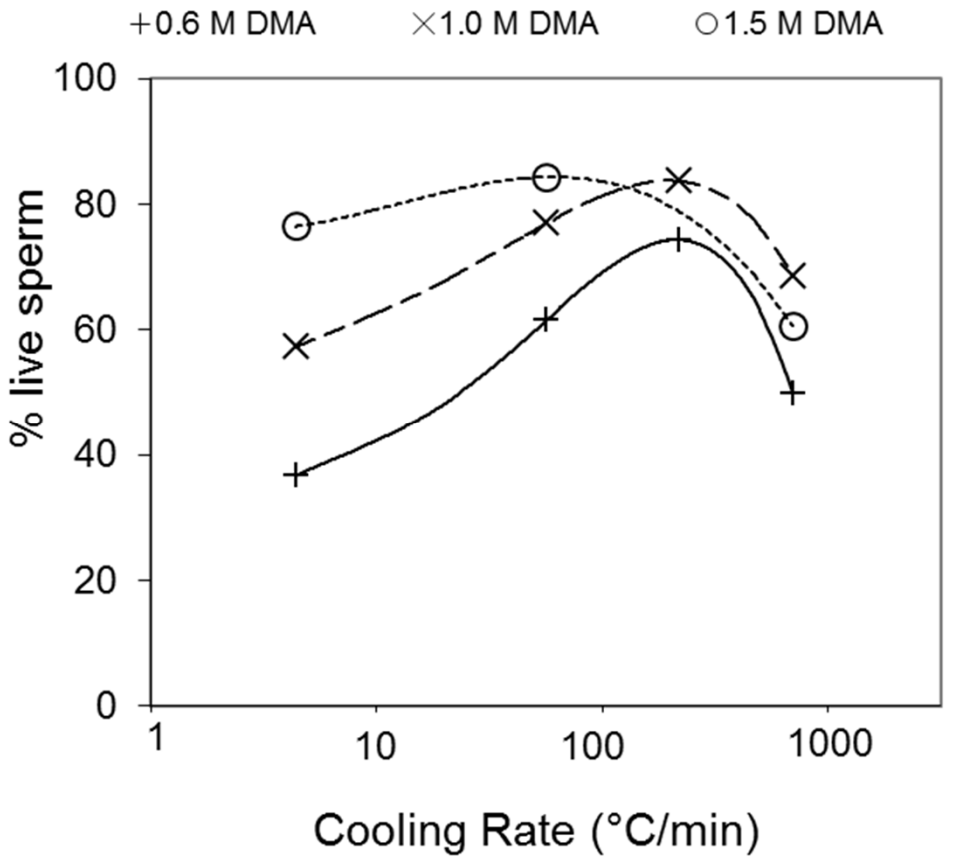
Freezing methods

- Tselutin et al.1999

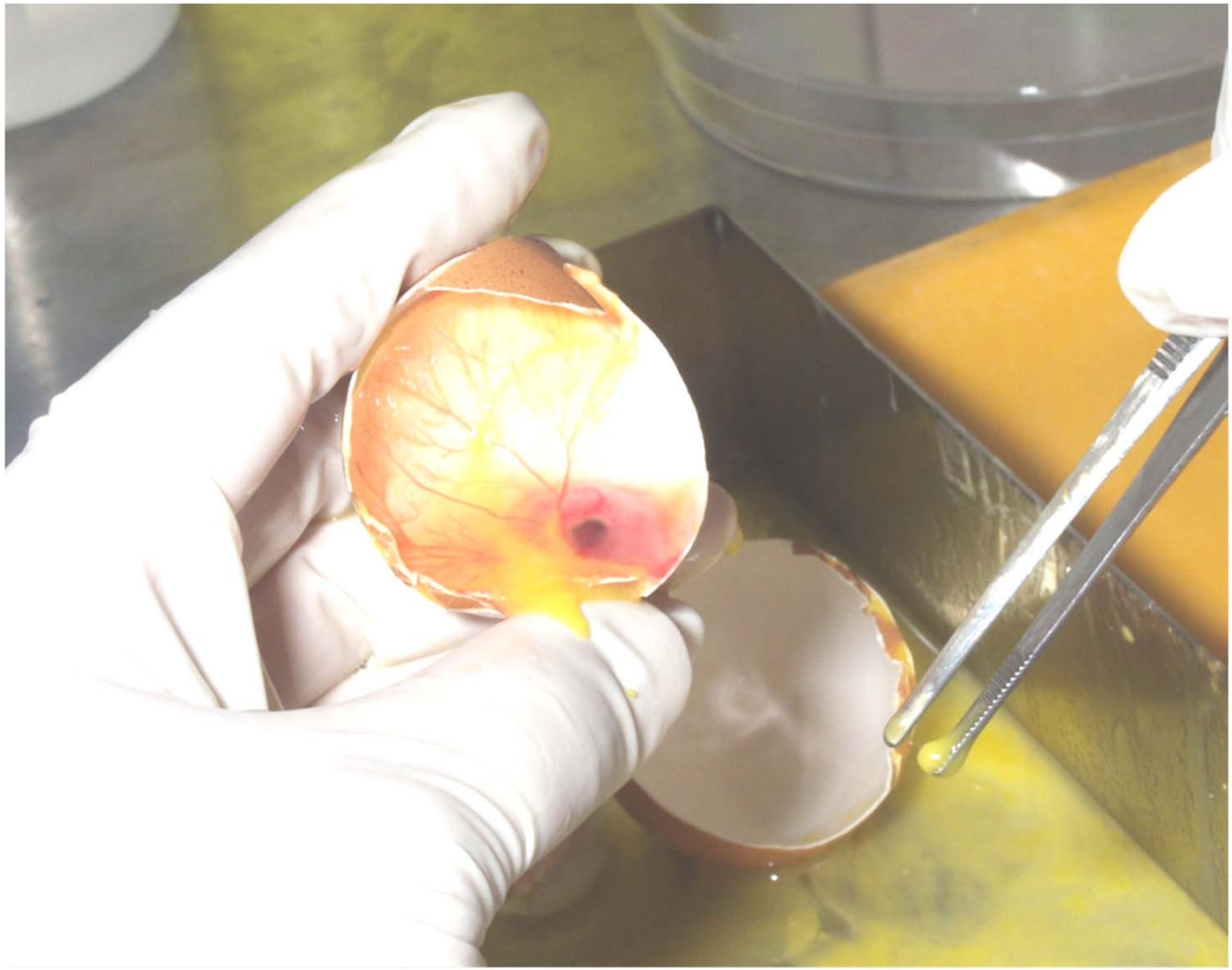
CPA	package	% fertilized eggs
glycerol	straws	63.9
DMA	pellets	84.7
DMA	straws	26.7

Interaction [CPA] x CR

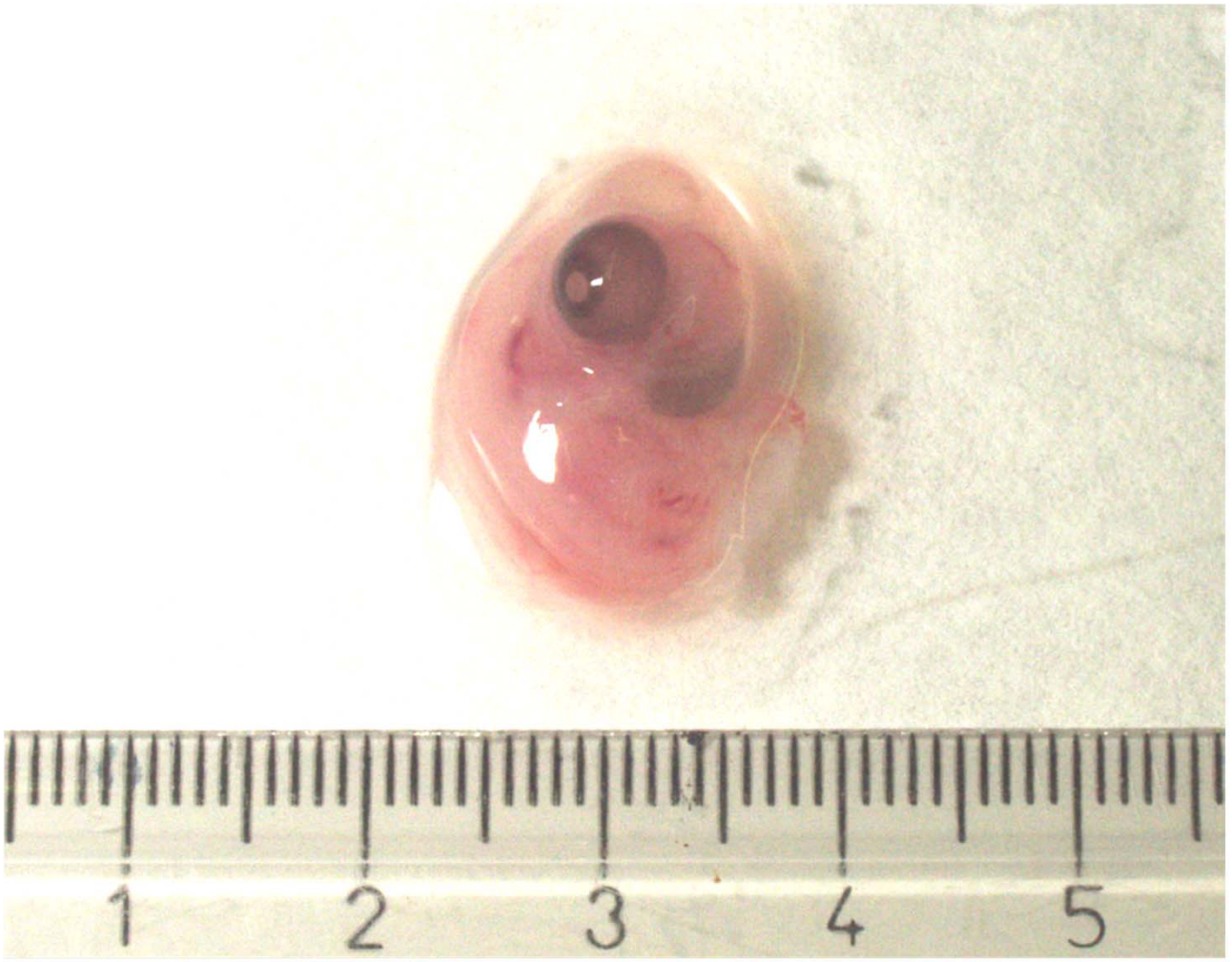
- Improved extender
- Optimised [DMA] x CR



Successful AI: fertile eggs



Successful AI: fertile eggs



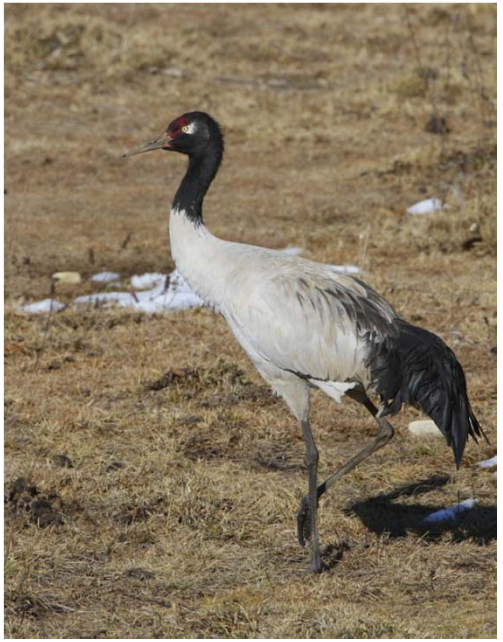
Insemination experiment

	Treatment	% fertile eggs	% eggs embryos	Days fertile after last insemination
1	Fresh semen in ASG medium	96.6[a]	90.5[a]	16.4[a]
2	Frozen in straws in ASG medium with DMA	87.6[b]	80.4[b]	12.7[b]
3	Frozen in straws in Lake's medium with DMA	78.1[b]	68.9[b]	9.9[c]
4	Frozen in pellets in Lake's medium with DMA	85.9[b]	77.8[b]	12.3[b]

- Improved freezing medium
- Improved freezing method for freezing in straws
- We now use this medium and method for poultry cryopreservation programme in the gene bank.

Tragopan pheasants and Cranes

- Our ASG extender improved longevity
- Semen frozen on site, to prevent transport time
- Portable freezer. Freezing on the kitchen table!
- Good post-thaw sperm quality



Other bird species

- Turkey semen:
  Low hatching rate with frozen semen
  but best results with our medium and method
  (Long et al., Cryobiology 2014)

- Goose and Duck: In CGN gene bank. Using same medium/method

Collaboration with Gene-bank Norway

NIBIO
NORSK INSTITUTT FOR BIOØKONOMI

Norsk genressurssenter

988 straws, 122 cocks

- post-thaw motility: 42 ± 5.5 %
- As yet disappointing % fertile eggs

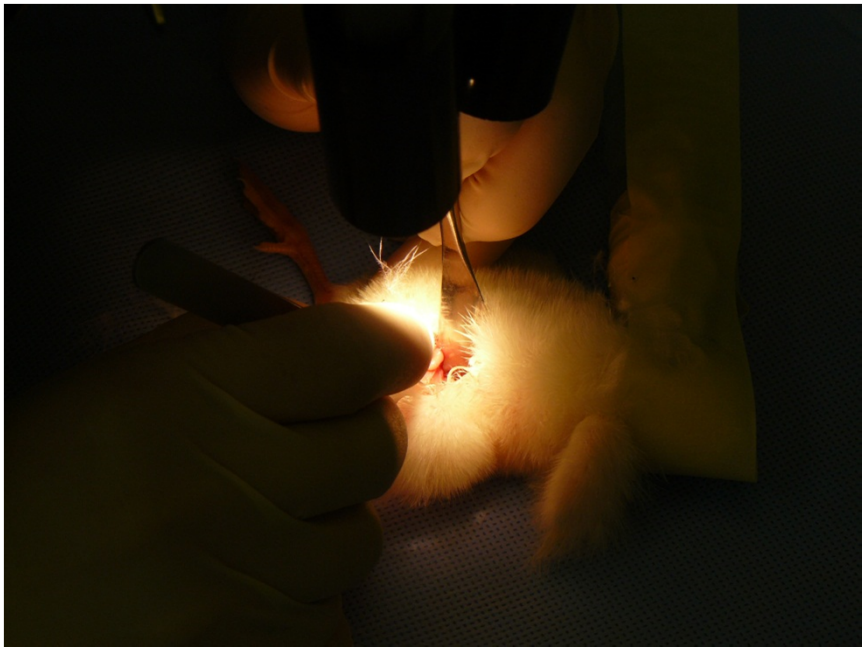
Birds, ovaries

BIOLOGY OF REPRODUCTION 83, 15–19 (2010)
Published online before print 17 March 2010.
DOI 10.1095/biolreprod.110.083733

Production of Donor-Derived Offspring from Cryopreserved Ovarian Tissue in Japanese Quail (*Coturnix japonica*)[1]

Jianan Liu,[3,4] Yonghong Song,[3,4] Kimberly M. Cheng,[3] and Frederick G. Silversides[4]

Faculty of Land and Food Systems,[3] University of British Columbia, Vancouver, British Columbia, Canada
Agassiz Research Centre,[4] Agriculture and Agri-Food Canada, Agassiz, British Columbia, Canada



- Vitrified ovaries. As good as fresh!.
- Easy and fast: Collection, vitrification, and grafting!
- In combination with frozen semen: → no backcrossing!
- And, also shown to be possible with testes.

Birds, ovaries

Preliminary results of the application of gonadal tissue transfer in various chicken breeds in the poultry gene conservation

Krisztina Liptoi[a,*], Gabriella Horvath[a], Janos Gal[b], Eva Varadi[a], Judit Barna[a]

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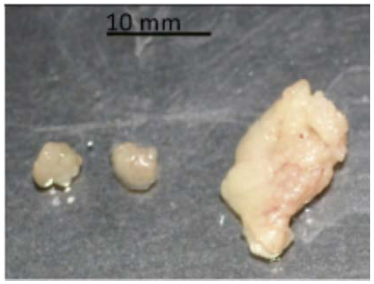
[b] Szent Istvan University, Istvan u. 2, H-1078 Budapest, Hungary

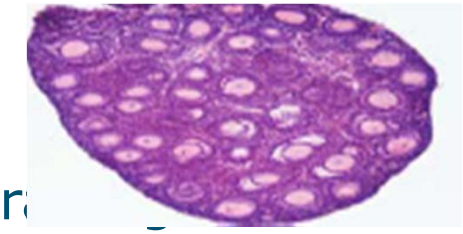


Horizon 2020
European Union funding
for Research & Innovation

IMAGE

Ovaries, Lab animals

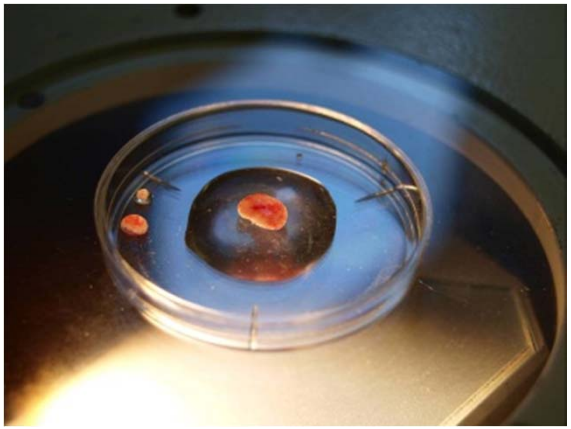
- Thousands of mouse strains
- Aim is a knock-out for each gene → >20,000 strains.

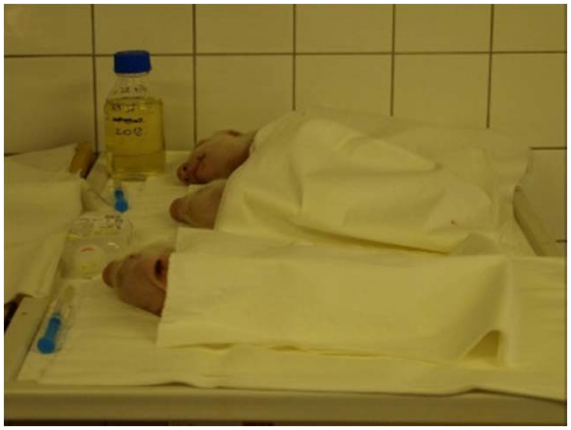


- Vitrification of juvenile mouse ovaries.
- Easy and fast: Collection, vitrification, and gr
- Very high success rate:
  - Banking a large series (22) of mutant mouse strains with various genetic backgrounds (C57BL/6, FVB, BALB/c)
  - Recovered the strains by grafting of the vitrified ovaries in recipient mice (Huang et al, 2010).

Other mammals?

- Pigs (collaboration with Herceghalom, Hungary)





Thank you for your attention

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