Induction of tetraploid gynogenesis in the European sea bass (*Dicentrarchus labrax* L.)

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Abstract

A preliminary study on tetraploid gynogenetic induction in the European sea bass was performed by pressure-blocking the second polar body release and the first cleavage in eggs fertilized with ultraviolet-irradiated sperm. Fertilization of eggs with genetically inactivated sperm produced only haploid development that terminated around hatching. Pressure treatments (8500 psi for 2 min) applied at 6 and 65 min after fertilization (a.f.) produced variable levels (7-95%) of tetraploid larvae at hatching. A small proportion of mosaics (3.8n/4.2n) was also recorded.

Keywords: Tetraploidy; Gynogenesis; Pressure shock; Sea bass; *Dicentrarchus labrax*

Introduction

Techniques for chromosome manipulation have been successfully applied to induce diploid gynogenesis and triploidy in the European sea bass (*Dicentrarchus labrax* L.) as reviewed by Zanuy et al. (2001). Another fundamental manipulation is the mitotic induction of tetraploidy. Induced tetraploids can be useful for mass production of triploid fish by mating them with normal diploids. Tetraploidy in fish is commonly produced by disrupting the first cleavage with thermal or hydrostatic pressure shocks in eggs fertilized with normal sperm. Viable tetraploids have been produced by these methods in a number of fish species (Pandian & Koteeswaran, 1998), including sea bass (Barbaro et al., 1998).

In this work we investigated a novel technique to induce tetraploidy in fish by pressure-blocking second meiosis and first mitosis in sea bass eggs fertilized with homologous irradiated sperm.

Materials and methods

Wild sea bass broodstock was maintained and spawned at our facilities. For each experiment, gametes were collected from two males and two females, microscopically checked for quality and pooled. Sperm inactivation, artificial insemination, and pressure treatments were performed according to Peruzzi & Chatain (2000). Timing of mitotic shock application was chosen according to the results on cytological examinations of early embryonic development and experimental mitotic inhibition in sea bass (B. Menu & J-C. Falguière, pers. comm.). Briefly, each insemination involved 75 ml of pooled eggs to be divided into two groups. One group (15 ml) used normal sperm (diploid control) while the remaining group (60 ml) used UV-irradiated

sperm. Batches of 15 and 60 ml of eggs were fertilized with 2.5 ml or 10 ml of diluted milt (1:20; milt:saline), respectively. Sperm was activated by adding 0.5 volume of seawater / volume eggs plus sperm. Three minutes after insemination, eggs were gently rinsed and the diploid control eggs incubated in 1L of filtered seawater at 13°C. Eggs fertilized with irradiated sperm were further divided into two groups. One group of 15 ml was used as haploid control and incubated as above, while the remaining eggs (45 ml) were pressure shocked during second meiosis (6 min a.f.). After treatment (8.500 psi for 2 min), eggs were equally divided into three groups and incubated at 13°C. For mitotic inhibition, each group was pressure shocked a second time (8.500 psi for 2 min) at 65, 70 and 75 min a.f., respectively. All groups were then transferred into individual 150 L tanks and incubated at 13-14°C. The experiments were conducted twice using a different pool of gametes from different pairs of breeders.

Survival of developing eggs and viable larvae was recorded at different developmental stages: gastrulation (24 h a.f.), embryonation (48 h a.f.), and hatching (96 h a.f.). Survival was expressed as relative to control after adjustment of the latter to 100%. Ploidy level was determined on dissociated cells from 1 to 2 days old hatched larvae by flow cytometry. For this purpose, collected larvae were rinsed in distilled water, individually placed into disposable test tubes containing a drop of distilled water and deep frozen (-80°C) until use. For analysis, single larvae were thawed in 0.5 ml of propidium iodide solution (Thiersch et al., 1989) and rapidly desegregated by use of a pipette, vortexed, and filtered through a 20µm nylon mesh. Experimental and control samples were first run separately. Certified polyploid larvae were further verified by adding aliquots of haploid and/or diploid standards as internal references. Ploidy levels were calculated in relation to control values. All hatched larvae from treated

groups and randomly selected controls (n=20 / replicate) were sampled. Haploid control groups were collected before complete mortality occurred (72-96 h a.f.).

Results

The hatching rate of diploid control groups within the two replicates at 24 h a.f. was 83% and 58% respectively. Table 1 shows percent survival of eggs and larvae relative to control groups (RC) between day 1 and hatching. Pressure treatments generally resulted in a sharp decrease of egg survival at day 1. Percent survival at hatching within treated groups ranged from 4 to 12%. In Replicate 1, only eggs double-shocked at 6 and 65 min a.f. yielded viable larvae. In the haploid control groups, larvae exhibited a typical haploid morphology and did not developed beyond hatching stage (Peruzzi & Chatain, 2000).

Ploidy levels of larvae recorded by flow cytometry are reported in Table 2. Only the groups shocked at 6 and 65 min a.f. yielded variable proportions of tetraploids (7-95%). The same treatment resulted in 5% of mosaics (3.8n/4.2n). Figure 1(A) reports the typical nuclear DNA content of haploid, diploid and tetraploid larvae. A fluorescence histogram showing an hypo- and hyper-tetraploid mosaic is reported in Figure 1(B). Only diploid larvae were scored among the other treatments and the diploid control groups. The flow cytometric analysis further confirmed the haploid state of embryos and larvae from the haploid gynogenetic groups**.**

Discussion

Variable levels of tetraploid gynogenetic sea bass larvae were produced by meiosis II and mitosis I blocking in gynogenetically activated eggs with pressure shocks. This variability was

possibly due to different egg quality between the two experiments, as indicated by the hatching rate of controls (83% and 58%), and to individual responses of females to pressure treatments. In the past, gynogenetic production coupled with cold shock to inhibit the second meiotic division has yielded viable tetraploid offspring in loach (Arai, Matsubara & Suzuki, 1993). These authors used diploid gametes from a spontaneously occurring tetraploid female and UV-irradiated carp (*Cyprinus carpio*) sperm.

In sea bass, the only published report on conventional tetraploid induction is provided by Barbaro et al. (1998). In their work, pressure shocks of 4-7 min duration at 69-83 MPa (approx. 10.000 - 12.000 psi) applied 60-90 min a.f. gave 56% tetraploid larvae. Under their conditions, the survival of tetraploid sea bass at hatching was 6-25%. None of the mitotic tetraploids survived to 8 months after fertilization. In the present work, treatments optima used for the retention of polar body II (Peruzzi & Chatain, 2000) were applied for the disruption of both meiotic and mitotic divisions. Pressure shocks applied at 6 min a.f. and 65' min a.f. at 13°C produced 7-95% tetraploids and 5% mosaics (3.8n/4.2n) at hatching. The other treatments were either totally disruptive, or produced diploids only. Mosaic larvae were most likely produced by unbalanced chromosome segregation patterns following sub-optimal shock conditions.

The experimental design and results leave little doubt that the diploids and tetraploids were indeed gynogenetic but final identification of the gynogenetic origin might only be obtained using molecular approaches. Confirmation of uniparental inheritance by microsatellite DNA analysis has been provided for gynogenetic sea bass produced under our experimental conditions in the past (Peruzzi & Chatain, 2000).

Further studies are needed to validate our method and to confirm the viability of tetraploid gynogenetic larvae beyond hatching. Also, the use of gynogenetic or other inbred sires should be investigated as a possible way to improve yield of tetraploid gynogens. Nevertheless, this preliminary study provides the first evidence of tetraploid larvae produced by such method and encouragement for future research on this subject therefore exists.

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Legends

Table 1.

Percentage of surviving eggs and larvae in control and pressure-shocked groups.

Table 2.

Ploidy levels of newly hatched larvae from control and pressure-shocked groups.

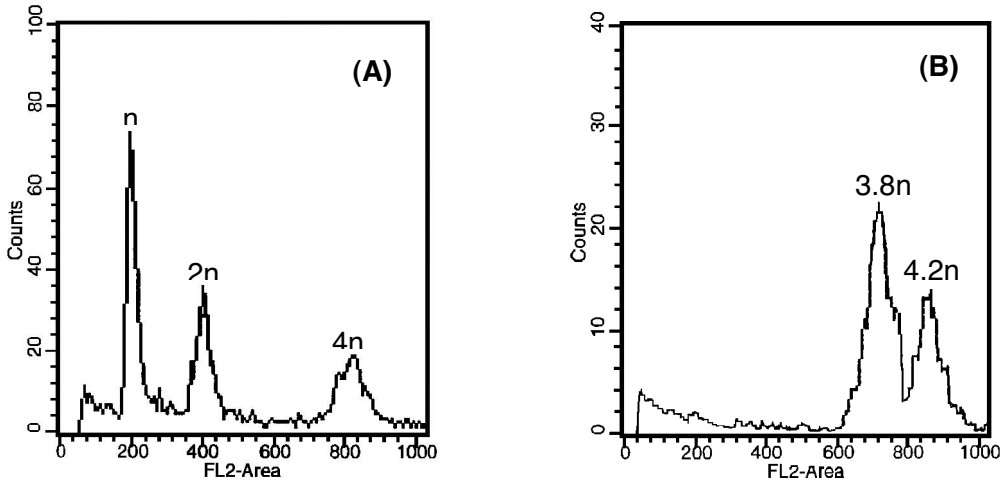
Figure 1.

Nuclear DNA content of 1-2 day-old D.labrax larvae as measured by flow cytometry. (A) cell suspension from one tetraploid (4n) larva with added haploid (n) and diploid (2n) controls ; (B) cell suspension from one mosaic (3.8 /4.2) larva. DNA values are reported in arbitrary units expressed as fluorescence channel numbers (FL2-Area).

Replicate and treatment	Percent survival RC*		
	24h (a.f.)	48h (a.f.)	96h (a.f.)
Replicate 1			
Eggs + UV sperm (haploid control)	25	6	0
Eggs + UV sperm + meiotic + mitotic (65' a.f.)	53	16	6
Eggs + UV sperm + meiotic + mitotic (70' a.f.)	24	0	0
Eggs + UV sperm + meiotic + mitotic (75' a.f.)	22	0	0
Replicate 2			
Eggs + UV sperm (haploid control)	24	9	0
Eggs + UV sperm + meiotic + mitotic (65' a.f.)	10	2	4
Eggs + UV sperm + meiotic + mitotic (70' a.f.)	12	9	8
Eggs + UV sperm + meiotic + mitotic (75' a.f.)	14	15	12

* Relative to diploid control

Replicate and treatment	Sample size	Percentage of ploidies observed			
		Haploid	Diploid	Tetraploid	Mosaic
Replicate 1					
Eggs + UV sperm (haploid control)	10	100	0	0	0
Eggs + sperm (diploid control)	10	0	100	0	0
Eggs + UV sperm + meiotic + mitotic (65’ a.f.)	44	0	0	95	5
Eggs + UV sperm + meiotic + mitotic (70’ a.f.)	-	-	-	-	-
Eggs + UV sperm + meiotic + mitotic (75’ a.f.)	-	-	-	-	-
Replicate 2					
Eggs + UV sperm (haploid control)	10	100	0	0	0
Eggs + sperm (diploid control)	10	0	100	0	0
Eggs + UV sperm + meiotic + mitotic (65’ a.f.)	29	0	93	7	0
Eggs + UV sperm + meiotic + mitotic (70’ a.f.)	20	0	100	0	0
Eggs + UV sperm + meiotic + mitotic (75’ a.f.)	13	0	100	0	0


(A)
n
2n
4n
Counts
FL2-Area
0
20
40
60
80
100
200
400
600
800
1000
(B)
3.8n
4.2n
Counts
FL2-Area
0
10
20
30
40