PRODUCTION OF HAPLOID AND DIPLOID GYNOGENETIC TURBOT *(Scophthalmus maximus L)*

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INTRODUCTION

The induction of gynogenesis was investigated in the turbot (Scophthalmus maximus L). Gynogenesis includes the production of diploid individuals with both sets of chromosomes from the female parent. Gynogenesis can be valuable as a rapid method for inbreeding of fish, positional mapping of genes in relation to their centromere, and production of exclusively female populations (Thorgaard and Allen 1987).

The production of gynogenetic fish was carried out by fertilizing eggs with sperm which had its DNA previously inactivated using UV light radiation so that the genetic material of spermatozoa would does not contribute to the genome of the zygote.

The radiation dose needs to be adjusted, so that the DNA of the sperm is completely inactivated without losing its mobility and therefore its capacity to fertilize the eggs and trigger embryonic development (Hertwig effect). The resulting zygotes are usually haploid, and therefore not viable after hatching. Diploidy must be restored by applying thermal shock or pressure treatments shortly after fertilization (Felip et al, 1999).

In this work, we have studied the appropriate conditions for production of diploid gynogenetic turbot. Different methods (karyotype analysis, nucleoli per nucleus counts, and microsatellite markers) were evaluated for gynogenesis checking. Survival, growth, and sex ratio, were analysed in one family of gynogenetics with relation to their corresponding diploid controls.

MATERIAL AND METHODS

I. Induction to gynogenesis

The induction of meiogynogenesis was carried out by fertilizing the eggs with sperm whose genome had been previously irradiated with UV ligth. Diploidy was restored by applying thermal shock treatment to the eggs minutes after fertilization (Piferrer et al., 2000; 2002). To achieve optimum induction of gynogenesis, three experiments were designed:

Experiment 1: Was performed to determine the effect of UV ligth on sperm mobility. The sperm, diluted in Ringer-200 (Chereguini et al., 1997), was divided into aliquots which were irradiated with increasing intensities of UV ligth from 5000 to 50000 erg.mm^{-2} except in the case of those used as a control. The mobility of the sperm treated was verified after its reactivation in sea water, according to the percentage of mobile cells (Sánchez-Rodríguez, 1975) and the duration of mobility.

Experiment 2: Was carried out to determine the quantity of UV ligth necessary to inactive the sperm's DNA without affecting its capacity to fertilize the egg and trigger embryos development (Hertwig effect).

Aliquots of eggs, were fertilized with irradiated sperm with UV ligth at increasing intensities from 300 to 100000 erg.mm^{-2}, them incubated at 13-14ºC. At 4, 5.72 and 144 hours, survival rates and the state of the larvae or embryos were determined and their haploidy condition was confirmed by counting chromosomes and nucleoli.

Experiment 3: Was carried out to establish the effect of UV ligth on the sperm in combination with thermal shock treatment applied to the newly fertilized eggs.

The eggs were fertilized with sperm irradiated with UV ligth to 30000-100000 erg.mm^{-2}, and then a cold shock treatment was applied (Piferrer et al., 2002). At the end of incubation, the fertilization, embryo genesis and hatching rates were determined.

II. Verification

The ploidy was examined in larvae collected 1 day after hatching, except for the sperm irradiated group, where the embryos were used because of the non- viability of haploid larvae. Metaphase plates were obtained according Kligerman and Bloom (1977), and were stained following the procedure of Howell and Black (1980). Chromosome counts and the number of nucleoli was examined in 10 plates and 50 nuclei, respectively in all individuals analysed.

The exclusively maternal inheritance of diploid gynogenetic, was determined by applying a set of 11 microsatellite loci, as genetic markers (Bouza et al., 2002). The potential of the microsatellite loci used for monitoring the production of meiogynogenetic turbot was evaluated by computing the two cumulative exclusion probabilities using the data from an Atlantic turbot population (Bouza et al., 2002). This population was selected as a reference among those analysed to obtain the potential of exclusion of microsatellites, because the brood stock kept at the IEO were founded with native individuals from this area.

III. Growth of gynogenetic turbot

One group of gynogenetic diploid turbot and their control, were cultivated separately for two years according to the established protocol for this species. Survival, growth and the percentage of sexes were monitored. The exclusively maternal inheritance was determined in larvae, two days after hatching, and confirmed at the end of the experiment in each of the fish in the meiogynogenetic group.

CONCLUSIONS

The production of gynogenetic diploid turbot was achieved by fertilizing eggs with sperm diluted 1:10 and irradiated with UV ligth at a dose of 30000-35000 erg.mm^{-2}. Diploidy was restored by applying a cold shock shortly after the fertilization, according to (Piferrer et al., 2002).

The hatching rate of meiogynogenetics diploids larva was 8,6 % compared to the control. with great mortality being observed during embryogenesis. However, after the larval phase, they exhibited similar survival, resistance to diseases and tolerance to treatment and vaccines as the controls, although their growth was 21% lower at 24 months of age.

Gynogenetic diploid turbot had a higher proportion of females (25% male and 75% female) than the controls (50 % male and 50% female).

Policy determination has been the traditional approach to evaluate gynogenesis in fish, however the obtention of good metaphases plates in fish is often hampered by the small size of chromosomes as compared and the lack of appropriate protocols for obtaining chromosomes of adequate quality. Nevertheless, the estimation of the number of nucleoli is a straightforward method, but the existence of a NOR-site polymorphism in the case of turbot, disturbs ploidy estimation by this approach. The use of hyper variable microsatellite loci described here constitutes a powerful and sound method to confirm the exclusive maternal inheritance in turbot gynogenetics.

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RESULTS

I INDUCTION OF GYNOGENESIS

Experiment 1

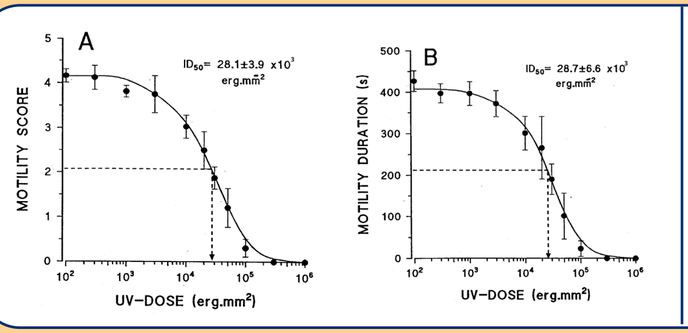

Fig. 1. Effect of exposure to different doses of UV light on the duration of the mobility of turbot spermatozoa diluted 1:10 with Ringer-200. A, effect on the mobility score, class 0 to 5, according to Chereguini et al. (1997). B, effect on the duration of mobility. The ID^{50} dose was calculated as the dose in which the mobility score on duration was reduced to 50% of the control.

It was observed that as the radiation dose increased, there was a decrease in sperm mobility, both in the percentage of mobile spermatozoids and in the duration of the mobility.

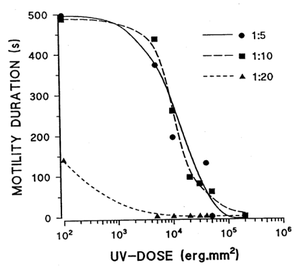

Fig.2. Effect of exposure to different doses of UV light on the duration of the mobility of turbot spermatozoa and according to different dilutions with Ringer-200.

Sperm from the same male at different dilutions showed that the greater the dilution, the lesser duration of mobility.

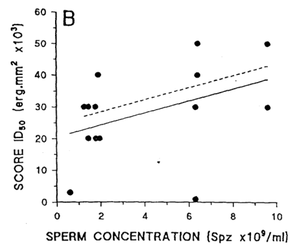

Fig.3. Relationship between sperm concentration and dose of UV light for which the score of mobility is reduced to 50%.

Sperm from different males with similar concentrations of spermatozoids did not respond in the same way to the application of UV radiation. This indicates that although the dilution of sperm is a factor to be taken into account there are other factors, like the quality of each sperm, which are more important than the concentration in determining the response to the effects of UV light.

Experiment 2

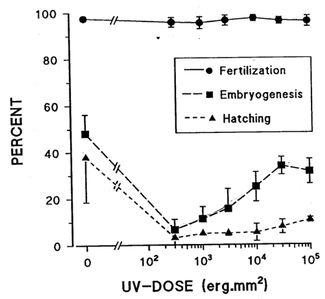

Fig. 4. Representation of a typical Hertwig effect curve in turbot. Survival is depicted at three different times: 4,5 h after fertilization (AF), embryogenesis (72 h AF) and hatching (144 h AF). The Hertwig effect takes place between 3 x 102 and 3 x 104 erg.mm^{-2}.

In the fertilisations made with sperm irradiated with increasing doses from 300 erg.mm^{2} onwards an increase of survival of larvae and embryos at 72 hours was seen between doses of 30 000-35 000 erg.mm^{-2}. These results are typical of the Hertwig effect, and for this reason it was considered appropriate the dose of 30 000-35 000 erg.mm^{-2} to inactivate the turbot sperm while maintaining in most cases their capacity to activate embryo development.

Experiment 3

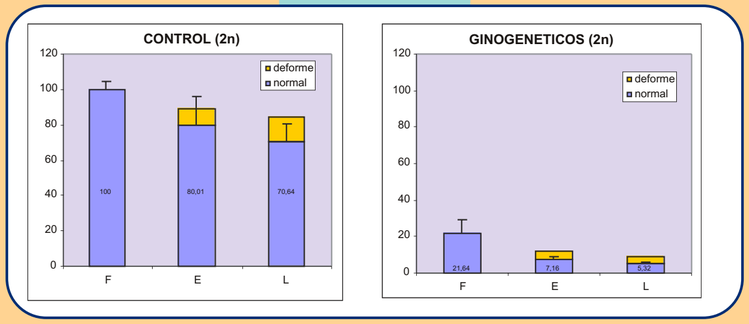

Fig. 5. Effect of gamete manipulation, fertilization with irradiated sperm with UV light , (30000-35000 erg.mm^{-2}) applied in combination with a cold shock on the viability of turbot during early developmental stages: fertilization 4,5 HPF (F); embryogenesis, 72 HPF (E); and 2 days post hatch, 144 HPH (L). (a), control diploid group, made with diluted sperm (control of gamete quality, with survival at fertilization set to 100%, to which the other treatments were compared). (b), gynogenetic diploid group made with diluted and irradiated sperm (30000-35000 erg.mm^{-2}) and thermally shocked (0ºC, applied at 6,5 min. post fertilization, during 25 min.) to restore diploidy (effect of UV light + thermal shock).

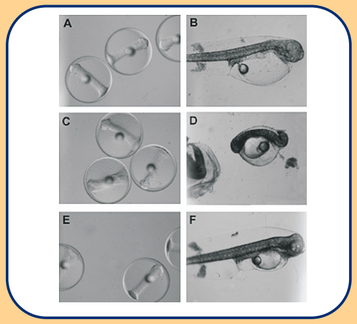

Fig. 6. Embryos (Fig. 2a) and hatched larvae (Fig. 2b) from de diploid control group, had a morphologically normal appearance, as expected. In contrast, embryos originating from eggs fertilized with sperm exposed to 30000-35000 erg.mm^{-2} , of UV light exhibited aberrant development (Fig. 2c). The few larvae that hatched were deformed, exhibing a typical " haploid syndrome" (Fig. 2d) . Group fertilized with UV-irradiated sperm, and thermally shocked had a external morphology similar to that of control diploids. (Fig. 2e, f).

II VERIFICATION

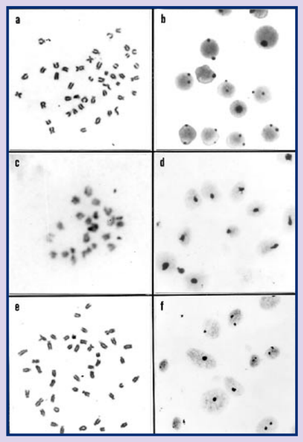

Fig. 7. Ploidy verification in turbot obtained after UV-irradiation of sperm. Typical karyotype and Ag-stained nuclei of cells obtained from control diploid (a and b, respectively; 2n= 44), gynogenetic haploid (c and d, respectively, 2n=22), and gynogenetic diploid (e and f, respectively, 2n=44) turbot embryos and larvae at 2 days after hatching and analysed in this study.

Locus	na	H(E)	PIC	Excl(1)	Excl(2)
Smax-01	13	0.742	0.704	0.355	0.532
Smax-02	24	0.845	0.829	0.545	0.707
Smax-03	9	0.635	0.581	0.227	0.390
Smax-04b	17	0.825	0.800	0.483	0.655
Sma3-8INRA	12	0.870	0.853	0.579	0.735
Sma3-12INRA	10	0.808	0.781	0.452	0.628
Sma4-14INRA	14	0.794	0.774	0.451	0.631
Sma5-111INRA	14	0.896	0.884	0.643	0.784
Sma1-125INRA	9	0.768	0.732	0.378	0.558
Sma3-129INRA	20	0.920	0.911	0.713	0.833
Sma1-152INRA	9	0.733	0.689	0.330	0.505
Mean/Total	13.73	0.803	0.776	0.999368	0.999992

Table I: Measures of genetic diversity and probabilities of exclusion for the 11 microsatellite loci used in the present work in an Atlantic natural population of turbot (Scophthalmus maximus; Bouza et al., 2002), where breeders used in the present work putatively proceeded. Calculations were performed with the computer program CERVUS 2.0 (Marshall et al., 1998). Primer sequences and PCR amplification conditions for the all the loci are described in Bouza et al. (2002).

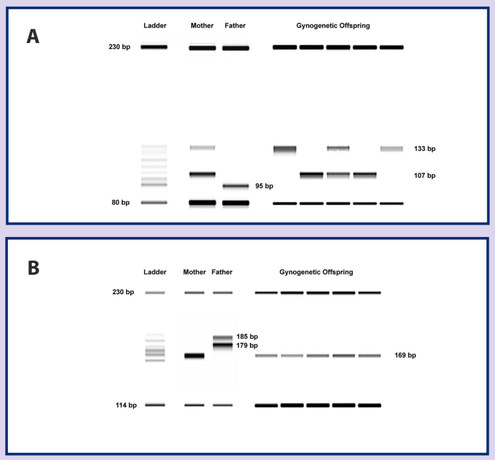

Fig. 8. Demonstration of the exclusive maternal contribution to gynogenetic offspring using the diagnostic microsatellite loci Smax-02 (a) and Sma3-129INRA (b) in each of the two families analysed, respectively. The electrophoretic patterns presented show in lane 1 the external standard size marker including an allelic ladder. Additionally, each lane contains two internal standard size markers (80-230bp (a); 114-230bp (b)) for genotyping accuracy. Lanes 2 to 8 contain correlatively the electrophoretic patterns of the two parents (a: 107/133 _ and 95/95 _; b: 169/169 _ and 179/185 _) and a sample of five gynogenetic offsprings (a: 107/107, 133/133, or 107/133; b: all 169/169).

III GROWTH AND DEVELOPMENT OF DIPLOID GYNOGENETIC TURBOT

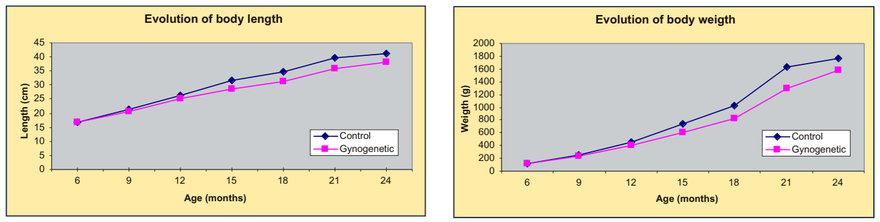

Fig. 9. Evolution from 6 to 24 months of age, of body weigth (a) and body length (b) of diploid and meiogynogenetic diploid turbot.

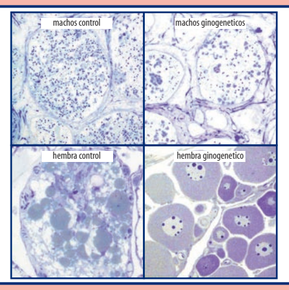

Fig. 10. Showing the microscopic appearance of testes (a, b) and ovaries (c, d) from diploid and meiogynogenetic diploid turbot at 24 months of age. (Stained with toluidine blue. Original magnification 200 x.).

Note the presence of spermatids and spermatozoa in the testis of both males diploid (a) and meiogenetic diploid (b), however a lower number of spermatozoa were evident in males meiogenetic diploid compared with those observed in males control diploid.

In contrast to males, clear differences in the gonadal development were observed in females between the control diploid group and the meiogynogenetic diploid. Whereas it was possible to identify mature oocytes in the ovary of females control diploid, in females meiogynogenetic diploid only perinucleolar oocytes were recognized.

Since it is not surprising the existence of undeveloped gonads in 24 month old turbot, the abnormal gonadal development in females meiogynogenetic diploid is an intriguing possibility worthy of further investigation.