Federal Ministry of Agriculture, Food and Regional Identity

Deutsche Genbank Landwirtschaftliche Nutztiere
Vielfalt bewahren

FRIEDRICH-LOEFFLER-INSTITUT
FLI
Bundesforschungsinstitut für Tiergesundheit
Federal Research Institute for Animal Health

Optimising ram semen cryopreservation first practical experience

Heiko Henning & Patrick Aldag

ERFP - Ad hoc workshop
Small Ruminant Semen Cryopreservation
Uppsala, 11.06.2025

History

Locations of Friedrich-Loeffler-Institut

2009 Decree of Ministry for Agriculture

A gene bank to be installed at Friedrich-Loeffler-Institut, Institute of Farm Animal Genetics

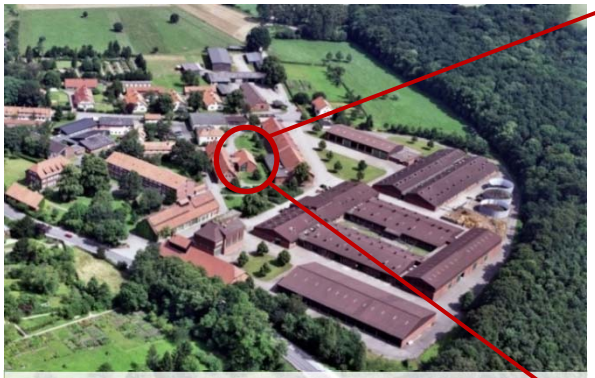
2016 officially founded & opened
contract between Federal Government & Federal States

Federal Government provides infrastruture & personnel

- *Core collection located in Mariensee*
  - *EU-registered AI and ET station (pig, cattle, sheep)*
  - *Quarantine stables*
  - *Cryo lab*
  - *Sperm lab*
  - *Embryo lab*
  - *Storage of cells in liquid nitrogen & at -80°C*
- Managing director at FLI (Prof. Steffen Weigend)

Federal States provide donor animals and/or material (semen, oocytes, embryos, etc.)

Location



Institut für Nutztiergenetik (ING)
Institute of Farm Animal Genetics

Deutsche Genbank
Landwirtschaftliche
Nutztiere
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Lagerraum mit Kryobehältern
storage with cryo containers

Außenansicht
external view

Eingangsbereich
entrance

FRIEDRICH-LOEFFLER-INSTITUT
FLI
Bundesforschungsinstitut für Tiergesundheit
Federal Research Institute for Animal Health

Organization and people

Daily operations

Managing Director

Prof. Steffen Weigend
(since 2021)

Legal & hygienic aspects

Prof. Claudia Klein

Breeding management

Christian Reimer (pig, sheep, goat)
Johannes Geibel (cattle, horse)
Steffen Weigend (poultry, small animals)

Cryopreservation/ Spermatology

Dr. Heiko Henning
Patrick Aldag
Vivian Hensel
Birgit Sieg

OPU, ET & IVF

Dr. Rebecca Herbicht
Dr. Stefanie Kurtz
Dr. Gregor Neufeld
Petra Hassel

Data management

Dr. Johannes Geibel
Dr. Christian Reimer
Robin Garcia

Associated research areas

Population genetics

Prof. Steffen Weigend
Dr. Johannes Geibel
Dr. Christian Reimer

Reproductive biology

Prof. Claudia Klein, Dr. Rebecca Herbicht (female repro)
Dr. Heiko Henning (male repro)
Dr. Stefanie Altgilbers (primordial germ cells = PGC)
Dr. Stefanie Kurtz (genome editing)

Bioinformatics

Dr. Gregor Neufeld
Robin Garcia

Selection of species and breeds

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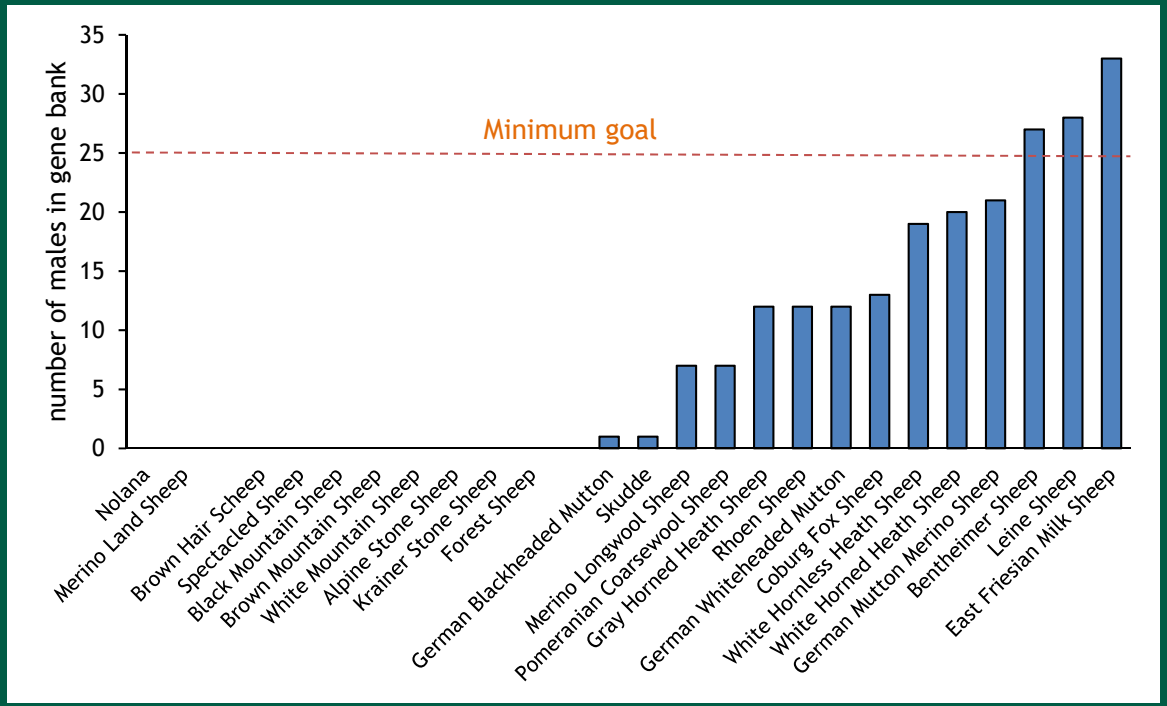
What is stored?

Data from 09/2024



Goal: 25 sires/breed, 100 AI doses/sire

Basic strategy for increasing the collection

Till 2023

- 1x year
- 10-15 rams after breeding season
- Quarantine, slaughther, isolation of epididymal sperm & freezing

Pro:
- stringent planning
- Defined, short, busy period for personnel

Contra:
- sometimes old rams (health/fertility?)
- Sometimes limited sperm source
- Hygienic status (?)



Since 2024

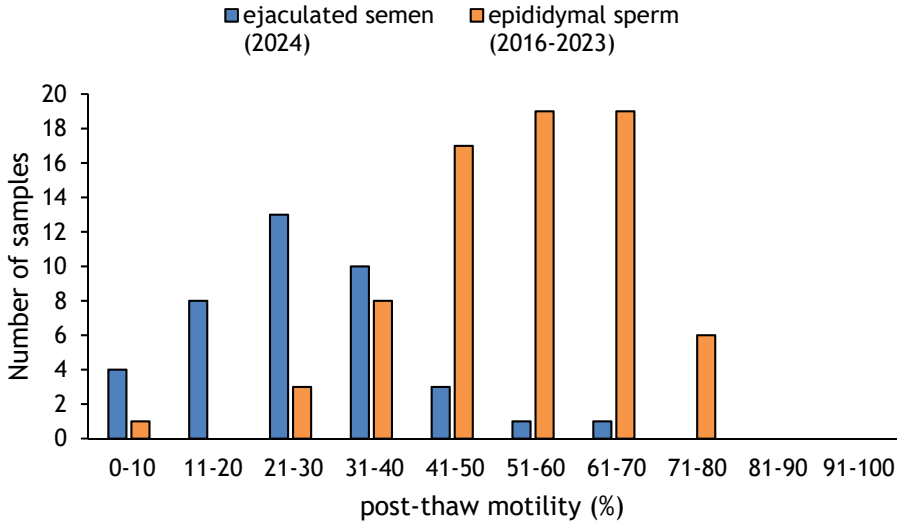
- Continously
- Up to 20 rams on site
- Quarantine, regular semen collection & freezing

Pro:
- More samples per ram can be frozen
- Rotation of rams between FLI & farms
- Hygienic status is well-defined

Contra:
- More investment in ressources, time & personnel
- Training for semen collection may fail

Revisiting our cryopreservation protocol

Our results from the past ….



Epididymal sperm:
200 x10^6 sperm/mL = 50 x10^6/0.25 mL straw

Ejaculated semen:
800 x10^6 sperm/mL = 200 x10^6/0.25 mL straw

Results from recent literature on ejaculated sperm ….

59-82% motility

Zhang et al. (2024)
https://doi.org/10.1038/s41598-024-61947-x

42-58% motility

Galarza et al. (2021)
https://doi.org/10.1016/j.cryobiol.2019.10.007

52-73% motility

Paul et al. (2020)
https://doi.org/10.1016/j.cryobiol.2020.07.013

But: preselected ejaculates
(volume, concentration, motility)

In practice, we freeze what we get.
Genetic value overrules semen quality.

How do we freeze?

- Arrival of sample
  (≈30°C)

- Add pre-warmed cooling extender
  (≈ 0.5-1.0 mL)
- Adjust sperm concentration
  double of final concentration

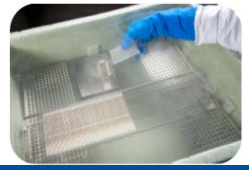
- Chilling in incubator

RT to 14°C (1 h)
14°C to 8°C (1h)
8°C to 5°C (1h)

Continue in work bench at 4°C

- Mix 1:1 with freezing extender
- Automated filling of straws (0.25 mL)

- Freeze in IceCube* or Styrofoam box**



Cooling extender/Freezing extender

TRIS
Citric acid
D-Fructose

Egg yolk 20 % (w/w)
Glycerol *(currently 12 % (w/w))*

Oxaloacetic acid
Catalase

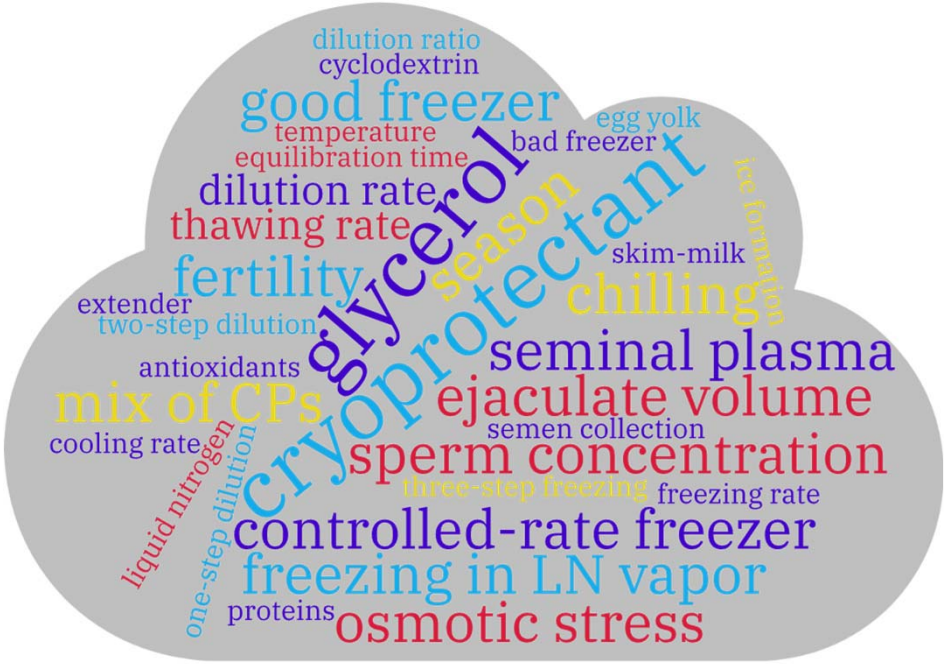
Penicillin
Streptomycin
Lincospectin

pH 6.75/ pH 6.85
320 mOsmol/kg / n.d.

*current freezing program
+5°C for 5 min
+5°C to -12°C @ 3°C/min
-12°C for 2 min
-12°C to -120°C @ 10°C/min
-120°C to -130°C @ 2°C/min

** 4 cm above liquid nitrogen, 30 min

Which factors have an impact?



Reviews (list not exhaustive)

Khalil at el. (2023) *Recent approaches in the use of antioxidants and proteomic modifications in ram semen*
doi: 10.1111/rda.14485

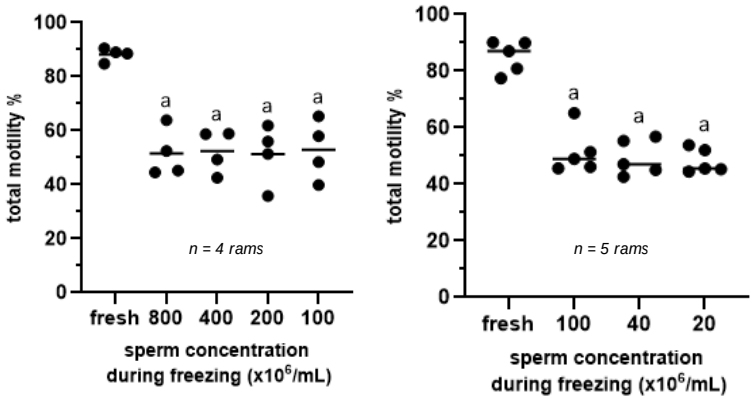
Saha et al. (2020) *Cryopreservation Techniques for Ram Sperm*
doi: 10.1155/2022/7378379

Larbi et al. (2018) *Supplementation of ram semen extender to improve seminal quality and fertility rate*
doi: 10.1016/j.anireprosci.2018.03.019

Salamon & Maxwell (2000) *Storage of ram semen*
doi: 10.1016/s0378-4320(00)00155-x

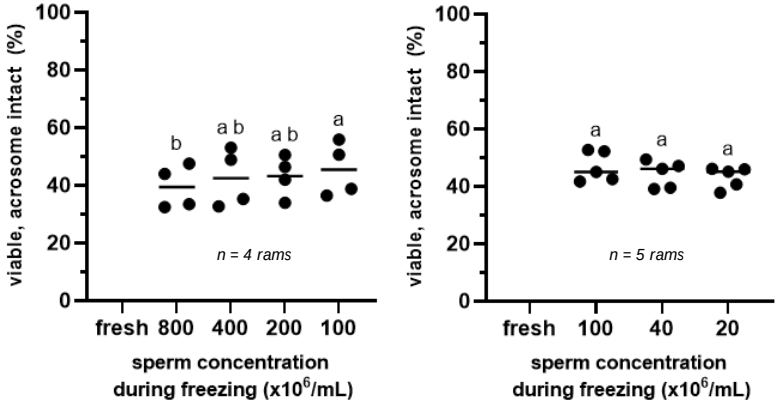
Sperm concentration

No effect on motility



Glycerol: 5.75%
Egg yolk: 20%
IceCube
P<0.05

minor effect on membrane integrity



Alvarez at al. (2012)
doi: 10.1016/j.theriogenology.2011.10.013

Post-thawing motility (CASA) for the four sperm concentrations.

Motility variables	Concentration (× 10^6 mL^{-1})			
	200	400	800	1600
TM (%)	65.2 ± 4.5[a]	63.4 ± 3.5[a]	58.3 ± 4.2[a]	41.3 ± 4.6[b]
PM (%)	39.7 ± 3.5[a]	35.9 ± 2.4[ab]	33.0 ± 2.9[b]	22.2 ± 2.4[c]
VCL (μm/sec)	120.3 ± 4.0[ab]	123.7 ± 5.1[ab]	126.8 ± 4.6[a]	107.6 ± 3.3[b]
LIN (%)	63.5 ± 1.6	62.3 ± 1.7	63.0 ± 1.5	59.2 ± 1.2
ALH (μm)	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.1 ± 0.1

Fertility of ewes after intrauterine insemination with semen doses frozen at different sperm concentrations (lambing rates).

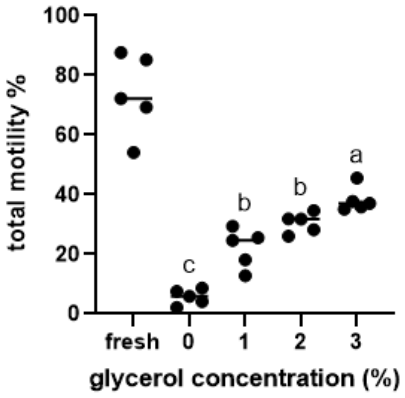
Concentration (× 10^6 mL^{-1})	Lambing rate (%)	200 vs. higher concentration	
		Odds ratio (95% CI)	P value*
200	154/268[a] (57.5)	Referent†	—
400	135/248[a] (54.4)	0.88 (0.62–1.25)	0.489
800	112/246[b] (45.5)	0.62 (0.44–0.88)	0.007

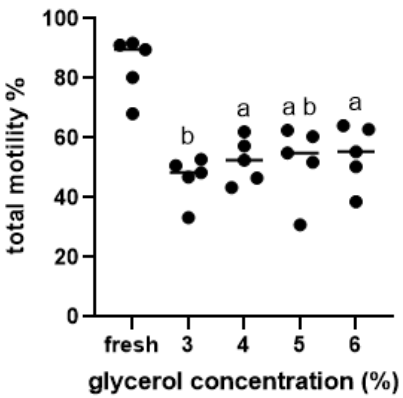
Insemination dose was fixed at 25 × 10^6 spermatozoa per uterine horn in all cases. Odds ratios are given taking 200 × 10^6 mL^{-1} as the reference group. Fertility values with different superscripts differ $P < 0.05$ (χ^2 test).

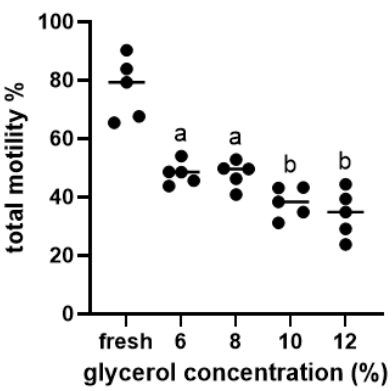
D'Allessandro et al. (2001)
doi: 10.1016/s0093-691x(01)00474-5

- Test range: 50, 100, 200, 400, 500, 800 x10^6 sperm/mL
- Lower motility and viability post thaw (800 x10^6 sperm/mL)
- No statistically lower lambing rate

Glycerol concentration



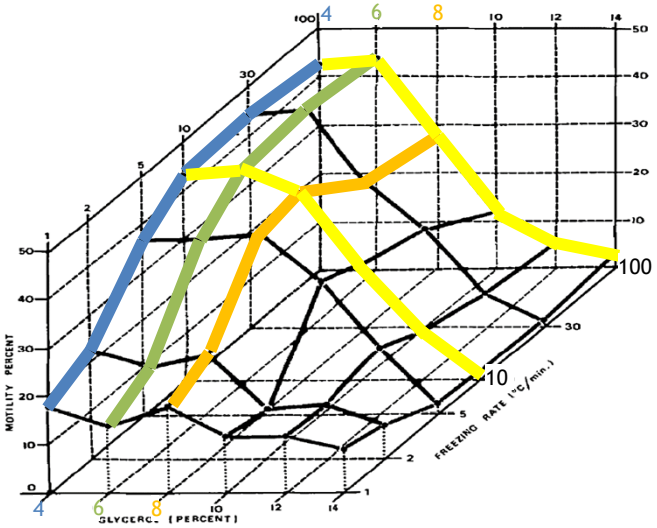




200 x10^6 sperm/mL
20 % egg yolk
IceCube
n = 5 rams
P<0.05

Fiser & Fairful (1984)
doi: 10.1016/0011-2240(84)90053-1

- 4 - 8 % glycerol in a two-step protocol performed best
- One-step freezing rates between 10 & 100°/min are tolerated (controlled-rate freezer)
- Glycerol level has more impact than freezing rate



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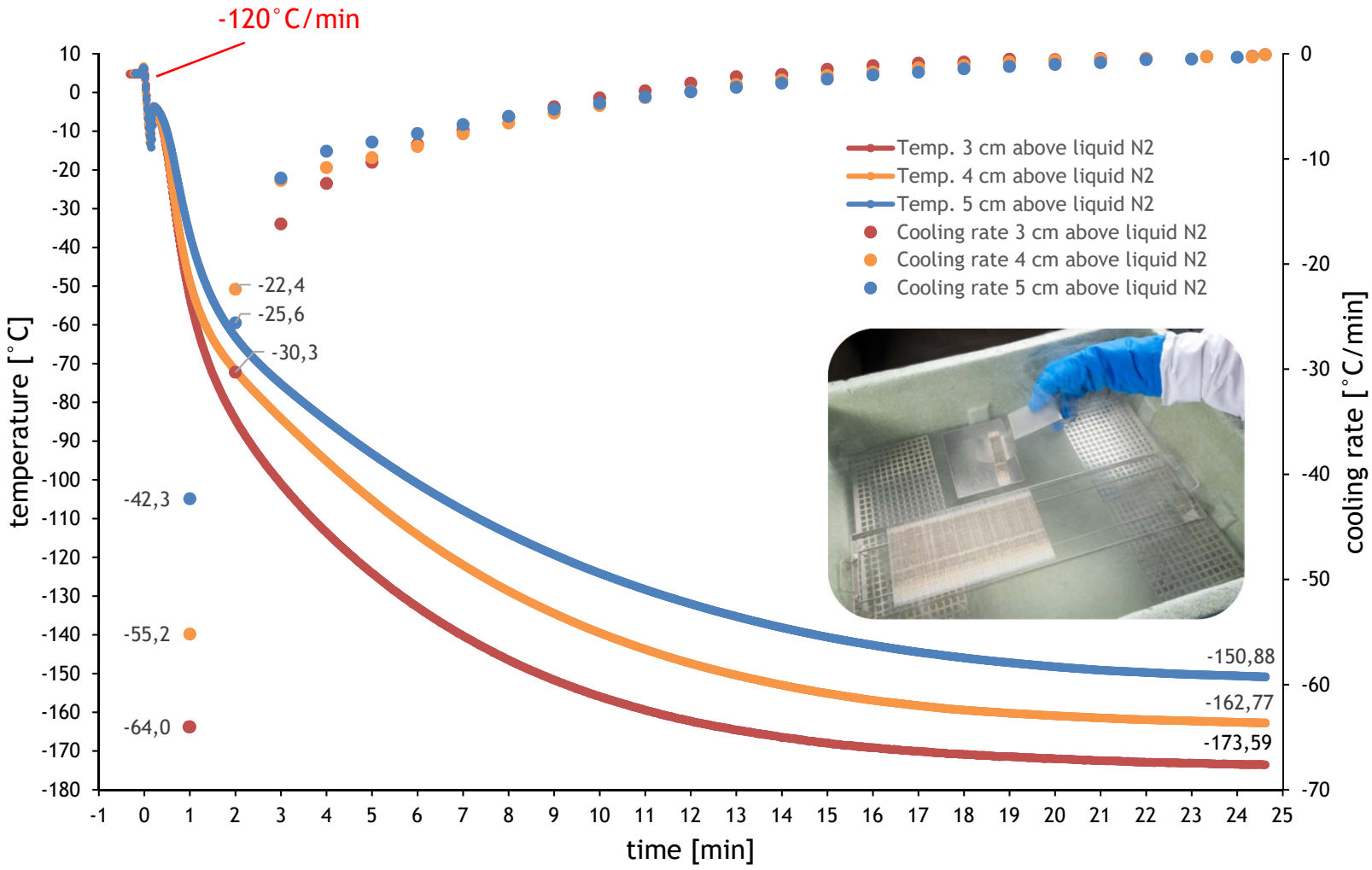
Cooling rates in styrofoam boxes



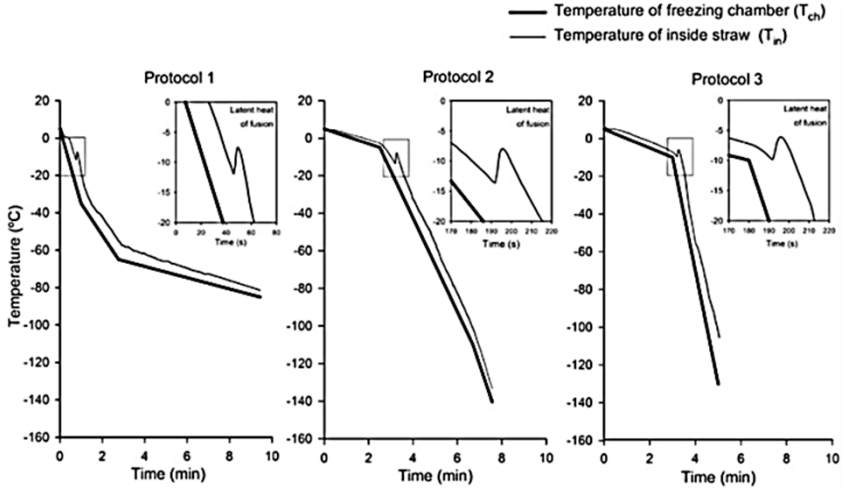
Are high freezing rates beneficial?

Zhang et al. (2024)
https://doi.org/10.1038/s41598-024-61947-x

Fumigation height (cm)	TM (%)	PM (%)	VSL (μm/s)	VCL (μm/s)	VAP (μm/s)	ALH (μm)	MAD (°/s)
2	78.33 ± 1.12[a]	63.95 ± 1.03[a]	42.06 ± 1.54	64.31 ± 2.57	45.48 ± 1.81	18.84 ± 0.75	55.18 ± 2.00[a]
4	70.78 ± 1.04[b]	54.30 ± 1.06[b]	39.99 ± 0.68	59.16 ± 0.74	41.83 ± 0.52	17.33 ± 0.22	44.16 ± 2.29[b]
6	70.42 ± 2.51[b]	53.66 ± 1.86[b]	39.17 ± 0.58	59.36 ± 1.16	41.97 ± 0.82	17.38 ± 0.34	46.42 ± 2.33[ab]
8	68.37 ± 3.13[b]	52.70 ± 1.67[b]	39.51 ± 1.15	59.52 ± 1.02	42.09 ± 0.72	17.43 ± 0.30	45.01 ± 4.07[b]

3 % glycerol
20 % egg yolk
?? sperm/mL

Galarza et al. (2021)
https://doi.org/10.1016/j.cryobiol.2019.10.007



Sperm parameters	Fresh samples (n = 98)	Protocol 1 (n = 78)	Protocol 2 (n = 79)	Protocol 3 (n = 79)
SM (%)	87.8 ± 0.8[a]	44.5 ± 1.9[c]	47.9 ± 2.0[c]	61.4 ± 1.9[b]
PSM (%)	34.8 ± 1.0[a]	18.0 ± 1.0[c]	20.8 ± 1.1[c]	27.2 ± 1.0[b]
VSL (μm/s)	85.1 ± 1.5[a]	55.1 ± 2.0[c]	59.2 ± 1.7[bc]	62.8 ± 2.1[b]
LIN (%)	51.1 ± 0.7[b]	63.3 ± 0.8[a]	63.5 ± 0.8[a]	63.5 ± 0.7[a]
STR (%)	66.5 ± 0.7[b]	75.1 ± 0.7[a]	76.1 ± 0.6[a]	75.1 ± 0.5[a]
ALH (μm)	4.7 ± 0.1[a]	2.5 ± 0.1[b]	2.7 ± 0.1[b]	2.6 ± 0.1[b]

Protocol 3
+5°C to -12°C @ 5°C/min
-10°C to -130°C @ 60°C/min

4.8 % glycerol
5.8 % egg yolk
100 x 10^6 sperm/mL

Practical considerations for the daily work

Overnight equilibration not harmful

Paul et al. (2020)
https://doi.org/10.1016/j.cryobiol.2020.07.013

- One-step dilution
- Equilibration at 5˚C

approx. 4.8 – 6.0 % glycerol
approx. 12 – 15 % egg yolk
800 x 10^6 sperm/mL

Freezing curve
+5°C to -125°C @ 25°C/min

Seminal attributes	Length of equilibration period (h)		
	3	10	22
Total motility (%)	52.44 ± 3.56^{a}	54.94 ± 3.90^{a}	72.89 ± 3.82^{b}
Rapid motility (%)	50.83 ± 3.55^{a}	53.25 ± 3.90^{a}	70.73 ± 3.78^{b}
VCL (µm/s)	249.74 ± 9.83^{a}	234.60 ± 5.95^{b}	257.41 ± 7.15^{a}
VAP (µm/s)	142.34 ± 4.04^{a}	131.26 ± 2.82^{b}	142.61 ± 3.40^{a}
ALH (µm)	9.39 ± 0.52^{a}	8.62 ± 0.27^{b}	9.90 ± 0.36^{a}
ELON (%)	47.73 ± 0.35^{a}	46.67 ± 0.20^{b}	47.46 ± 0.4^{ab}

VCL: curvilinear velocity, VAP: average path velocity, ALH: amplitude of lateral head displacement, ELON: elongation.
Means bearing different superscripts within a row differ significantly, $P < 0.05$.

Camara et al. (2011)
doi:10.1016/j.theriogenology.2011.02.013

- One-step dilution
- Equilibration at 5˚C

approx. 5.8 – 6.0 % glycerol
approx. 9.7 – 10 % egg yolk
100 x 10^6 sperm/mL

Freezing curve
+5°C to -120°C @ 12.5°C/min

Frozen-thawed samples	
Equilibration time 5 °C	
0 h	12 h
14.8 ± 5.6^{Bb}	38.1 ± 14.8^{Ba}

Purdy et al. (2010)
doi:10.1016/j.anireprosci.2009.06.014

- One-step dilution
- Equilibration at 5˚C

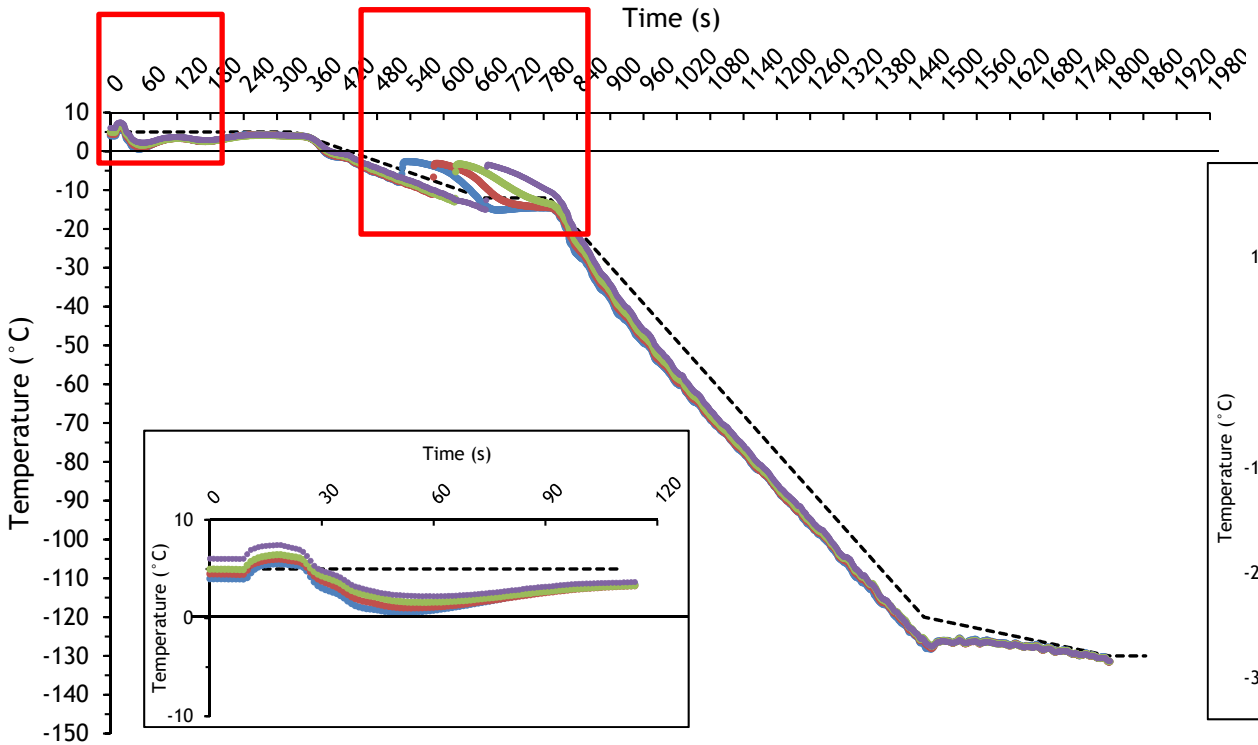
approx. 4.0 – 5.0 % glycerol
approx. 12 – 15 % egg yolk
200 x 10^6 sperm/mL

Freezing curve
+5°C to -5°C
-5°C to -110°C @ 25°C/min
-110°C to -140 °C @ 35°C/min

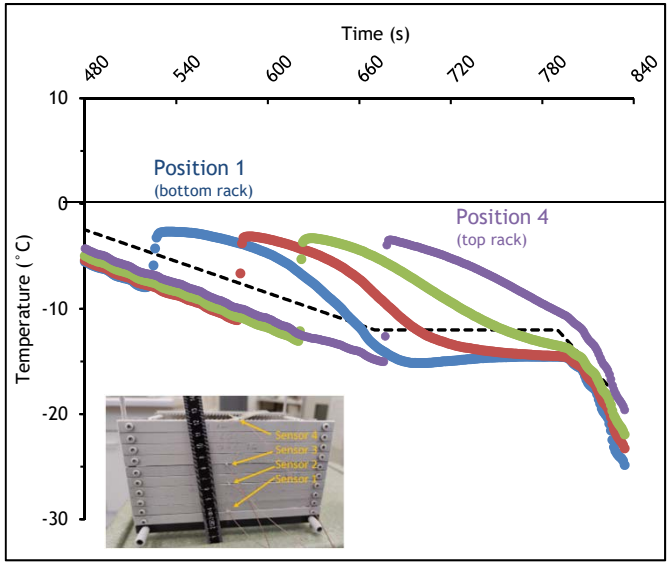
Treatment	MOT
T0	44
T24	46
SEM	2

Cooling rates in controlled-rate freezer

Only measuring is believing….

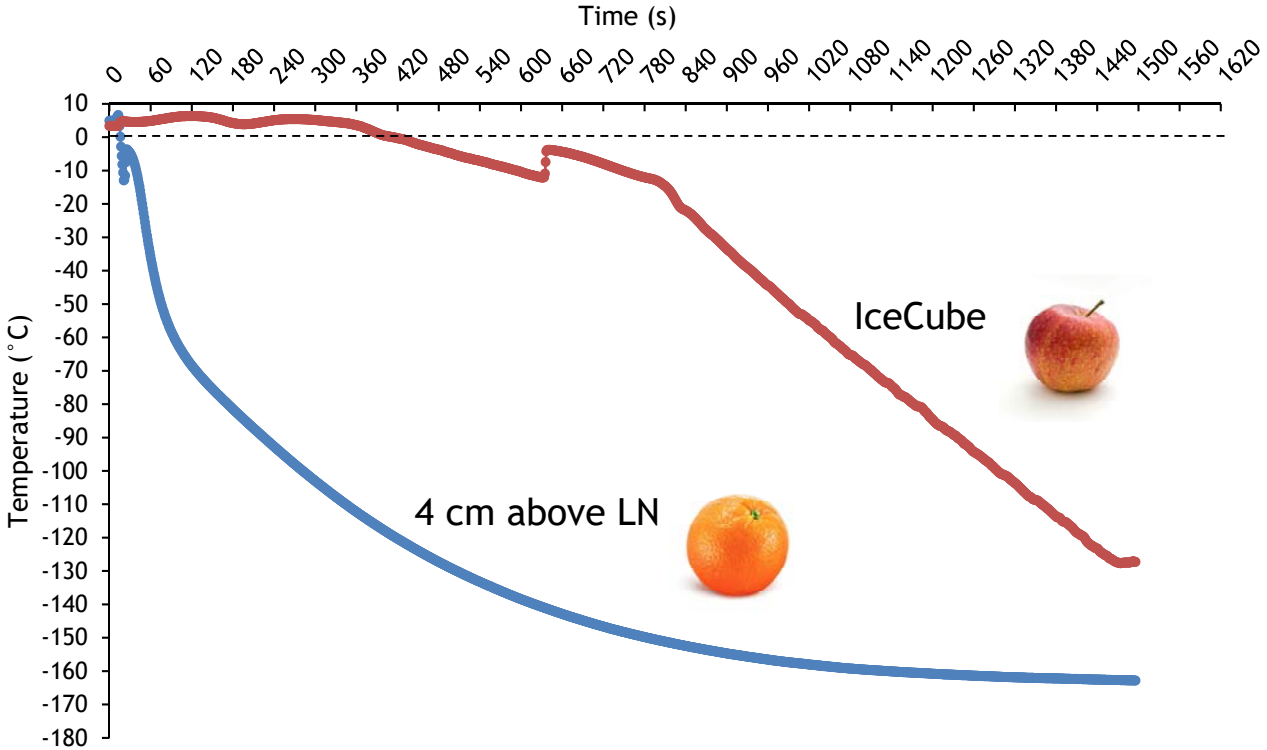


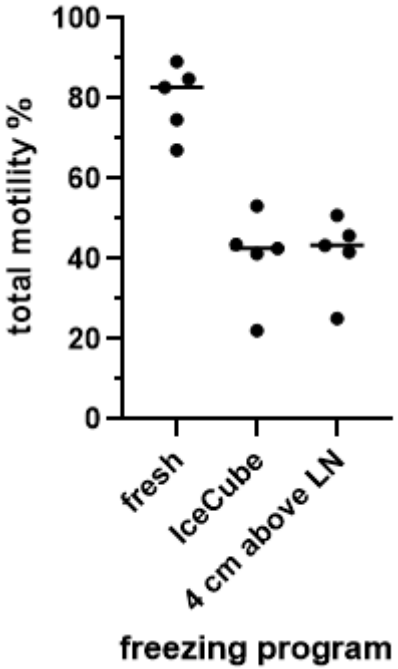
Temperature fluctuation after placing racks into IceCube



Time delay in ice formation depends on position of rack

Controlled-rate freezer vs. 4 cm above LN





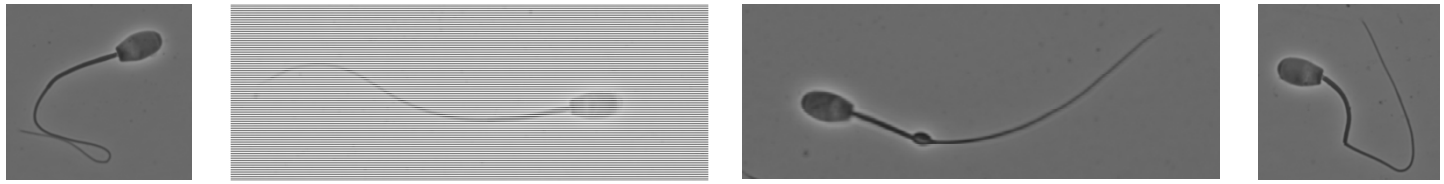
Either ram sperm tolerate very different conditions

or (an) other factor(s) has/have a major impact

200 x 10^6/mL
20% egg yolk
6% glycerol
n = 5 rams, P<0.05

Conclusions

- Ram spermatozoa can survive a wide a range of conditions for cryopreservation (good basis for preserving genetic material)
- 4-8% glycerol is optimal
- Freezing rates ≥ -60°C/min after start of ice formation appear promising
- Overnight equilibration after one-step dilution feasible alternative to freezing at day of semen collection
- A controlled-rate freezer may not necessarily provide stable and controlled temperature profiles for the samples
- Still ample space for improving post-thaw motility and harmonizing the reporting of experimental protocols



Acknowledgement

Federal Ministry of Agriculture, Food and Regional Identity

Bundesanstalt für Landwirtschaft und Ernährung

INFORMATIONS- UND KOORDINATIONSZENTRUM BIOLOGISCHE VIELFALT

Questions?

Suggestions?

Collaborations?

Contact us at genbank@fli.de

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