Protocol B - Induction of spawning and artificial fertilization [Pratical guide of protocols: broodstock management]

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I - Introduction

Control of reproduction is an important issue in aquaculture for the success of farming production of the species under cultivation. It enables supplies of eggs and fry any time during the year and not just in the natural spawning time of the species. In the Mediterranean area and under culture conditions, sexual maturation in sea bass males is reached during the second year of life, although some males reach an early puberty near the first year of life. They are called early-maturing or precocious males. In contrast, females usually reach puberty near the third year of life (Carrillo *et al.*, 1995a). Moreover, sea bass breed normally under captive conditions and natural spawning occurs in the winter months (i.e. December-March).

Nevertheless, the timing of spawning can be altered by environmental manipulations (i.e. photoperiod, temperature) (Carrillo *et al*., 1989; 1993). Thus, natural spawnings are generally advanced in cold years, while spawnings are retarded in warmer years (Carrillo *et al*., 1995a). In addition, since sea bass is a multiple spawner, several spawns can be collected from the same female (Mylonas *et al*., 2001).

Furthermore, methods for inducing spawning in this species are available and they are commonly used in order to synchronize the production of eggs and sperm in mature fish (Carrillo *et al*., 1995a,b). In fish, these methods are usually based on hormonal treatments, which act at the level of the pituitary gland releasing gonadotropin hormone, the luteinizing hormone (LH). This hormone modulates the control of many aspects of gonadal development, including oocyte growth and maturation, ovulation and spawning. Moreover, sustained-release delivery systems for gonadotropin-release hormone analogues (GnRHa) have also been developed and used in fish species for controlling reproduction (Mylonas and Zohar, 2000). These GnRHa-delivery systems release GnRHa for a period of time that goes from a few days to many weeks, stimulating the LH plasmatic levels and inducing final oocyte maturation. Nevertheless, most of the strategies for inducing spawning involve intraperitoneal injections of hCG (human chorionic gonadotropin) or LHRHa (luteinizing hormone releasing hormone analogue). The LHRHa is a synthetic analogue of GnRH that is usually used because it has longer-lasting actions than the naturally occurring hormone. The optimization of the induction of ovulation by LHRHa has been described in sea bass by Alvariño *et al.* (1992). This hormonal treatment has demonstrated to be effective in this species when it is administrated to females with ovaries showing egg diameters at least 650-800 µm. At water temperatures around 12-13ºC, spawning generally occurs 72 h after hormonal injection and thus, artificial fertilization can be conducted if necessary.

The dry method is a common approach used for artificial fertilization in fish. The eggs are stripped directly from the female into a dry and clean container where the sperm is added. A feather is used in mixing the gametes. For sea bass egg fertilization, seawater is subsequently added into the mixture while stirring it for about 30 sec. Finally, the fertilized eggs are transported to the incubation tank (around 100 eggs/litre) until hatching (about 72 h at 16ºC after fertilization) (Billard, 1984). Generally, sea bass fertilized eggs float in the water column (pelagic) while the unfertilized and dead eggs settle to the bottom of the incubation tank. Eggs at the bottom are removed by siphoning. The newly-hatched larvae are carefully collected and transferred to larval rearing tanks. The hatching rate of sea bass eggs by hormonal treatment can reach 80% (Felip *et al*., 1997).

II - Purpose

The aim of this Protocol is the induction of spawning in the sea bass by one injection in males and two injections (4 h apart) in females of LHRHa administrated during the reproductive season of this species (December-March). At water temperature around 12-13ºC, male and female gametes can be collected 72 h after the first injection by gentle abdominal massage and artificial fertilization can be conducted (Figs B.1 and B.2).

The present Protocol is a modification of that from Billard (1984) and Carrillo *et al*. (1995ab).

III - Procedure

(i) Anaesthetize broodstock with MS-222 or 2-phenoxyethanol. Clove oil can also be used. Induction of anaesthesia is rapid (1-3 min) in small fish although it is slower in larger fish. The above doses may be lethal after about 20 min, thus animals must be handled gently and rapidly. The use of gloves is recommended during this process.

(ii) Cannulate females with a plastic catheter, and select and inject only those females showing oocytes with migrating germinal vesicle. To check the migration of the germinal vesicle, use the Serra solution in a subsample of eggs under the stereoscope. Inject females with LHRHa at 5 µg kg^{-1} body weight. Inject males at the same doses.

(iii) Four hours later, inject females for a second time with LHRHa at 10 µg kg^{-1} body weight. Thereafter, house females with males (one female per two males).

(iv) Seventy-two hours later, collect gametes by abdominal massage as follows.

(v) Anaesthetize male fish as previously described and, after cleaning the genital area with fresh water and drying, collect total expressible milt by gentle abdominal massage pressure in a 15 ml glass vial. Store on ice until artificial fertilization.

(vi) Subsequently, anaesthetize female fish as previously described and, after cleaning the genital area with fresh water and drying, collect a subsample of eggs by a very gentle abdominal massage pressure into a 15 ml glass vial.

(vii) Check for gamete quality at small scale as follows: Fertilize a subsample of eggs (n ~100) adding a few µls of sperm. Activate the sperm adding a few mls of seawater. After 30 sec, gently rinse fertilized eggs with seawater. Check for egg floatability, percentage of fertilized eggs and symmetric divisions at 2-3 h post fertilization.

(viii) If gamete quality is acceptable, pass to artificial fertilization at large scale. Check for the egg-sperm ratio and the timing which are critical to succeed in the artificial fertilization and, in particular, for the application of chromosome set manipulation techniques (see Section iii).

(ix) Repeat step 6 in order to collect the remaining eggs by gentle abdominal massage pressure into a 500 ml glass vial.

(x) Put eggs into a dried tray and add 0.4 ml sperm per each 100 ml eggs. Mix well using a feather.

(xi) Activate sperm adding 2 vol of seawater/vol of eggs plus sperm. The moment of sperm activation is taken as time zero.

(xii) Thirty seconds later, rinse gently fertilized eggs with seawater for 30 sec and incubate in the incubation system until hatching.

IV - Materials and equipment

- 1-ml insulin syringes
- 0.4 mm x 13 mm needles
- Excavated glass slides
- Plastic catheter (cannula) of 1.2 mm in diameter
- Light microscope / stereoscope
- 15-ml glass vials
- 500-ml glass vials
- A feather
- Plastic tray for artificial fertilization
- A graduated cylinder
- Kitchen clothes
- Scale
- Timer
- Plastic containers to keep fish during broodstock management
- Available tanks to place fish after hormonal treatment
- Incubation tanks
- Gloves, lab coat and boots

V - Reagents and solutions

- Anaesthetic: MS-222 or 2-phenoxyethanol (0.1 g l^{-1} and 0.5 ml l^{-1} of seawater, respectively). Alternatively, the induction of anaesthesia can be carried out using clove oil. Clove oil has been evaluated as an effective anaesthetic in sea bass and it can be used at almost 10-fold lower doses than 2-phenoxyethanol.

- Aliquots of LHRHa prepared at 100 μg/ml in sterile water. Store at -20ºC until use.

- Serra solution: 60 ml of 96º ethanol, 30 ml of 40% formol and 10 ml of glacial acetic acid.

VI - Results and discussion

This Protocol presents a general procedure applicable to male and female breeders for induction of spawning in sea bass by injection of LHRHa. The induction of spawning and artificial fertilization are two important issues in aquaculture to obtain gametes and fry for both experimental and commercial purposes. It requires a previous selection from broodstock, mainly from female breeders, in order to succeed in the response to LHRHa administration of the injected fish. Sea bass females are generally selected according to oocyte development. Thus, females with postvitellogenic oocytes around 650-800 μm in diameter and migrating germinal vesicle are selected and injected (Fig. B.1). This selection is carried out taking an individual egg sample from each female breeder by intraovarian cannulation using a plastic catheter and then observed under the microscope. Male breeders are usually matured during the spawning season and sperm samples can be collected from December to March at IATS facilities (40ºN; 0ºE). The use of a hormonal treatment based on intraperitoneal injection of LHRHa is a

common procedure in the sea bass for inducing spawning (Carrillo *et al*., 1995ab). It generally takes place 72 hours after the hormonal treatment at water temperature around 12-13ºC.

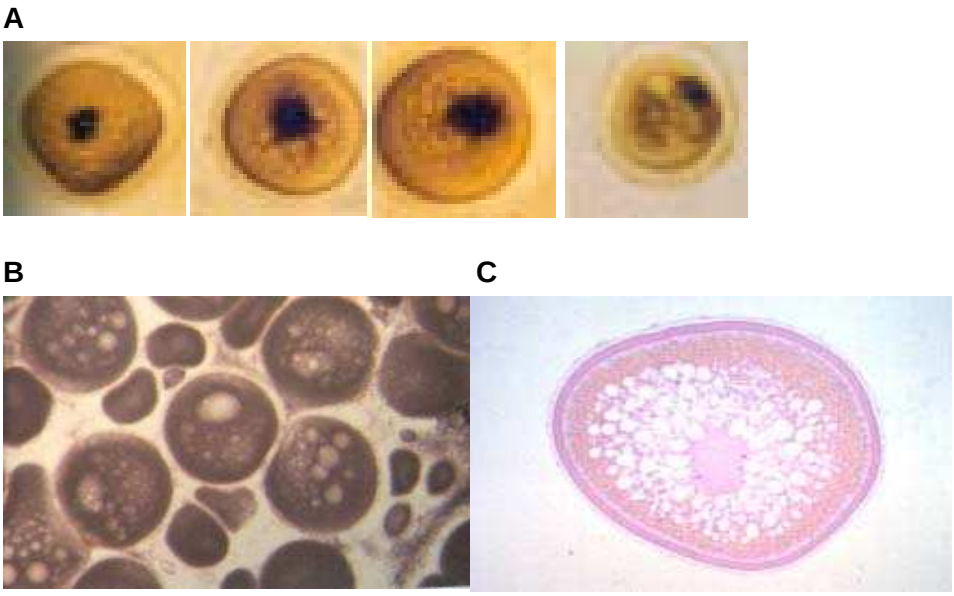

Fig. B.1. Estimation of oocyte development. (A) Different stages of germinal vesicle migration in maturing oocytes. (B) Fresh samples of postvitellogenic oocytes by cannulation. (C) Histological preparation of a postvitellogenic oocyte.

Quality of spawnings is crucial for experimentation. Thus, gamete quality and particularly egg quality from each injected female is individually checked. Several parameters for assessment of egg quality are considered following artificial fertilization of a subsample of eggs (i.e., 1 ml approx. 650-750 eggs; 1.2 mm egg diameter). These parameters include floatability, fertilized eggs and egg symmetric divisions. Only females in which the percentage of floatability and the percentage of fertilized eggs with regular divisions are >80% are considered for experimentation (i.e., chromosome set manipulation). The artificial fertilization approach is based on a dry method in order to control the timing after fertilization (Fig. B.2) (Billard, 1984). To control the timing of meiotic and mitotic divisions of the fertilized eggs (see Protocols *F* and *H*) is critical step for the application of chromosome set manipulation techniques. Finally, the temperature of incubation after artificial fertilization has to be gradually increased or decreased according to the experimental conditions. At 16ºC, sea bass eggs hatch 72 h after fertilization.

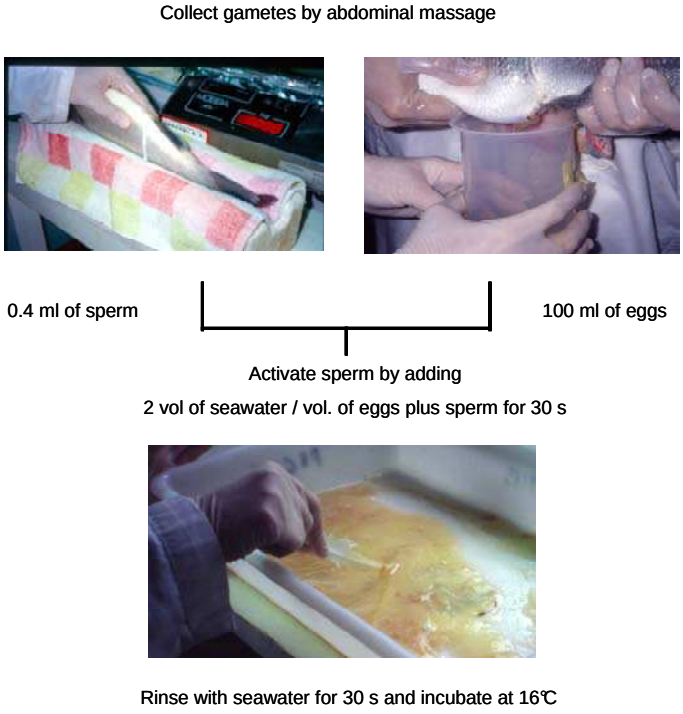

Fig. B.2. Artificial fertilization by the dry method in the sea bass.

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