Novel gene banking approaches in poultry and pigs

PGCs, gonads, embryos, semen

Including some highlights from the EU project “IMAGE”

Henri Woelders

WAGENINGEN UNIVERSITY & RESEARCH

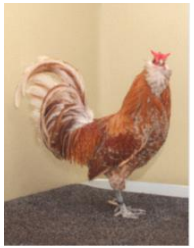
100years 1918 — 2018

IMAGE Innovative Management of Animal Genetic Resources

Objectives of EU project IMAGE (WP3)

- Improve possibilities for storage and use of germplasm
- Emphasis on “difficult species”: Sheep, Pigs, Poultry

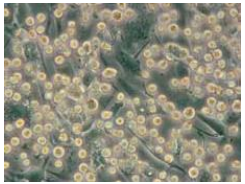
Chicken (bird) germplasm



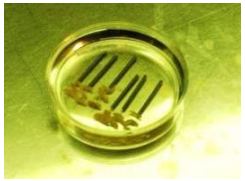
- Oocytes of birds cannot be frozen
- Same holds true for embryos

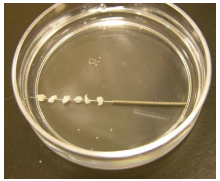
- Semen. Possible but ‘difficult’

- Primordial germ cells (PGCs)



- Juvenile gonads





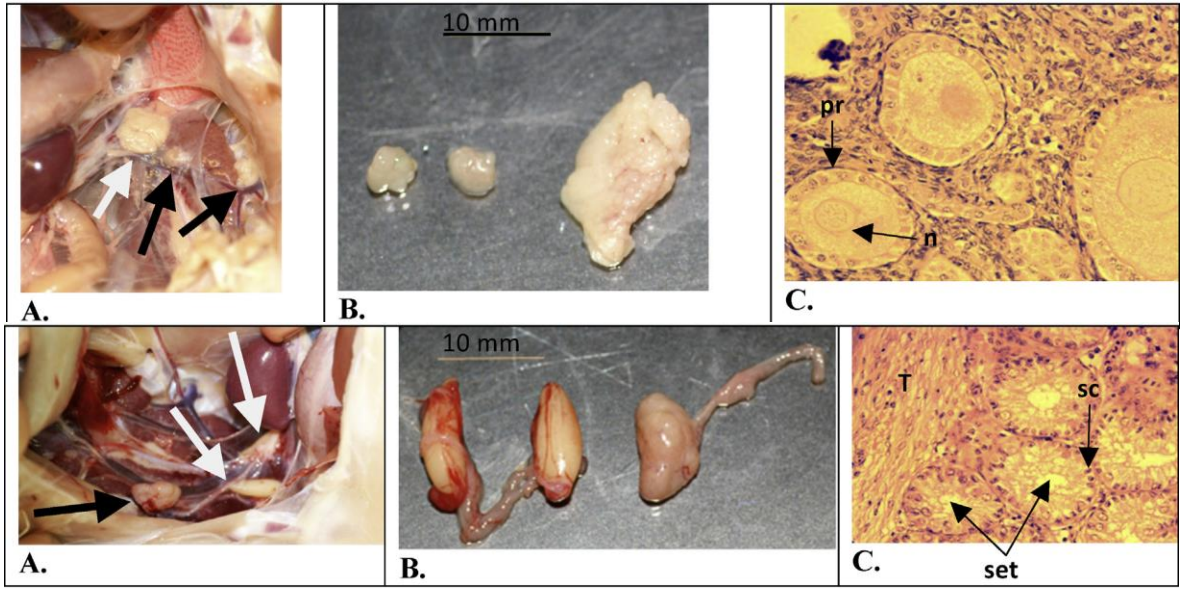
Transplantation of gonads

Y. Song and F. G. Silversides, 2006
The Technique of Orthotopic Ovarian Transplantation in the Chicken

Y. Song and F. G. Silversides, 2007
Offspring Produced from Orthotopic Transplantation of Chicken Ovaries
Production of Offspring from Cryopreserved Chicken Testicular Tissue

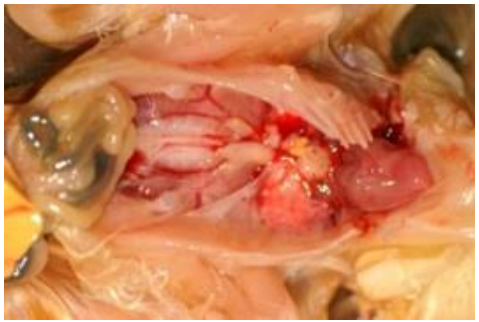
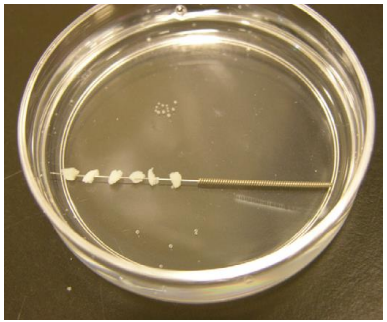
K. Liptói et al., 2013
Grafting of chicken ovaries and testes

Compatibility of donor
and recipient breeds



Transplantation of gonads

- Suitable donor – recipient pairs
- Cryopreservation of gonadal tissues
- Optimizing of pre- and post-operation treatments

Transplantation of gonads

- Success depends mainly on donor – recipient pair
- Not all breeds are suitable recipients

- All procedures and raising of the recipients post surgery can be carried out under simple „field” conditions
- Cryo method was easy and effective
  - Similar success Cryo and ‘fresh’ (72% vs. 80%)
- Suitable recipients shown for 5 indigenous breeds of 7.

RECIPIENT

White Leghorn

Novogen White

DONOR

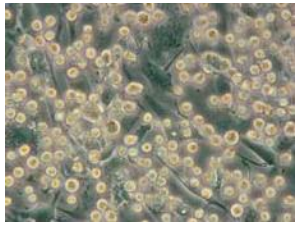
Yellow Hungarian

Partridge-color Hungarian

Speckled Hungarian

Black and Speckled Transylvanian Naked Neck

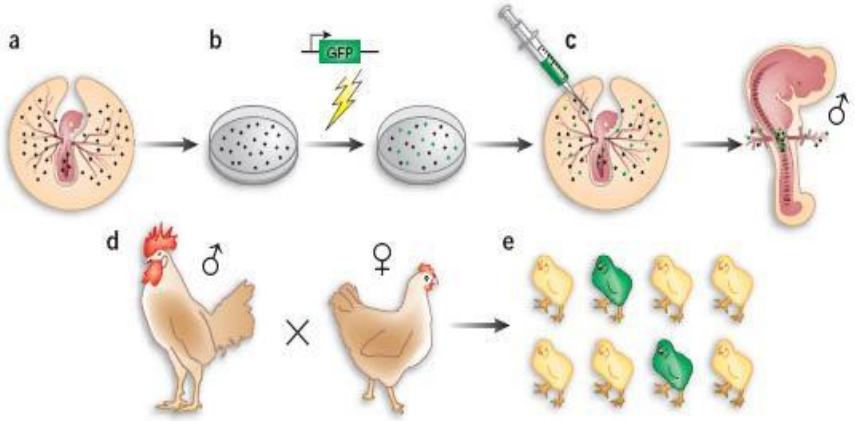
Primordial germ cells (PGCs)



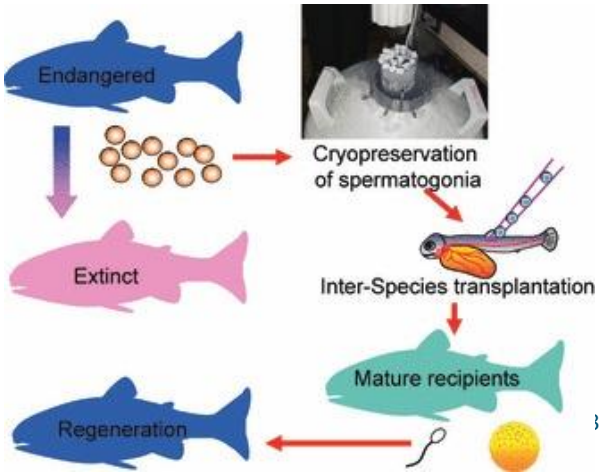
In birds and fish

- Full complement of chromosomes and mitochondrial DNA
- No need for back-crossing
- Donor and recipient are embryos. No ‘animal procedure’

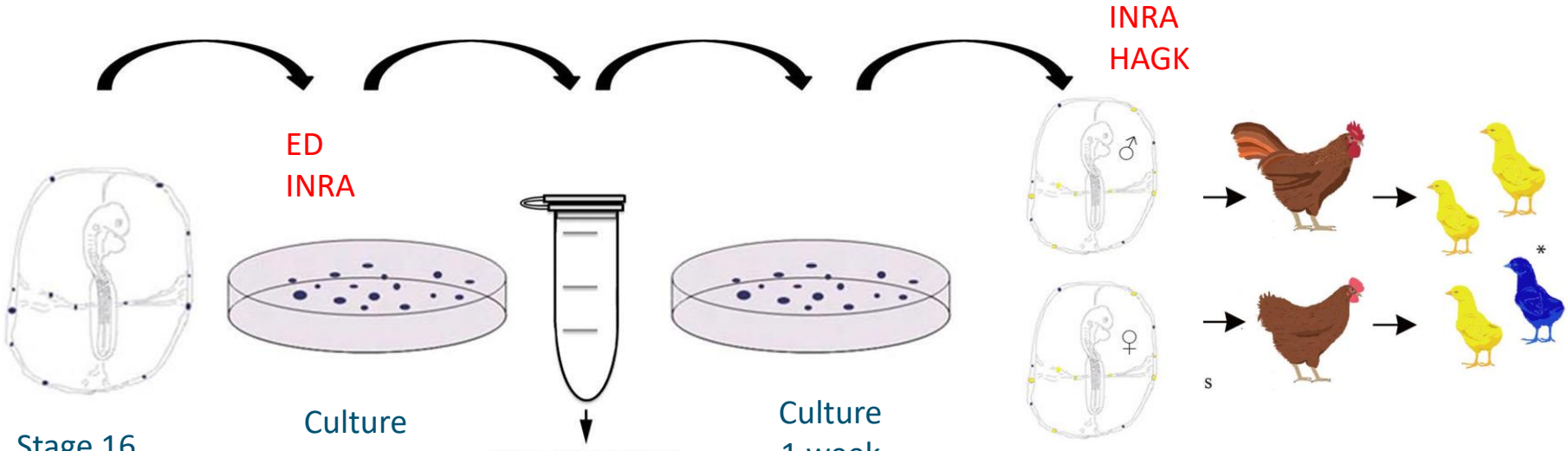
Esmaeilzadeh and Farhadi, 2011



Yoshizaki et al., 2012

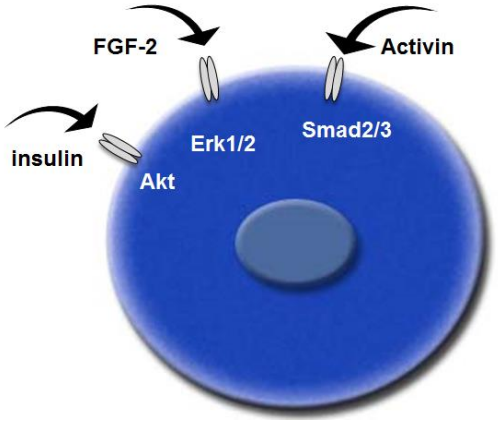


Primordial germ cells (PGCs)



Primordial germ cells (PGCs)

Proliferation of PGCs in vitro Whyte et al., 2015



Infertile recipients

Taylor et al., 2017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Science + Add to myFT

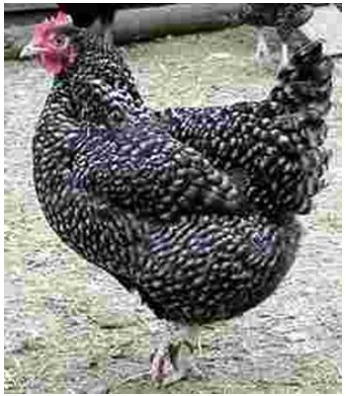
Scientists develop hens for use as surrogates

New gene-editing technology could help breed endangered birds

Researchers plan to implant primordial germ cells from other breeds in to sterile chickens

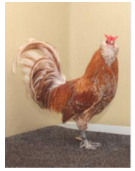
Successful biobanking

Indigenous Chicken breeds: Scotland (Roslin), Hungary, HáGK), and France (INRA)



Freezing of poultry semen

First Cryo success was: ...Poultry semen



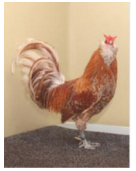
1949: Polge, Smith and Parkes

Nature 164, 666-666 (1949)
Revival of Spermatozoa after Vitrification and Dehydration at Low Temperatures

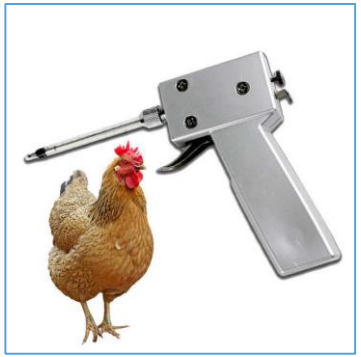
They had a bit of luck....

- Serendipitous 'invention' of glycerol as CPA

Use of AI and frozen semen in poultry

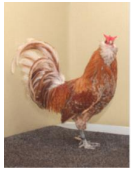


- Frozen semen quickly adopted in cattle
- Much less so in avian species



- AI in birds is largely restricted to ‘fresh’ semen
  - Frozen semen for routine purposes is not practical
- Fertility with frozen semen of ± 90% is possible
  - Largely unnoticed in the poultry industry
  - Possibilities underestimated?
- Frozen avian semen is used for gene banking
- May be used for maintaining breeding lines
  - Cost-effective compared with holding live birds

Fresh semen: The extender

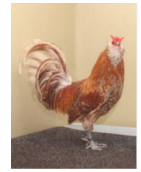


Industry use a low semen dilution.
Strange, as:

- Up to 40 times dilution yields a sufficient insemination dose
- Higher dilution means:
    - Sperm remain vital longer
    - Good fertility with semen stored 24 or 48h
    - But cf. Blesbois and Reviers, 1992

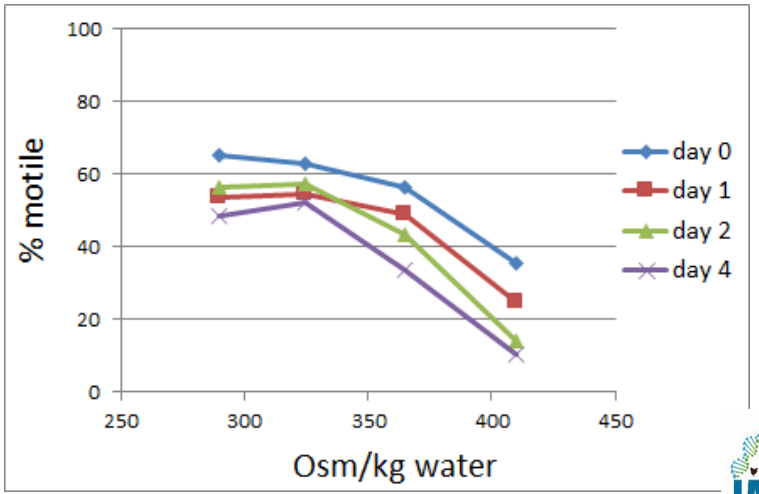
Perhaps ‘fear’ of diluting semen comes from inappropriate osmolality of extender used

Fresh semen: The extender



We have earlier compared all thinkable extender media.

- Best results: Lake and Ravie (1979) diluent "7.1"
- But...
  - Lake "7.1" is 411 mOsm/kg
  - **Too high!** Inhibits motility
  - We modified it: 325 mOsm/kg



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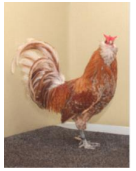
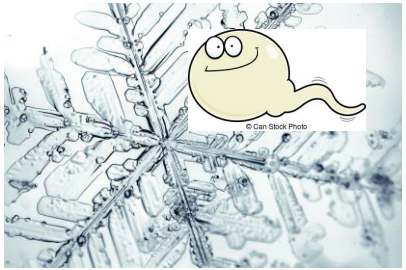
Frozen semen: Again ...the extender



"Happy sperm are freezable sperm"

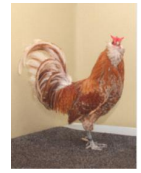
So, first a good extender!



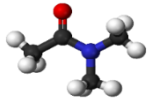
Next... A good CPA

Next ... Find optimal freezing rate *and* CPA concentration

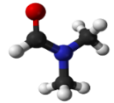
Type of CPA



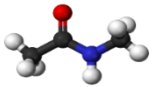
- Six different CPAs at 0.6M
- Semen frozen at 250°C/min
- 100 million sperm/ml
- 3 replicates x 5 cocks/replicate



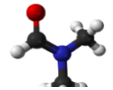
DMA



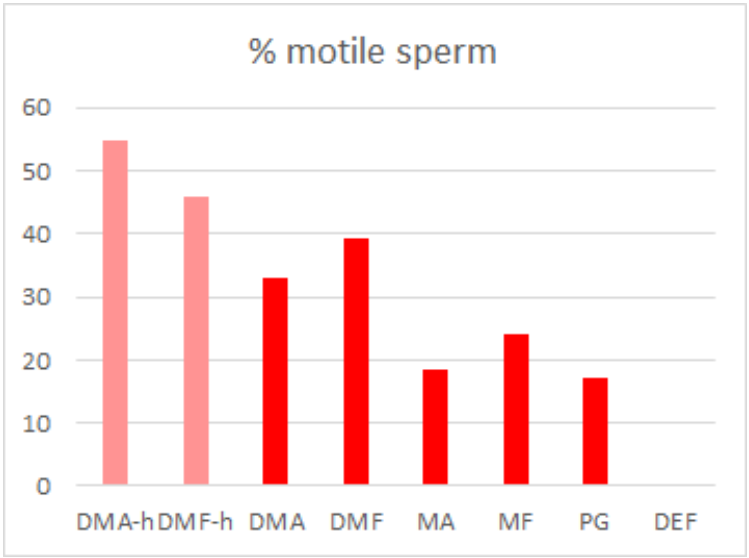
DMF

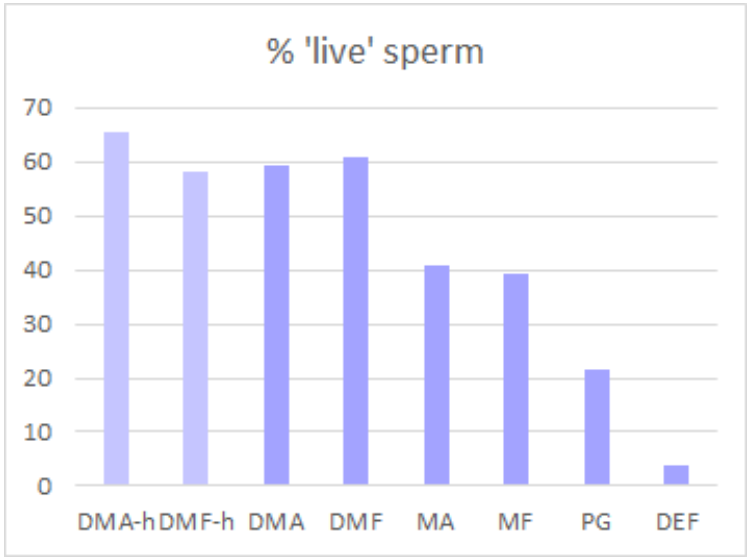


MA



MF





IMAGE
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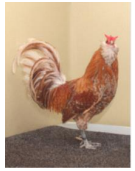
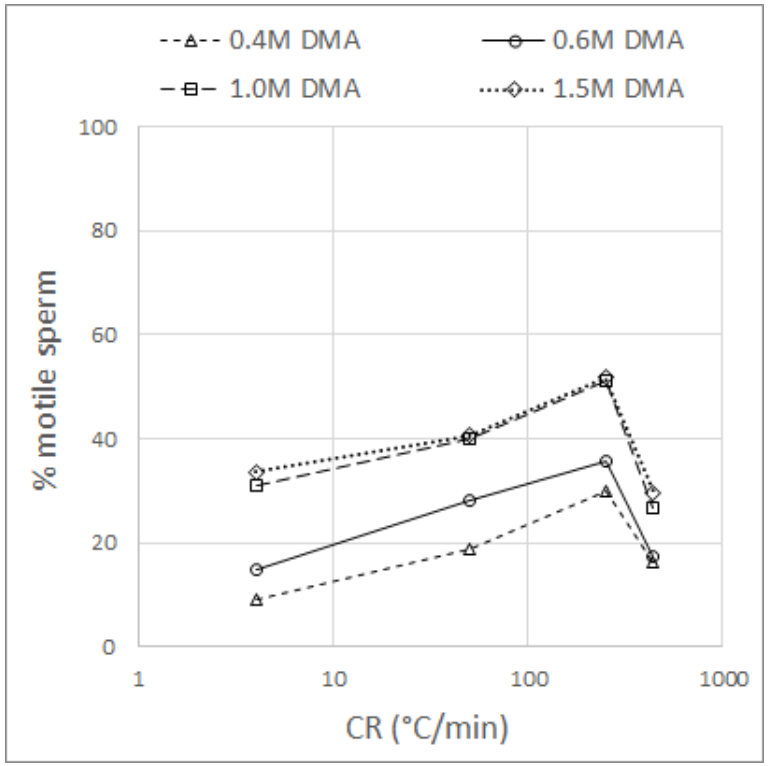
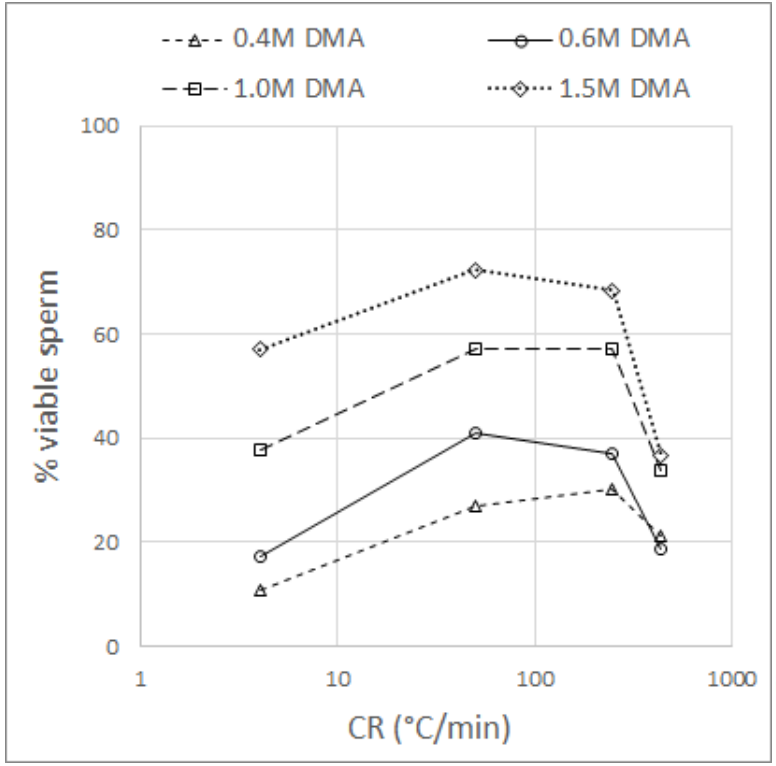
[CPA] x CR

- 4 cooling rates x 4 concentrations DMA
- Pooled semen
- 1200 million sperm/ml
- 4 replicates x 30 cocks/replicate







IMAGE
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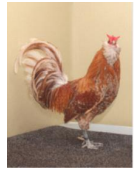
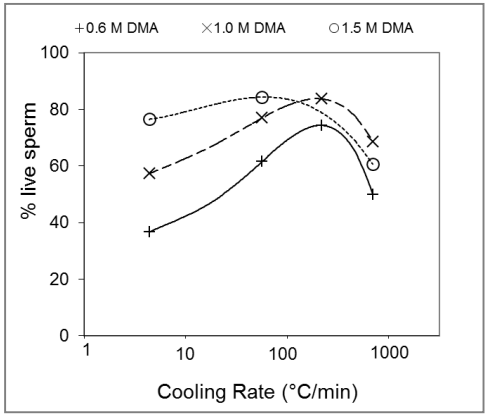
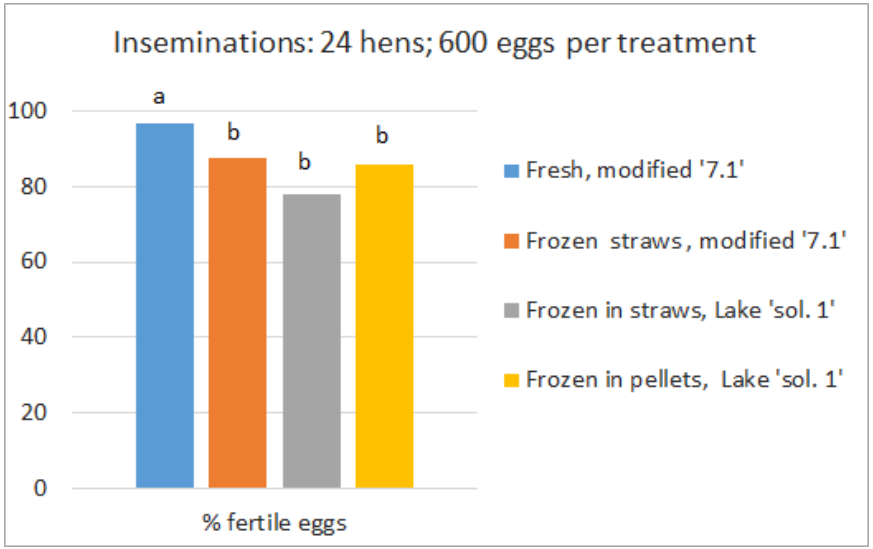
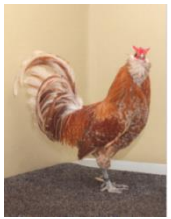
Fertility with frozen semen

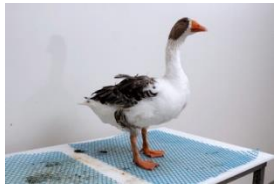




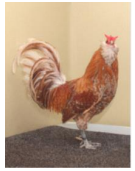


Woelders et al., 2006



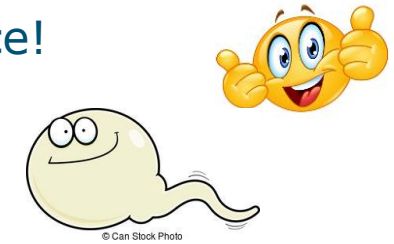


Take home



- Good extender is important!

- Our PE (modified Lake ‘7.1’) performs very good.
  - Important to give the sperm some space!
  - Cold storage; Longevity for days!
  - Good base medium for freezing!

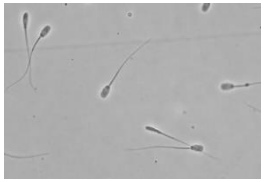


- Good freezing results after optimization of CR and [CPA].

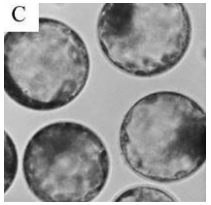
Pigs

Pigs

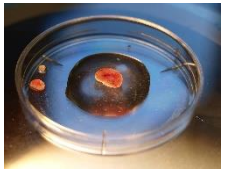
- Semen, possible ...



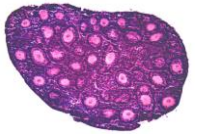
- Oocytes .... Unsuccessful yet
- Embryos. Difficult ...
  - But, live offspring (Matsunari et al., 2012)



- Juvenile gonads

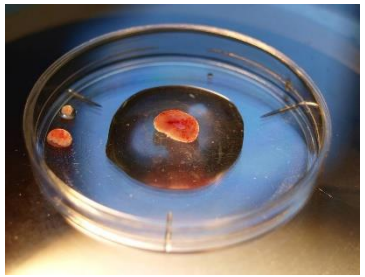


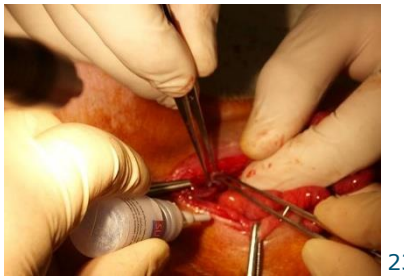
Juvenile gonads (mammals)



- Mice:
  - Mice: Live offspring
    (Parrott, 1960; Gunasena et al., 1997; Sztein et al., 1998; Huang et al., 2008)
- Human
  - More than 60 babies born (tally of 2015)
    (Donnez et al., 2015)
- Pig
  - Pilot study (Woelders, Ratky, Brüssow, et al.)

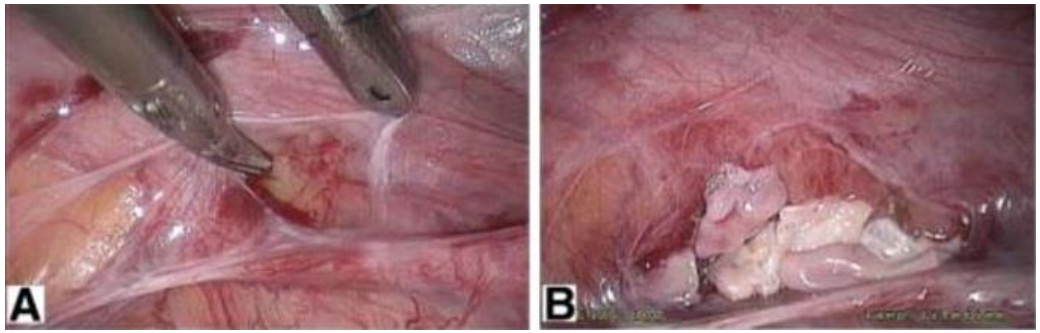
Genetic background	Mutation of donor
C57BL/6	*Clip1*$^{Gfp/Gfp**}$ (KI)[1] *mCry1/mCry2*** (DKO)[2] *BC1*^{3}*/Fmr1*** (DKO)[4] *FMR-Q203*** (KO)[5] *Fmr1ko(2)73*** (KO)[6] *FMR-Inb*** (KO)[7] *mHR6B** (KO)[8] *mOgg1** (KO)[9] *mNth1** (KO)[10] *Myh ex8–15** (KO)[11] *Smad4 E6sad** (KO)[12] *P53 P275S A** (KI)[13] *Apc1638T** (KO)[14] *Clip1*$^{-/+}$*Clip2*$^{-/+*}$ (DKO)[15] *Clip1*$^{-/+*}$ (KO)[16] *Transgenic tOVA line2** (Tg)[17] *act-EGFP** (Tg)[18]
FVB	*mHR6A** (KO)[19] *PSA-Cre-tg** (Tg)[20]
BALB/c	*CD2-GATA3#b X DO11.10** (Tg)[21]



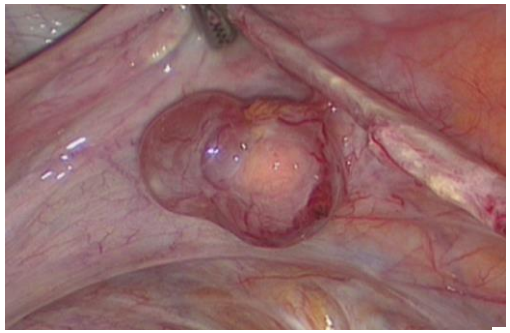


Improving pig ovary grafting

- Orthotopic grafting of juvenile ovary, still best option
- But now: Do not leave behind a part of the host ovary as ‘grafting bed’.
- Instead:
  - Cortex pieces transplanted onto the peritoneum (Interceed - fibrin glue)
  - Stimulate revascularization of the graft, VEGF, S1P, EPO, FSH/LH



Donnez and Dolmans, 2016



Dittrich et al., 2015

IMAGE
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Pigs, embryos

Vitrification

Slow Freezing

- Water will normally form ice when frozen
- Intracellular ice formation is killing

Vitrification

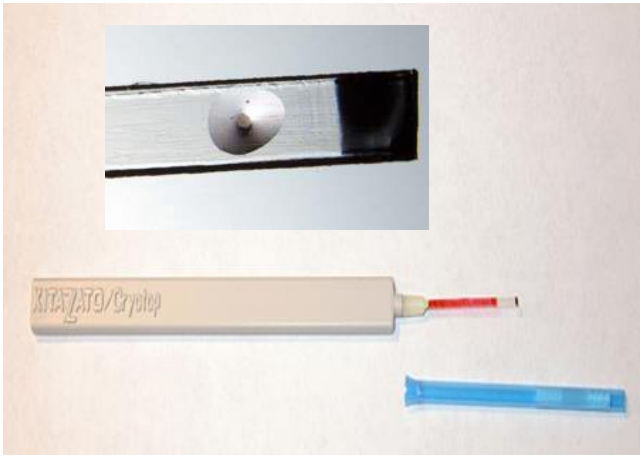
- Literally: formation of ‘glass’
- No ice crystals, yet solid.
- How?
    - Very high solute (CPA) concentration
    - Very high cooling and warming rates

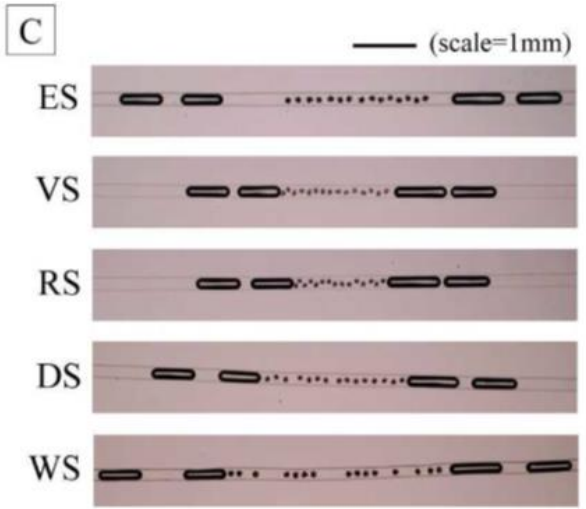
Image: Greg Fahy

Vitrification

Very high cooling and warming rate by:

- Minimal volume (o.p.s., grid, cryoloop, cryotop, QMC etc.
- Hollow fibre

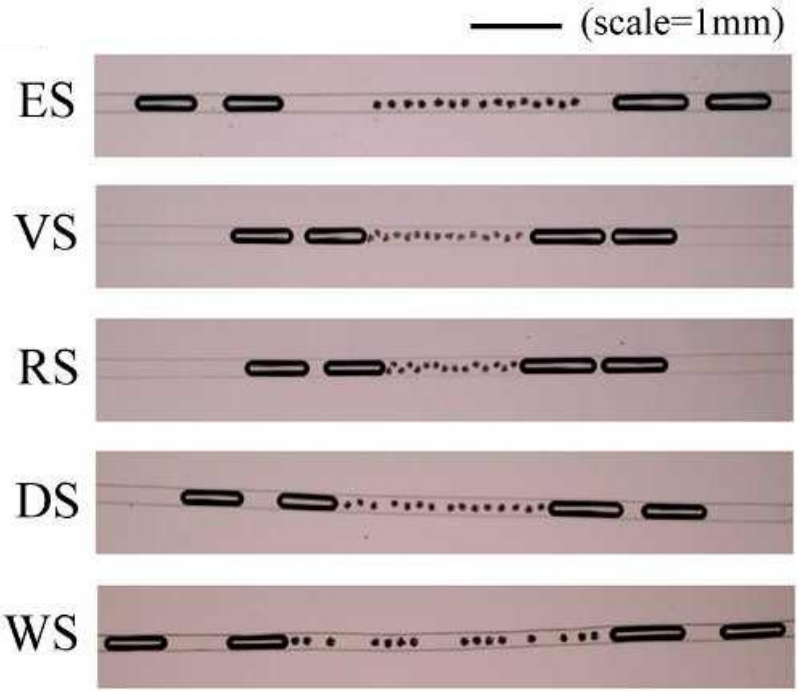


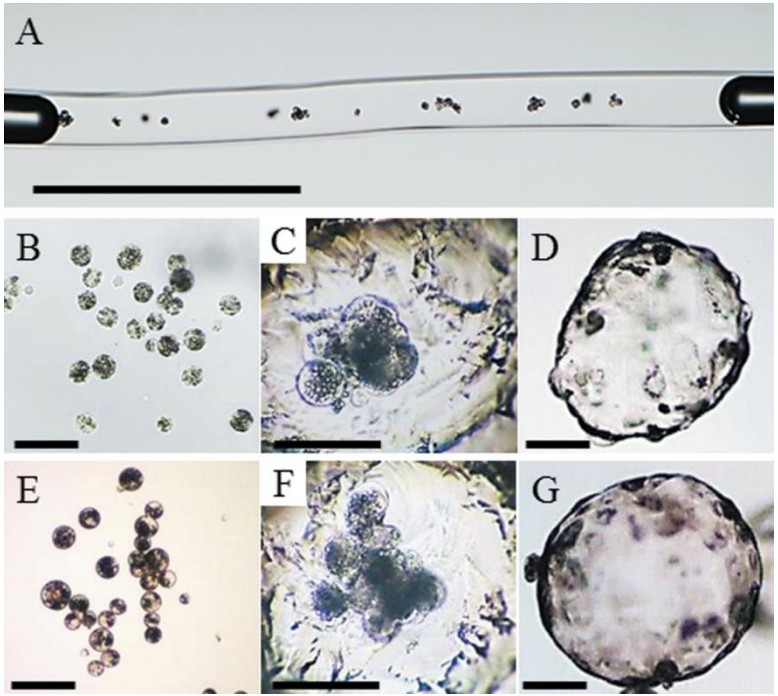


Pig embryo vitrification

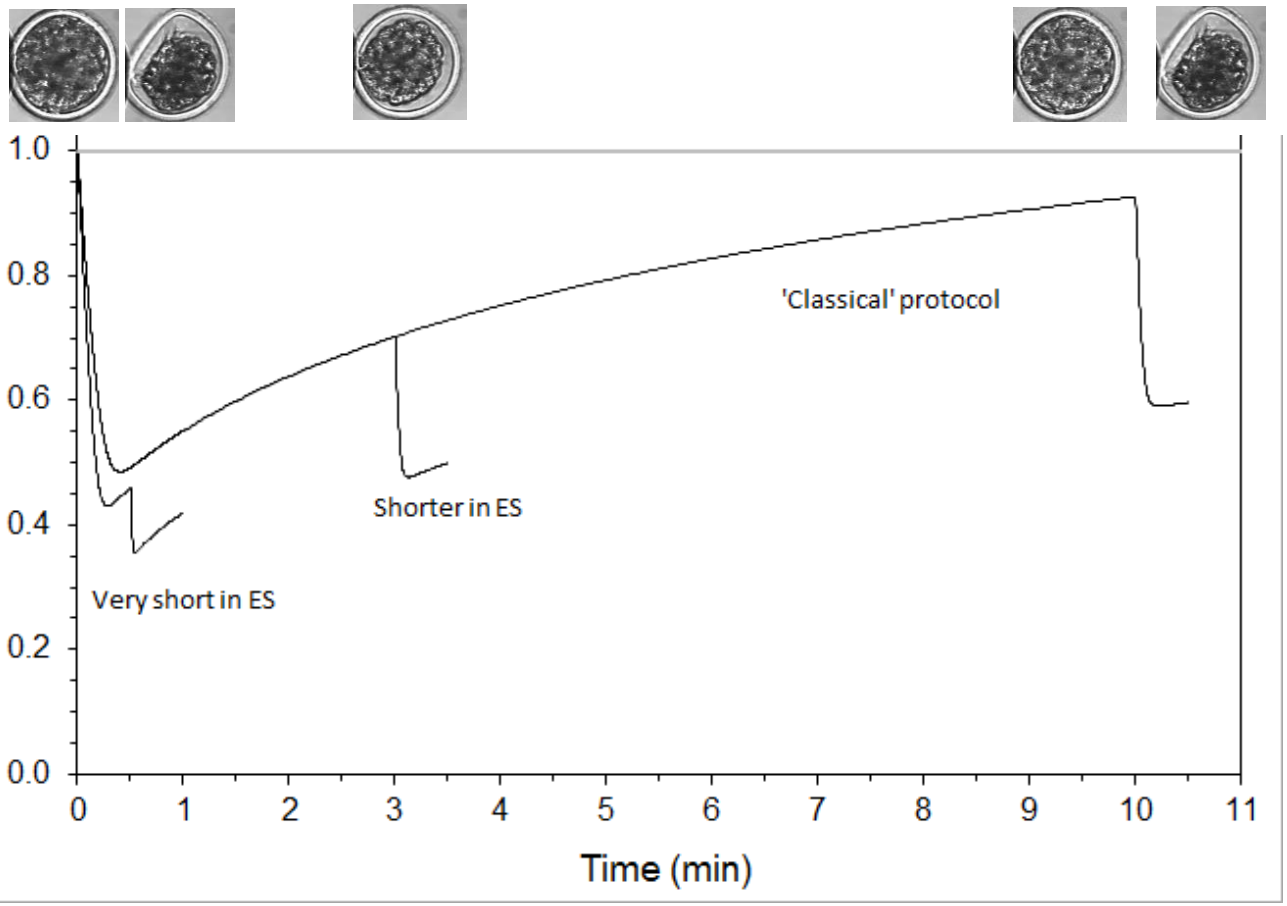
Hollow fibre vitrification Nagashima's group: 2012-2016

Inner diameter **185 µm**





Duration of exposure to CPAs



IMAGE
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Simulations (Woelders et al., 2018)

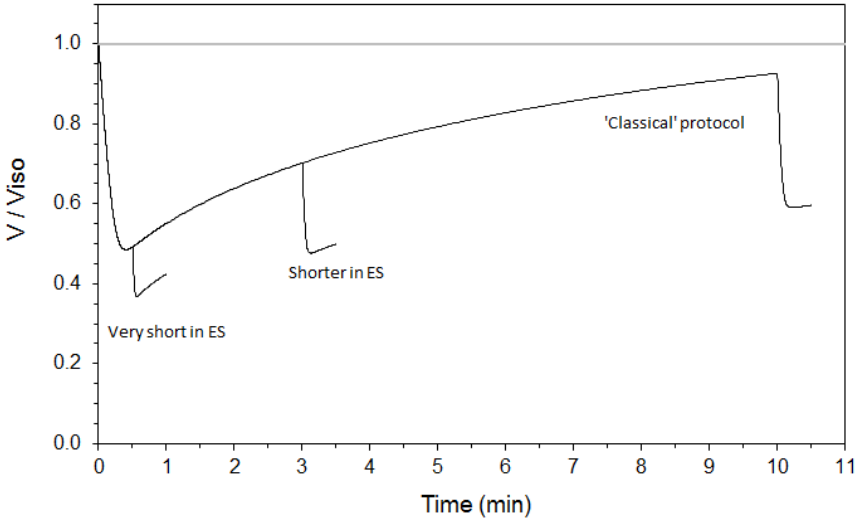
- 2P formalism (Jacobs, 1933; Kleinhans, 1998)

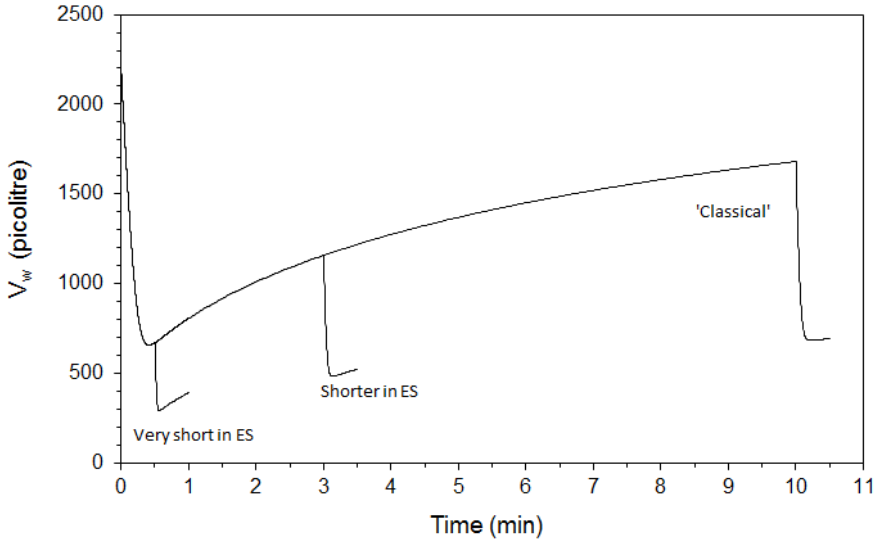
$$\frac{dV_w}{dt} = L_{p(T)}.A.R.T.\Delta M$$

$$\frac{dV_{CPA}}{dt} = P_{s(T)}.A.\Delta M_s$$

- Permeability coefficients based on published values.
- CPAs (EG + DMSO) in ES and VS
- In VS, varying ratios of sucrose / permeant CPAs
- The duration in ES was varied between 0.5-10 min.

Total volume and water volume





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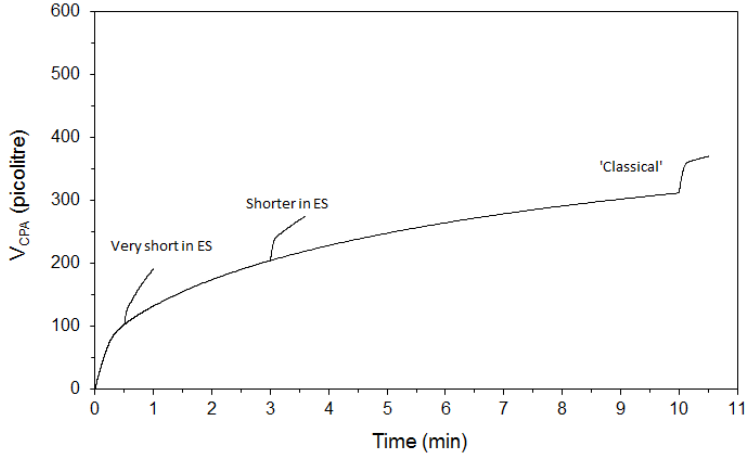
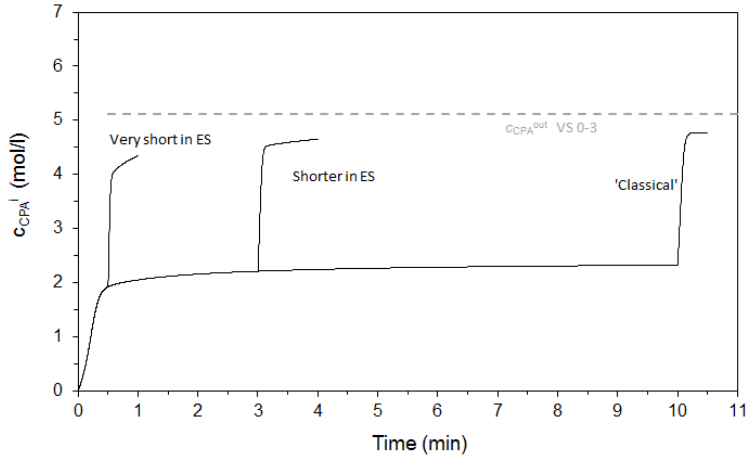
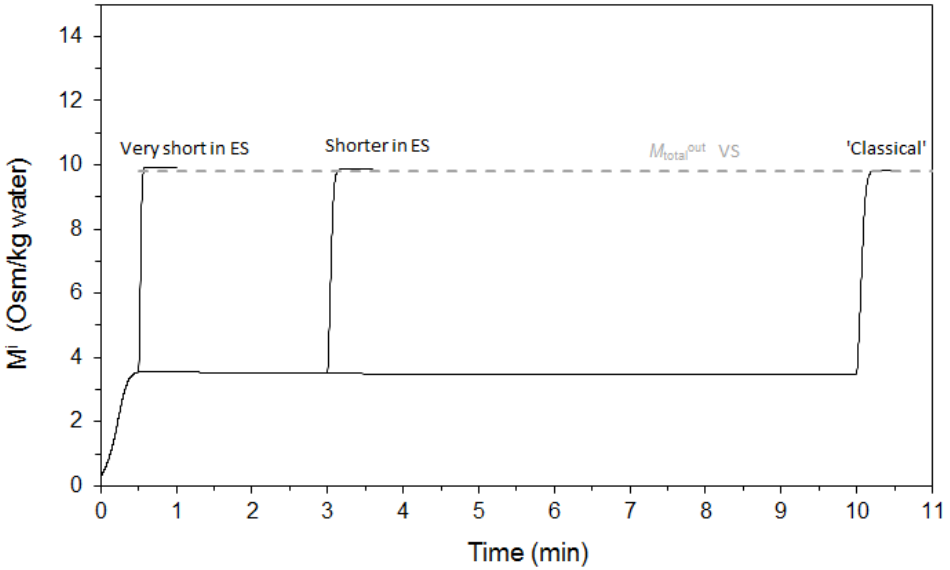
CPA entry


V_{CPA} (picolitre)
Very short in ES
Shorter in ES
'Classical'
Time (min)


c_{CPA}^{i} (mol/l)
c_{CPA}^{out} VS 0-3
Very short in ES
Shorter in ES
'Classical'
Time (min)


Mi (Osm/kg water)
Very short in ES
Shorter in ES
M_{total}^{out} VS
'Classical'
Time (min)

Simulations (Woelders et al., 2018)

- In VS, intracellular $\mu_{(H_2O)}$ gets equal to VS within seconds.
- 'Vitrifiability' should be O.K. within seconds.
- Best to reduce duration of VS stage to 10 seconds.

- Short ES stage → Less CPA enters the cells
- But, similar build-up of [CPA] and of 'vitrifiability' in cells.

- Short ES protocol successfully applied to horse oocytes
  - First reported live foal from immature vitrified oocytes! (Ghent)

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IMAGE Innovative Management of Animal Genetic Resources

Conclusions

- Current repro and cryo methods need further improvement for some species
  - Semen is non-invasive → remains currently key in cryobanking
  - PGCs allow recovery of breed or line in one generation
  - Same may apply to use of gonads
  - Regulations for the latter methods need be addressed
- Methods for porcine embryos are improving and seem to become practical
- Mathematical simulations can be valuable for cryobiology
  - understand mechanisms of cryoinjury
  - Predict outcomes, so we can better choose experiments to do

Thank you for your attention

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