Protocol F - Induction of triploidy by cold shock [Pratical guide of protocols: chromosome set manipulation]

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I - Introduction

Polyploidy is a term that describes the presence of more than two homologous sets of chromosomes in an organism or cell. Polyploids are not considered genetically modified forms. Particularly triploidy, the most common form of polyploidy, refers to those organisms or cells containing three sets of homologous chromosomes. In some invertebrates and vertebrates, including reptilians, amphibians and teleosts, triploidy can be easily induced in the laboratory resulting in viable individuals. In fish, the inhibition of the second meiotic division of the egg, shortly after fertilization, is effectively achieved by shock treatments of the eggs (Thorggard, 1983; Ihssen *et al*., 1990). These treatments are based on pressure, thermal or chemical shocks that cause destabilization of microtubules thus affecting centrosomes that are needed to form the mitotic spindle (Komen and Thorgaard, 2007). Consequently, cell division of the egg is interrupted, thus inducing the production of a triploid fish (3n) that has two sets of chromosomes of maternal origin (2n) and a set of chromosomes of paternal origin (1n).

Optimal treatment conditions for induction of triploidy are species-specific. Finding the adequate combination of type of shock, duration and timing after fertilization to be applied is crucial for each fish species (Ihssen *et al*., 1990; Felip *et al*., 2001a; Komen and Thorgaard, 2007). As triploid fish exhibit an extra amount of chromosomes, germ cells cannot correctly undergo meiotic divisions. Therefore these fish are usually genetically sterile animals showing significant alterations that affect their gonadal development. In general, triploid females exhibit a reduced gonado-somatic index with ovaries showing oogonia and primary oocytes, without any endocrine signs of maturation. On the other hand, triploid males show a similar gonado-somatic index to that of diploids with impaired spermatogenesis affecting late meiosis and thus blocking spermiogenesis. Although the endocrine profile in males is similar to that of diploids, triploid males rarely produce sperm. Nevertheless, the spermatozoa from triploid males, if produced, are aneuploid and thus, their fertilization ability is limited (Benfey, 1999).

Because triploidy is a method to produce sterile fish, it has been considered as a potential solution for the rearing improvement of fish farming species (Hulata, 2001). Due to this sterile condition, growth perfomances in triploids may result better than those of diploids (Ihssen *et al*., 1990; Felip *et al*., 2001a). Nevertheless, it should be noted that this statement cannot be genaralized for all fish species. Currently, the monosex culture production of all-female triploids is carried out in rainbow trout (Sheehan *et al*., 1999). Although polyploidy technology is applied in practical aquaculture involving many other species of commercial interest (Hulata, 2001). Additionally, triploidization may be considered as an alternative for the use of genetically modified organisms as well as for mitigating the genetic impact of escapees from farmed fish on wild populations. Protocols for the induction of triploidy have been described for several species of interest in aquaculture. They include several families of freshwater species (Salmonidae, Cichlidae, Siluridae and Cyprinidae) and marine species (Sparidae, Moronidae, Pleuronectidae) among others (Ihssen *et al*., 1990; Felip *et al*., 2001a; Piferrer *et al*., 2007).

An optimized protocol for the induction of triploidy in the sea bass based on cold shock of the eggs is presented and discussed in the present Protocol according to Felip *et al*. (1997).

II - Purpose

The goal of this Protocol is to induce triploidy in the European sea bass using an optimized protocol based on cold-shocking the eggs. The contribution of each cold treatment variable to

triploidy percentage and survival is discussed. Finally, the effect of triploidy on the gonadal development of triploid male and female sea bass is presented.

III - Procedure

(i) Keep a broodstock, including both males and females, to be induced to spawn according to Protocol *B* (Steps 1 to 6).

(ii) Check gamete quality according to Protocol *B* (Step 7).

(iii) If quality gametes are produced, prepare materials and equipment for the induction of triploidy as follows.

(iv) Collect gametes and fertilize eggs according to Protocol *B* (Steps 10 to 12).

(v) Gently, put fertilized eggs in a graduated cylinder. Only floating viable eggs are used for the induction of triploidy.

(vi) Five minutes after fertilization, apply a cold shock at 0ºC to fertilized eggs for 10 minutes. For cold shock, add prechilled seawater into a glass vial and place it in a tray containing a mixture of crushed ice and water in order to reach the desired temperature for shocking the eggs inside the vial. Use a mercury thermometer to check temperature variations during the shock treatment.

(vii) Transfer the shocked eggs to the incubation system.

IV - Materials and equipment

- 15-ml glass vials to collect sperm
- 500-ml glass vial to collect eggs
- Plastic tray for artificial fertilization
- Hen feather
- Pasteur pipettes
- Graduated cylinders
- Plastic trays and sieves
- Plastic trays for cold shock
- Glass vials to place fertilized eggs to be shocked
- Timer
- Thermometer
- Kitchen clothes
- Plastic containers to anaesthetize fish
- Available incubation system
- Gloves, lab coat and boots

V - Reagents and solutions

- Anaesthetic: MS-222 (0.1 g l^{-1} of seawater) or 2-phenoxyethanol (0.5 ml l^{-1} of seawater). 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.

- Crushed ice-water mixture for cold shocks.

VI - Results and discussion

The induction of triploidy is a type of polyploidy by which the zygote has three haploid sets of chromosomes, one coming from the father (1n, sperm) and two from the mother (1n, egg nucleus plus 1n, second polar body nucleus) (Fig. F.1). Thus, the retention of second polar body is critical to produce triploids (3n). Consequently, it is necessary to control the procedure for artificial fertilization (Protocol *B*) in order to elucidate the optimal timing to apply the shock and to prevent the second meiotic division in the newly fertilized egg (Felip *et al*., 1997). Thermal, chemical or pressure shocks have been successfully used in fish (Ihssen *et al*., 1990; Felip *et al*., 2001a).

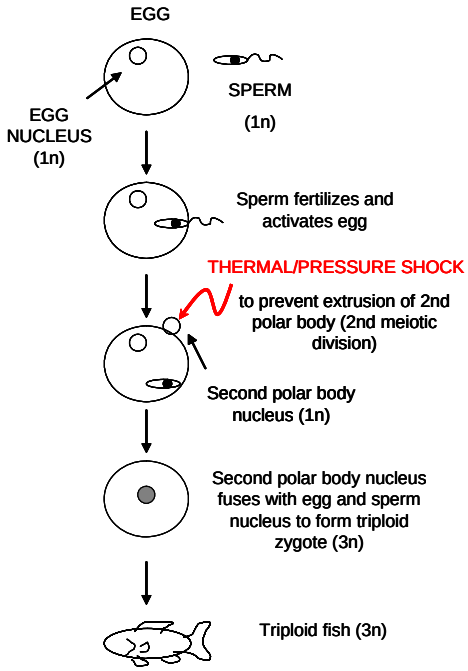

Fig. F.1. A schematic flow chart of triploidy induction.

In sea bass, thermal and pressure shocks have been applied (Carrillo *et al*., 1995; Felip *et al*., 1997). Although thermal manipulations have been standardized (Felip *et al*., 1997), pressure shocks have demonstrated to be an alternative approach for triploidization in sea bass (Carrillo *et al*., 1995). Pressure shocks may be more efficient and predictable than thermal shocks, although their application is limited in a large volume of eggs due to the reduced space of the hydrostatic pressure chambers. In addition, the three more important variables associated to the application of shock treatments for the suppression of the second meiotic division are the time after fertilization when the shock is applied (TAF), the intensity of the shock (TS) and the duration of the shock (DS). A study in sea bass has demonstrated that the TAF and TS significantly contribute to triploidy (Fig. F.2). On the other hand, the determination of egg quality (Protocol *C*) is also another important parameter to assess the chromosome set manipulation success.

Finally, the main objective in the induction of triploidy in fish is the production of sterile individuals, which are considered to have the ability to grow better than those of diploids. This is because triploid fish show a reduced gonadal development and thus it is considered that they can revert energetic investment from reproduction to somatic growth. Unfortunately, it depends on the species, the age of the fish and the rearing conditions. Currently, triploidy is only an experimental practice in marine fish but it is a commercial practice for rainbow trout and oysters. The induction of triploidy in sea bass produces gonadal and functional sterility (Figs F.3 and F.4). However, advantages of triploid sea bass for aquaculture purposes still need to be assessed in terms of growth enhancement (Piferrer *et al*., 2007).

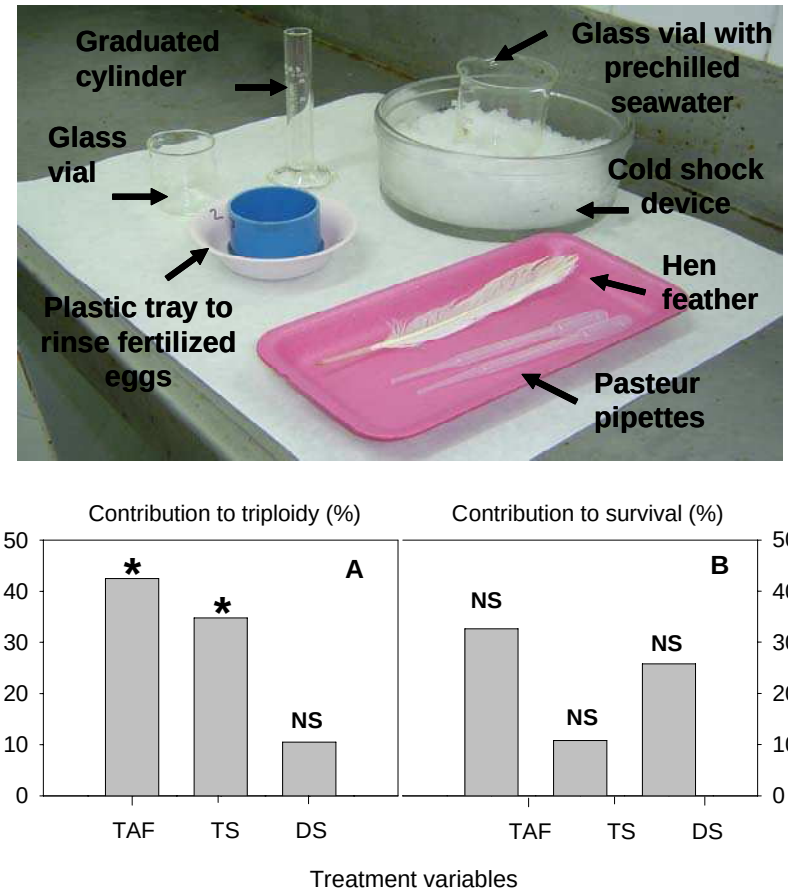

Fig. F.2. Equipment required for a cold shock treatment (above panel) and contribution to survival of each cold shock treatment variable on triploidy induction and survival of sea bass one-day post hatch (below panel). *P < 0.05. Abbreviations: TAF, time after fertilization; TS, temperature of the shock; DS, duration of the shock; NS, not significant.

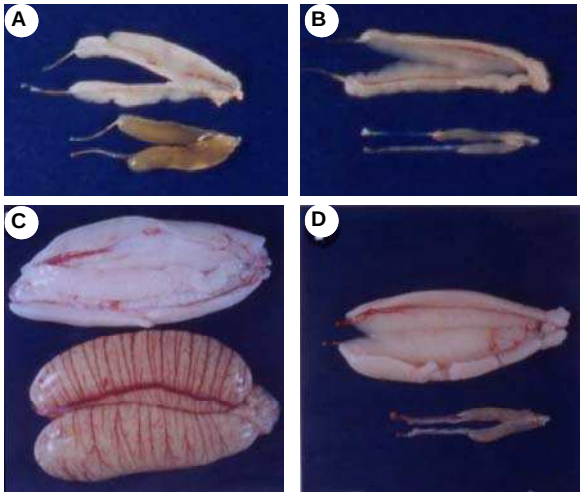

Fig. F.3. Gonadal development in 2 year-old (A, B) and 4 year-old (C, D) diploid (left) and triploid (right) sea bass. In each photograph, testes are placed above and ovaries below. Modified from Felip *et al*. (2001b).

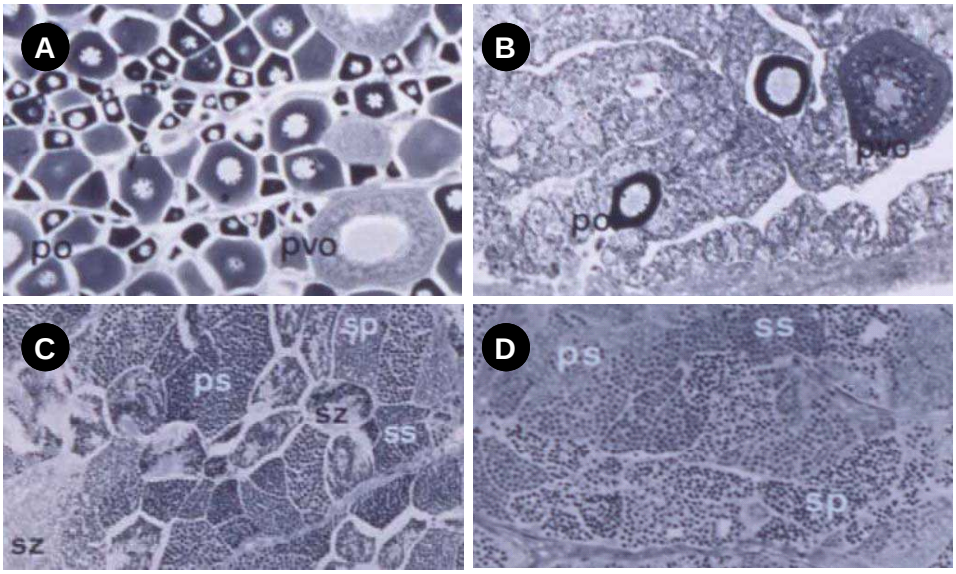

Fig. F.4. Photomicrographs of ovaries from diploid (A) and triploid (B) female sea bass at 2 years old, containing perinuclear oocytes (po) and previtellogenic oocytes (pvo) and testes from diploid (C) and triploid (D) male sea bass at 3 years old, exhibiting an apparently normal spermatogenesis, but note the absence of spermatozoa in triploid males. Primary spermatocytes (ps), secondary spermatocytes (ss), spermatids (sp) and spermatozoa (sz). Modified from Felip *et al.* (2001b).

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