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Protocol H
Induction of gynogenesis using UV-light and cold shock

I - Introduction

Gynogenesis is a term that describes exclusively maternal uniparental inheritance. In vertebrates, spontaneous gynogenesis occurs in reptilians, amphibians and teleosts. Although the diploidization mechanisms of the oocytes vary, genome duplication followed by normal cell division has become the most common form for restoring diploidy (Komen and Thorgaard, 2007). The production of gynogenetic fish in the laboratory has focused on genome inactivation of the sperm by ionizing irradiation or UV-irradiation and followed by suppression of meiotic division or suppression of mitotic division of the eggs by shocking treatments. The production of heterozygous meiotic diploids (meiogynogens) and homozygous mitotic diploids (mitogynogens) are obtained, respectively (Thorgaard, 1983; Ihssen *et al*., 1990; Felip *et al*., 2001; Piferrer *et al*., 2007).

In general, the yields of hatched gynogenetic fish are usually low. In sea bass, yields of meiogynogenetic fish vary between 17 and 35% (Felip *et al*., 1999; Francescon *et al*., 2005), although it can be as high as 76% (Peruzzi and Chatain, 2000) with an effectiveness of 100% of gynogenetics. On the contrary, the yields of mitogynogenetic fish are not up to 27% (Francescon *et al.*, 2004). One explanation for these low yields, despite the application of optimized protocols, is the drastic handling to which male and female gametes are exposed. On the other hand, the inbreeding depression of gynogenetic fish as a consequence of the expression of homozygous deleterious mutations can influence their survival rates during early development stages (Leary *et al*., 1985; Don and Avtalion, 1988).

Gynogenesis can be used to elucidate the sex-determining mechanism operating in a fish species. Thus, in species where female is the homogametic sex, gynogenetic progeny should result in 100% females. If so, the induction of gynogenesis in combination with hormonal sex reversal approaches can be feasible to produce female monosex stocks in species with XX-XY sex determination (Hulata, 2001). In practice however, sex ratios from gynogenetic fish are variable from all females to a high proportion of males even when a XX-XY genetic system is operating. The role of environmental factors, mainly temperature, or the existence of recessive genes affecting sex determination can explain these results (Baroiller and D'Cotta, 2001; Hulata, 2001).

II - Purpose

In gynogenesis, only the female genome contributes in offspring. The main goal of this Protocol is to induce gynogenesis in the European sea bass using an optimized protocol based on: (i) UV-irradiation for sea bass sperm genome inactivation (Protocol G); and (ii) cold-shocking of the eggs to inhibit the extrusion of the second polar body (i.e., restoration of diploidy) to produce partially inbred fish, called meiogynogen diploids.

The contribution of several factors to mortality of meiogynogenetic sea bass during early development and sex ratios in meiogynogenetic sea bass are also discussed. A schematic flow chart for the meigynogenetic fish production is described in this Protocol (Fig. H.1).

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* (Steps 7).

(iii) If so, prepare the facilities for the induction of gynogenesis as follows using the optimal conditions in each species.

(iv) Inactivate DNA sperm by UV-irradiation according to Protocol *G*.

(v) Prepare the cold shock device by adding prechilled seawater at the desired temperature into a glass vial.

(vi) Place the glass vial in a tray containing crushed ice and water at the appropriate temperature. Measure with a mercury thermometer to check that the temperature for shock is maintained.

(vii) Collect gametes and fertilize eggs according to Protocol *B* (Steps 10 to 12). It should be noted that some modifications need to be made for artificial fertilization: add 4 ml of irradiated and diluted (1:10) sperm to 100 ml of eggs.

(viii) Gently, put fertilized eggs in a graduated cylinder. Only floating viable eggs will be used for the induction of gynogenesis.

(ix) Five minutes after fertilization, cold shock fertilized eggs at 0ºC for 10 min.

(x) After cold shock, transfer the eggs to the incubation system.

IV - Materials and equipment

- 15-ml glass vials to collect sperm, previously irradiated
- 500-ml glass vial to collect eggs
- Plastic tray for artificial fertilization
- Hen feather
- 2-l graduated cylinder
- Plastic trays for cold shock
- Glass vials to put fertilized eggs to be shocked
- Timer
- Thermometer
- Plastic containers to anaesthetize fish
- Available incubation system
- Kitchen clothes
- Gloves, lab coat, boots

V - Reagents and solutions

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

- Ice-water mixture for cold shock.

VI - Results and discussion

The induction of the gynogenesis requires the manipulation of both gametes, the sperm and the egg (Fig. H.1). The DNA of the sperm needs to be genetically inactivated (Protocol *G*) before egg fertilization and the diploidization needs to be restored in the egg to produce a viable zygote. It can be achieved using shock treatments similar to those described in Protocol *F*. Although comparable treatments based on the inhibition of the first mitotic division of the fertilized egg are also effective to induce gynogenesis in fish (Thorgaard, 1983; Ihssen *et al*., 1990; Komen and Thorggard, 2007). Accordingly, gynogenetic fish maintain an exclusively maternal inheritance.

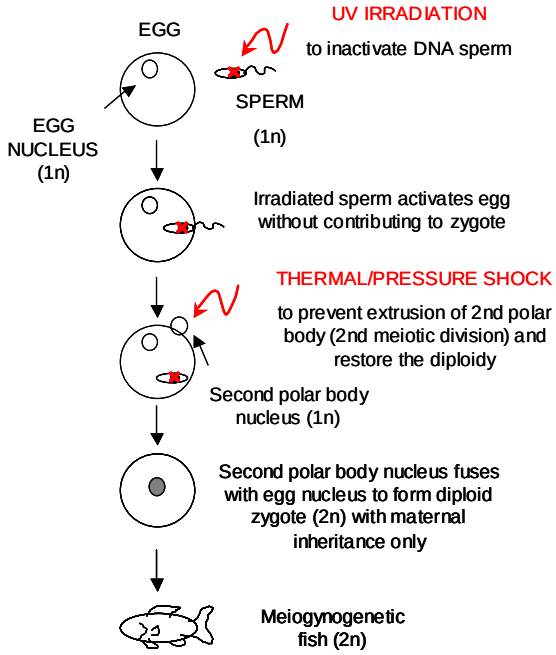

Fig. H.1. A schematic flow chart of gynogenesis (meiogynogenesis).

The induction of gynogenesis negatively affects the survival of embryos and larvae during the early developmental stages in fish (Felip *et al*., 1999). Particularly, the UV-irradiation treatment has a significant contribution on mortality of early developmental stages, resulting more pronounced during hatching and thereafter. On the other hand, the effect of the shock treatment is more evident after fertilization and it decreases by the time of hatching (Fig. H.2).

Finally, the higher homozygosity of gynogenetic fish compared to those of diploids also contributes to the low viability of gynogenetics in comparison to that of triploids and diploids (Leary *et al.*, 1985; Don and Avtalion, 1988). The production of gynogenetic fish is a tentative

approach to elucidate the mechanism of sex determination operating in fish. In those species where the female is the homogametic sex (XX), the induction of gynogenetic fish results in all-female progenies. In the sea bass, the production of a considerable number of males in gynogenetic progenies has suggested that another genetic system different from a XX-XY mechanism operates in this species for the genetic sex control. Currently, it has been known that sea bass is a thermosensitive species with parental influence on the sex-ratio of progenies and a genetic variation that shows that sex may be under polygenic control (Vandeputte *et al*., 2007).

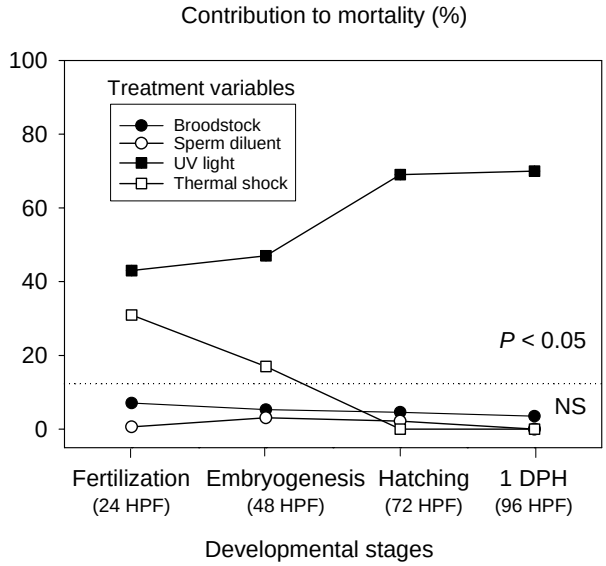

Fig. H.2. Contribution of several factors to mortality of gynogenetic sea bass during early development. DPH, day post hatching; HPF, hours post fertilization.

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