European eel sperm cryopreservation

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Traditional fisheries of glass eel and eels

Glass eel fishing (Basque Country)

Eel fishing (East Spain)
Aquaculture industry Europe and Asia

- Recirculation systems
- Decline in aquaculture from 2000'
- Greenhouse systems
- Increase in aquaculture since 1990' (China)
- 50-times > European production

Global Aquaculture Production for species (tonnes)
Source: FAO FishStat

Depends on glass eel fisheries
Conservation status European eel: critically endangered (IUCN)

- ICES (2011): recruitment of glass eels reduced -95 % (-99 %) of the levels before the 1980’s
- CITES (2009): included in appendix II. Trade out EU forbidden.
  - 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

Graphs showing the drop in biomass of glass and silver eels from 1965 to 2008 (source: Brand, 2007).
Peculiar and complex lifecycle through the Atlantic ocean...

- Teleost fish
- Wide distribution
- Marine & Continental
- Peculiar migration
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?

- 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.

<table>
<thead>
<tr>
<th>Week</th>
<th>n</th>
<th>Head length (μm)</th>
<th>Head width (μm)</th>
<th>Area (μm²)</th>
<th>Perimeter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>471</td>
<td>3.99±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.07±0.009&lt;sup&gt;g&lt;/sup&gt;</td>
<td>4.90±0.03&lt;sup&gt;g&lt;/sup&gt;</td>
<td>13.63±0.10&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>1560</td>
<td>4.11±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.19±0.004&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.19±0.02&lt;sup&gt;f&lt;/sup&gt;</td>
<td>14.13±0.05&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>3007</td>
<td>4.11±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.21±0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.14±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.94±0.05&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>3147</td>
<td>4.31±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.13±0.003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.44±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.32±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>2357</td>
<td>4.28±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.13±0.003&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.38±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.06±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>3060</td>
<td>4.20±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.17±0.004&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.46±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.10±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>1375</td>
<td>4.38±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.10±0.003&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5.51±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.09±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>1514</td>
<td>4.09±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.15±0.004&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.27±0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.37±0.06&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c,d,e,f,g</sup> 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

- Hoechst 33258
- SYBR GREEN / IP
- JC-1
Variation in the percentage of viable spermatozoa along the maturation process

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\(^{+}\)

\[
\begin{align*}
\text{Ca} & : 0,0 \quad 0,5 \quad 1,0 \quad 1,5 \\
\text{Mg} & : 0 \quad 2 \quad 4 \quad 6 \quad 8 \\
\text{K} & : 20 \quad 25 \quad 30 \quad 35 \quad 40 \quad 45 \\
\text{Na} & : 80 \quad 90 \quad 100 \quad 110 \quad 120 \quad 130
\end{align*}
\]

Development of our extender P1

Freezing media: comparison of extender types

<table>
<thead>
<tr>
<th>(mM)</th>
<th>TNK</th>
<th>P1</th>
<th>P2</th>
<th>K30</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>137</td>
<td>125</td>
<td>70</td>
<td>134.5</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>76.2</td>
<td>20</td>
<td>75</td>
<td>20</td>
</tr>
<tr>
<td>KCl</td>
<td>--</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>--</td>
<td>2.5</td>
<td>2.5</td>
<td>1.6</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>--</td>
<td>1</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td>TAPS</td>
<td>20</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>pH</td>
<td>8.2</td>
<td>8.5</td>
<td>8.5</td>
<td>8.1</td>
</tr>
</tbody>
</table>

- **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-α-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 lecythtin

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

SATORU TANAKA,1† TOMOKO UTOH,1 YOSHIKI YAMADA,1 NORIYUKI HORIE,1 AKIHIRO OKAMURA,1 ATSUSHI AKAZAWA,1 NAOMI MIKAWA,1 HIDEO OKA1 AND HISASHI KUROKURA2

1IRAGO Institute, Atumi, Aichi 441-3605 and 2Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan

ABSTRACT: In order to find out the role of sodium bicarbonate (NaHCO3) on the initiation of sperm motility in the Japanese eel Anguilla japonica, interactions were investigated between NaHCO3 and various reagents (K+ channel blocker 4-aminopyridine [4-AP], ammonium chloride [NH4Cl], sodium bicarbonate inhibited the initiation of sperm motility in the Japanese eel. However, NaHCO3 restored the motility of immotile sperm that 4-AP inhibited. The inhibitory effect of NaHCO3 disappeared with the addition of NH4Cl, which raised [pH], but the promoting effect was not affected by [pH]. Although NaHCO3 recovered motility in the presence of 4-AP, this recovery was also observed with the addition of CaCl2 instead of NaHCO3. In the initiation of sperm motility in the Japanese eel, two roles for NaHCO3 are suggested: an inhibitory role relating to the regulation of [pH] and a promoting role relating to the uptake of another initiation factor, which could be Ca2+

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

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
Testing our cryopreservation protocol in fertilization of eel eggs.

Sperm motility evaluation

Sperm selection (pools, dilution)

Cryopreservation

20 °C

4 °C

Fertilization
Number of fertilized eggs (5 h)

Female A

<table>
<thead>
<tr>
<th>Pool and treatment</th>
<th>Female A</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 4 Cryo</td>
<td>a</td>
</tr>
<tr>
<td>20 4 Cryo</td>
<td>a</td>
</tr>
</tbody>
</table>

Female B

<table>
<thead>
<tr>
<th>Pool and treatment</th>
<th>Female B</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 4 Cryo</td>
<td>b</td>
</tr>
<tr>
<td>20 4 Cryo</td>
<td>b</td>
</tr>
</tbody>
</table>

Pool 1

| 20 4 Cryo | a |

Pool 2

| 20 4 Cryo | a |

Pool and treatment

Percentage

0 20 40 60 80 100
We need to improve the protocols
First “cryolarvae”
Comparison of “spanish” and “hungarian” cryopreservation methods

<table>
<thead>
<tr>
<th>Asturiano et al., 2003</th>
<th>Peñaranda et al., 2009</th>
<th>Müller et al., 2004</th>
<th>Szabó et al., 2005</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Farmed fish</strong></td>
<td><strong>Wild fish</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Seawater</strong></td>
<td><strong>Freshwater</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hormonal treatment: hCG rec</strong></td>
<td><strong>Hormonal treatment: natural hCG</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Dilution medium: P1</strong></td>
<td><strong>Dilution medium: Tanaka</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sperm dilution ratio: 1:2</strong></td>
<td><strong>Sperm dilution ratio: 1:9</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cryoprotectant: DMSO</strong></td>
<td><strong>Cryoprotectant: Methanol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Container: 250 µl straws</strong></td>
<td><strong>Container: 500 µl straws</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Comparison of “spanish” and “hungarian” methods

- **Spanish methods**
  - Asturiano et al., 2003
  - Peñaranda et al., 2009

  - Wild fish
  - Freshwater
  - Hormonal treatment: natural hCG
  - Dilution medium: Tanaka
  - Sperm dilution ratio: 1:9
  - Cryoprotectant: Methanol
  - Container: 500 μl straws

- **Hungarian methods**
  - Müller et al., 2004
  - Szabó et al., 2005

  - Farmed fish
  - Seawater
  - Hormonal treatment: HCG rec
  - Dilution medium: P1
  - Sperm dilution ratio: 1:2
  - Cryoprotectant: DMSO
  - Container: 250 μl straws

- Comparison of “spanish” and “hungarian” methods

  - 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

Straws filling

Freezing (vapor of LN)

Thawing (immersion into water bath)

Pre-cryopreservation

Post-cryopreservation

> 50% motile cells
## PRELIMINARY RESULTS

<table>
<thead>
<tr>
<th>MOTILITY</th>
<th>Protocol</th>
<th>Percentage motile spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td></td>
<td>87.0 ± 0.014 a</td>
</tr>
<tr>
<td>Pre-cryopreservation</td>
<td>Asturiano et al. (2003)</td>
<td>54.1 ± 0.043 c</td>
</tr>
<tr>
<td></td>
<td>Müller et al. (2004)</td>
<td>69.9 ± 0.105 b</td>
</tr>
<tr>
<td>Post-cryopreservation</td>
<td>Asturiano et al. (2003)</td>
<td>1.48 ± 0.005 e</td>
</tr>
<tr>
<td></td>
<td>Müller et al. (2004)</td>
<td>32.49 ± 0.024 d</td>
</tr>
</tbody>
</table>
## PRELIMINARY RESULTS

<table>
<thead>
<tr>
<th>Mmorphometry</th>
<th>Protocol</th>
<th>Percentage motile spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perimeter (mm)</td>
<td>Fresh</td>
<td>19.68 ± 0.67 a</td>
</tr>
<tr>
<td></td>
<td>Asturiano et al. (2003)</td>
<td>17.56 ± 0.21 b</td>
</tr>
<tr>
<td></td>
<td>Müller et al. (2004)</td>
<td>16.56 ± 0.16 b</td>
</tr>
<tr>
<td>Area (mm²)</td>
<td>Fresh</td>
<td>8.99 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>Asturiano et al. (2003)</td>
<td>9.08 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>Müller et al. (2004)</td>
<td>8.22 ± 0.13</td>
</tr>
</tbody>
</table>
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 attention!

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