European eel sperm cryopreservation

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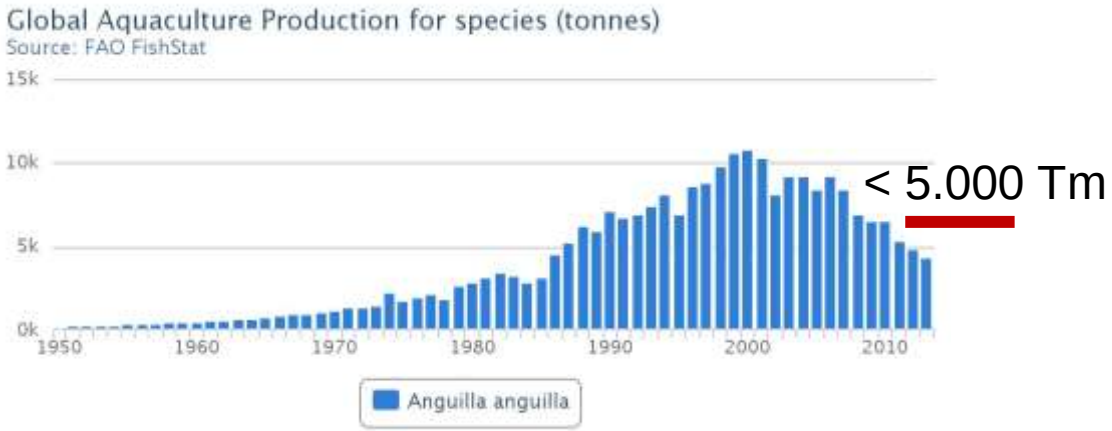
2nd IMPRESS Workshop & 5th AQUAGAMETE Training School

Traditional fisheries of glass eel and eels

Glass eel fishing
(Basque Country)

Eel fishing (East Spain)

Aquaculture industry Europe and Asia



< 5.000 Tm

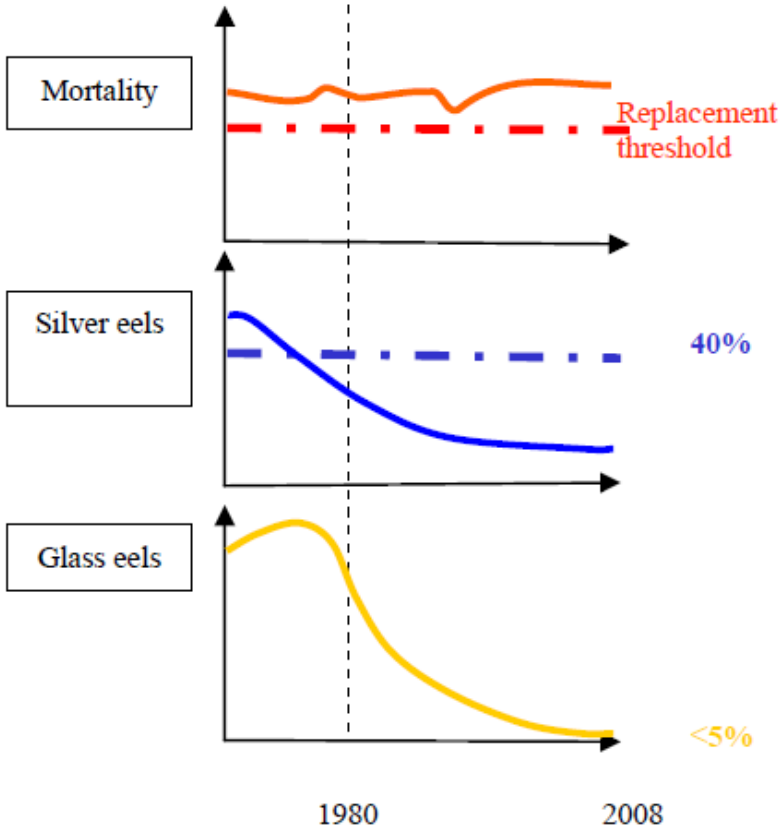
- Recirculation systems
- Decline in aquaculture from 2000'

250.0000 Tm

- Greenhouse systems
- Increase in aquaculture since 1990' (China)
- 50-times > European production

Depends on glass eel fisheries

Conservation status European eel: critically endangered (IUCN)



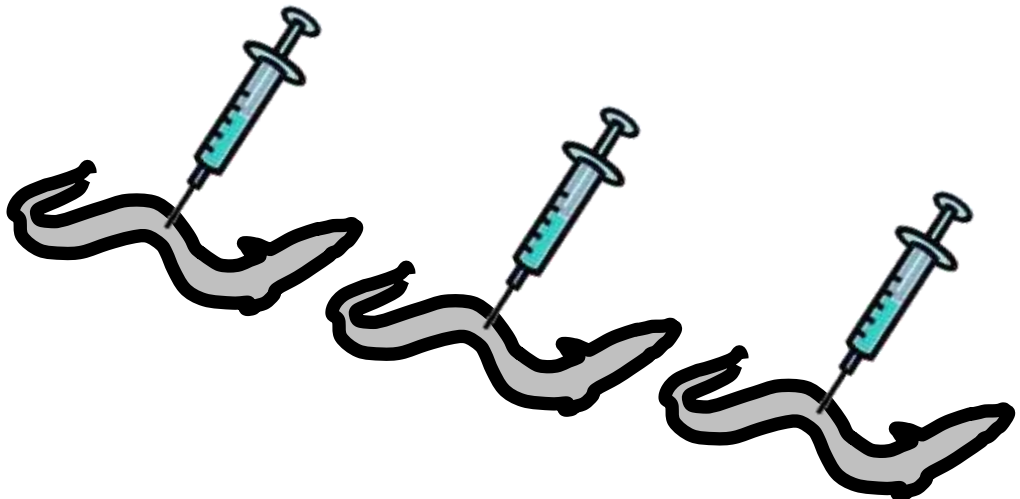
Graphs showing the drop in biomass of glass and silver eels from 1965 to 2008 (source: Brand, 2007).

- ICES (2011): recruitment of glass eels reduced -95 % (-99 %) of the levels before the 1980'
- CITES (2009): included in appendix II. Trade out EU forbidden.
- EU (2007): Regulation establishing measures for the recovery of the stock of European eel (EC 1 100/2007: European Council, 2007)
  - **allow 40 % of adult eels to escape from inland waters to the sea**
  - reserve 60 % of glass eel catches for restocking within the EU
  - Habitat restoration (barriers, pollution), fishery restrictions, restocking

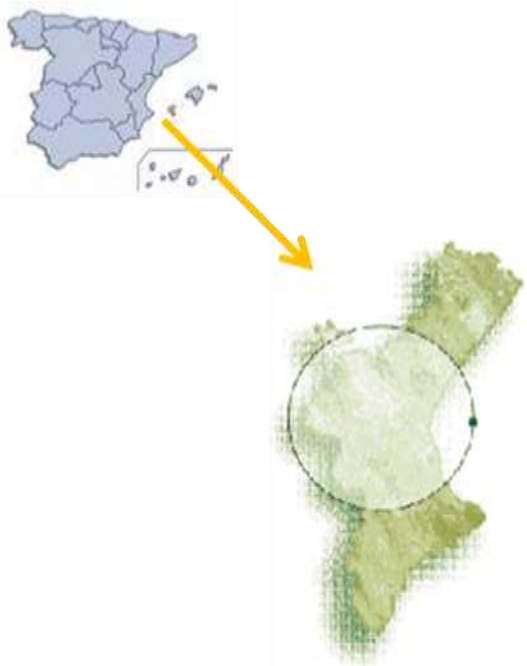
Peculiar and complex lifecycle through the Atlantic ocean…

Reproduction in captivity is important to decrease the pressure on the wild populations

- Sex maturation is blocked in captivity
- Chronic hormonal treatments to obtain sperm and eggs



Where, how and when get the fish?

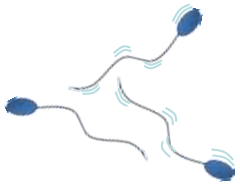


Valenciana de Acuicultura, S.A. (Puzol)

Males, 100-150 g. All over the year

Albufera de Valencia
(Fishermen El Palmar Association)

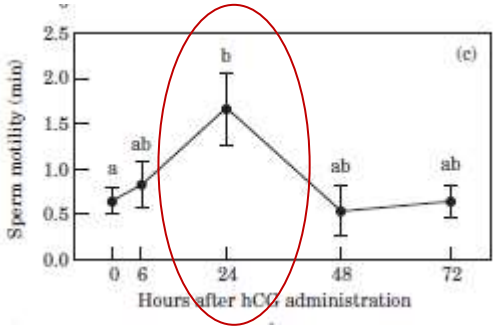
Females, 600 - 1500 g. October-March

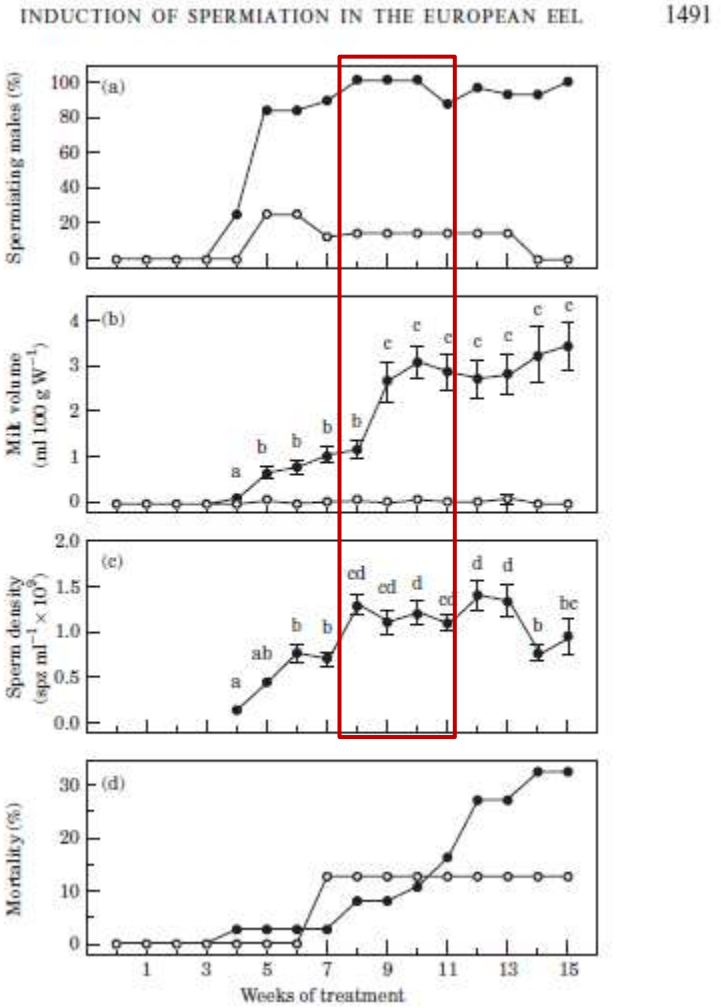


How long can we obtain the sperm? What is the best time to strip the males?

- Weekly injections hCG

- Best sperm motility: weeks 8-12
- 24 hours after hCG administration



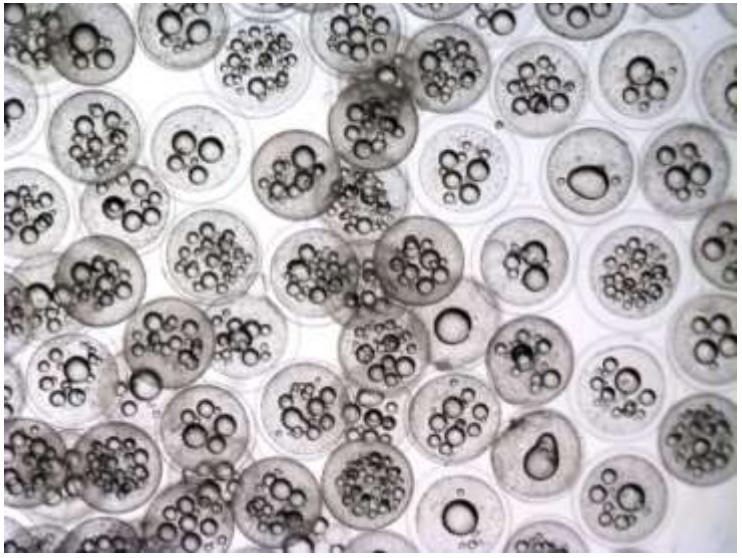


Pérez et al. 2000. J. Fish Biol. 57

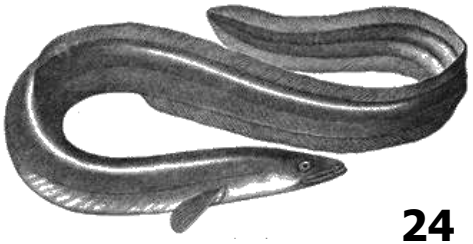
And the females?

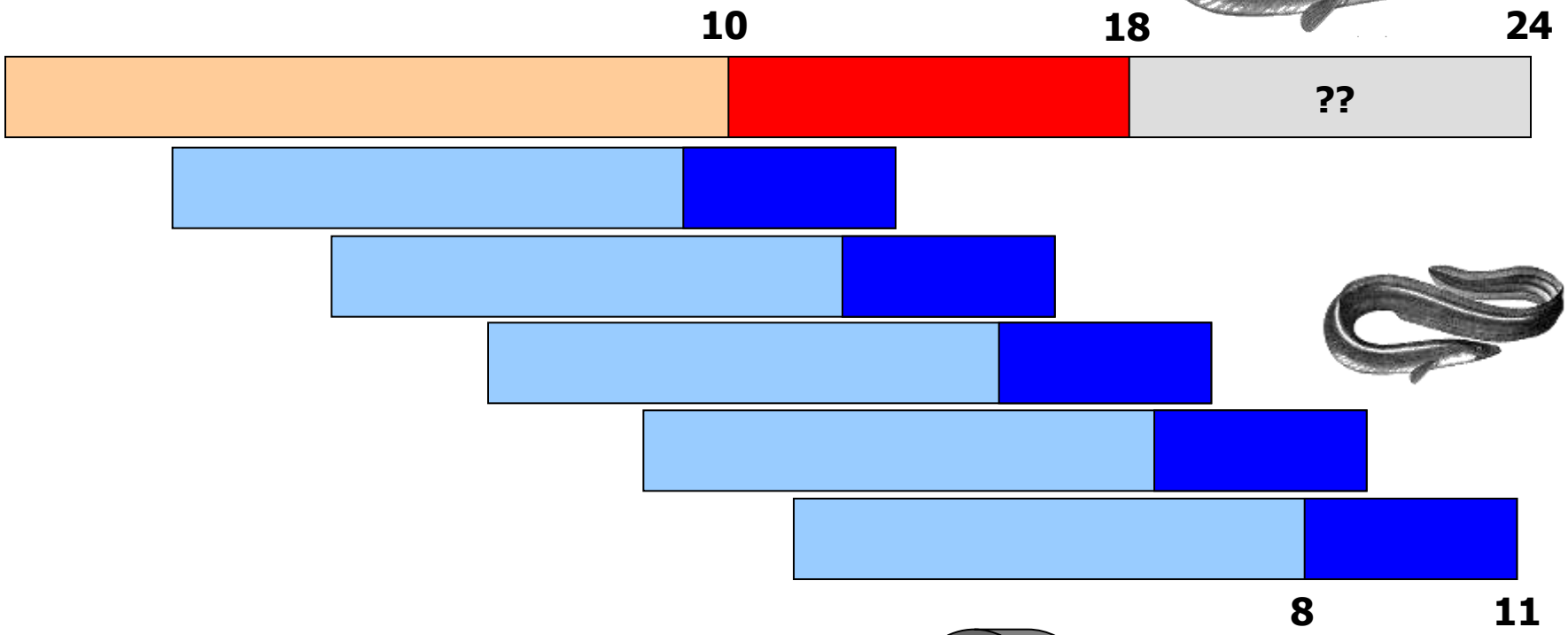
- 12-15 weeks (Asturiano et al. 2005. Boletin IEO): NO MALES!!
- 10-17 weeks (Pérez et al. 2008, Cybium)

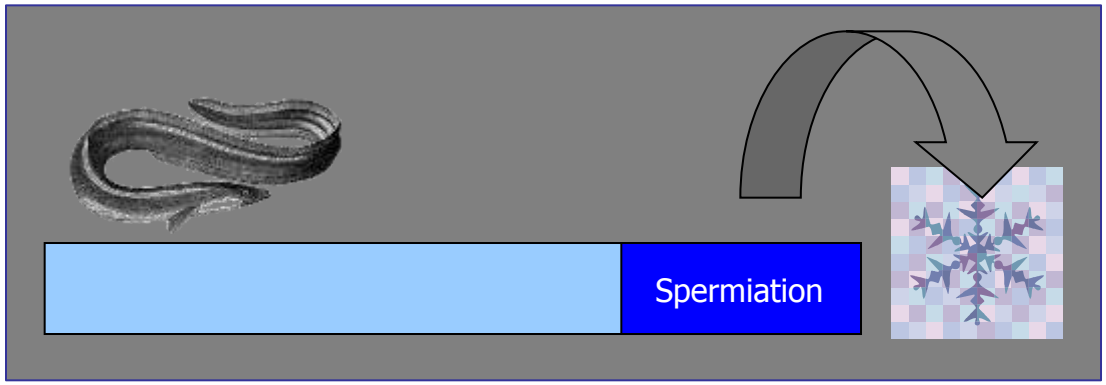
- **Longer times to mature**
- **Higher individual variation in sex maturation**
- **Higher difficulty in handling (size, diseases)**
- **Egg quality more unpredictable**



Synchronization vs cryopreservation







What to freeze?

Development of sperm quality evaluation techniques

Spermatozoa motility parameters (CASA)

Spermatozoa morphometry parameters (ASMA)

Percentage of alive cells (fluorescent stainings)

How to freeze?

Development of cryopreservation methods

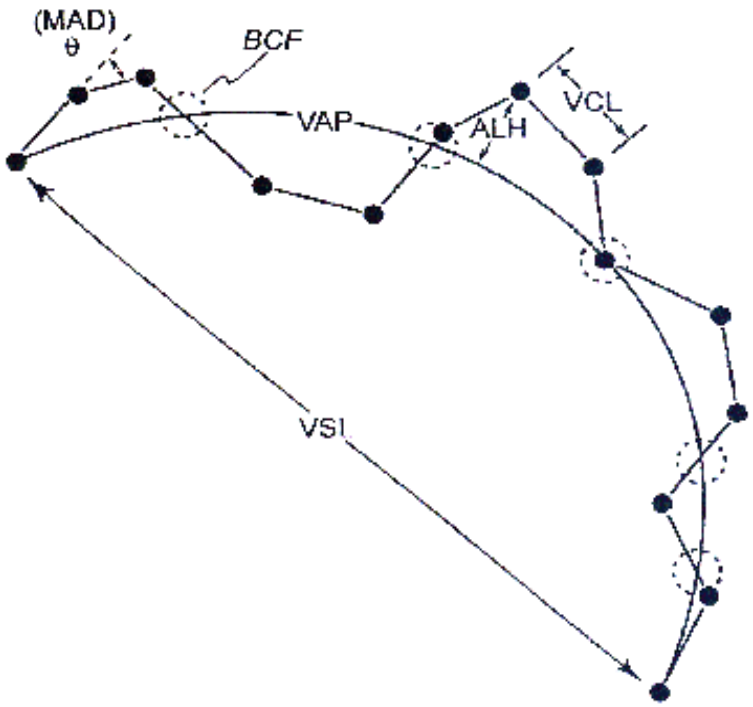
Physio-chemical characteristics of seminal plasma for sperm diluents design

Cryopreservation media, cryoprotectants and cell membrane stabilisers

Freezing-thawing protocols

Containers and dilution factor

Sperm quality evaluation by CASA

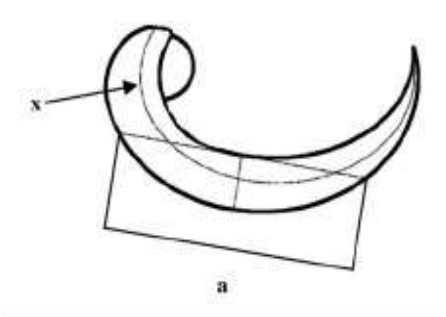


- **VCL**: Curvilinear velocity
- **VSL**: Straight line velocity
- **VAP**: Average path velocity
- **BCF**: Beating cross frequency

Data from fast and medium-velocity spermatozoa (VCL >40 mm/s)

- **Percentage of motile cells, progressive motility**

Sperm quality evaluation by ASMA

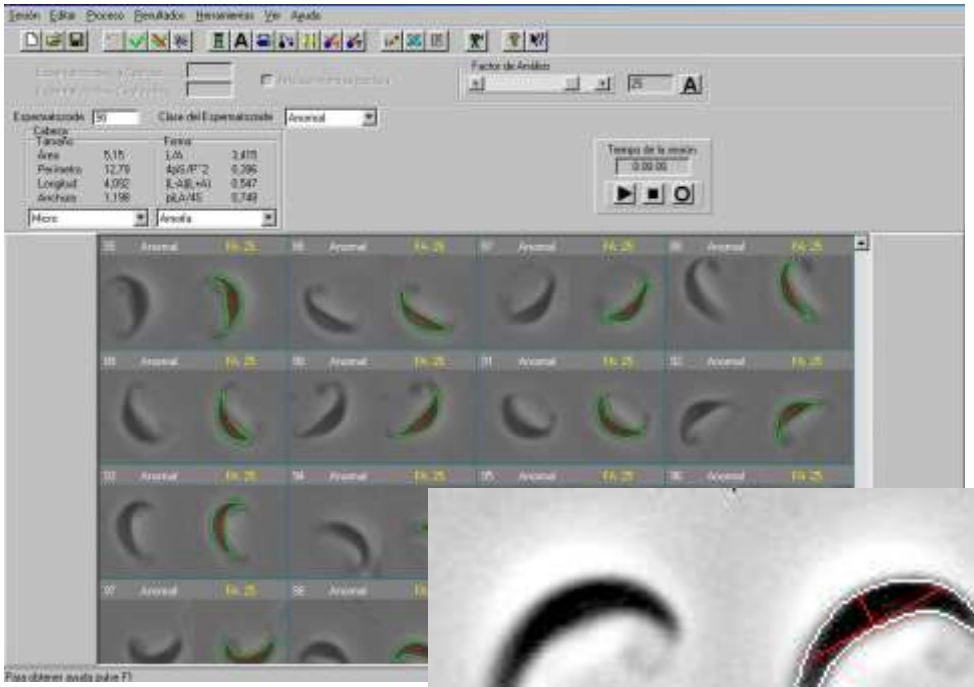


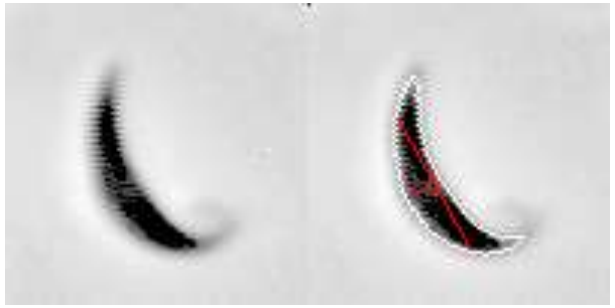
Width: 1.1 µm

Length: 4.3 µm *

Perimeter: 17.4 µm

Area: 6.3 µm²

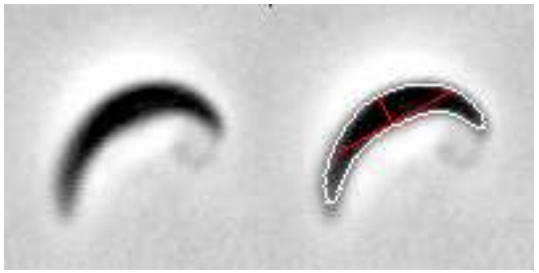




n: 15.000 spermatozoa

Marco-Jiménez *et al*., 2006 Theriogenology
Asturiano *et al*., 2007 Reproduction in Domestic Animals

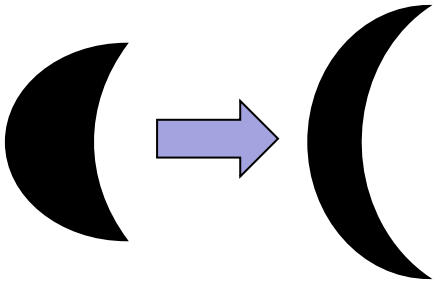
Variation of spermatozoa head morphometry throughout the maturation treatment



Least square means ± standard error of the means for each of the measured parameters (head length, width, perimeter and area) from 5th to 12th weeks of treatment.

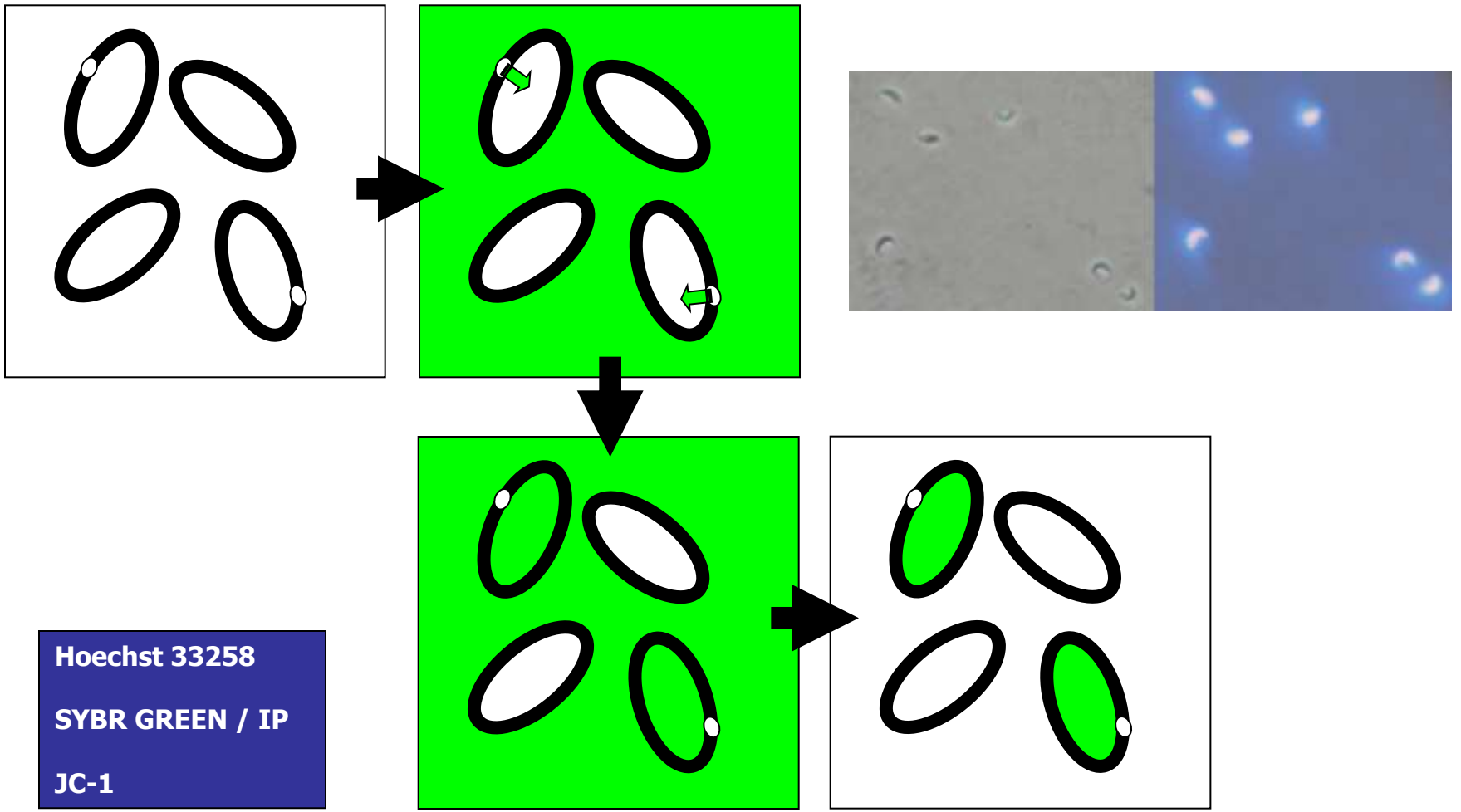
Week	n	Head length (µm)	Head width (µm)	Area (μm^2)	Perimeter (µm)
5	471	3.99±0.03^{e}	1.07±0.009^{g}	4.90±0.03^{g}	13.63±0.10^{f}
6	1560	4.11±0,01^{d}	1.19±0.004^{b}	5.19±0.02^{f}	14.13±0.05^{d}
7	3007	4.11±0.01^{d}	1.21±0.003^{a}	5.14±0.01^{e}	13.94±0.05^{e}
8	3147	4.31±0.01^{b}	1.13±0.003^{e}	5.44±0.01^{b}	15.32±0.04^{a}
9	2357	4.28±0.01^{b}	1.13±0.003^{e}	5.38±0.01^{c}	15.06±0.05^{b}
10	3060	4.20±0.01^{c}	1.17±0.004^{c}	5.46±0.01^{b}	15.10±0.05^{b}
11	1375	4.38±0.01^{a}	1.10±0.003^{f}	5.51±0.01^{a}	15.09±0.05^{b}
12	1514	4.09±0.01^{d}	1.15±0.004^{d}	5.27±0.02^{d}	14.37±0.06^{c}

^{a,b,c,d,e,f,g} Values in the same column with different superscripts are statistically different ($P<0.05$). n: number of spermatozoa considered in every case.

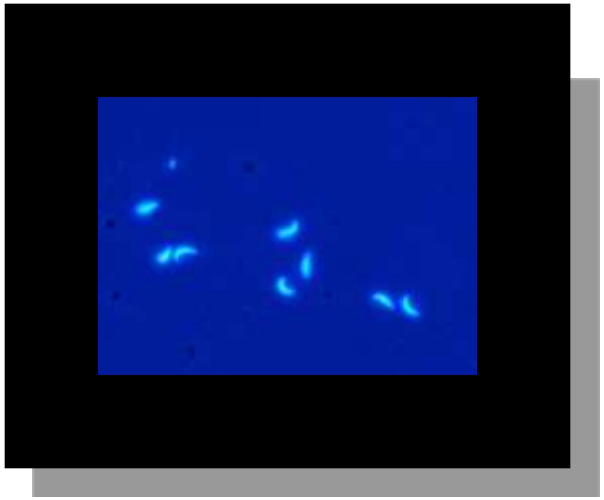


Asturiano *et al.*, 2006 Theriogenology

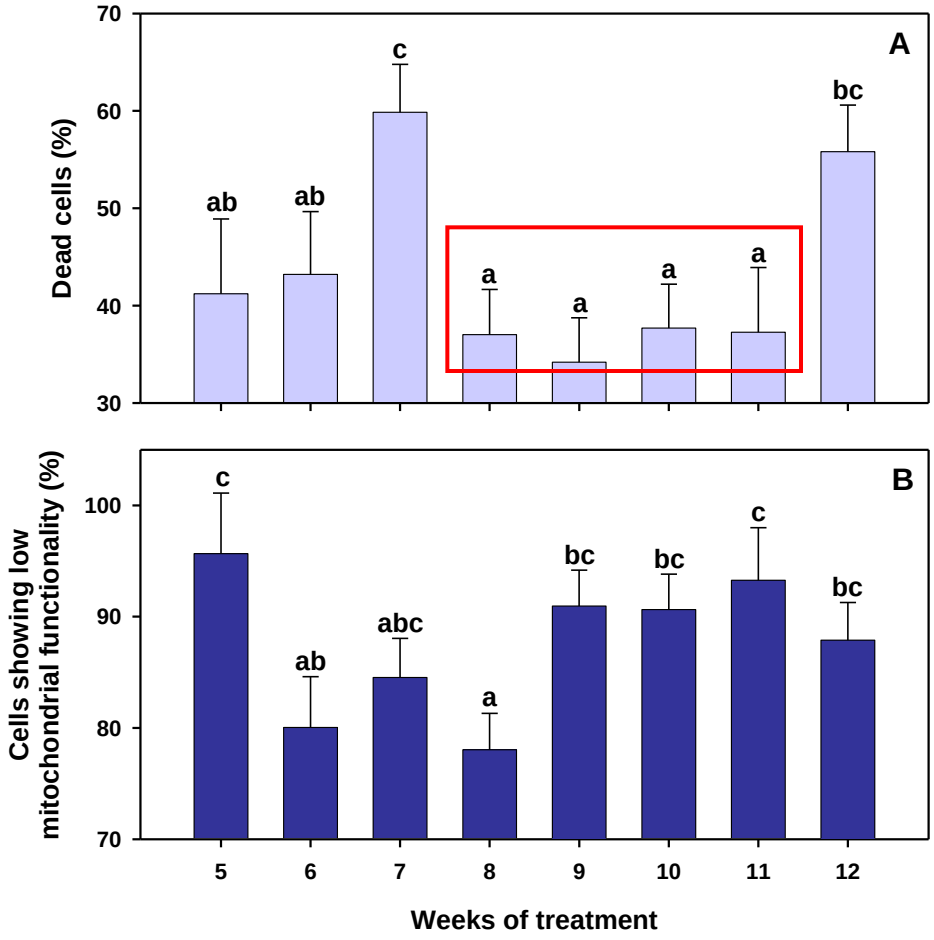
Fluorescent staining: dead/alive cells



Variation in the percentage of viable spermatozoa along the maturation process



Hoechst 33258



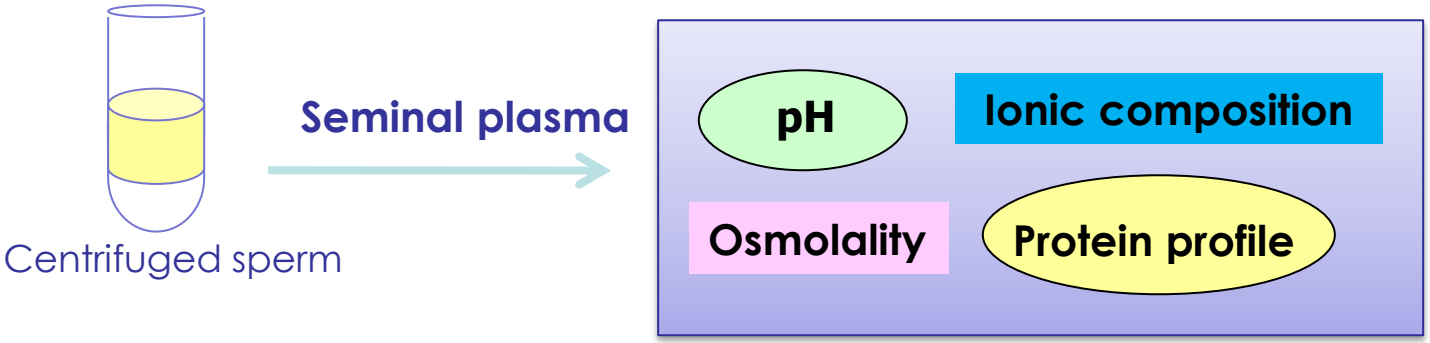
JC-1

A) Sperm viability obtained by Hoechst 33258 staining, expressed as percentage of dead spermatozoa. B) Mitochondrial function determined by JC-1 staining, showed as percentage of cells showing low mitochondrial functionality. Different letters indicate significant differences.

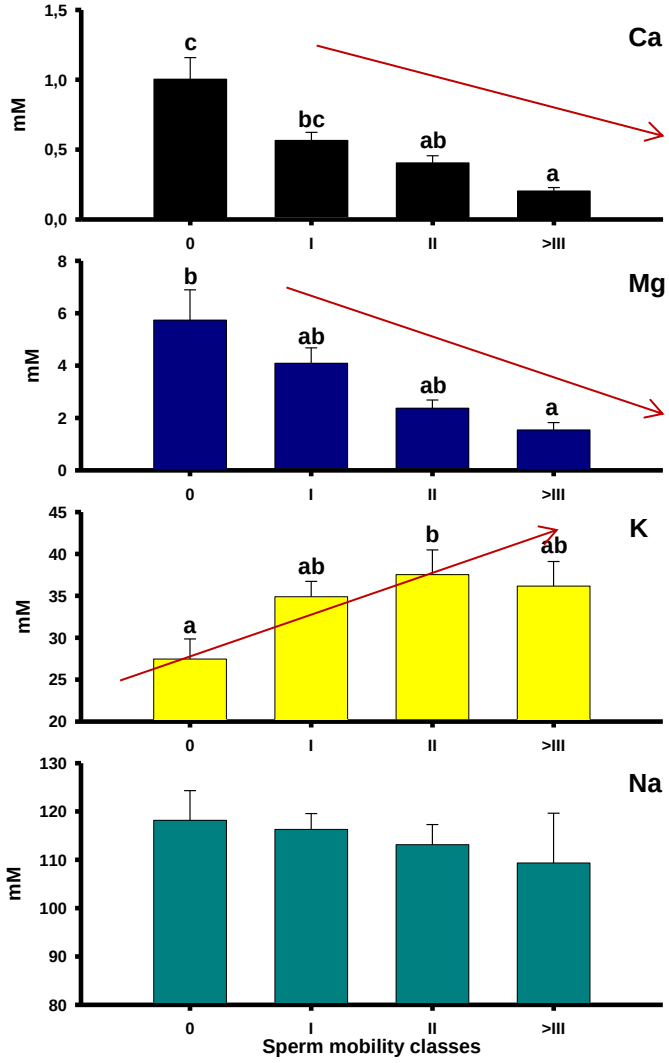
Asturiano et al., 2006 Theriogenology

2. Development of cryopreservation methods

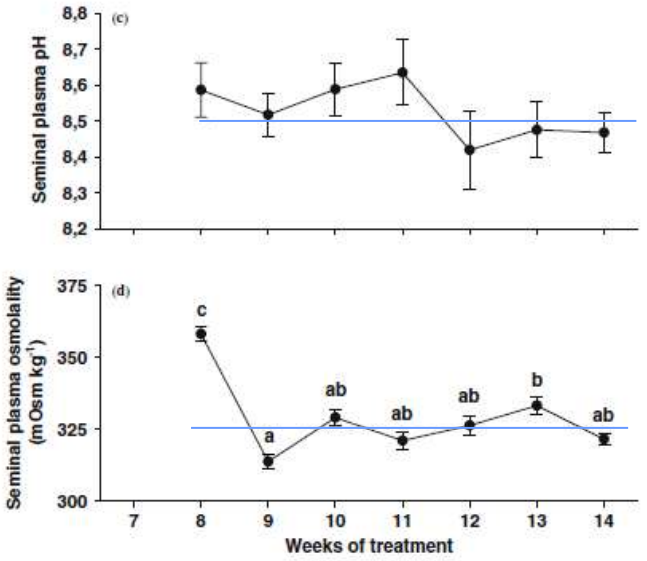
Study of the seminal plasma biochemical composition: ions, pH, osmolality



Study of the seminal plasma biochemical composition: ions, pH, osmolality



In the sperm samples with higher motility,
-lower levels of Ca^{2+}, Mg^{2+}
-high concentration of K^{+}



pH=8,5

Osm= 325 mOsm

Development of our extender P1

Pérez et al. 2003. Fish Physiol. Biochem.
Asturiano et al. 2004. Fish Physiol. Biochem.

Freezing media: comparison of extenders

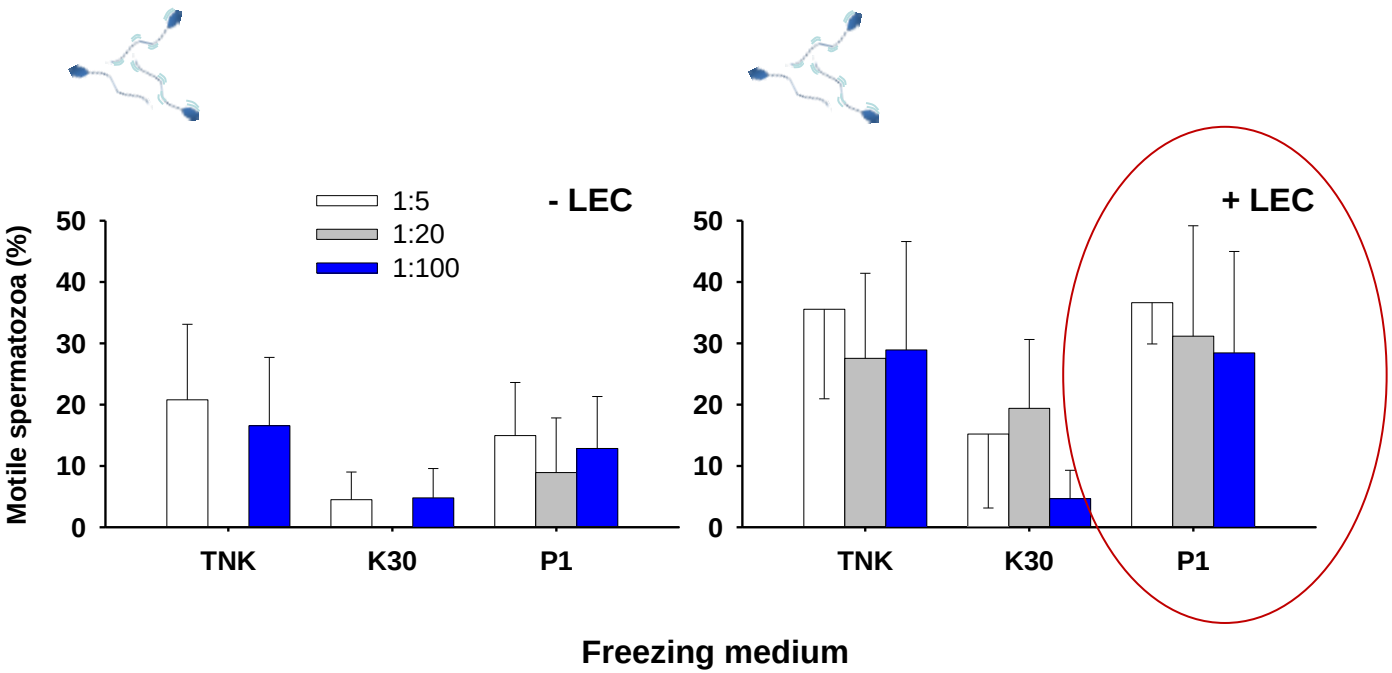
(mM)	TNK	P1	P2	K30
NaCl	137	125	70	134.5
$NaHCO_3$	76.2	20	75	20
KCl	--	30	30	30
$MgCl_2$	--	2.5	2.5	1.6
$CaCl_2$	--	1	1	1.3
TAPS	20	--	--	--
pH	8.2	8.5	8.5	8.1

-Tanaka (TNK): extender Japanese eel (Tanaka *et al*., 2002)
-P1 and **P2,** isoionics with European eel seminal plasma (Pérez *et al*., 2003)
-K30: extender Japanese eel (Ohta *et al*., 2001); good sperm motility

+ 10% v/v DMSO

+/- L-a-phosphatidylcholine (1.4 g/100 ml)

Dilution factors (1:5, 1:20, 1:100)



Post-thawing motile cells: aprox. 20-25%

Trends:

Better results with lower dilution factors

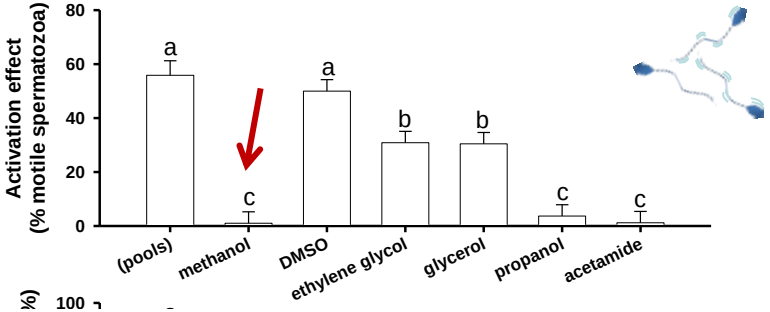
Positive effect of lecythin

Asturiano *et al.*, 2004 Fish Physiology and Biochemistry

Freezing media: comparison of cryoprotectants

Motility activation caused by different cryoprotectants (osmolality)?

Best (lowest activation): methanol

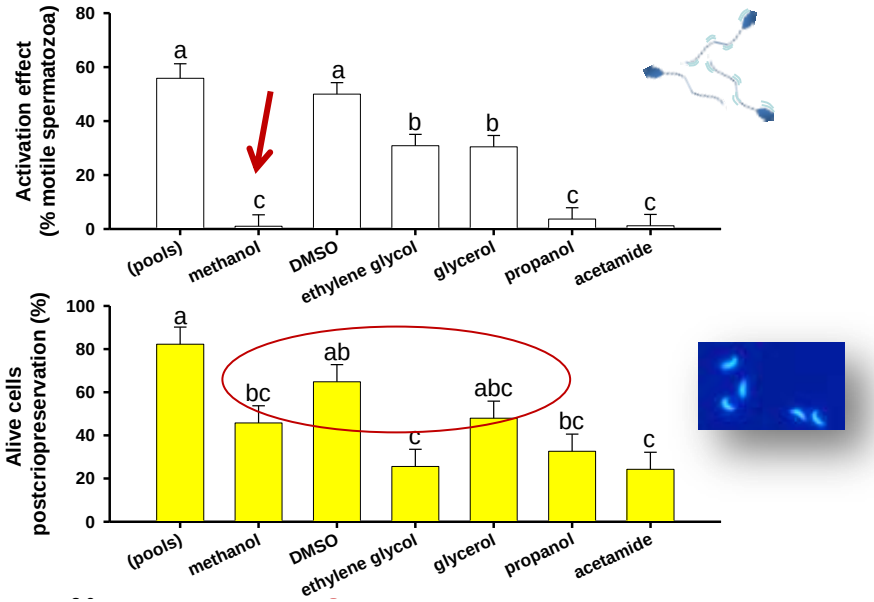


Freezing media: comparison of cryoprotectants

Motility activation caused by different cryoprotectants (osmolality)?

Best survival?

Methanol, DMSO, glycerol



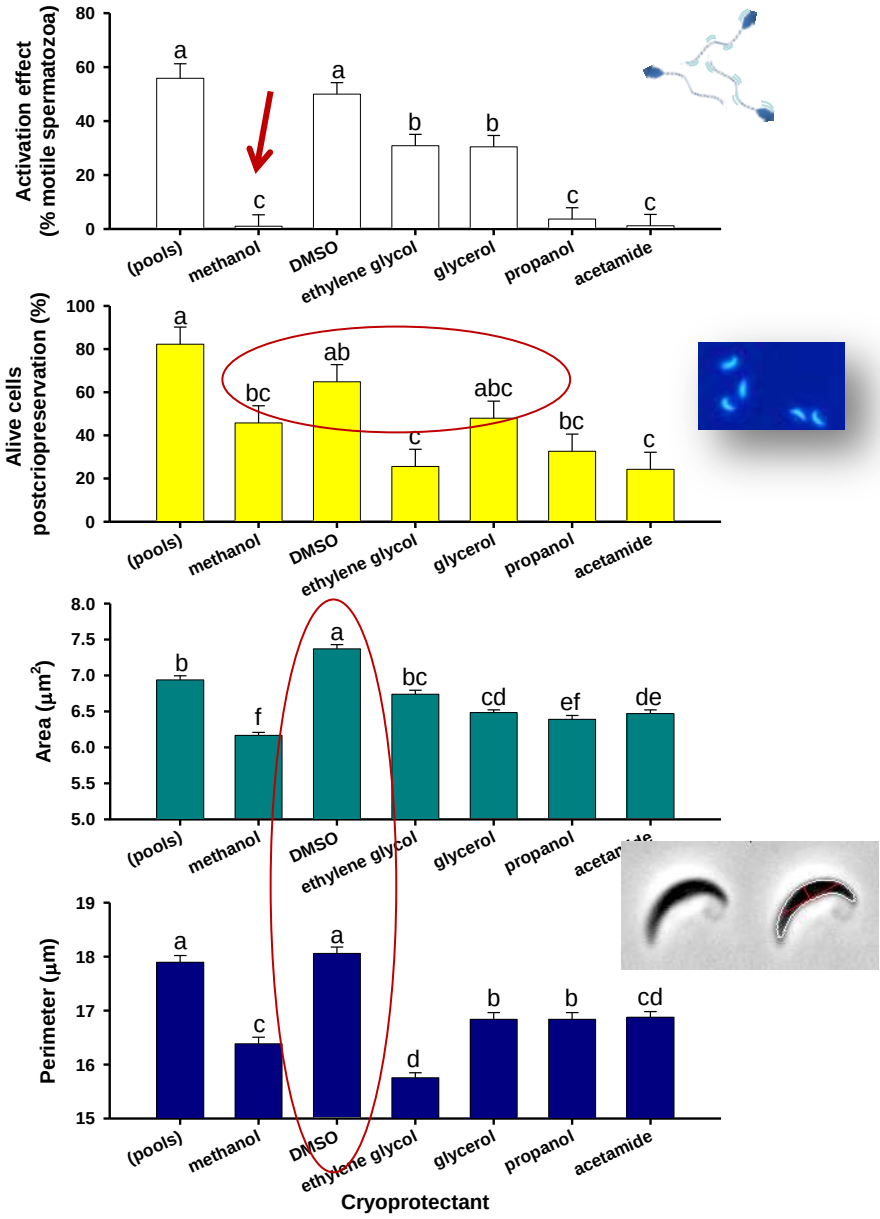
Freezing media: comparison of cryoprotectants

Motility activation caused by different cryoprotectants (osmolality)?

How many cells survive?

Effect on cell morphology?

Best: DMSO



Freezing media: comparison of cryoprotectants

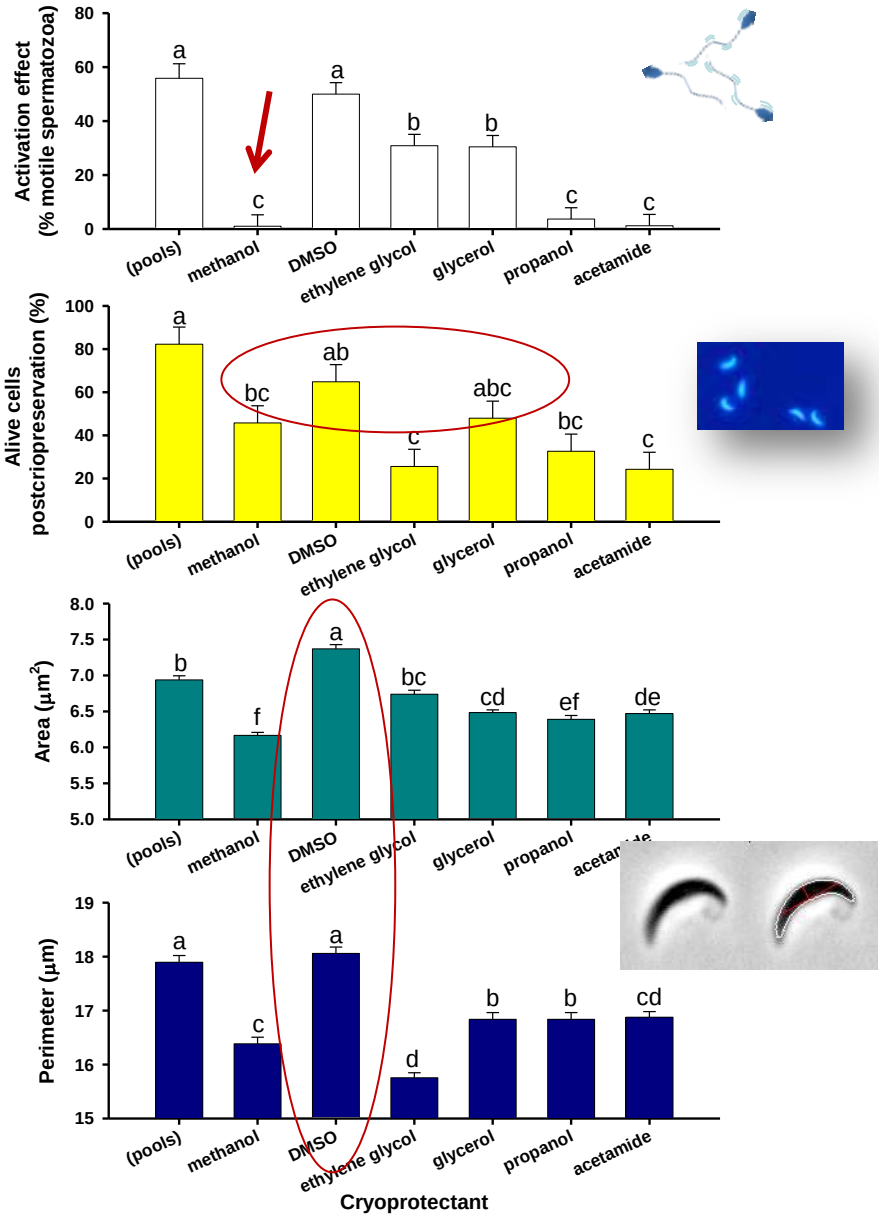
Motility activation caused by different cryoprotectants (osmolality)?

How many cells survive?

Effect on cell morphology?

Best candidates:

DMSO
Methanol
glycerol



Marco-Jiménez *et al.*, 2006 Cryobiology

Garzón *et al.*, 2008 Reproduction in Domestic Animals

FISHERIES SCIENCE 2004; **70**: 780–787

Role of sodium bicarbonate on the initiation of sperm motility in the Japanese eel

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ABSTRACT: In order to find out the role of sodium bicarbonate ($NaHCO_3$) on the initiation of sperm motility in the Japanese eel *Anguilla japonica*, interactions were investigated between $NaHCO_3$ and various reagents (K^+ channel blocker 4-aminopyridine [4-AP], ammonium chloride [NH_4Cl], sodium

Sodium bicarbonate inhibited the initiation of sperm motility in the Japanese eel.

Sodium bicarbonate inhibited the initiation of sperm motility in the Japanese eel. However, $NaHCO_3$ restored the motility of immotile sperm that 4-AP inhibited. The inhibitory effect of $NaHCO_3$ disappeared with the addition of NH_4Cl, which raised $[pH]_i$, but the promoting effect was not affected by $[pH]_i$. Although $NaHCO_3$ recovered motility in the presence of 4-AP, this recovery was also observed with the addition of $CaCl_2$ instead of $NaHCO_3$. In the initiation of sperm motility in the Japanese eel, two roles for $NaHCO_3$ are suggested: an inhibitory role relating to the regulation of $[pH]_i$ and a promoting role relating to the uptake of another initiation factor, which could be Ca^{2+}.

KEY WORDS: ***Anguilla japonica*, 4-aminopyridine, initiation of sperm motility, sodium bicarbonate.**

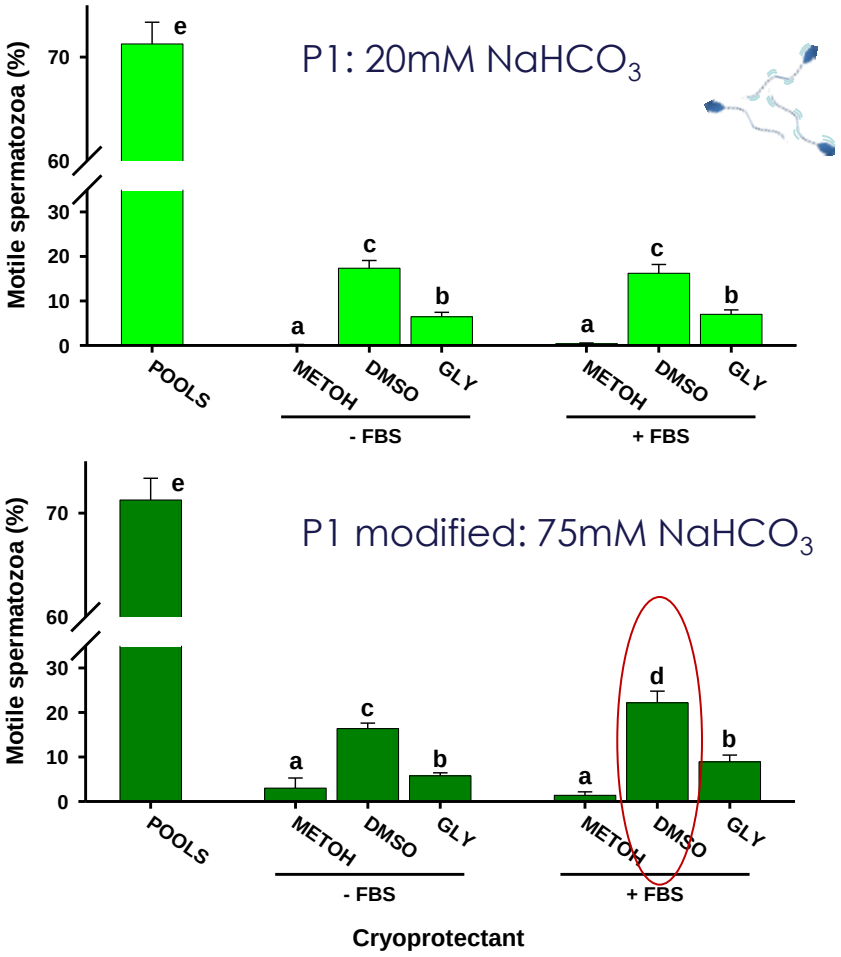
Freezing media: bicarbonate, Foetal bovine serum

Higher concentrations of bicarbonate could reduce the "activation effect" caused by cryoprotectants?

Could FBS help protecting cells?

The combination of higher bicarbonate concentration, DMSO and FBS caused best survival

However, the percentage of post-thawing motile cells is still low (aprox. 22%)



Garzón *et al.*, 2008 Reproduction in Domestic Animals

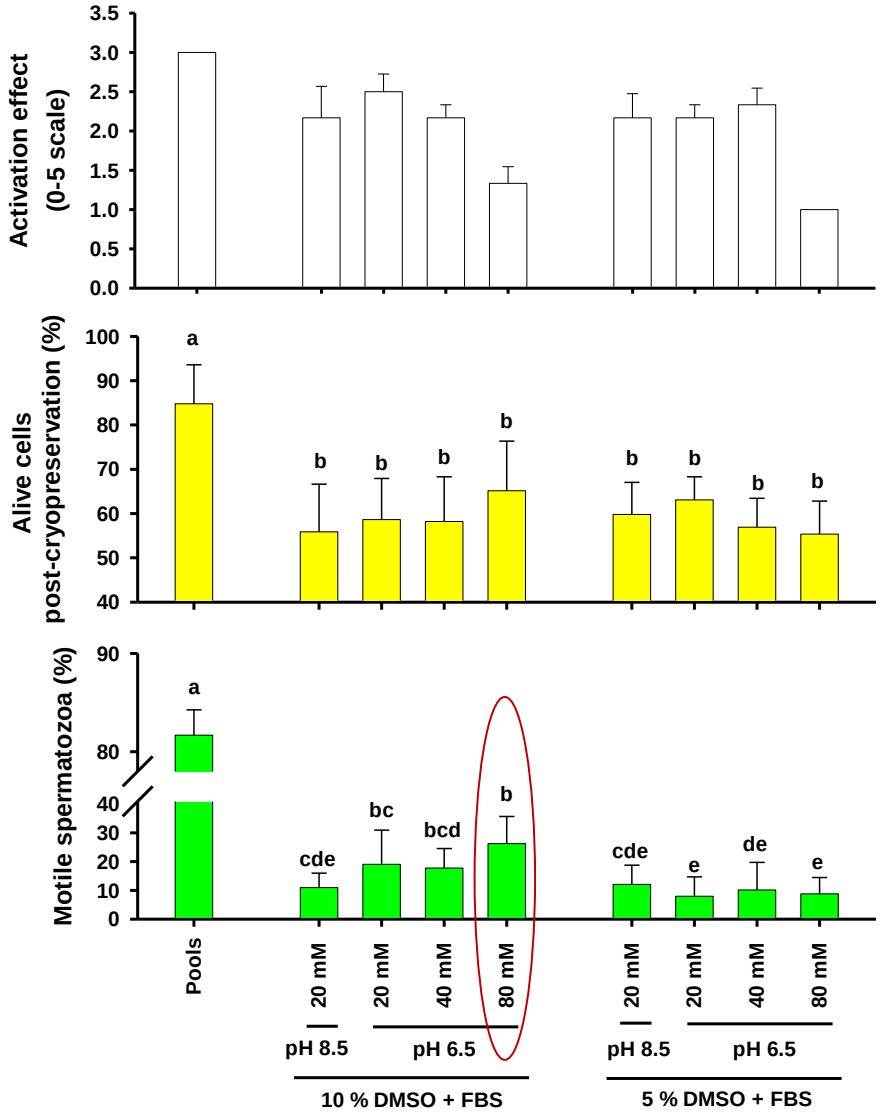
Could lower DMSO concentrations reduce the "activation effect" without reducing survival?

What is the effect of pH? Low pH is better?

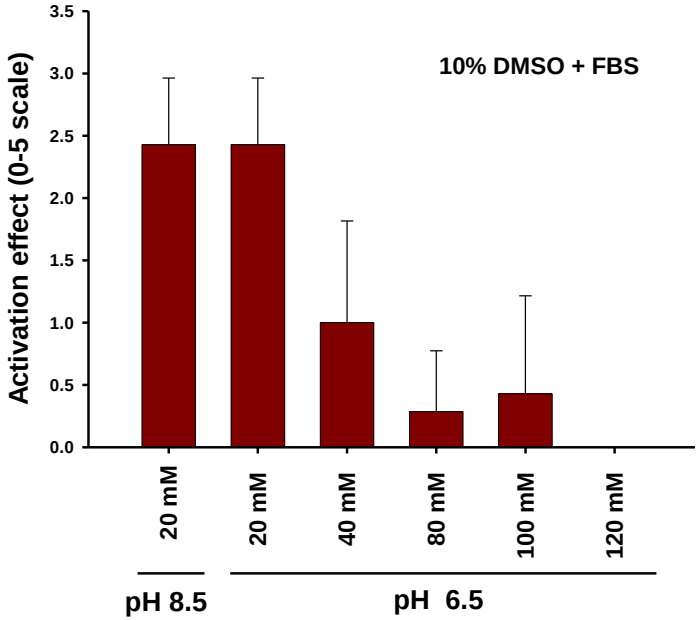
5 and 10% DMSO caused similar "activation effect"

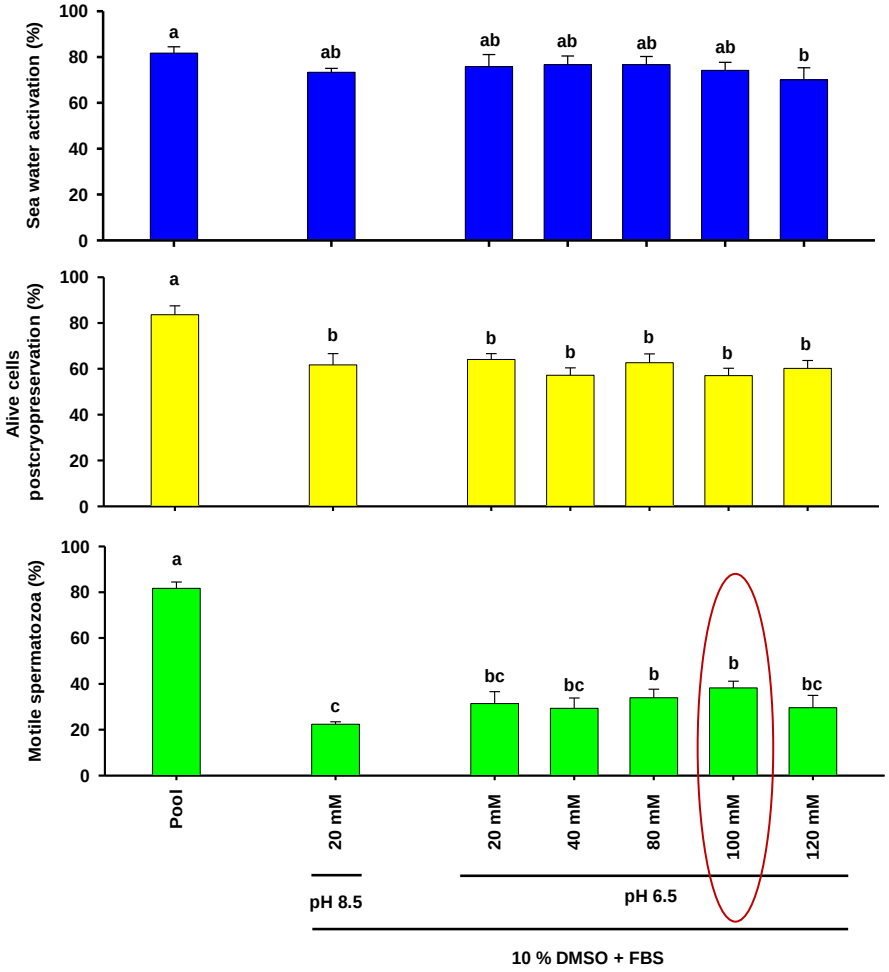
Higher bicarbonate concentrations together with lower pH causes lower "activation effect"

Percentage of post-thawing motile cells (26%) is higher with 10% DMSO, low pH and the highest bicarbonate concentrations



Peñaranda et al. 2009. Cryobiology 59, 119-126



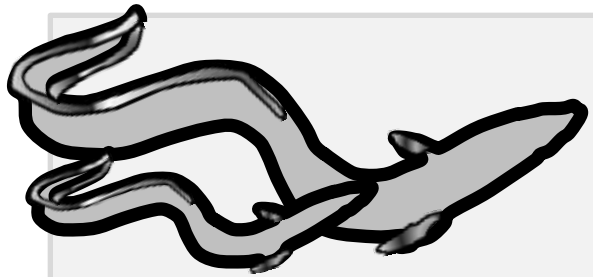


DMSO "activation effect" pre-cryopreservation can be eliminated with high bicarbonate concentrations and low pH (without killing cells)

Increase of survival post-cryopreservation aprox. **40%**

Defined our protocol

Peñaranda et al. 2009. Cryobiology 59, 119-126



Testing our cryopreservation protocol in fertilization of eel eggs.

Sperm motility evaluation

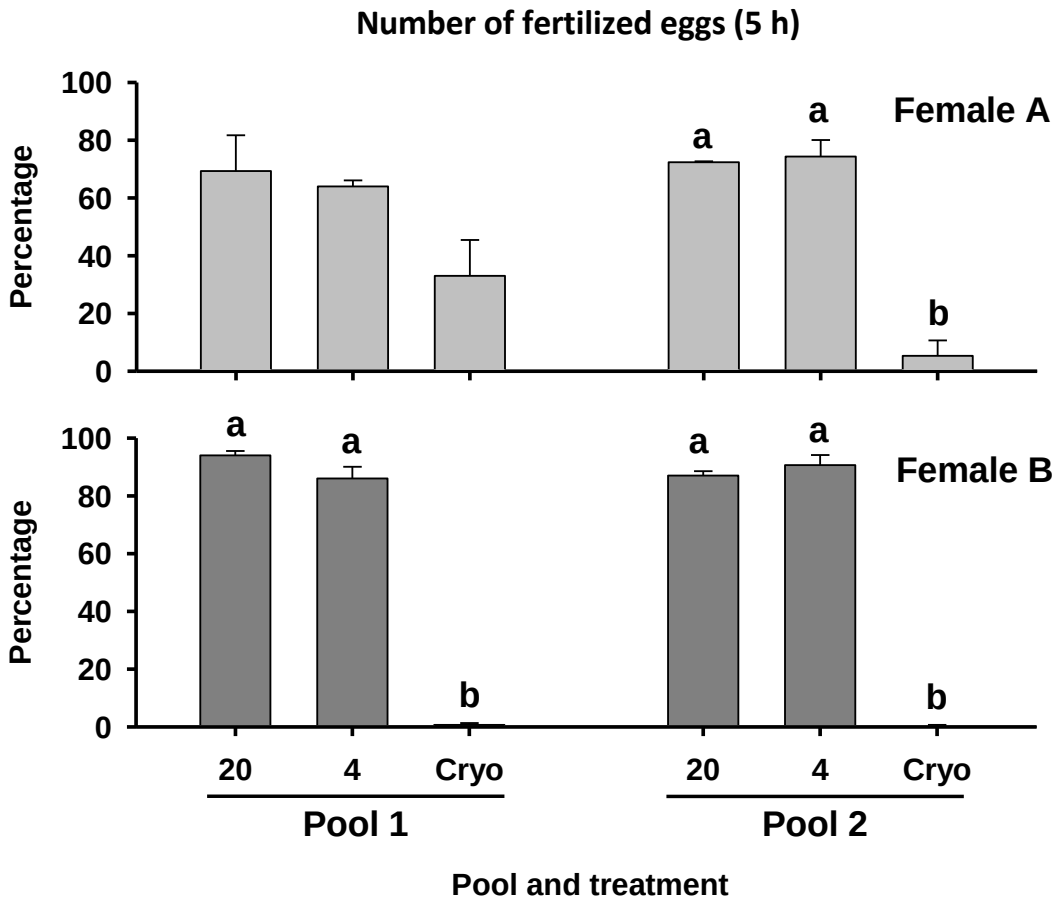
Sperm selection (pools, dilution)

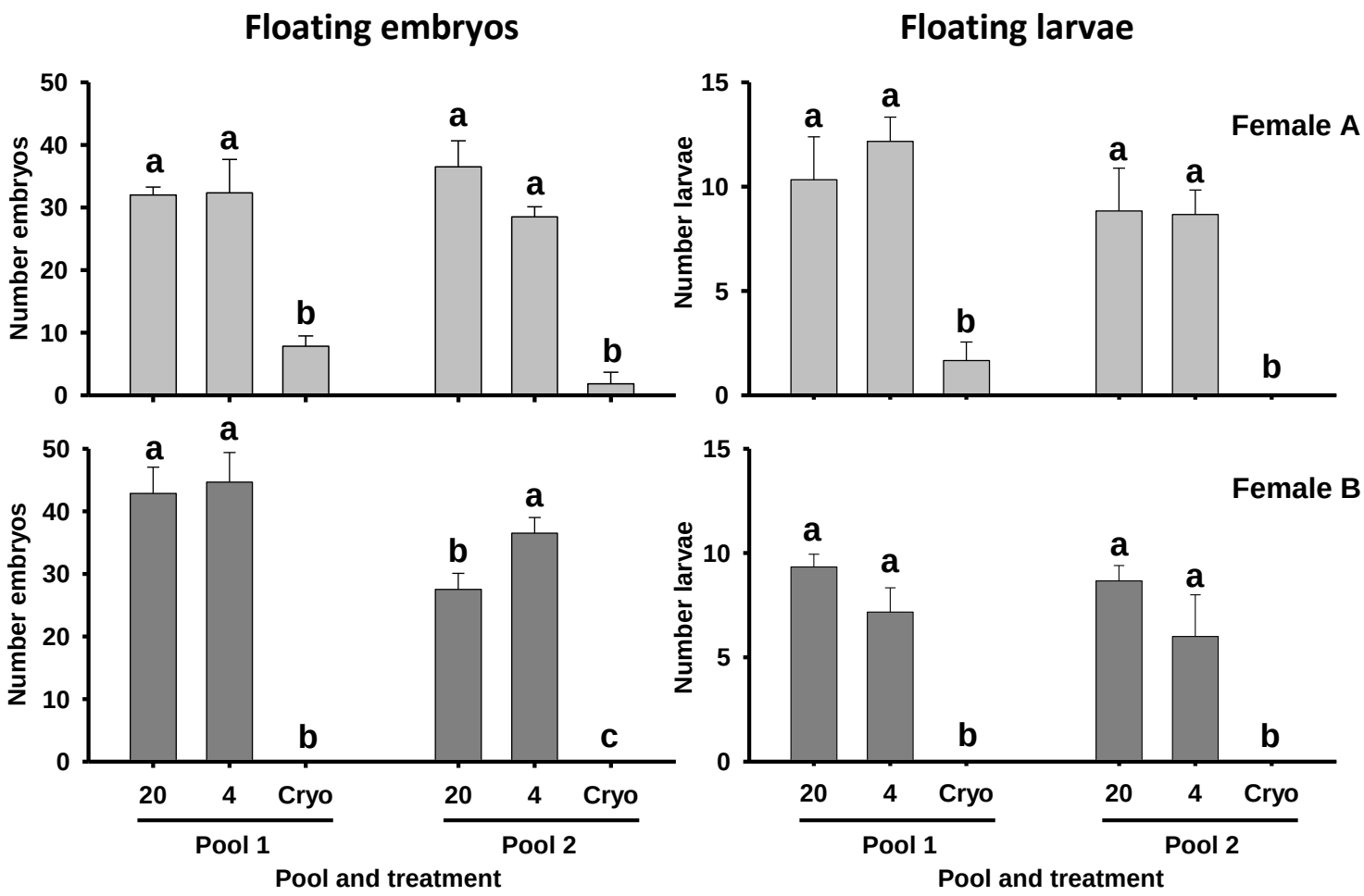
20 °C

4 °C

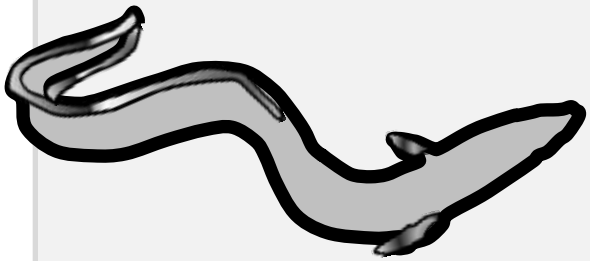
Cryopreservation

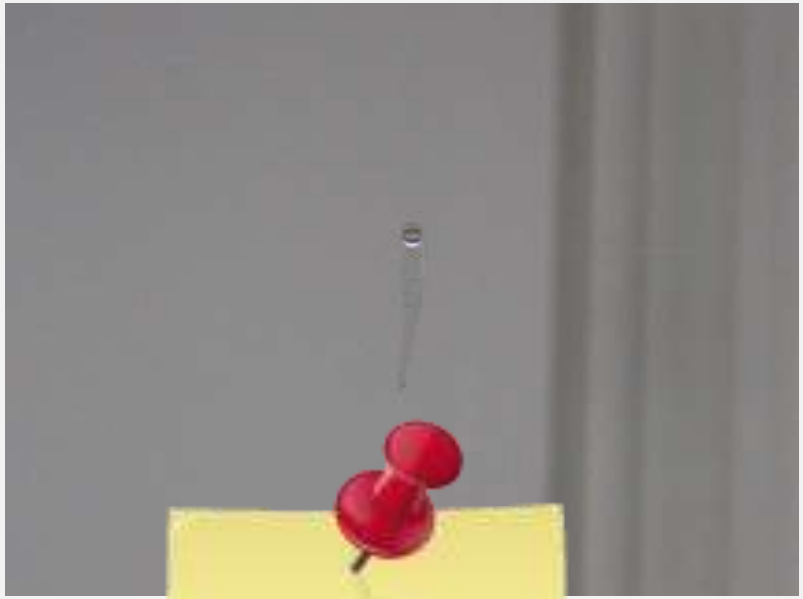
Fertilization


Number of fertilized eggs (5 h)
Percentage
100
80
60
40
20
0
a
a
b
Female A
a
a
b
Female B
20
4
Cryo
Pool 1
Pool 2
Pool and treatment

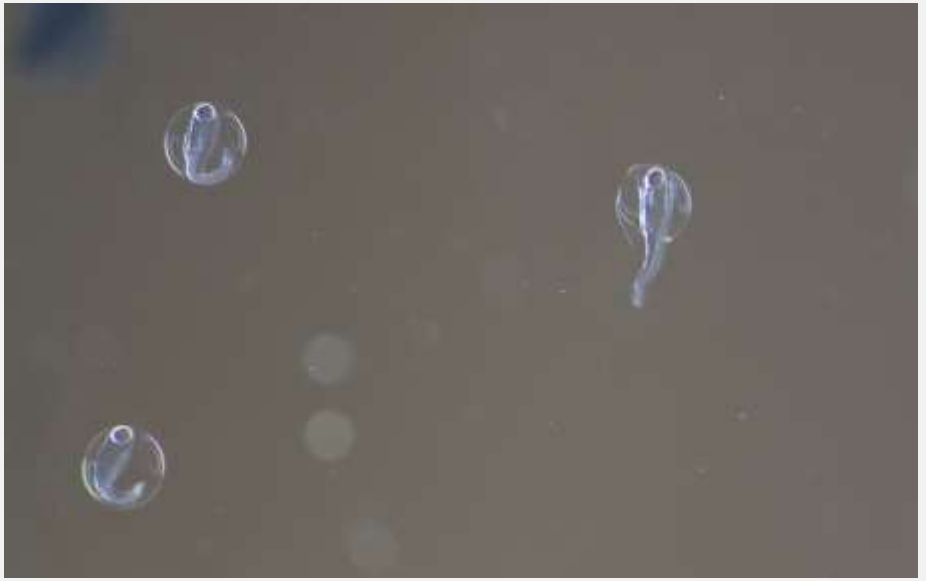


We need to improve the protocols





First
“cryolarvae”



Comparison of “spanish” and “hungarian” cryopreservation methods

Asturiano *et al.*, 2003	Peñaranda *et al.*, 2009	Müller *et al.*, 2004	Szabó *et al.*, 2005
- Farmed fish		- Wild fish	
- Seawater		- Freshwater	
- Hormonal treatment: hCG rec		- Hormonal treatment: natural hCG	
- Dilution medium: P1		- Dilution medium: Tanaka	
- Sperm dilution ratio: 1:2		- Sperm dilution ratio: 1:9	
- Cryoprotectant: DMSO		- Cryoprotectant: Methanol	
- Container: 250 µl straws		- Container: 500 µl straws	

Comparison of “spanish” and “hungarian” methods

Asturiano *et al.*, 2003

Peñaranda *et al.*, 2009

Müller *et al.*, 2004

Szabó *et al.*, 2005

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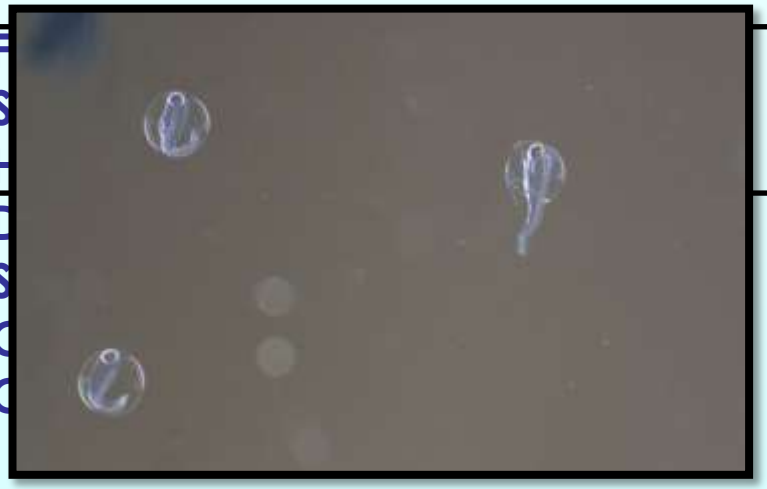
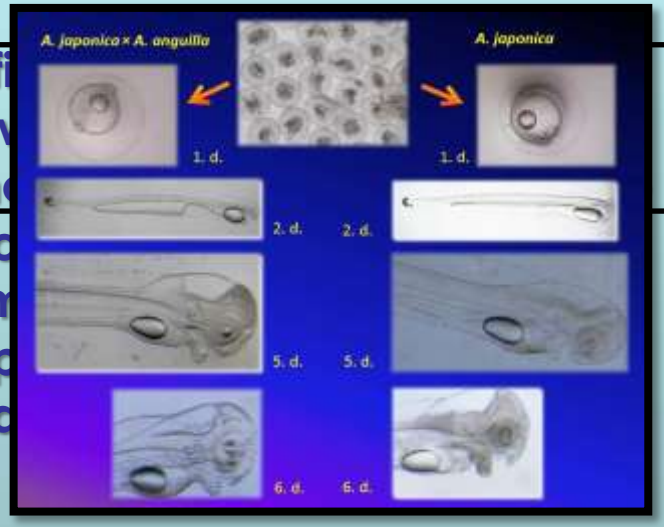
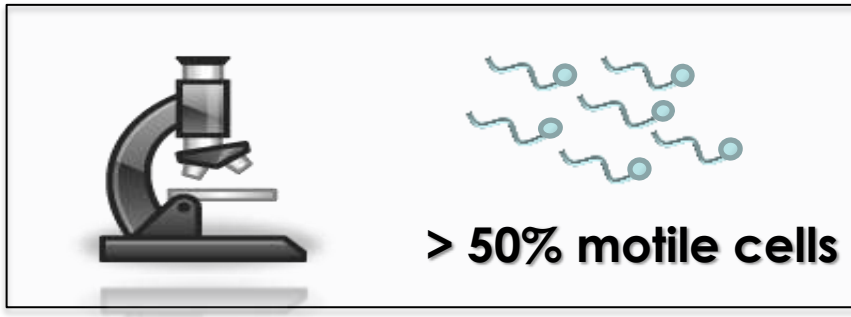


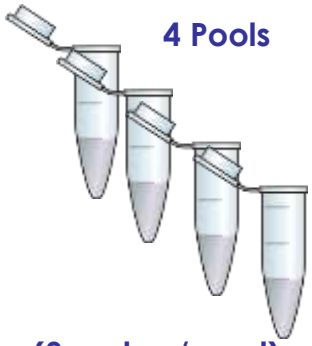
First cryolarvae
(*A. anguilla* x *A. anguilla*)



First hybrids
(*A. anguilla* x *A. japonica*)

Comparison joint experiments





4 Pools

(3 males / pool)

Fresh samples

Dilution with a mixture of extender and cryoprotectant

Pre-cryopreservation

Straws filling

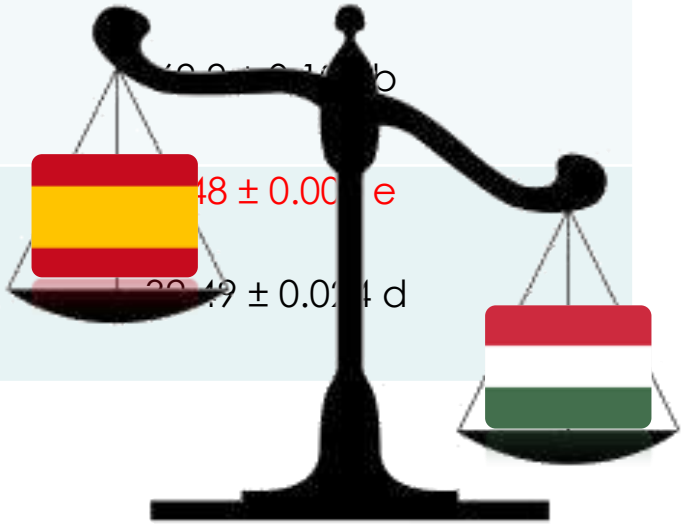
Freezing (vapor of LN)

Thawing (immersion into water bath)

Post-cryopreservation

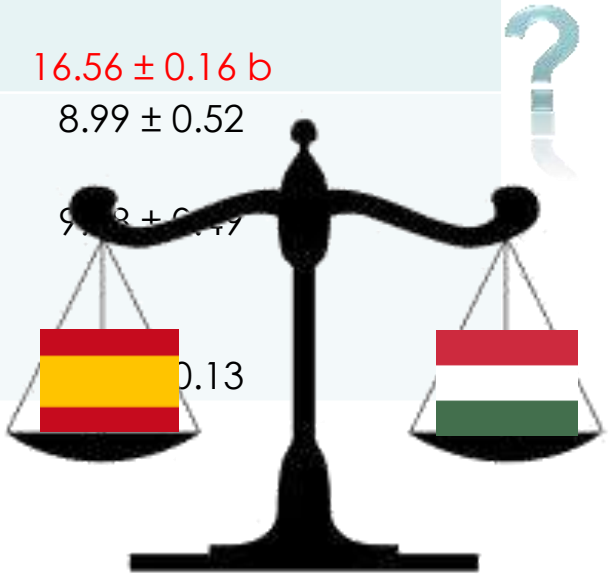
PRELIMINARY RESULTS

MOTILITY	Protocol	Percentage motile spermatozoa
Fresh		87.0 ± 0.014 a
Pre-cryopreservation	Asturiano et al. (2003) Müller et al. (2004)	54.1 ± 0.043 c b
Post-cryopreservation	Asturiano et al. (2003) Müller et al. (2004)	48 ± 0.00 e ± 0.0 d



PRELIMINARY RESULTS

MORPHOMETRY	Protocol	Percentage motile spermatozoa
Perimeter (mm)	Fresh	19.68 ± 0.67 a
	Asturiano et al. (2003)	17.56 ± 0.21 b
	Müller et al. (2004)	16.56 ± 0.16 b
Area (mm^2)	Fresh	8.99 ± 0.52
	Asturiano et al. (2003)	[illegible]
	Müller et al. (2004)	[illegible]0.13



Conclusions

- **Develop a isoosmotic, specific extender for your species**
  - Research: extend the sperm survival (days)
  - Basis for cryopreservation media
  - Physiological sperm studies: ions, pH, etc.
  - Dilute the sperm for fertilization

- **Techniques: cryoprotectants, timing, etc.**
- **Eel sperm vitrification (Esther Kasa, Akos Horvath)**
- **European eel: Hungarian method seems better**

Thanks for your atention!

Funded by COST Action FA1205: AQUAGAMETE

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