

Introduction: State of the art

Spawning protocol:

- Sex differentiation strategy - Gonochoric
- Size or age at first maturity -> 8kg wild 5-6 kg in culture
- The period of gametogenesis, and environmental conditions during the period - Winter - Spring 15+ °C feeding sardines, squid and Skretting vitalis-reprod
- The spawning period, and environmental conditions during the period – spring-summer 16-23 °C optimal 18-20°C
- Information hormonal induction of spawning.
 - Induction date - March-September
 - Characteristics of fish - 6-30 kg
 - Oocyte Size -> 0.56 mm
 - Hormone and dose. Single injection GnRHa 15 µg / kg
 - Spawn details: 0.2-3.5 million / spawn (mean 1.7 million) fertilization > 80%
- Latency Period – 48-72 hours (from application)
- Types of eggs – 0.9-1 mm pelagic
- Fecundity data - 282.000 to 498.000 per kg (130,000 per induced spawning)





Meagre

Introduction: State of the art



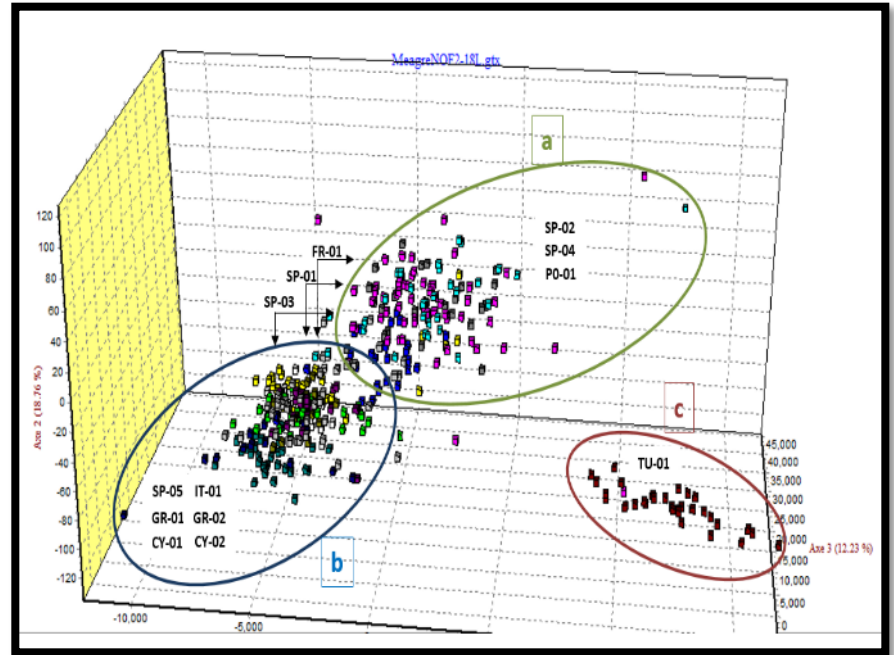
Introduction: Bottlenecks

Need for breeding programs.

Tools for the implementation of breeding programs

Reproductive control

To produce families from selected breeders that have the desired phenotype for future generations

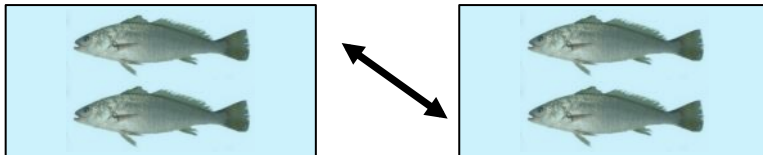


Diversify examined 2 approaches:

**PRODUCE DESIRED FAMILIES FOR
GENETIC BREEDING PROGRAMS**

**PAIRED BREEDING WITH A
CROSS MATING DESIGN**

***IN VITRO* FERTILIZATION**





Meagre

**INDUCED SPAWNING OF PAIRED MEAGRE
(*ARGYRO SOMUS REGIUS*) WITH MALE ROTATION: AN
APPROACH TO PRODUCE MULTIPLE FULL AND HALF-
SIB FAMILIES FOR GENETIC BREEDING PROGRAMS**

DIVERSIFY - EU PROJECT

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Meagre

INDUCED SPAWNING OF PAIRED MEAGRE (*ARGYROSOMUS REGIUS*) WITH MALE ROTATION. AN

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Paired spawning with male rotation of meagre *Argyrosomus regius* using GnRH α injections, as a method for producing multiple families for breeding selection programs



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^e CONACyT-UAN-Nayarit Centre for Innovation and Technological Transference, Av. Emilio M. González s/n, C.P. 63173, Tepic, Mexico

Experiments completed:

An experiment that examined the maximum number of spawnings in response to weekly GnRHa injections (HCMR in 2014)



Four experiments to examine paired spawnings with male rotation on a weekly basis (IRTA in 2014 and 2015; HCMR in 2015)



Common methods, induced spawning:

1. Females , > 550 μ m oocytes induced with single injection 15 μ g / kg GnRH α
2. Males with sperm, either:
 - single injection 15 μ g / kg GnRH α ,
 - single injection 7.5 μ g / kg GnRH α or
 - implant ~ 50 μ g / kg GnRH α

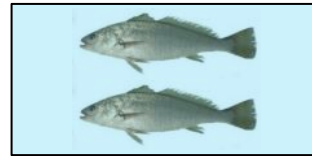
Common methods, egg quality:

1. Count number of eggs spawned, volumetric sub-samples
2. Fertilization, from egg (n>100) development when collected
3. Hatching and 5 day larval survival in 96 well plates, 2 plates / spawn



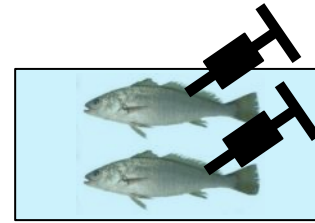
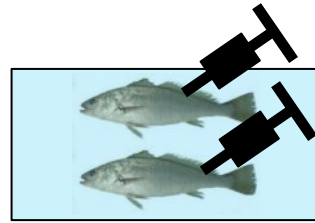
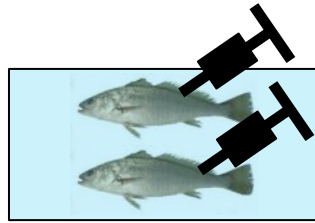
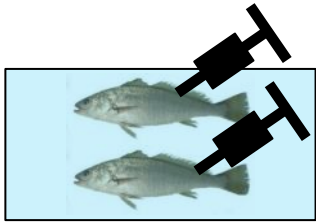
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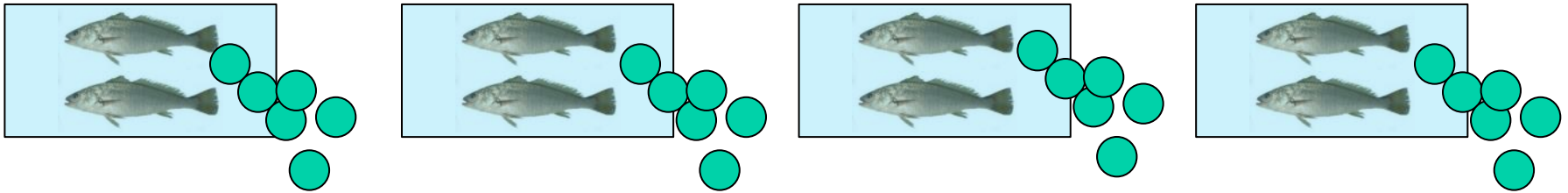
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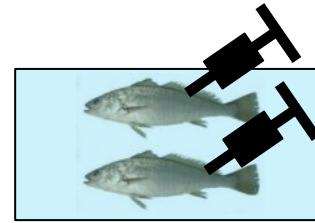
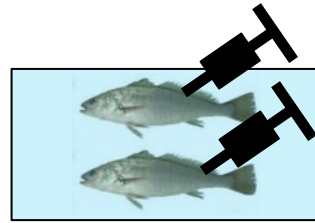
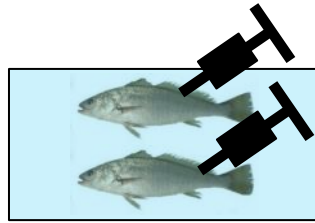
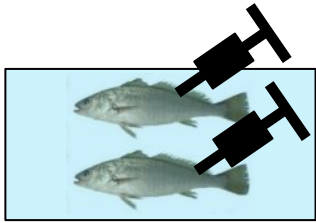
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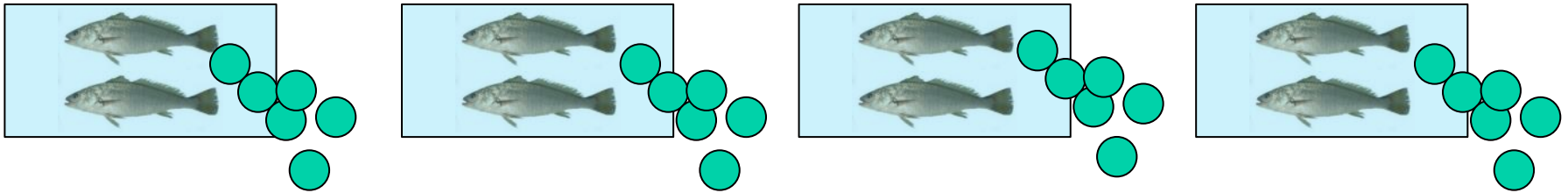
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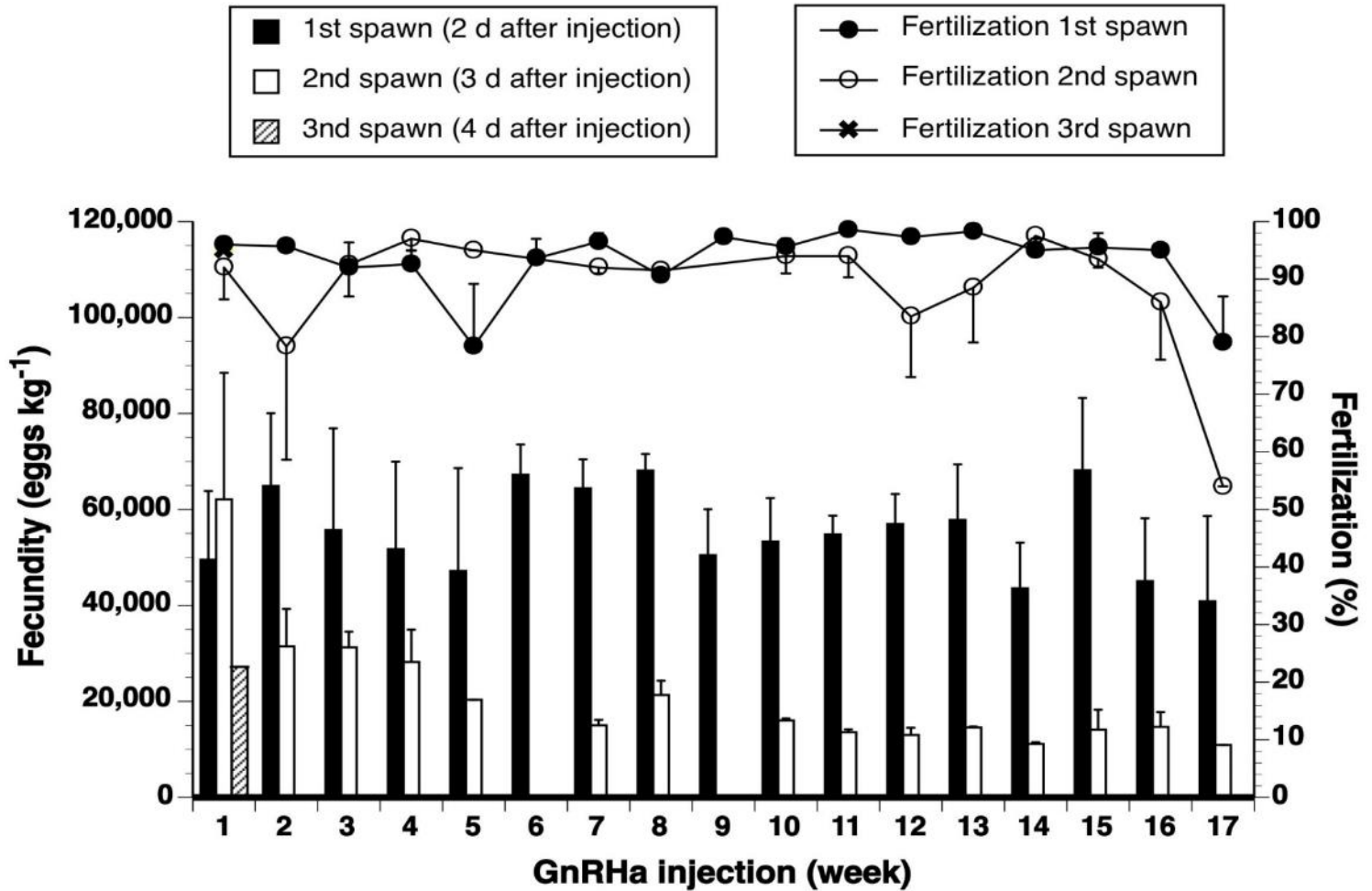


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Results: Fecundity



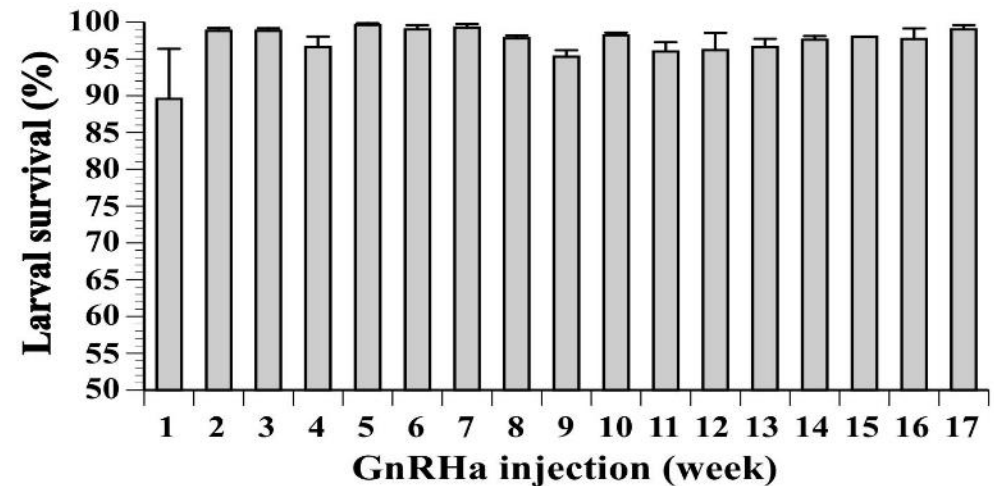
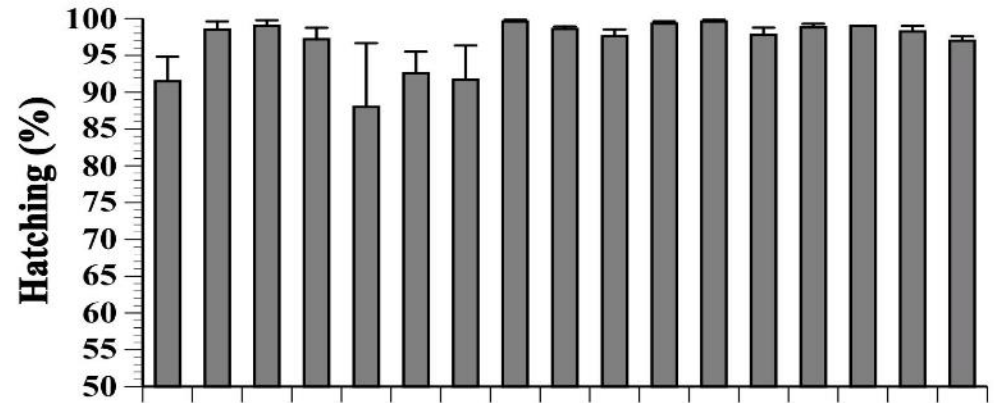
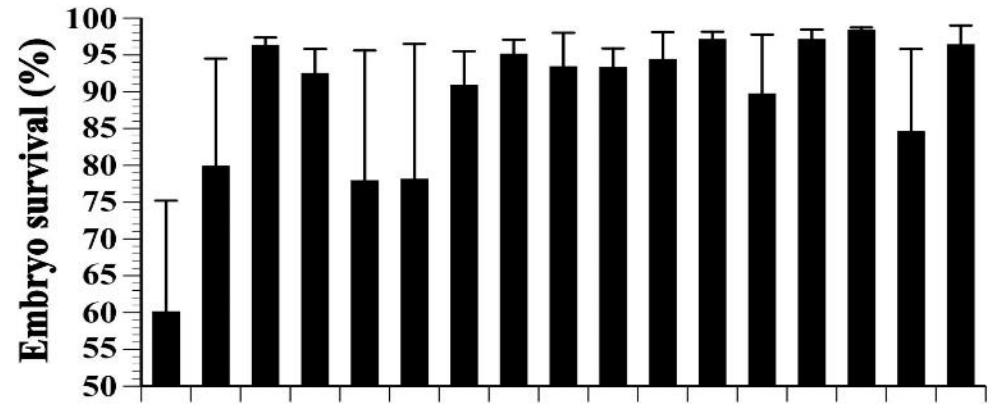


Results: Egg quality

High fertilization

High hatching %

High 5 day larval survival





Meagre

Conclusion

**Pairs of meagre
can be induced to spawn
high quality and quantity of eggs
on a weekly bases
for up to 17 weeks**



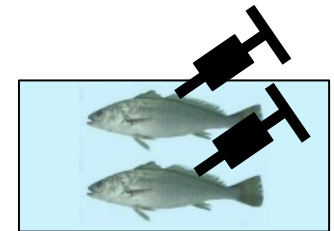
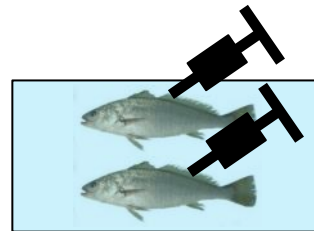
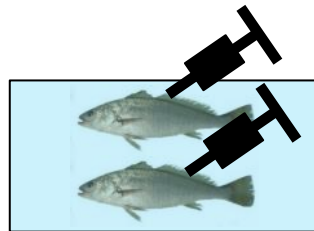
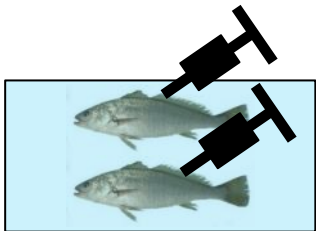
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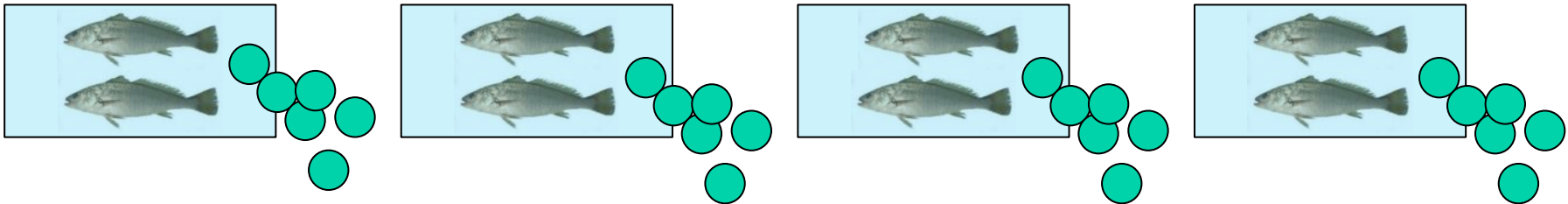
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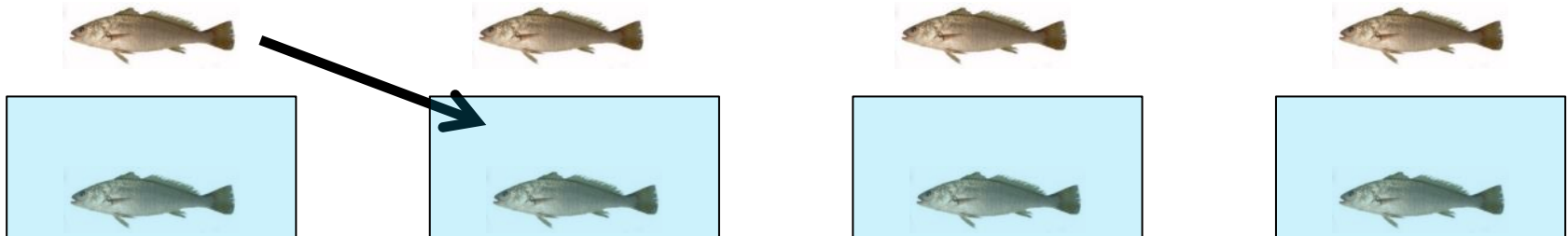
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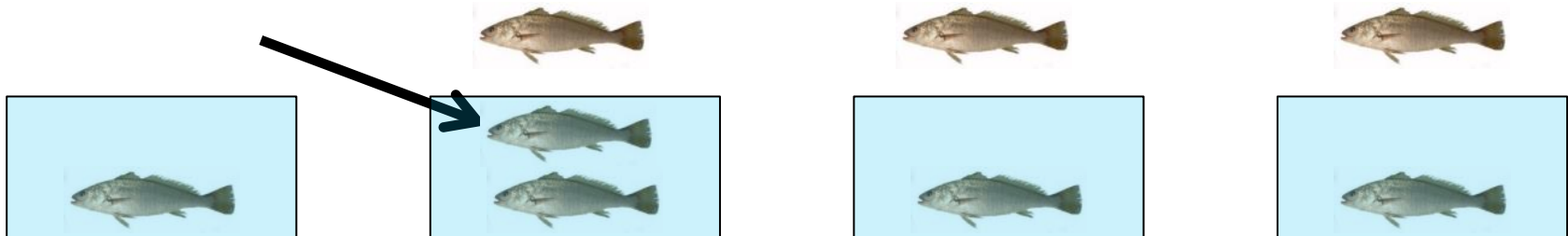
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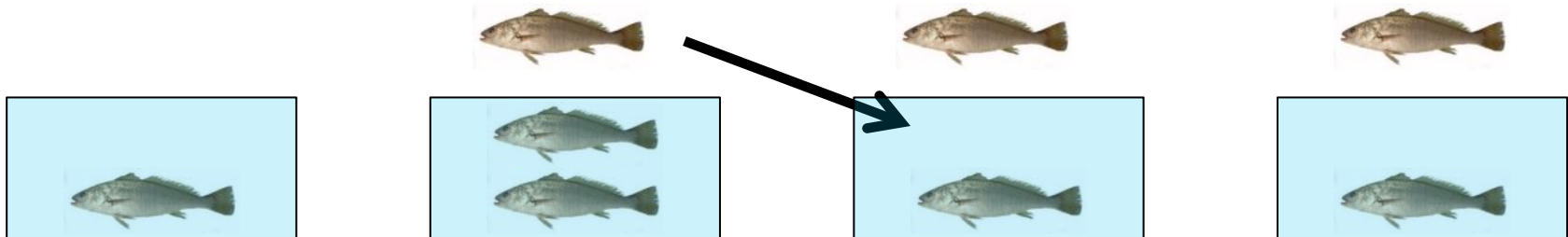
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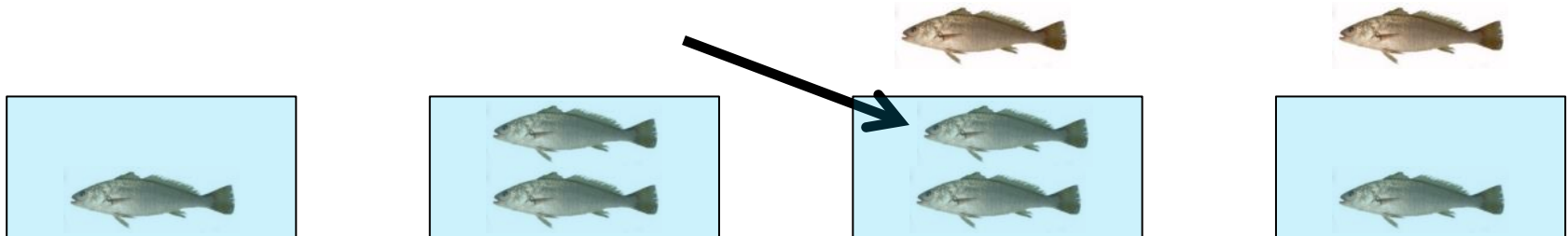
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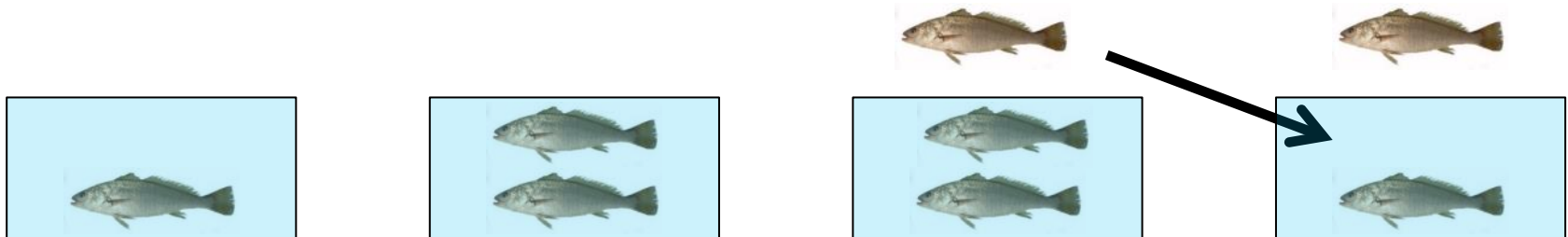
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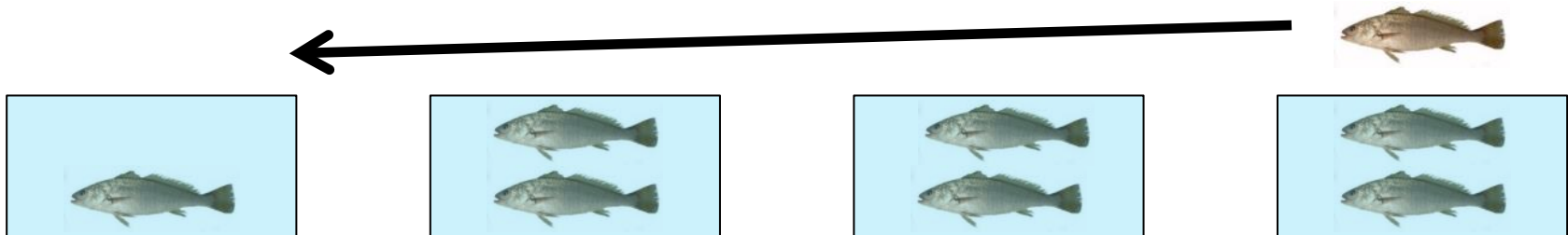
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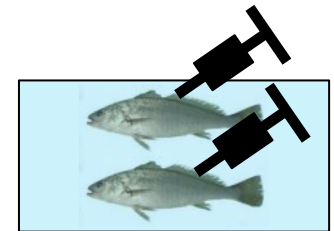
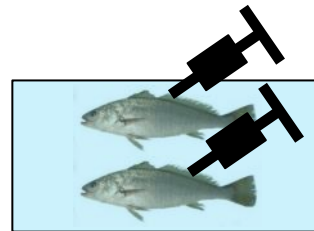
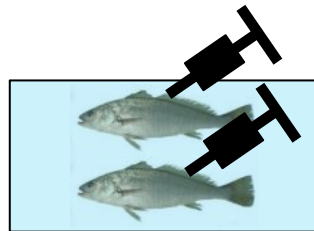
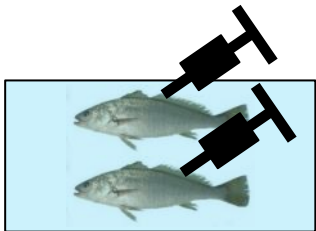
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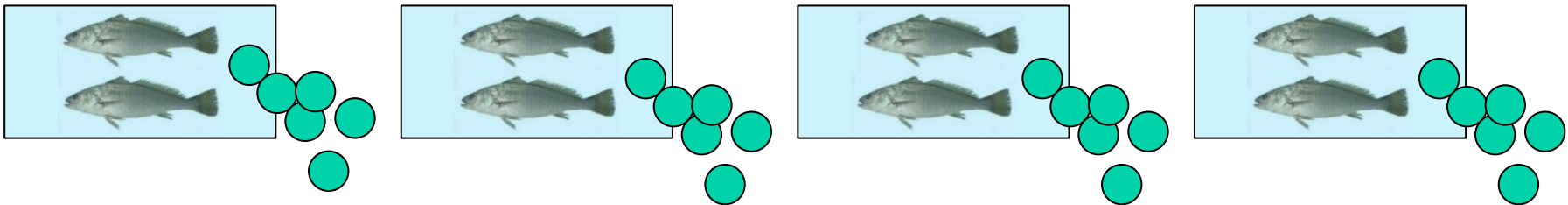
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Repeat each week to pair each male with each female

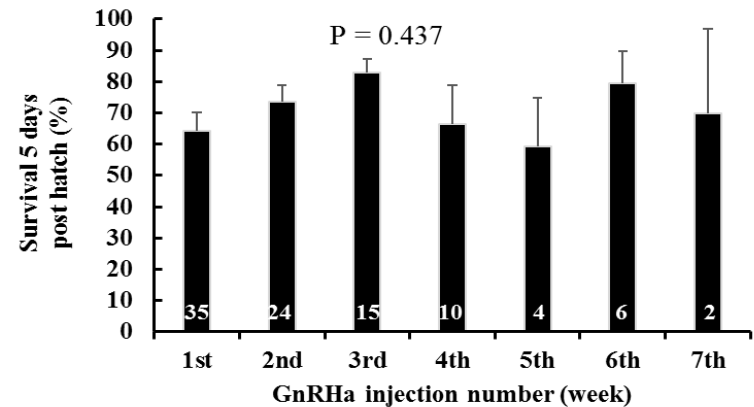
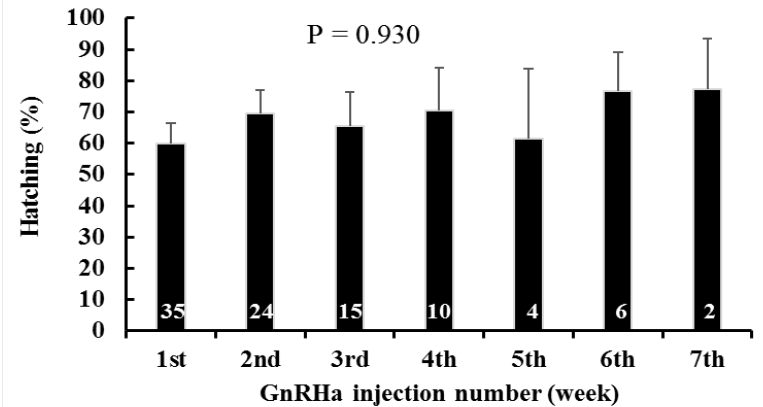
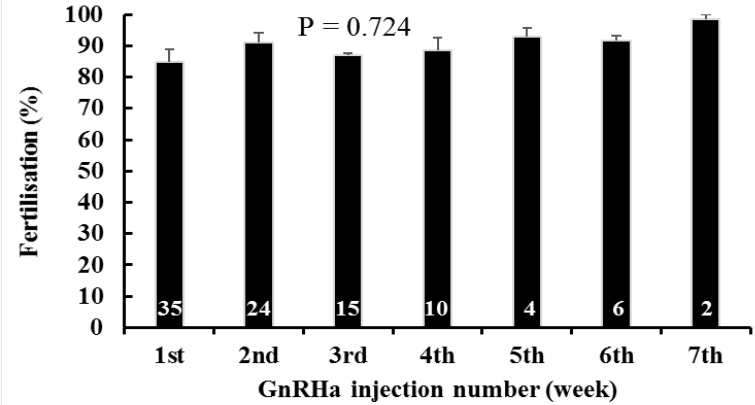


Results: Egg quality

High fertilization

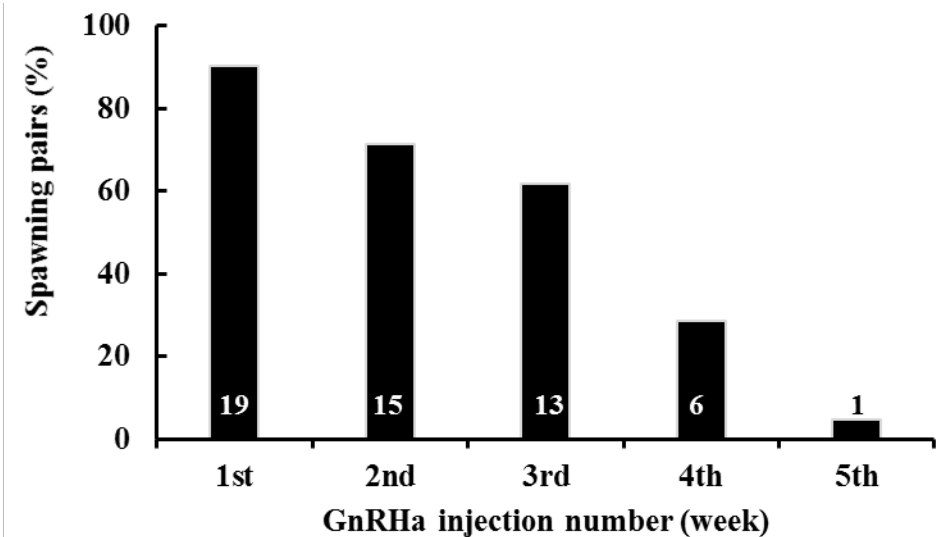
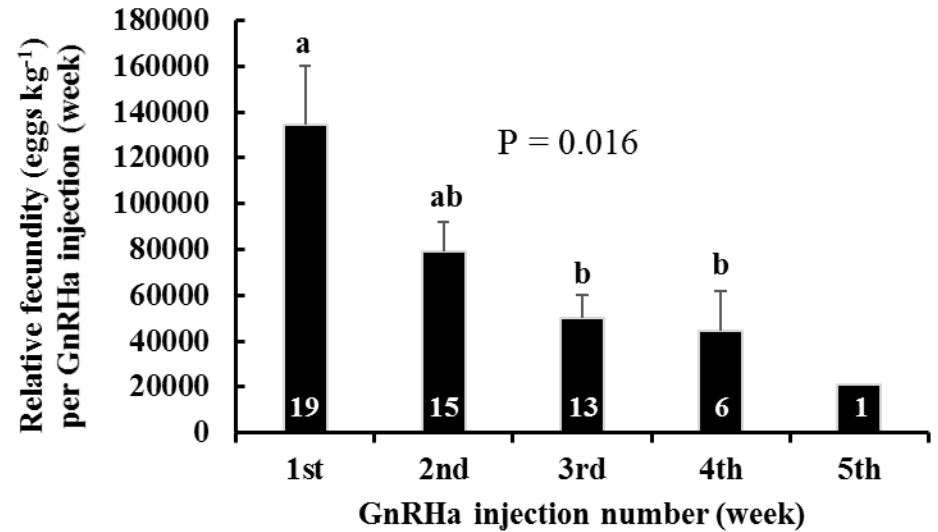
High hatching %

High 5 day larval survival



Results: Fecundity

Significant decline in fecundity

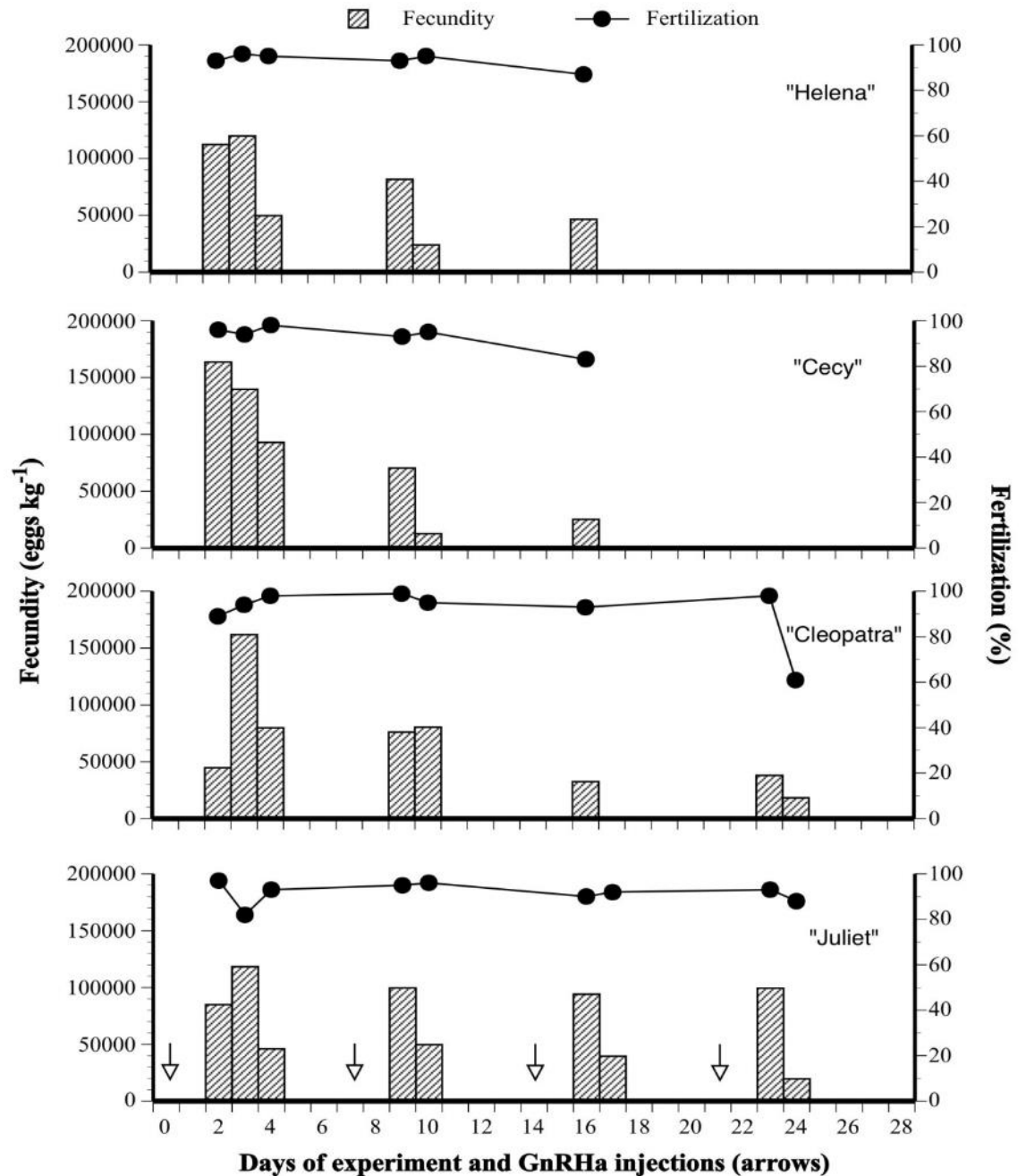




Results: Fecundity

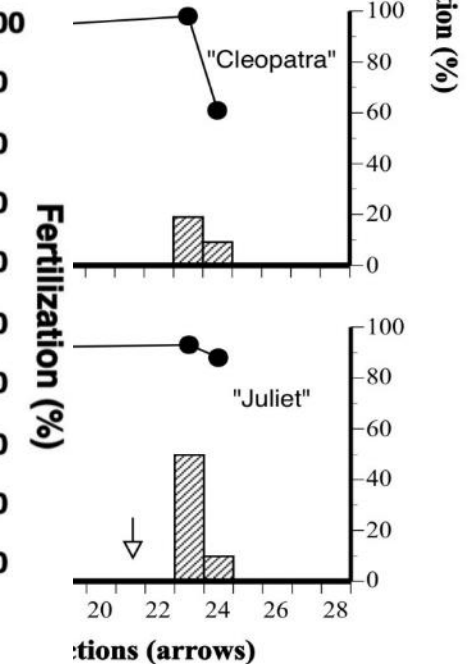
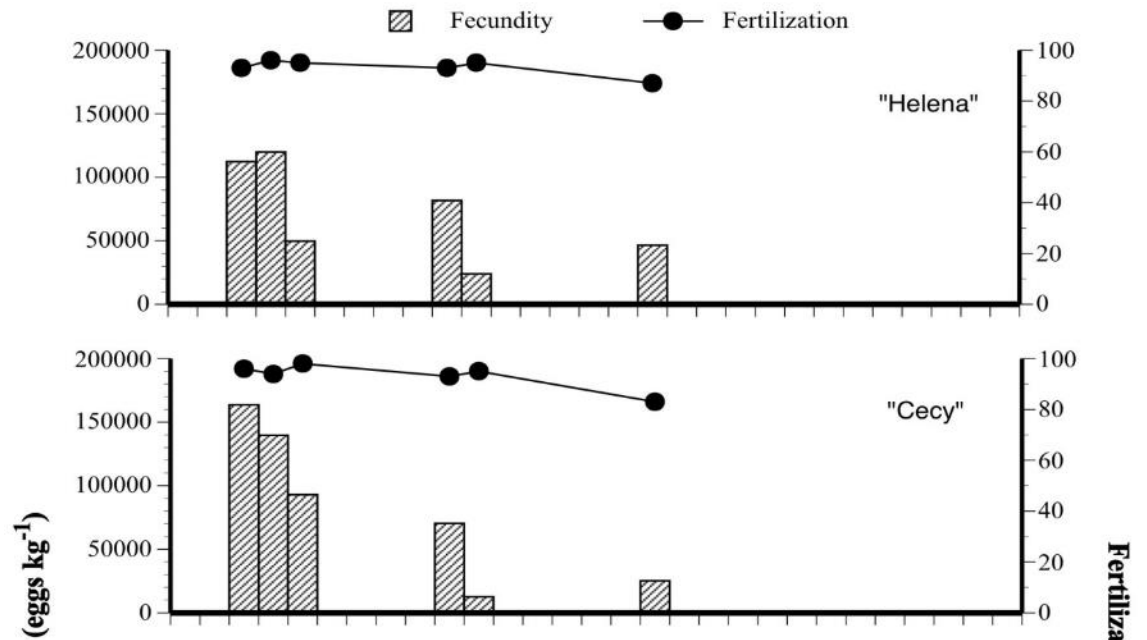
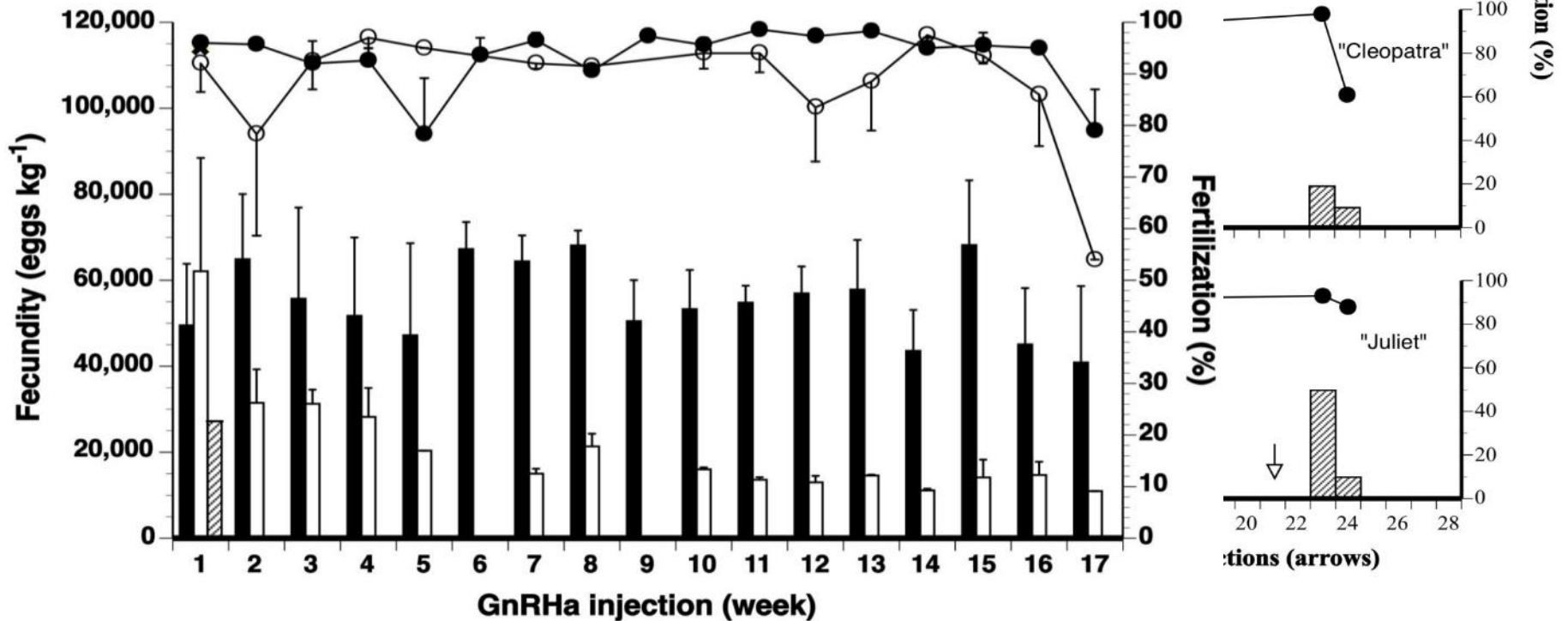
Two pairs did not spawn after 4th injection of GnRH_a
Data from HCMR

Significant decline in fecundity



Results: Fecundity

Effect of male rotation?
Stress?





Meagre

Conclusion

Paired spawning of meagre was possible for the production of known families from parents with known phenotypes

The success of spawning pairs with male rotation was 76% to produce 61 families (full and half-sib) that had >200,000 good quality eggs.





Meagre

Conclusion

Females produce 3 half-sib families before problems with maturity status

Males more flexible and did not lose maturity status





Meagre

Conclusion

A successful “proof of concept” for the paired spawning approach, highlighting the both the positive potential of the approach and possible drawbacks





Co-funded by the Seventh Framework Programme of the European Union



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This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration (KBBE-2013-07 single stage, GA 603121, DIVERSIFY).



**Thank you,
for all technical help
In HCMR and IRTA**



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

Sandra Ramos (IRTA), 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

DEVELOP A PROTOCOL FOR ARTIFICIAL FERTILIZATION BY:

- 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
- EXPERIMENT 1** Determining the optimum time at which the egg is ready to be fertilized, establishing the time of ovulation after hormonal treatment.
- EXPERIMENT 2** Establishing the optimal sperm:egg ratio.

MATERIAL AND METHODS

Breeder selection

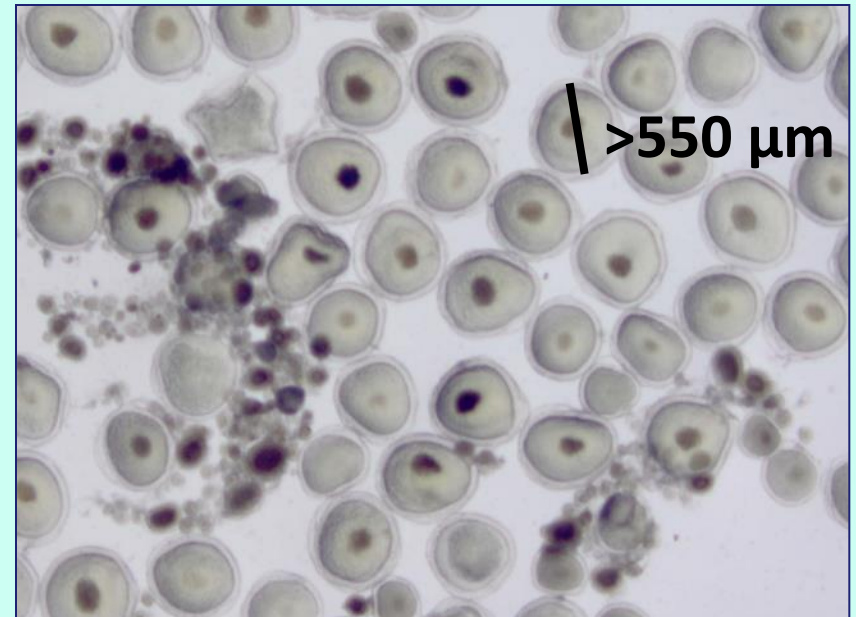
by the maturity status



Anaesthesia
(70.6 mg/L MS-222)



- Ovarian biopsies by cannulation
- **Selected females:** Oocytes in full vitellogenesis (diameter $>550 \mu\text{m}$)



MATERIAL AND METHODS

Breeder selection

by the maturity status



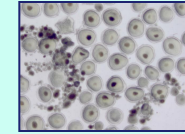
Anaesthesia
(70.6 mg/L MS-222)



Total: 14
females
20.45 ± 6.22 kg
Total: 5 males
15.94 ± 2.75 kg



- 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

Sperm analysis

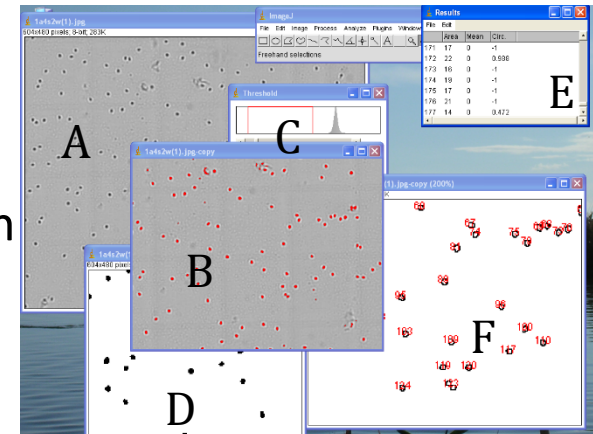


- **Before and after hormonal treatment.**
- 10 μ L, 20 μ L, 40 μ L aliquots of **diluted sperm** in Leibovitz L-15 cell culture medium modified (1:4) + 1 mL of **sea water with BSA** (6.6 mL BSA/100mL sea water) in Eppendorf tubes for activation.
- 1 μ L **sample immediately pipetted into ISAS** counting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



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

- **Duration of sperm motility (min)**
 - **Initial sperm motility (%)**
 - **Initial average path velocity (VAP, μ m/s)**
 - **Variation of motility and VAP after activation**
-
- Using a THOMA cell chamber,
 - **sperm concentration** (n° spzoa/mL of milt)

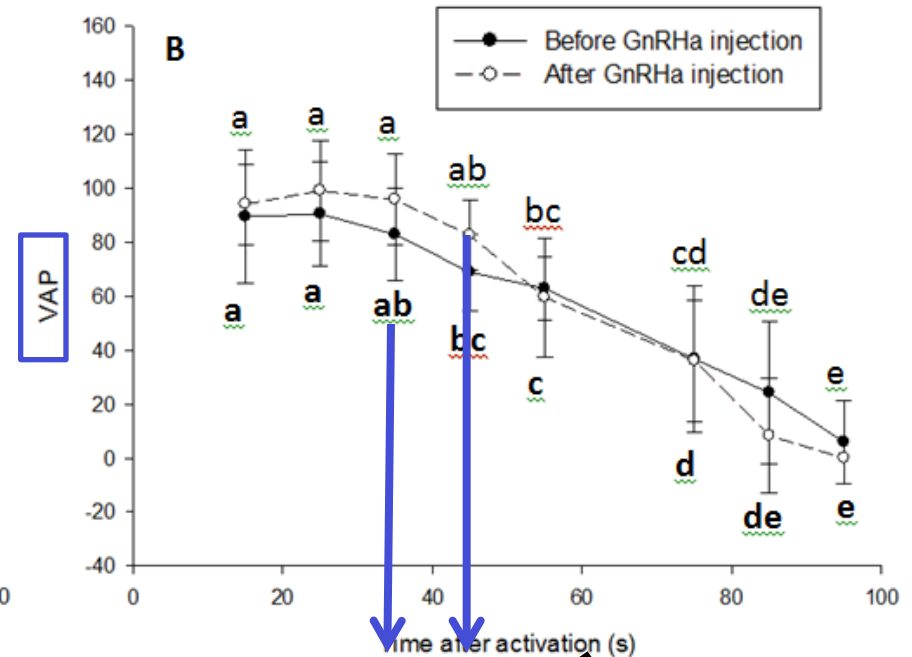
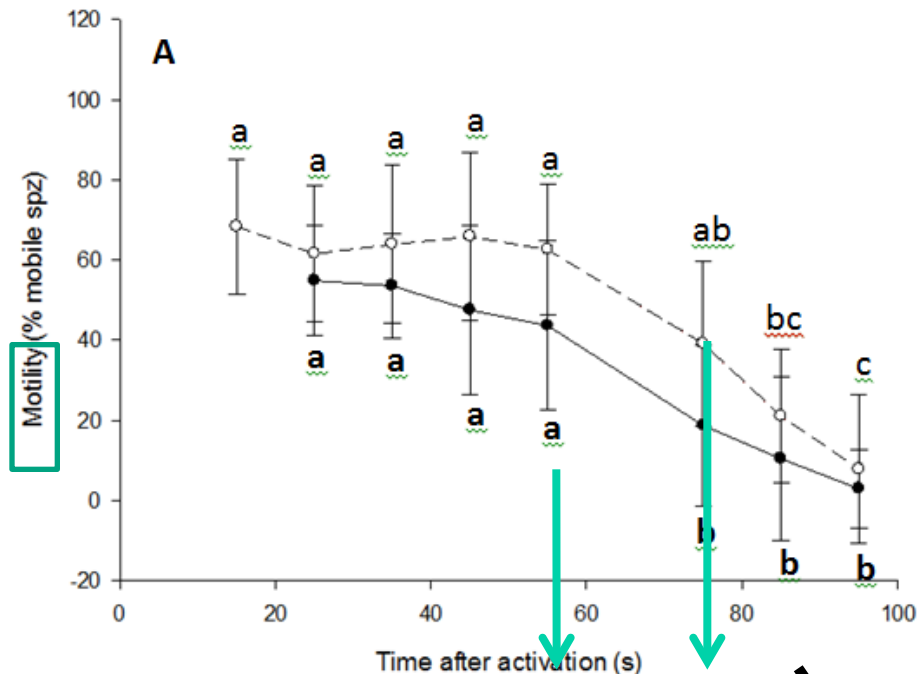


RESULTS

SPERM CHARACTERISATION ♂

BEFORE vs AFTER HORMONAL TREATMENT

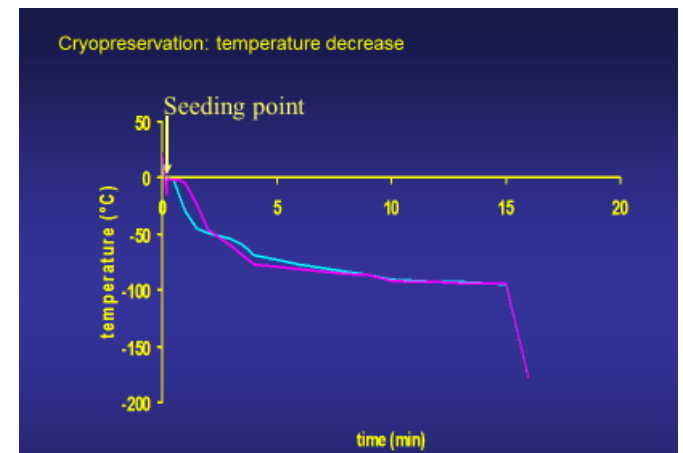
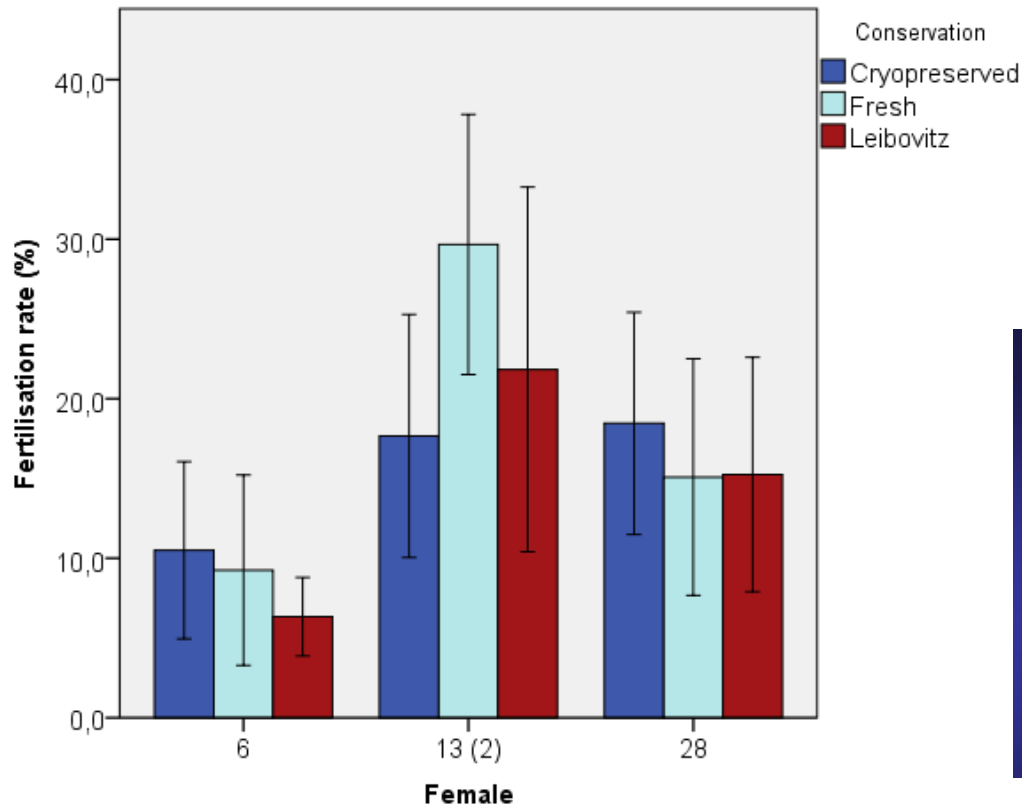
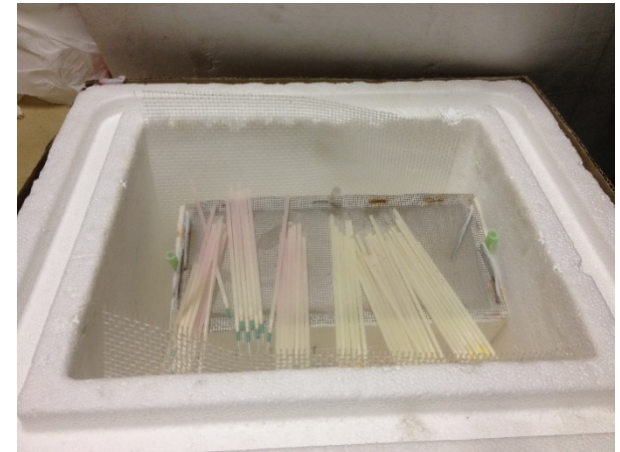
Before/ after GnRHa injection	Sperm concentration (spermatozoa/ mL)	Sperm duration (min)	Initial motility (%)	Initial VAP (µm/s)
Before	$3.21 \cdot 10^{10} \pm 1.18^a$	$1.71 \pm 0,29^a$	48.17 ± 2.80^a	90.69 ± 5.76^a
After	$2.76 \cdot 10^{10} \pm 0.62^a$	1.57 ± 0.50^a	66.76 ± 15.83^a	98.07 ± 11.68^a



RESULTS

Sperm storage

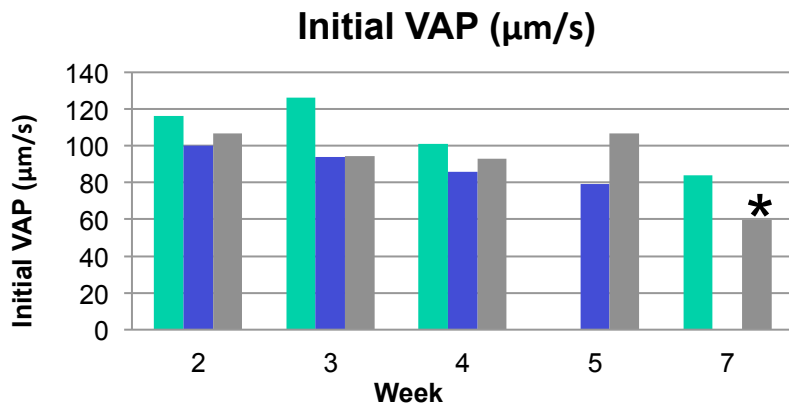
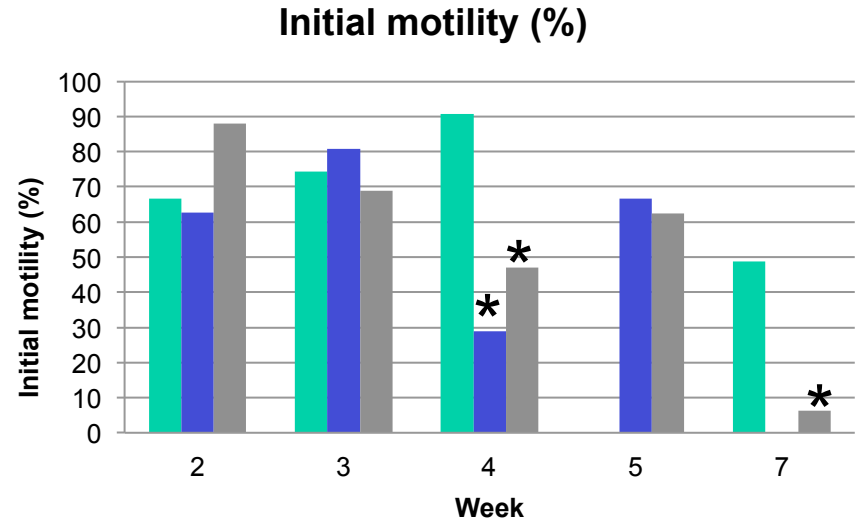
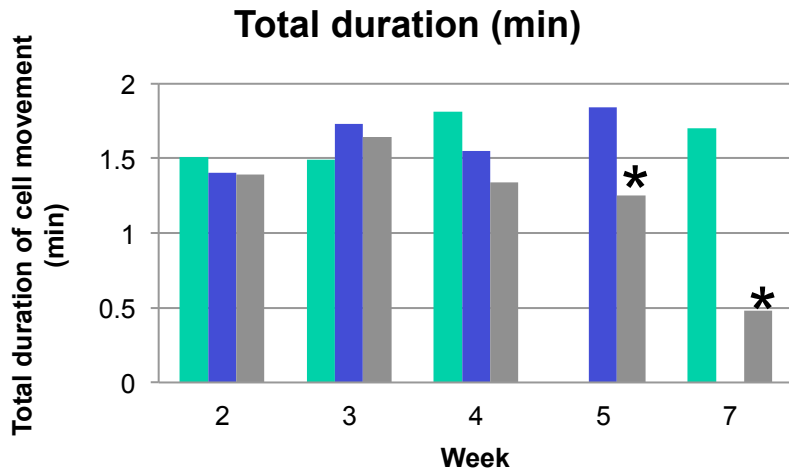
- Cryopreserved: Leibovitz plus 10% dimethyl sulfoxide (DMSO)
- Stored 1:4 in Leibovitz for 24 hours
- Fresh sperm



RESULTS

SPERM CHARACTERISATION ♂

SHORT TERM STORAGE



Analysis time (h)

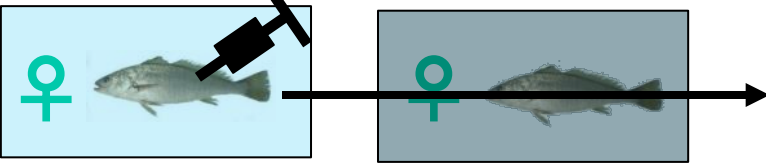
- 10:30 h (fresh sperm)
- 13:30 h
- 17:30 h



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

EXPERIMENT 1: TIMING OF OVULATION

After 35 hours until
ovulation was detected



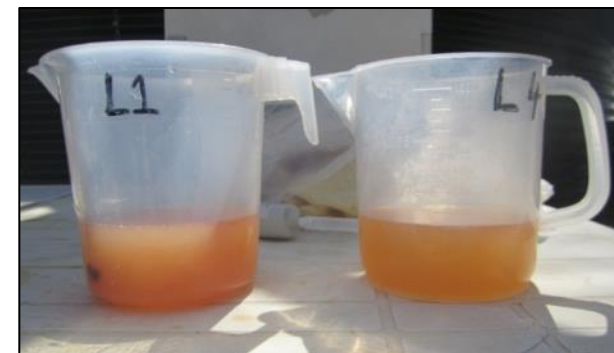
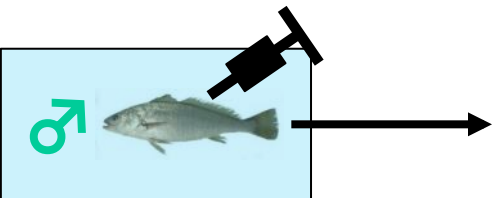
- Received abdominal massages every 2 and ½ hours.
- Time of ovulation= time ovulated eggs were first detected.



Sperm collection

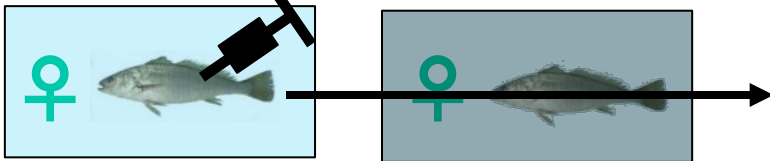
- Diluted in Leibovitz.
- Stored above ice until required.

9-10 h after
females



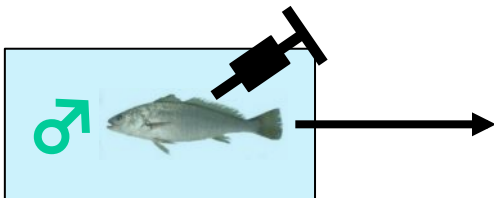
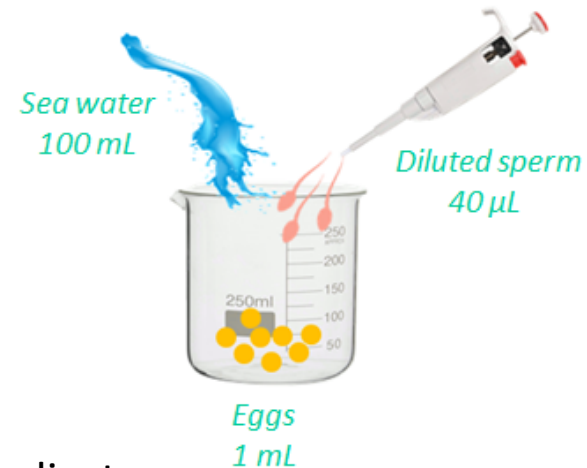
**EXPERIMENT 1:
TIMING OF OVULATION**

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



Sperm collection

9-10 h after females

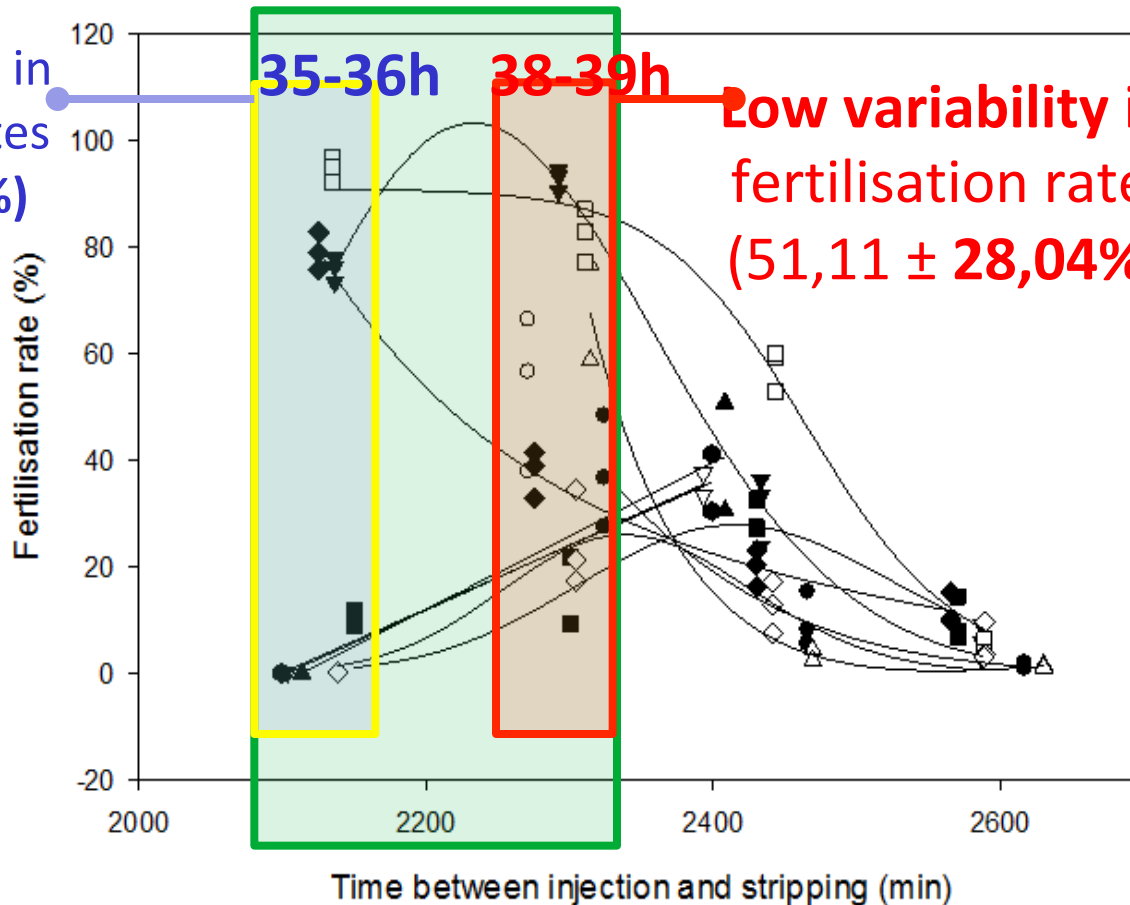
- Diluted in Leibovitz.
- Stored above ice until required.

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

EXPERIMENT 1: TIMING OF OVULATION

Ovulation between 35-39 hours

High variability in fertilisation rates
($32,5 \pm 43,50\%$)



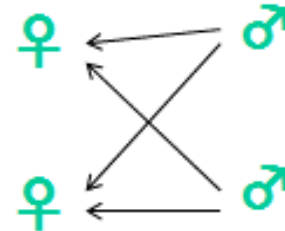
Low variability in fertilisation rates
($51,11 \pm 28,04\%$)

- Female 2 R = 0,9426
- Female 6
- ▼ Female 11 R = 0,9951
- △ Female 13 R = 0,9863
- Female 28 R = 0,7295
- Female 11(2) R = 0,9895
- ◆ Female 13 (2) R = 0,996
- ◇ Female 2 (2) R = 0,8794
- ▲ Female 5 (2) R = 0,9178
- ▽ Female 6 (2) R = 0,9950
- Female 13 (3) R = 0,9675

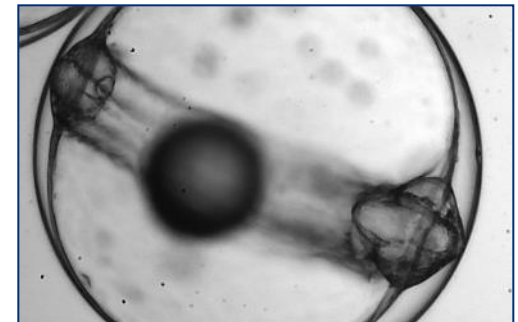
FERTILIZATION



- 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.

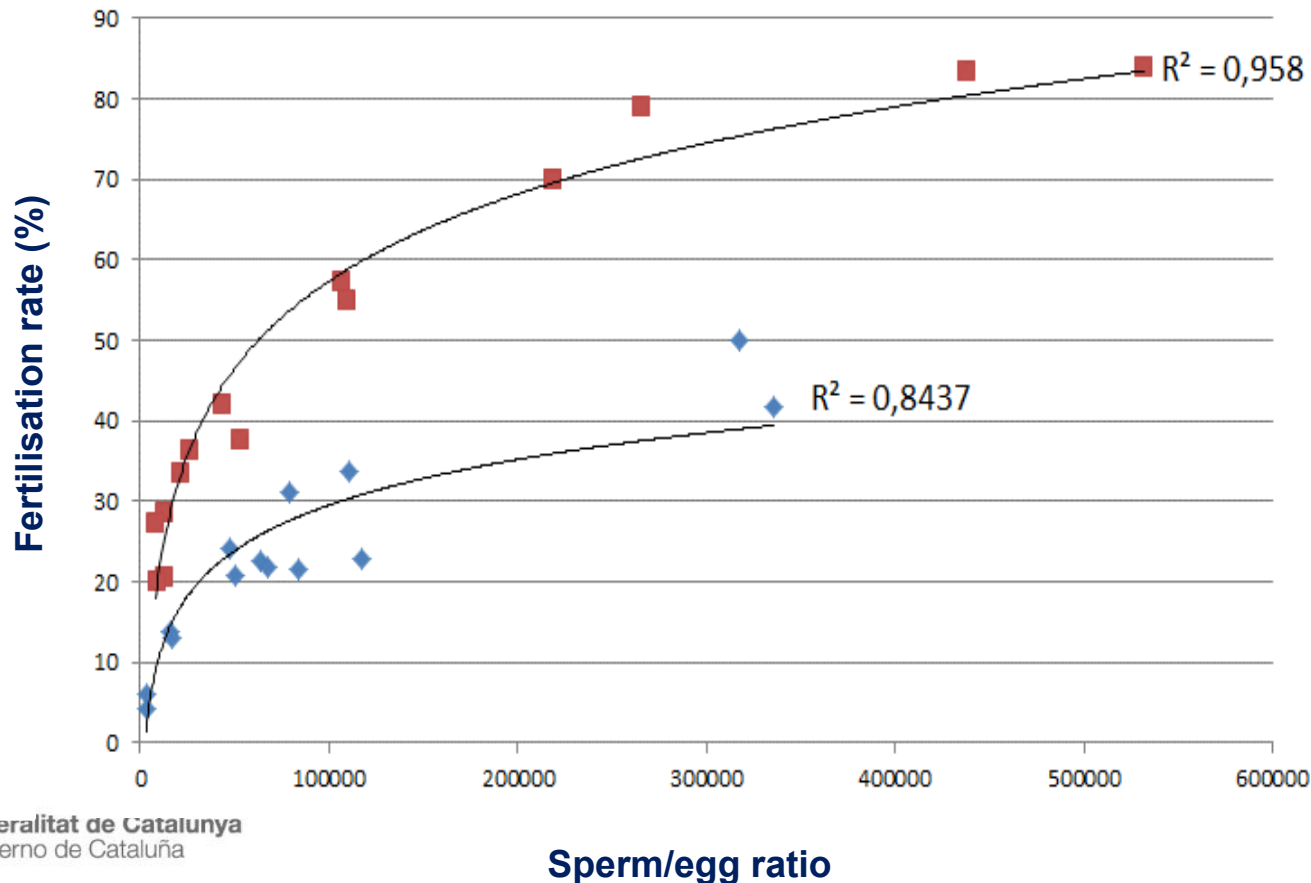
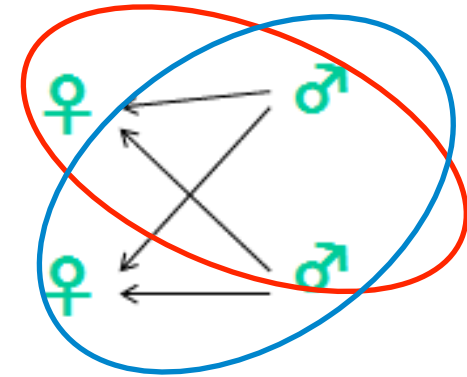


- After 2 hours: content poured onto a 200µm sieve and both floating and sinking eggs placed into a petri dish.
- **FERTILIZATION RATE** of 100 randomly selected eggs from each beaker.



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.



PROTOCOL FOR THE ARTIFICIAL FERTILISATION OF MEAGRE



- The broodstock should be examined at 38 h post-injection at 18°C 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 GnRH α 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

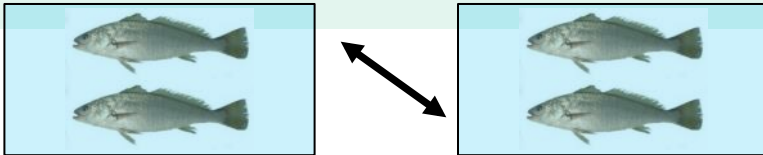
Thanks to work in Ifremer and IRTA



PRODUCE DESIRED FAMILIES FOR GENETIC BREEDING PROGRAMS

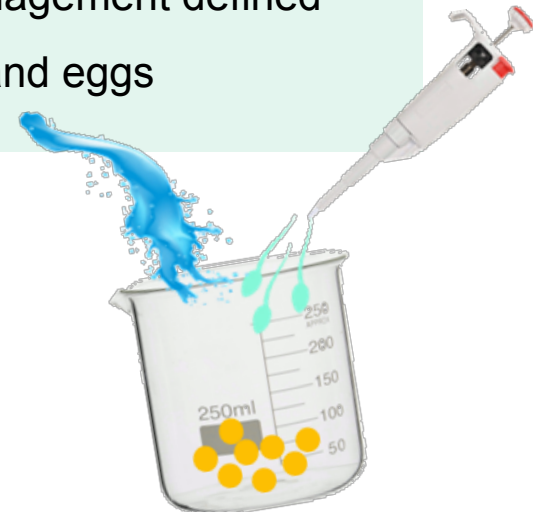
PAIRED BREEDING WITH A CROSS MATING DESIGN

- Successful paired spawning
- 3 half-sib families / female
- High fecundity – multiplication
- Based on normal induce spawning



IN VITRO FERTILIZATION

- High number of families
- Less period of time
- Gamete management defined
 - Sperm and eggs



**Thank you for your
attention**



DIVERSIFY

