# Protocol for the strip spawning of meagre females and *in vitro* fertilisation

Presenter: Sandra Ramos

Neil Duncan, (IRTA), Christian Fauvel, (IFREMER),

Gilberto Dutto, (IFREMER), Wendy Gonzalez, (IRTA).

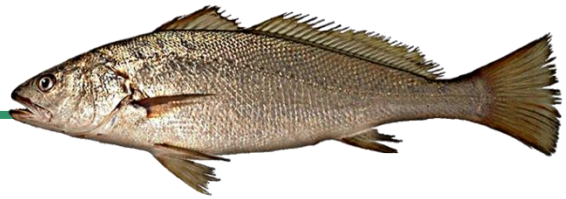
DIVERSITY, Work Package 2 – Meagre Reproduction

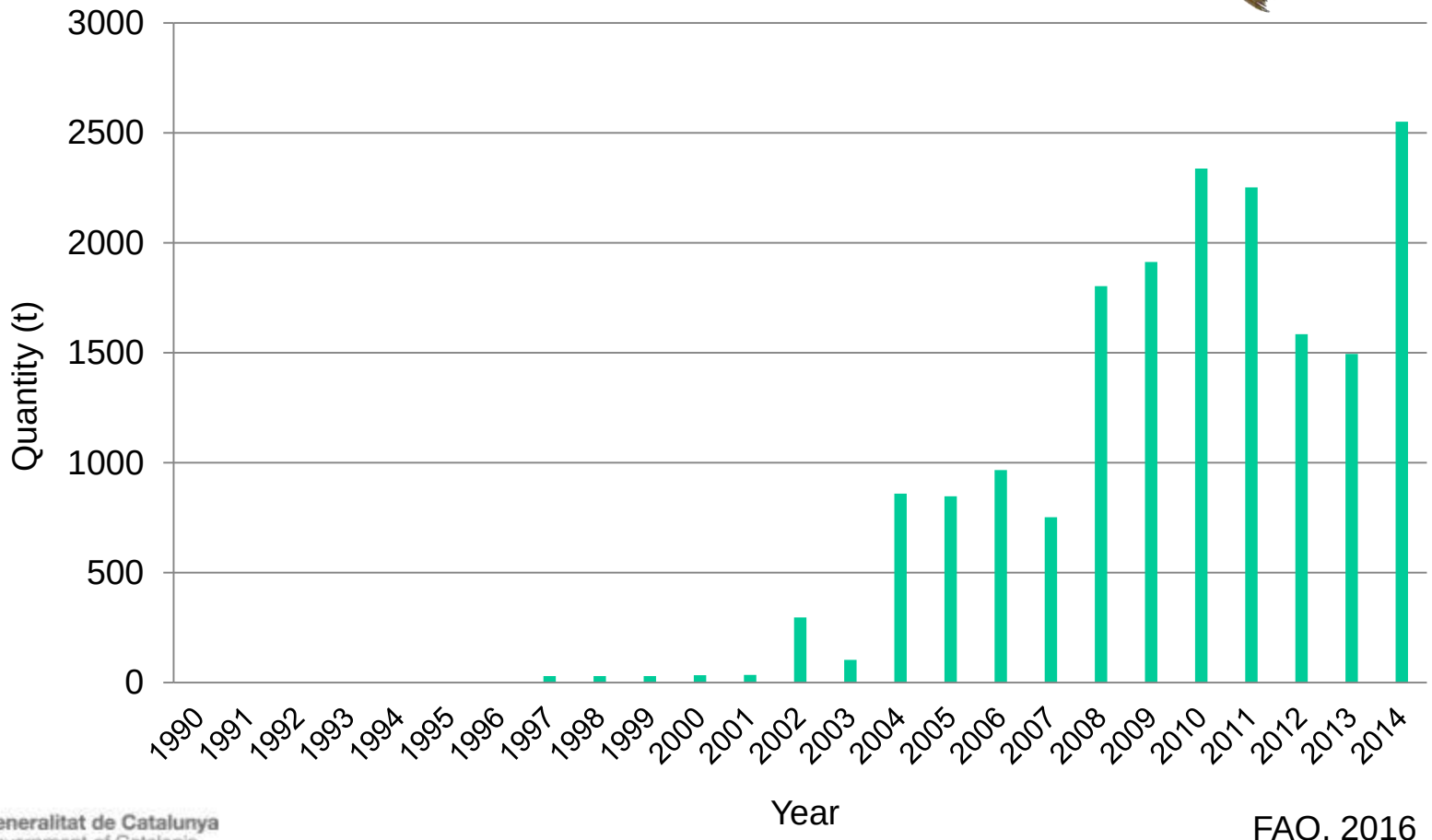
Task 2.4 Development of in vitro fertilization methods for planned crosses

Deliverable 2.7 Protocol for the strip spawning of meagre females and in vitro fertilization

Generalitat de Catalunya
Government of Catalonia

# European aquaculture production (t)



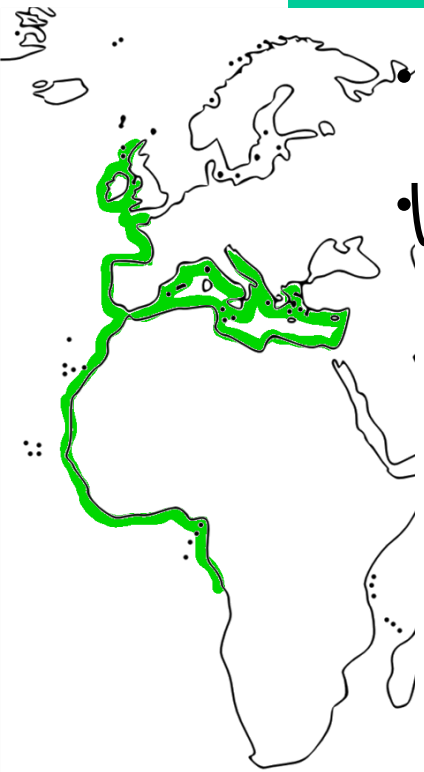



FAO, 2016

IRTA
RESEARCH & TECHNOLOGY
FOOD & AGRICULTURE

# Aquaculture production

Small genetic pool in base population for genetic breeding programs



Generalitat de Catalunya
Government of Catalonia

- Wild breeders selected from few populations from few specific areas.
- Seasonality in capture.

Main origin of aquaculture breeders has been from 1 commercial French hatchery established in 1997, which increases the possibility of inbreeding

Enrich the variability and establish
GENETIC BREEDING PROGRAMS
selecting the reproductive broodstock

# PRODUCE DESIRED FAMILIES FOR GENETIC BREEDING PROGRAMS

## PAIRED BREEDING WITH A CROSS MATING DESIGN

## *IN VITRO* FERTILIZATION

- Less stress of individuals
- High number of families
- Less period of time

IRTA
RESEARCH & TECHNOLOGY
FOOD & AGRICULTURE

## DEVELOP A PROTOCOL FOR ARTIFICIAL FERTILIZATION BY:

1. **EXPERIMENT 1** Determining the optimum time at which the egg is ready to be fertilized, establishing the time of ovulation after hormonal treatment.

2. **EXPERIMENT 2** Establishing the optimal sperm:egg ratio.

3. **Sperm analysis** Describing quantitative sperm parameters useful for quality assessment before and after hormonal treatment.
   - Concentration
   - Initial motility
   - Initial velocity
   - Variation of motility and velocity after sperm activation

## MATERIAL AND METHODS

### Breeder selection

### by the maturity status

♀

♂

- Total: 14 females
- Mean weights: 20.45 ± 6.22 kg

- Total: 5 males
- Mean weights: 15.94 ± 2.75 kg

# MATERIAL AND METHODS

## Breeder selection

## by the maturity status


Anaesthesia
(70.6 mg/L MS-222)

♀

♂

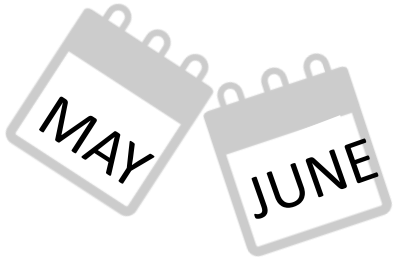
- Total: 14 females
- Mean weights: 20.45 ± 6.22 kg

- Total: 5 males
- Mean weights: 15.94 ± 2.75 kg

## MATERIAL AND METHODS

### Breeder selection

### by the maturity status



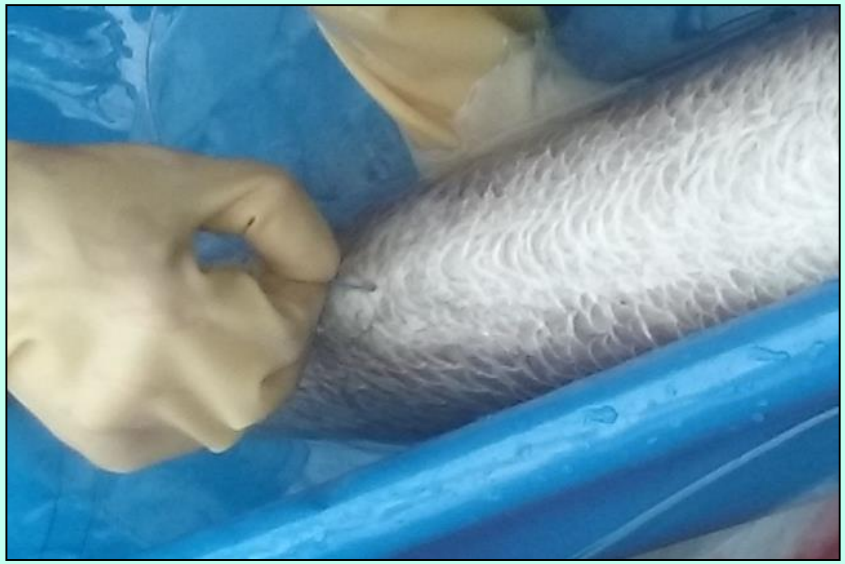

Anaesthesia
(70.6 mg/L MS-222)

♀

♂

♀

- Ovarian biopsies by cannulation.



## MATERIAL AND METHODS

### Breeder selection

### by the maturity status

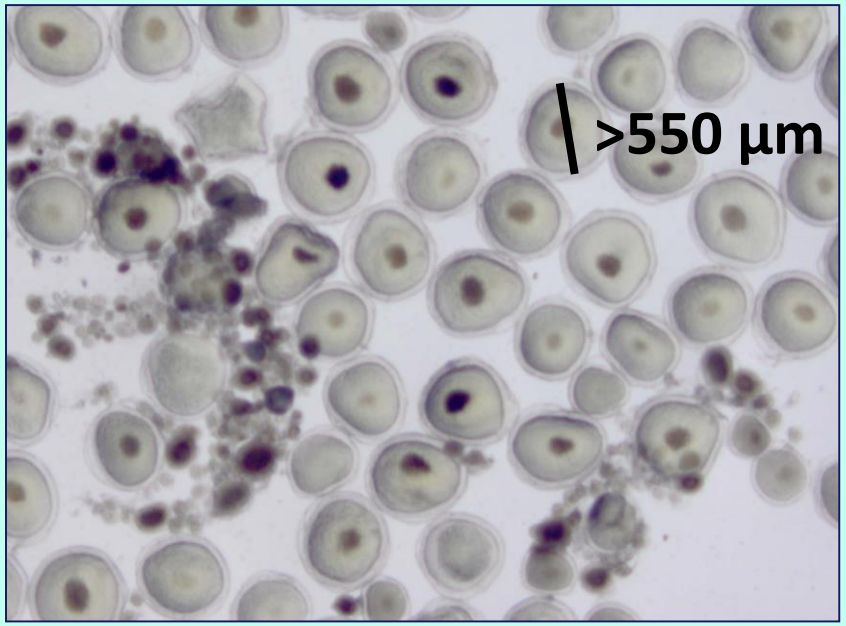

Anaesthesia
(70.6 mg/L MS-222)

♀

♂

♀

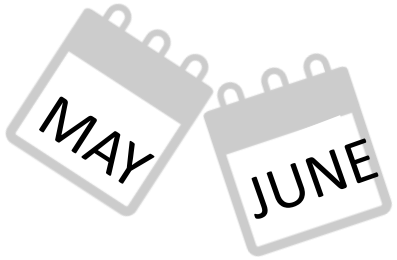
- Ovarian biopsies by cannulation
- **Selected females:** Oocytes in full vitellogenesis (diameter >550 µm)



# MATERIAL AND METHODS

## Breeder selection

## by the maturity status




Anaesthesia
(70.6 mg/L MS-222)

♀

♂

♀

- Ovarian biopsies by cannulation
- **Selected females:** Oocytes in full vitellogenesis (diameter >550 µm)

♂

- Release of sperm by abdominal pressure
- **Males in a spermiation stage of 2 and 3**

0 = not fluent
1 = fluent but no sample can be obtained
2 = fluent
3 = very fluent)

**Sperm analysis**

Generalitat de Catalunya
Government of Catalonia

# MATERIAL AND METHODS

## Sperm analysis

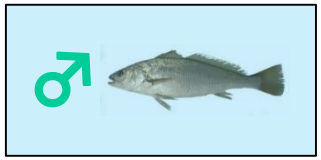
- **Before and after hormonal treatment.**
- 10 µL, 20 µL, 40 µL aliquots of diluted sperm in Leibovitz L-15 cell culture medium modified (1:4) + 1 mL of sea water with BSA (6.6 mL BSA/100mL sea water) in Eppendorf tubes for activation.
- 1 µL sample immediately pipetted into ISAS counting chamber.

Video recorded and sequences analysed with the Computer Assited Sperm Analysis (CASA) plugin, with open source software Image J.

- **Duration of sperm motility** (min)
- **Initial sperm motility** (%)
- **Initial average path velocity** (VAP, µm/s)
- **Variation of motility and VAP** after activation

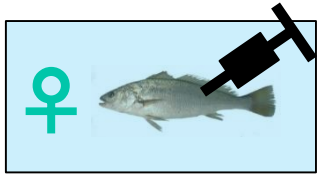
- Using a THOMA cell chamber, **sperm concentration** (number of spzoa/mL of milt)

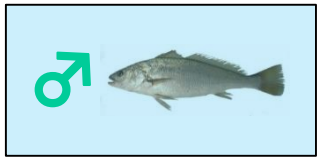
# MATERIAL AND METHODS



# MATERIAL AND METHODS

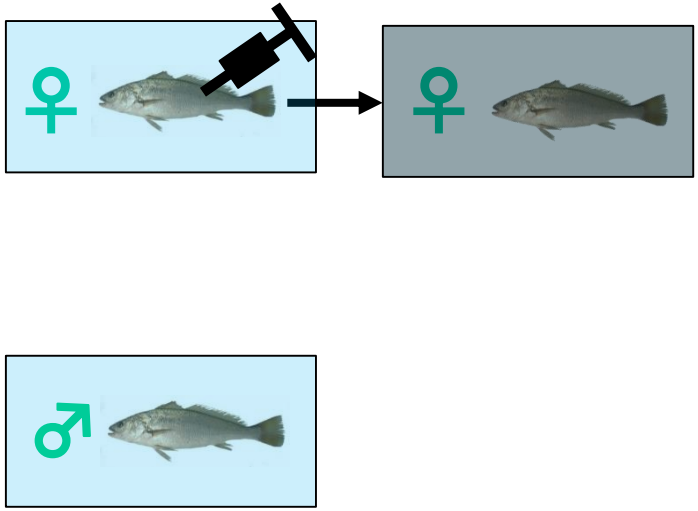
GnRHa intramuscular injection of 15 µg/kg



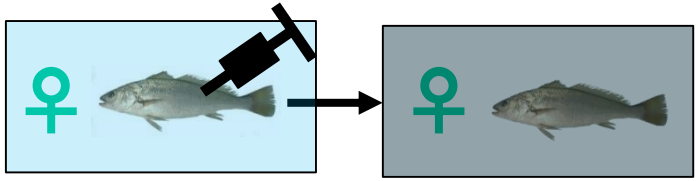
# MATERIAL AND METHODS

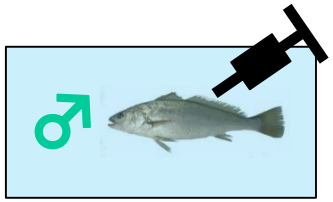
GnRHa intramuscular injection of 15 µg/kg



# MATERIAL AND METHODS

GnRHa intramuscular injection of 15 µg/kg

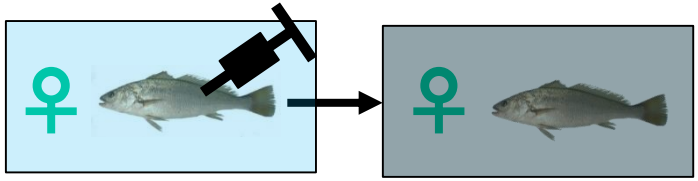


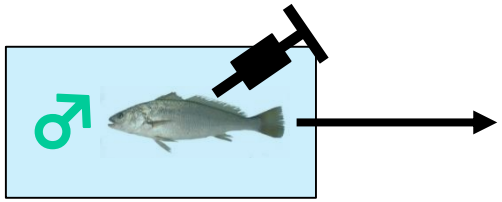


9-10 h after females

# MATERIAL AND METHODS

GnRHa intramuscular injection of 15 µg/kg





9-10 h after females

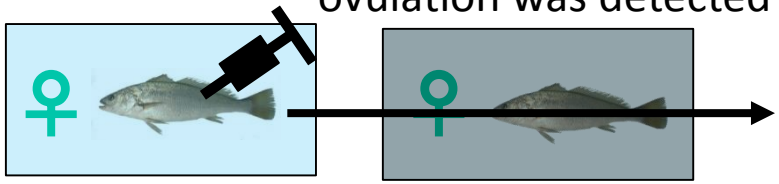
**Sperm collection**

- Diluted in Leibovitz.
- Stored above ice until required.

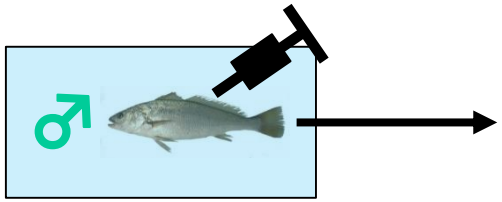
# MATERIAL AND METHODS

## EXPERIMENT 1: TIMING OF OVULATION

After 35 hours until ovulation was detected



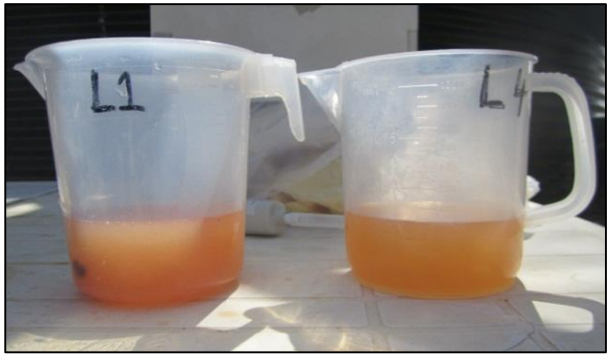
- Received abdominal massages every 2 and ½ hours.
- Time of ovulation= time ovulated eggs were first detected.



9-10 h after females

**Sperm collection**

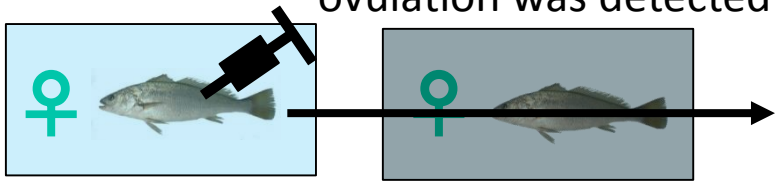
- Diluted in Leibovitz.
- Stored above ice until required.



# MATERIAL AND METHODS

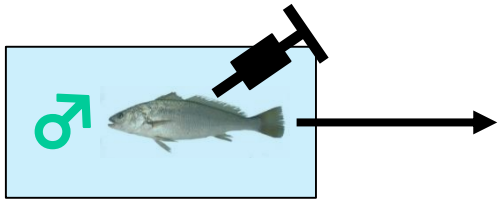
## EXPERIMENT 1: TIMING OF OVULATION

After 35 hours until ovulation was detected



- Received abdominal massages every 2 and ½ hours.
- Time of ovulation= time ovulated eggs were easily stripped.

→ **FERTILIZATION**



9-10 h after females

**Sperm collection**

- Diluted in Leibovitz.
- Stored above ice until required.

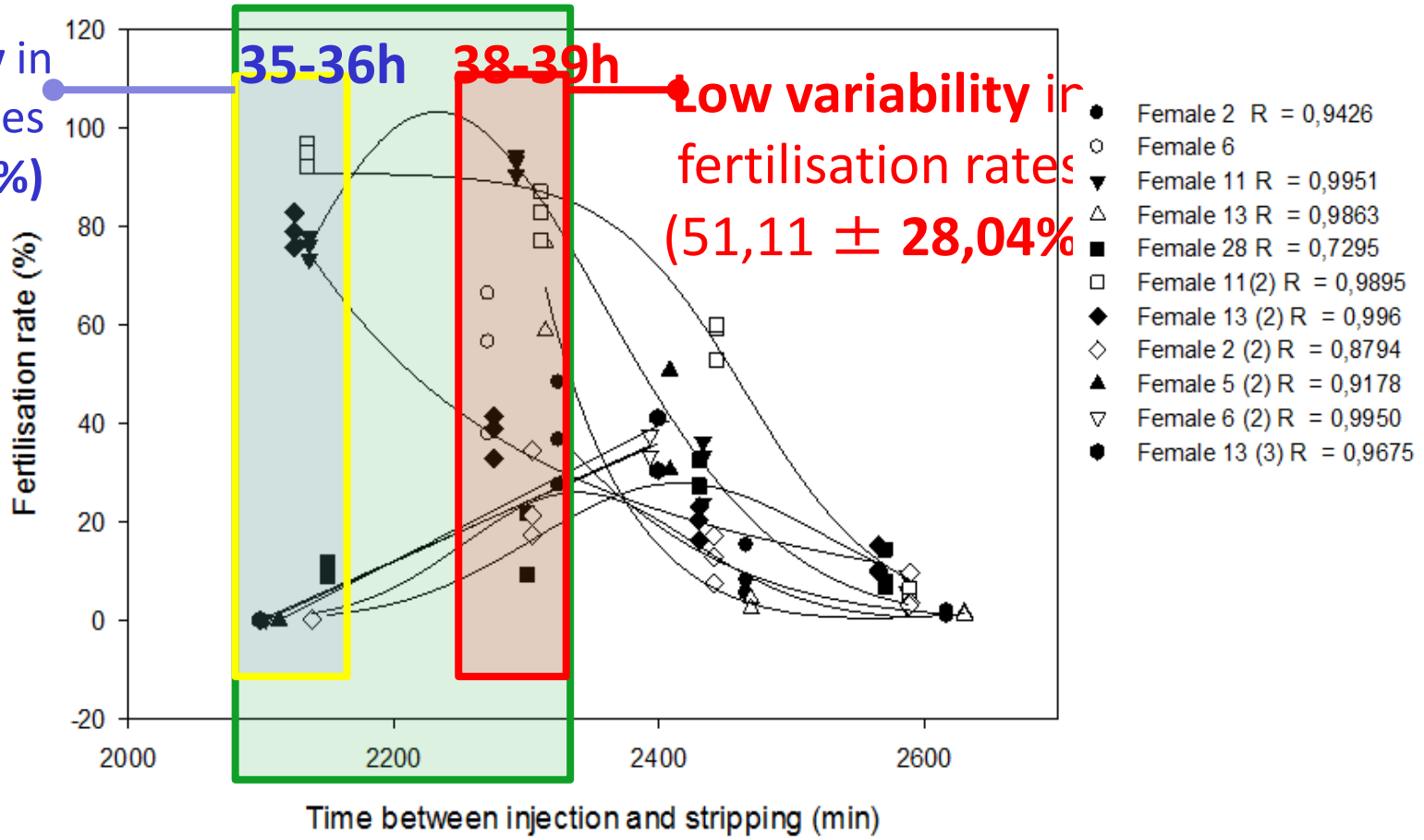

- Duplicates
- Batches of eggs were incubated (17,8 ºC to 18,4 ºC ) during 30h
- 400 eggs/incubator were examined under a binocular
- **SURVIVAL RATE** (% of number of eggs with embryos)

## RESULTS

## EXPERIMENT 1: TIMING OF OVULATION

### Ovulation between 35-39 hours

**High variability** in fertilisation rates (32,5 ± **43,50%**)



## RESULTS

## EXPERIMENT 1: TIMING OF OVULATION

The best stripping time was estimated to lie within 38-39 h following GnRHa injection at 18ºC, and corresponds to the completion of ovulation.

## EXPERIMENT 1: TIMING OF OVULATION

- Optimal timing of stripping established. ✓

## EXPERIMENT 1: TIMING OF OVULATION

- Optimal timing of stripping established. ✓

## EXPERIMENT 2: SPERM:EGG RATIO

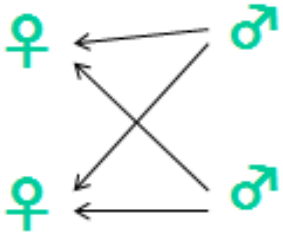
- Fertilization was carried out at **different sperm concentrations** in order to establish the minimum number of sperm to obtain maximal success in fertilization.

# MATERIAL AND METHODS

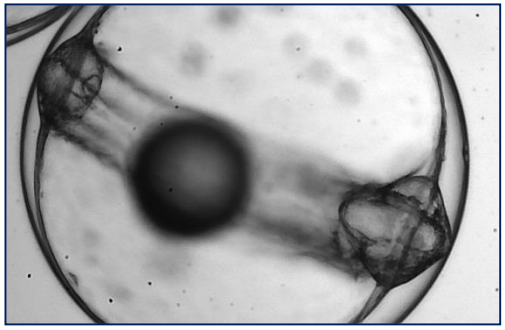
## EXPERIMENT 2: SPERM:EGG RATIO

### FERTILIZATION


- Carried out at different sperm concentration (number of sperm ranged from 2,675,000 to 407,500,000)
- 100 mL of sea water was added for activation.
- 200 mL added for the early embryonic development stages.

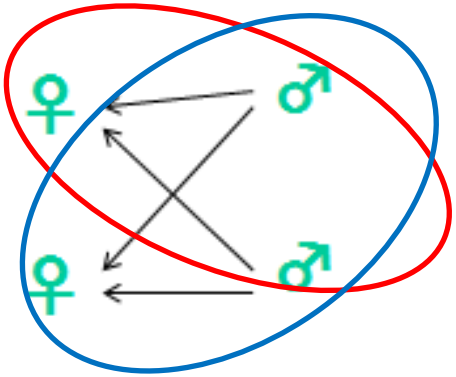



- After 2 hours: content poured onto a 200µm sieve and both floating and sinking eggs placed into a petri dish.
- **FERTILIZATION RATE** of 100 randomly selected eggs from each beaker.

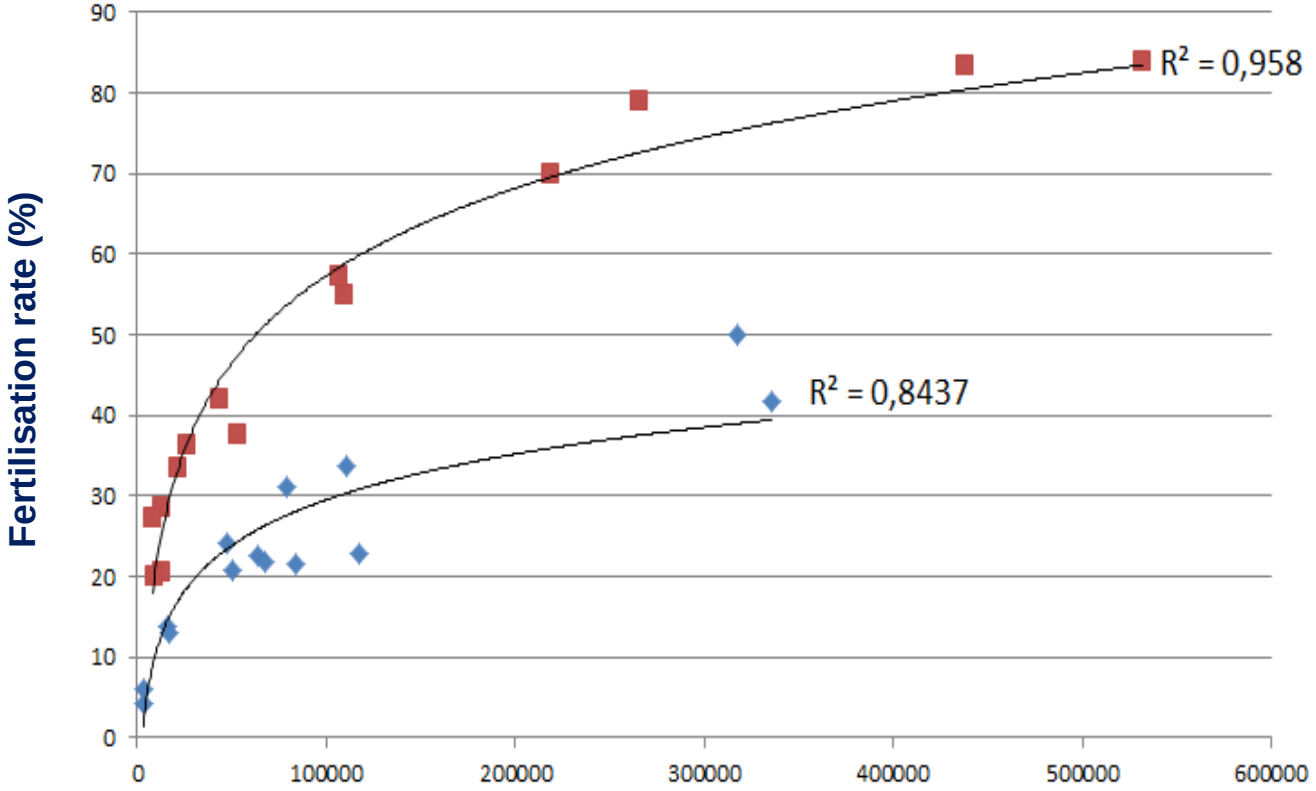


## RESULTS

## EXPERIMENT 2: SPERM:EGG RATIO



- No significant differences (P > 0.05) in the F.R. between males.
- Significant differences (P < 0.05) between females (different egg quality).
- Combined data in each female to obtain regressions.

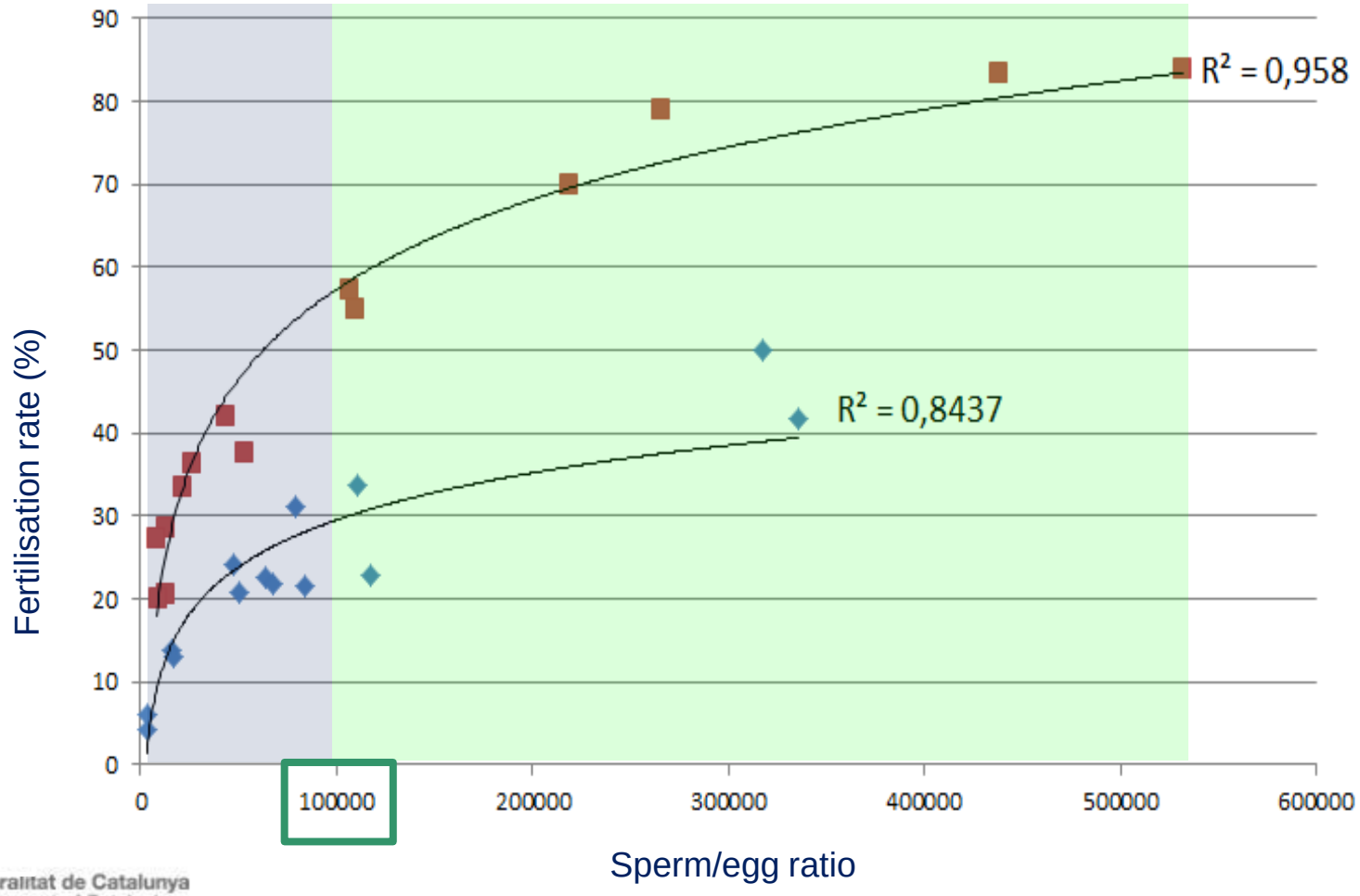


IRTA
RESEARCH & TECHNOLOGY FOOD & AGRICULTURE

# RESULTS

## EXPERIMENT 2: SPERM:EGG RATIO

Increase in F.R. Stabilised regression equations

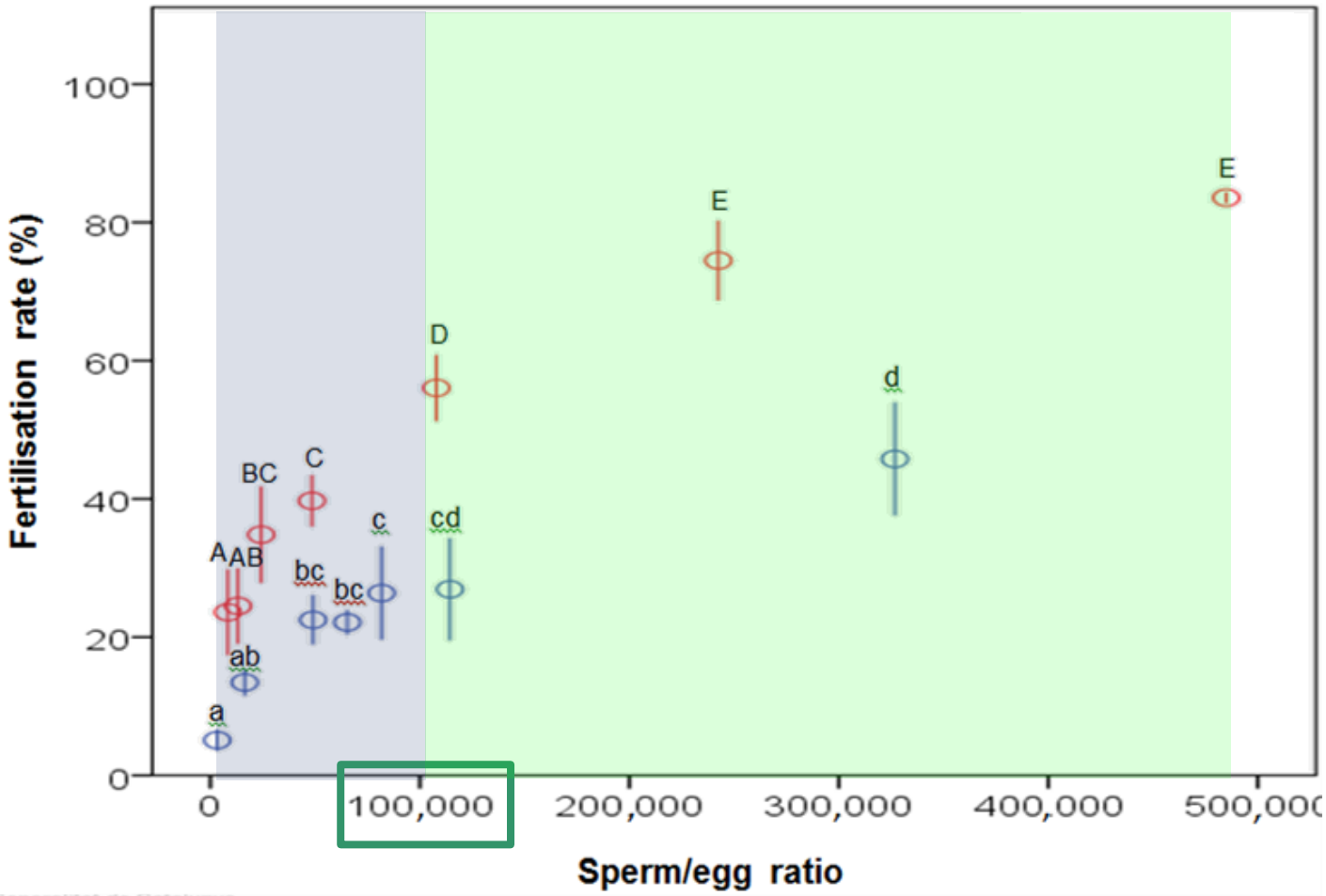


## RESULTS

## EXPERIMENT 2: SPERM:EGG RATIO

Stabilised regression equations

Increase in F.R. > 100,000 sperm:egg ratio → optimal fertilization

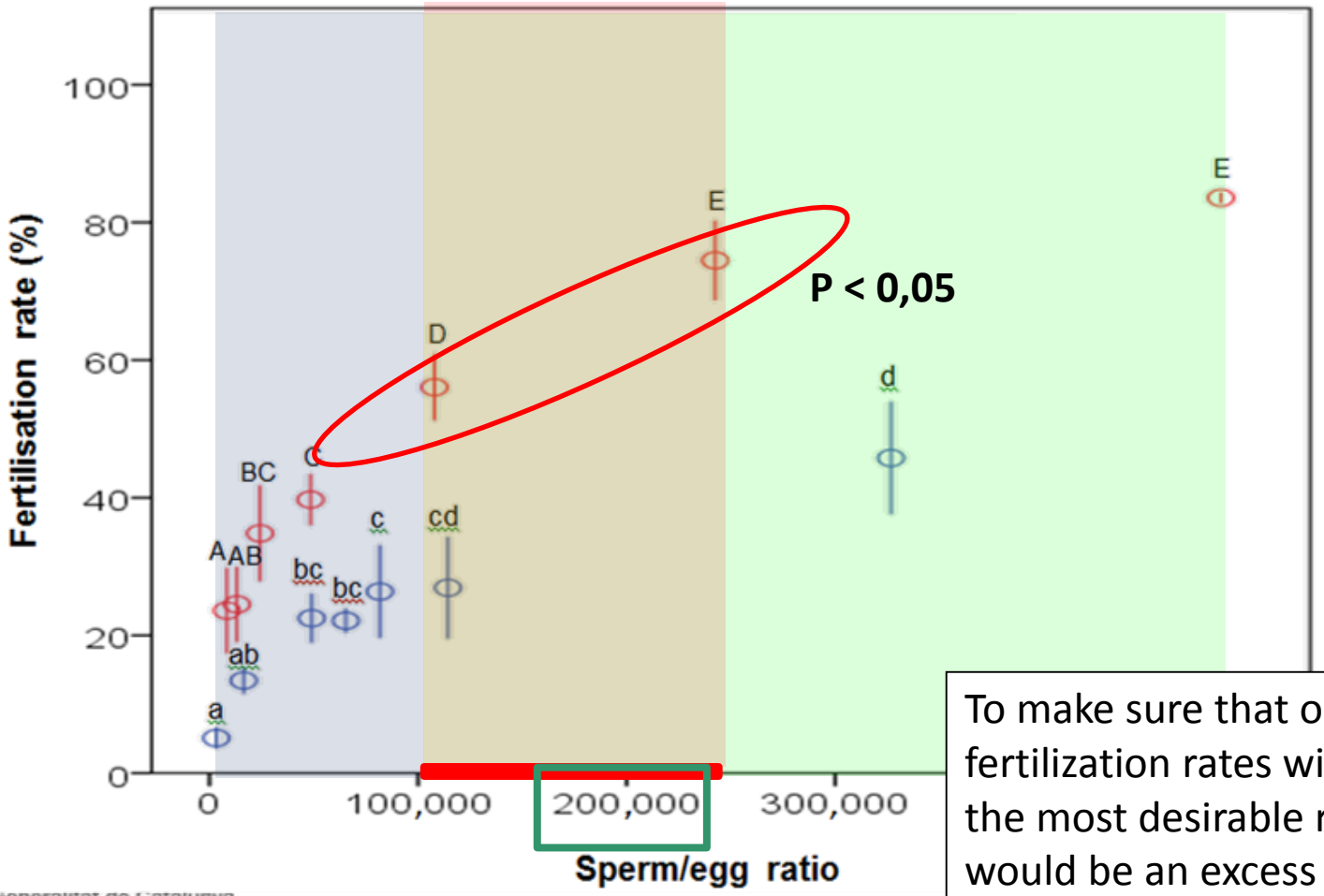


## RESULTS

## EXPERIMENT 2: SPERM:EGG RATIO

Stabilised regression equations

Increase in F.R. > 100,000 sperm:egg ratio → optimal fertilization




74.51 ± 3.81%

**159.08 ± 12.95 µm/ s**

66.61 ± 3.94%

**79.08 ± 4.25 µm/ s**

To make sure that optimal fertilization rates will be obtained, the most desirable ratio to use would be an excess of **200,000 sperm to egg ratio.**

## RESULTS

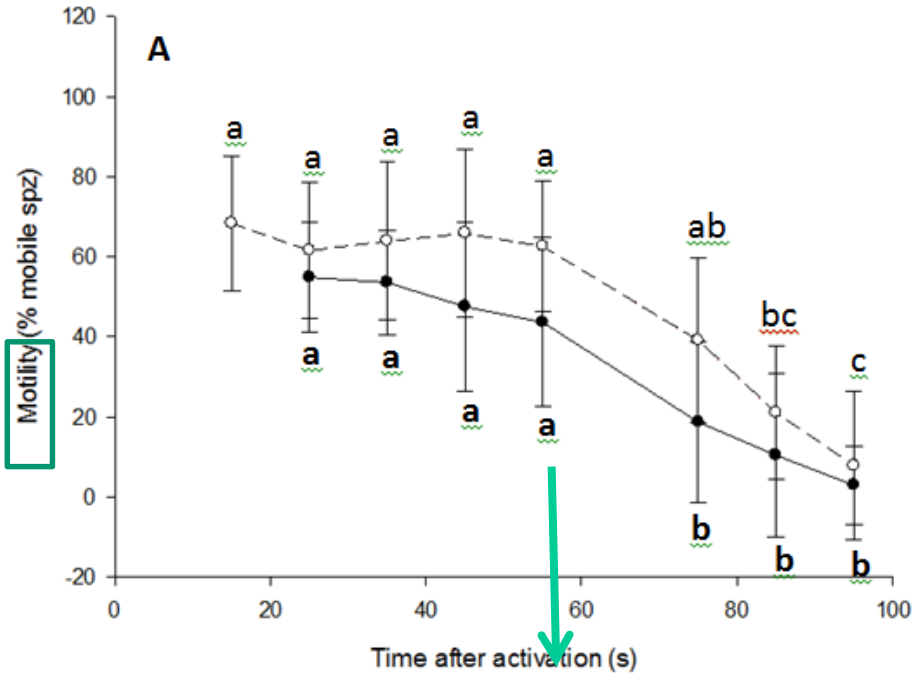
## SPERM CHARACTERISATION ♂

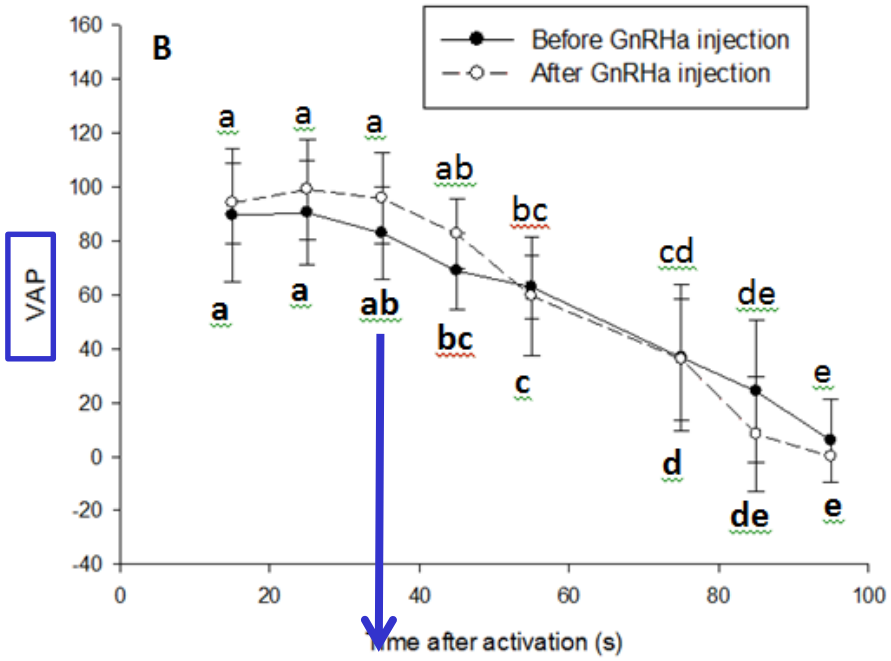
BEFORE HORMONAL TREATMENT

| Sperm concentration (spermatozoa/ mL) | Sperm duration (min) | Initial motility (%) | Initial VAP (µm/s) |
|---|---|---|---|
| $3.21 \cdot 10^{10} \pm 1.18^{a}$ | $1.71 \pm 0,29^{a}$ | $48.17 \pm 2.80^{a}$ | $90.69 \pm 5.76^{a}$ |

Similar to other studies

17 – 24 µm/ s [3]



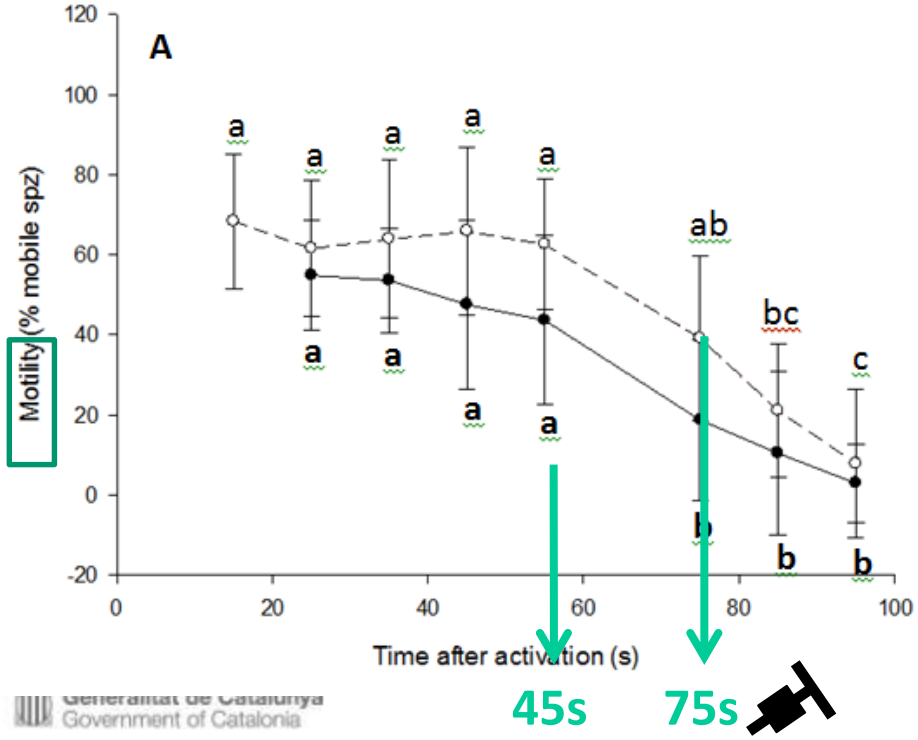

Generalitat de Catalunya
Government of Catalonia

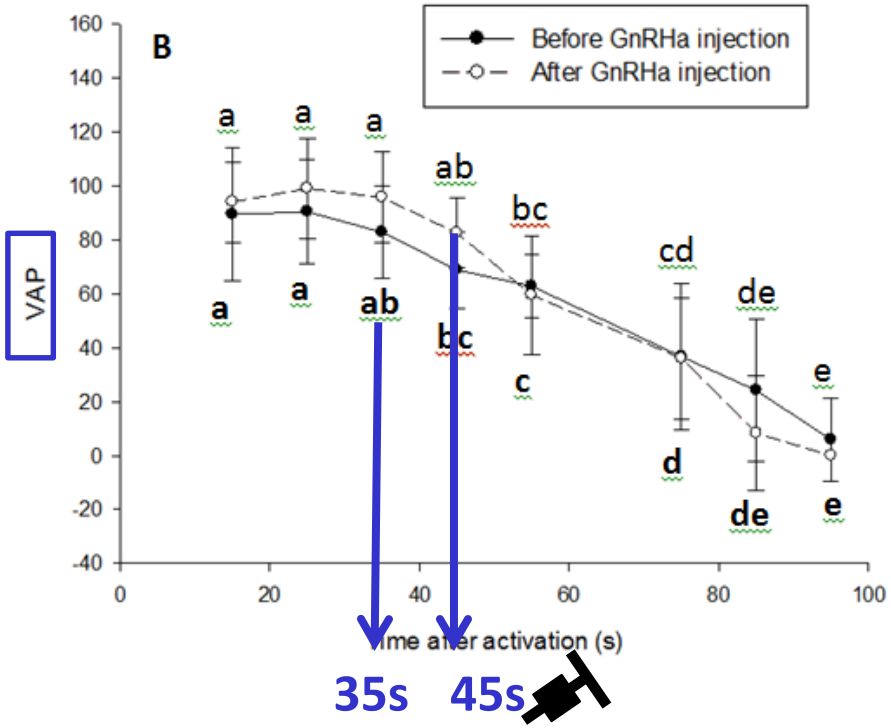
## RESULTS

## SPERM CHARACTERISATION ♂

BEFORE vs AFTER HORMONAL TREATMENT

| Before/ after GnRHa injection | Sperm concentration (spermatozoa/ mL) | Sperm duration (min) | Initial motility (%) | Initial VAP (µm/s) |
|---|---|---|---|---|
| Before | $3.21 \cdot 10^{10} \pm 1.18^{a}$ | $1.71 \pm 0,29^{a}$ | $48.17 \pm 2.80^{a}$ | $90.69 \pm 5.76^{a}$ |
| After | $2.76 \cdot 10^{10} \pm 0.62^{a}$ | $1.57 \pm 0.50^{a}$ | $66.76 \pm 15.83^{a}$ | $98.07 \pm 11.68^{a}$ |



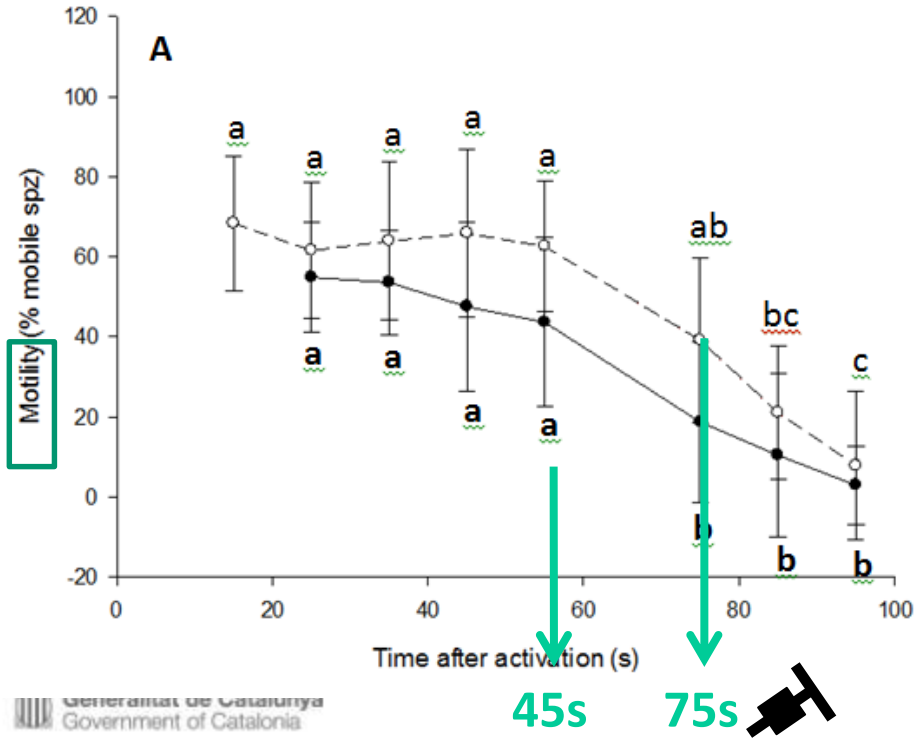



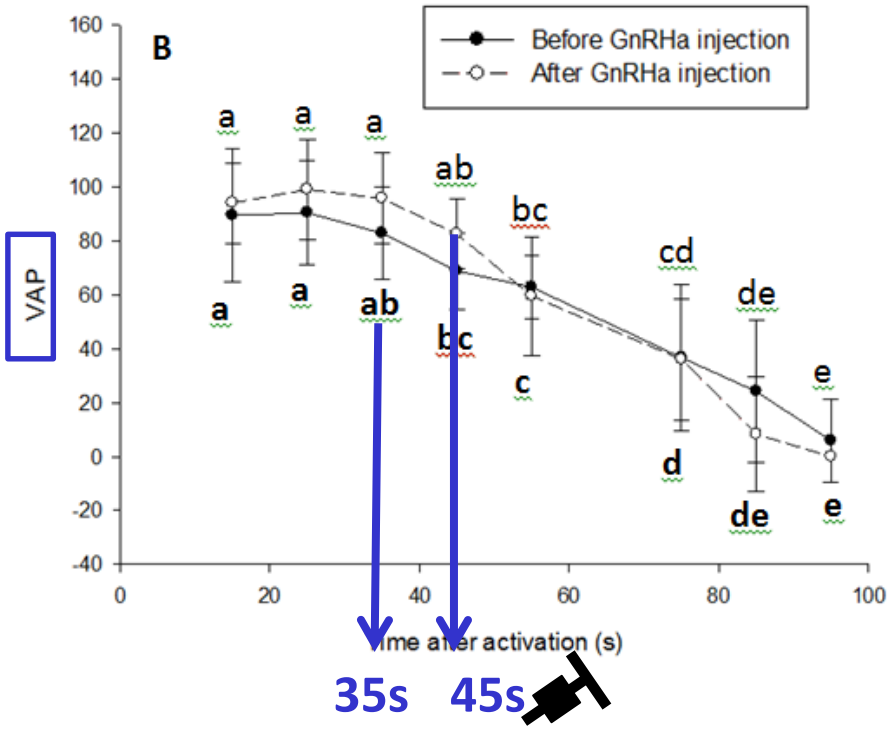
## RESULTS

## SPERM CHARACTERISATION ♂

### BEFORE vs AFTER HORMONAL TREATMENT

- Sperm **motility** and **velocities** serve as predictors of **fertilisation ability**. In some studies the highest coefficients of correlation were found for VAP (Gallego et al., 2013).
- If VAP is highly correlated with fertilizing ability, the optimal period for fertilization could be reduced to the **first 35 s**.
- **Hormonal treatment** induced probably **higher fertilization** success.



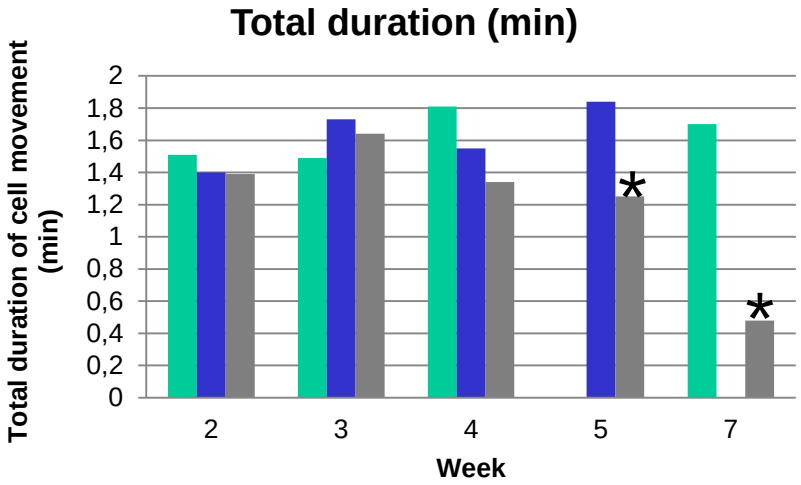



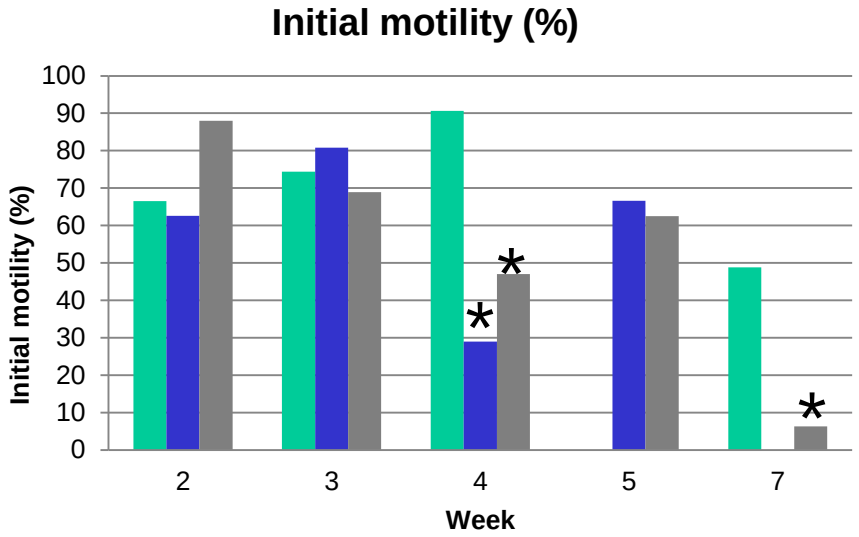

Generalitat de Catalunya
Government of Catalonia

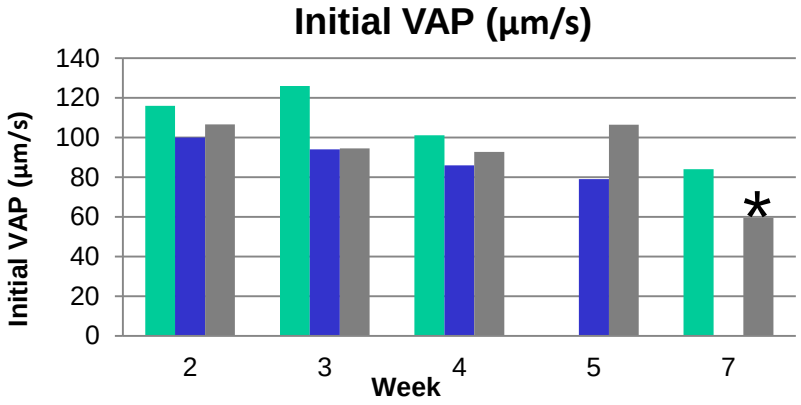
# RESULTS

## SPERM CHARACTERISATION ♂

### STORAGE




**Analysis time (h)**

- 10:30 h (fresh sperm)
- 13:30 h
- 17:30 h

Sperm was successfully stored in Leibovitz culture medium for 7 h with no loss of fertilisation ability compared to fresh sperm.

IRTA
RESEARCH & TECHNOLOGY
FOOD & AGRICULTURE

# PROTOCOL FOR THE ARTIFICIAL FERTILISATION OF MEAGRE

- The broodstock should be examined at 38 h post-injection at 18ºC to obtain optimum egg quality.
- For conventional production, a minimum of 200,000 spermatozoa per egg is recommended to ensure high fertilisation rates.
- The application of GnRHa should be recommended to induce males to extend sperm motility and velocity and facilitate sperm collection, especially towards the end of the spawning season.
- Similar protocols used in hatchery to make crosses: 3 females each with 40 males for a breeding program

Generalitat de Catalunya
Government of Catalonia

# Thank you for your attention