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Study of the treatment conditions leading to the mass-production of triploid and gynogenetic sea bass

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SUMMARY - The appropriate conditions to prevent the extrusion of the second polar body in eggs fertilized with normal sperm for triploidy induction, or to restore diploidy in eggs activated with UV-irradiated sperm for gynogenesis induction in the sea bass were investigated. Shocks of 10 min duration at 0°C starting 5 min after fertilization resulted in 100% triploidy. Ploidy was determined by counting the number of nuclear organizing regions (NOR) of cells. Using these shock conditions, triploid sea bass were mass-produced, having an overall survival about 10% lower than that of the diploid controls at one day post hatch (1 DPH). In this case, ploidy was identified by karyotyping. At 2 years of age, triploid sea bass juveniles had a specific growth rate similar to that of diploids. Similarly, the sex ratio in triploids was not different from diploids. To induce gynogenesis, the most suitable UV-dose to inactivate the sperm DNA was investigated. Sperm was diluted 1:10 prior to irradiation. Results showed that 35,000-40,000 erg mm^{-2} was the best dose range to inactivate sperm DNA while maintaining sufficient sperm motility to activate the eggs. Eggs fertilized with irradiated sperm but not shocked showed a typical haploid syndrome. The restoration of diploidy was carried out by application the same optimized shock as described above to induce triploidy. Diploid gynogenetic sea bass exhibited 1 or 2 NOR and 48 chromosomes like their diploid counterparts. Survival in the gynogenetics was about 30% of that of the control diploids at 1 DPH. Survival in haploids was always ~2.5% at hatch, indicative that some spermatozoa escaped UV-irradiation. Currently, it remains to be determined whether triploid sea bass will outgrow diploids when the latter approach sexual maturation and also the percentage of females in the gynogenetic offspring.

Key words: Triploidy, gynogenesis, cold shock, UV-irradiation, teleost, sea bass, *Dicentrarchus labrax*.

RESUME - *"Etude des conditions de traitement menant à la production en masse de loup triploïde et gynogénétique". Nous avons étudié les conditions les plus appropriées pour prévenir l'émission du $2^{ème}$ globule polaire dans des oeufs qui avaient été fertilisés avec des spermatozoïdes irradiés aux UV pour produire des gynogénétiques diploïdes. Des chocs de 10 min à 0°C, appliqués 5 minutes après la fertilisation, ont donné comme résultat une production de triploïdes à 100%. La triploïdie a été déterminée par comptage du nombre de régions organisatrices du nucléole (NOR) dans des cellules isolées. Ces conditions ont été utilisées pour induire la triploïdie à grande échelle dans les oeufs de loup. Un jour après l'éclosion (1DDE), la survie larvaire a été pour les triploïdes de 10% inférieure à celle des contrôles. En ce cas le degré de triploïdization a été déterminé par le comptage du nombre de chromosomes. A deux ans, les juvéniles triploïdes de loup et les contrôles diploïdes avaient le même taux de croissance. La proportion des sexes était identique. Nous avons étudié la dose de radiation qui convenait le mieux pour inactiver l'ADN du sperme et induire la gynogénèse. Avant l'irradiation, le sperme a été dilué au $1/10^{ème}$. Les résultats ont démontré que la meilleure dose pour inactiver l'ADN, sans perte de sa capacité d'activation de l'oeuf, se situait entre 35.000-40.000 erg mm^{-2}. Les oeufs fertilisés avec le sperme irradié, mais qui n'avaient pas reçu le choc thermique, montraient le typique syndrome d'aploïde. Les conditions de choc froid utilisées pour restaurer la diploïdie ont été les mêmes que celles décrites antérieurement pour induire la triploïdie. De la même façon que leurs frères diploïdes, les individus gynogénétiques avaient 1 ou 2 NOR et 48 chromosomes. La survie des gynogénétiques à 1 DDE, a été environ de 30% par rapport à celle des*

contrôles. La survie des individus haploïdes au moment de l'éclosion a été toujours inférieure à ≈ 2,5% indiquant qu'il y avait quelques spermatozoïdes qui avaient échappé à l'irradiation ultraviolette. Nous ne savons pas si, au moment de la maturation sexuelle, les loups triploïdes ont une croissance supérieure a celle des contrôles et nous ne connaissons pas le pourcentage de femelles gynogénétiques obtenues.

Mots-clés : *Triploïdes, gynogénèse, choc froid, irradiation ultraviolette, téléostéens, loup,* Dicentrarchus labrax.

Introduction

Recently, the interest to improve the aquaculture of Mediterranean marine species has increased considerably. In the sea bass and sea bream, genetic techniques, including selective breeding, chromosome set manipulation, hybridization and genetic engineering have begun to be carried out. Specifically, chromosome set manipulation in fish can be used to obtain sterile stocks (by inducing triploidy) and in some cases all-female stocks (by inducing gynogenesis). For triploidy induction it is necessary to duplicate the maternal genome by preventing the extrusion of the second polar body in eggs fertilized with normal sperm. To optimize triploidy induction protocols, the study of three variables must be carried out: (i) the time after fertilization when the shock is applied; (ii) the temperature; and (iii) the duration of the shock. To induce gynogenesis, the restoration of diploidy in eggs activated with UV-irradiated sperm needs to be achieved. Thus, a further aspect, the genetic inactivation of the sperm DNA, needs to be investigated.

Triploidy and gynogenesis induction has been carried out in many species (Benfey, 1989; Solar *et al.*, 1991). Triploid fish are in general, mostly genetically sterile and have reduced reproductive capacity, specially in the females and it is thought that these fish do not suffer the negative effects that sexual maturation produces, i.e., (i) reduction in growth rate; and (ii) increased mortality. Depending on the species and the age of fish (juvenile or adult), triploid fish might exhibit increased or decreased growth performance than diploid fish (Ihseen *et al.*, 1990). Furthermore, triploid males in many species mature sexually, maintaining the problems named above (Lincoln and Scott, 1984; Benfey *et al.*, 1986; Nakamura *et al.*, 1993; Hussain *et al.*, 1995). This fact suggests that triploid females have a potential economic interest for aquaculture. Gynogenesis induction may result in all-female stocks in those species where the female is the homogametic sex. In this latter case, however, the low survival of gynogenetic fish (Leary *et al.*, 1985) jeopardizes their use for production. However, neomales (genetic females masculinized into phenotypic males by androgen treatment) could be mated with normal females and obtain monosex stocks in those species with a XX/XY female: male sex determination mechanism.

Considering the importance of the sea bass in the European aquaculture and to be aware of the problems that this species usually presents under rearing conditions, i.e., (i) males outnumber females; (ii) males appear to grow more slowly than females; and (iii) a proportion of males mature before reaching marketable size (Carrillo *et al.*, 1995), an accurate investigation of the conditions leading to the mass-production of triploids and gynogenetics in this species has been carried out.

Materials and methods

Triploidy and gynogenesis induction

Triploidy was induced according to Felip *et al.* (in press). Briefly, eggs were cold shocked at 0°C, for 10 min starting 5 min after fertilization. Ploidy was determined by counting the number of nuclear organizing regions (NOR) of cells (Rufas *et al.*, 1982). Under these conditions triploid sea bass were mass-produced and ploidy was identified by karyotyping (Kligerman and Bloom, 1977). Diploid and triploid larvae thus obtained were reared in duplicate using current sea bass farming practices (Barnabé, 1991). Periodically, triploid and diploid juvenile fish were weighed to determine the specific growth rate in weight. Diploid and triploid fish were sacrificed to monitor sex ratio.

Gynogenesis induction was carried out from eggs fertilized with irradiated sperm at a range dose between 35,000-40,000 erg mm^{-2} and subsequently shocked using the same optimized shock as described above. Ploidy was determined by NOR analysis and karyotyping.

Ploidy level and survival

Gametes from only one female and only one male were manipulated to obtain four different ploidy levels: (i) diploids; (ii) triploids; (iii) gynogenetic diploids; and (iv) haploids. Survival in each ploidy level was calculated at 1 DPH as the percentage of normal larvae in the total number of inseminated eggs. Survival in the diploid control group was set at 100%, where survival in the other groups was expressed relative to the controls.

Statistical analysis

Survival rate were transformed to percentages and arc-sine transformed before statistical analysis (Sokal and Rohlf, 1981). Analysis of variance (ANOVA) was used to compare treatment effects on survival and ploidy among groups. Student's test was used to compare the specific growth rate between diploids and triploids. A Chi-square test was used to determine significant difference from 3:1 (males:female) sex ratio. Differences were accepted as significant when $P<0.05$. Experiments were conducted at least 2-3 times and each group was carried out at least in triplicate.

Results and discussion

Although combination of different temperatures and different durations of shock were investigated to ascertain the treatment with the highest capacity for triploidy induction, cold shocks at 0°C exhibited the highest percentage of triploidy (Table 1). The percentage of triploidy were consistently 100% when the shock lasted 10 min. Although there were no significant differences between treated groups and control groups, the survival at 1 DPH in the former groups decreased when the duration of

the shock increased. Using a cold shock of 0°C, for 10 min, starting 5 min after fertilization, triploid sea bass were mass-produced.

Table 1. Treatment variables for the induction of triploidy in sea bass using cold shocks

Temperature of shock[1]	Survival[2] (%)	Triploidy[3] (%)	Yield[4] (%)	Optimum shock duration (min)
0°C	59 - 95	91 - 100	55 - 94	10
2°C	71 - 98	38 - 93	37 - 67	20
4°C	74 - 100	8 - 63	8 - 55	>20

[1]Shocks started 5 min after fertilization and lasted 5-20 min.
[2]Survival of controls set at 100% and determined 1 day post hatch
[3]Triploidy determined by NOR analysis
[4]Yield determined by combination of percentage of survival and triploidy

Although the survival in triploids was about 10% lower than that of the diploid controls at 1DPH (Fig. 1), from 170 DPH triploid sea bass had a performance similar to that of diploids (Table 2). At least until 2 years of age, there were no significant differences in specific growth rate in weight between juvenile diploid and triploid sea bass. Nevertheless, it remains to be determined the interest of these type of stocks for the aquaculture of this species.

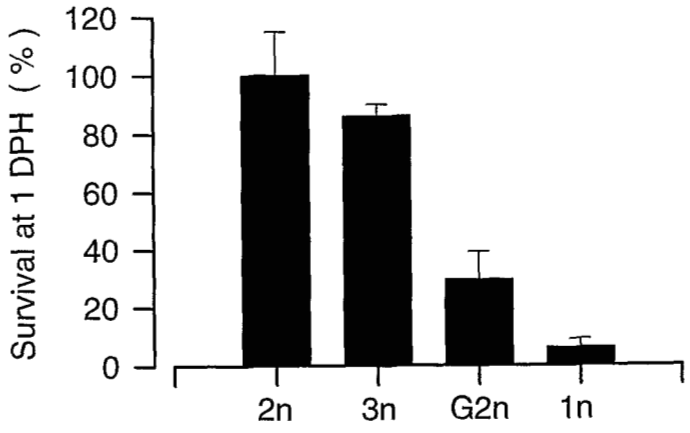

Fig. 1. Survival of seabass normal larvae at 1 DPH depending on ploidy level. Values relative to control 2n, set at 100%.

Table 2. Specific growth rate from juvenile diploid (control) and triploid (cold shock) sea bass

Month of year	Temperature range (°C)	Age (DPH)	Elapsed time (days)	Specific growth rate (%)		Ratio (3n /2n)
				Diploids	Triploids	
Aug	24 → 27	170-215	46	2.36 ± 0.02	2.42 ± 0.02	1,02
Nov	19 → 15	254-277	24	0.51 ± 0.11	0.48 ± 0.03	0,94
Jan-Mar	12 → 13	333-391	59	0.21 ± 0.04	0.16 ± 0.01	0,76
Jun-Jul	21 → 23	467-546	80	0.63 ± 0.03	0.59 ± 0.07	0,94
Jul-Oct	23 → 19	546-623	78	0.31± 0.03	0.38 ± 0.01	1,22

Values as mean ± SEM of two replicates per ploidy level (N=130-200 fish per replicate)

Similarly, triploidy induction did not affect the proportions of sexes. The sex ratio in triploid sea bass was similar to diploids, i.e., 2:1 *vs* 3:1 (not significant), respectively, in accordance to that reported for this species under farming conditions (Carrillo *et al.*, 1995). The percent males in diploids was 72.9% where in triploids was 65.5%. There were 27.1% females in diploids and 34.5% females in triploids (Table 3).

As reported by Sola *et al.* (1993), the sea bass has only one chromosome with 1 NOR per haploid genome. Therefore, counting of nuclear organizer regions (NOR) was used as technique to determine the ploidy level, where 1 or 2 NOR were indicative of diploidy and up to 3 NOR indicative of triploidy. However, since Ag-silver staining only detects active nucleoli the results obtained with this technique were confirmed by karyotyping. Juvenile triploid fish exhibited metaphase spreads with 72 chromosomes *vs* 48 in diploids.

Table 3. Sex ratio in juvenile diploid and triploid sea bass from 11 to 23 months of age

Ploidy level	Number sexed	Males (%)	Females (%)	Sex ratio
Diploid fish	95	72.9 ± 5.6	27.1 ± 5.6	3 ♂ : 1 ♀
Triploid fish	99	65.5 ± 2.3	34.5 ± 2.3	2 ♂ : 1 ♀

Values as mean ± SEM of two replicates per ploidy level

Inactivation of sea bass sperm DNA was obtained with a dose of UV-irradiation between 35,000-40,000 erg mm^{-2}. Embryos and larvae thus obtained exhibited a typical haploid syndrome, and determination of their ploidy showed 1 NOR per cell and 24 chromosomes. As expected under these treatment conditions, irradiated sperm maintained the ability to activate the development of eggs without genetically contributing to the future zygote. However, the expected haploid group still showed 5% of surviving normal larvae relative to control group (~2.5% in absolute values) (Fig. 1). This suggests that some spermatozoa escaped from irradiation. The extender used for diluting sperm did not affect the process of insemination. Finally, induction of gynogenesis was carried out by activating eggs with irradiated sperm at 35,000-40,000 erg mm^{-2} and subsequently shocked under the same optimal conditions used to retain the second polar body. The restoration of diploidy in gynogenetic larvae was corroborated by observing 1 or 2 NOR per cell and 48 chromosomes, similar to diploid larvae. Gynogenetics had a survival about 30% of that to the control diploid at 1 DPH (Fig. 1), probably due to the high homozygosity level that affects the development stability of these individuals (Leary *et al.*, 1985).

Conclusions

On the basis of the results obtained in the present work, we can concluded that the mass-production of triploid sea bass using cold shocks can successfully be carried out without affecting survival of these fish. At least until 2 years old, triploid sea bass exhibited a performance in growth and a proportion of sexes similar to that of diploid sea bass. However, it remains to be determined whether adult triploids will outgrow diploids when the latter approach sexual maturation. On the other hand, a method for induction of gynogenesis in the sea bass has been developed but it still remains to know the percentage of gynogenetic females obtained. Nevertheless, these potential improvements provide gains for short-term. The development of a adequate level of domestication in sea bass (and other marine species) needs to be established as a long-term breeding programme in addition to the short-term gains achieved with the present manipulations.

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