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Induction of mitotic gynogenesis in turbot *Scophthalmus maximus*

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ABSTRACT

All-female production will maximize the profit potential for the aquaculture of the turbot *Scophthalmus maximus*, which exhibits significant sexually dimorphic growth. However, the basis for the technology of all-female production, i.e. the genetic mechanism of sex determination in turbot has been obscure so far, and might be ascertained by analysis of the sex ratio of its diploid mitotic gynogens. In this study, artificial mitotic gynogenesis in turbot was induced by hydrostatic pressure shock of its eggs activated by ultraviolet (UV)-irradiated sperm of red sea bream *Pagrus major*. Before the activation of turbot eggs, cryopreserved sperm of red sea bream were thawed, diluted 1:20 with Hank's solution, and UV-irradiated at a dosage of 6480–7200 erg/mm^2. Under water temperature of 14.5 $\pm$ 0.5 °C, treatments were tested by shocking the activated eggs at designed protocols: timings of 70–95 maf (min after fertilization) with an intensity of 70 MPa for 6 min; intensities of 60–85 MPa with a timing of 90 maf for 6 min; durations of 2–10 min with an intensity of 75 MPa initiated at 90 maf. The optimal protocol of the induction was determined to be pressure-shocking activated eggs initiated at 85–90 maf with 75 MPa for 6 min. A mitotic gynogen of 124 juveniles survived by 150 days after hatching. The ploidy of these juveniles was confirmed to be diploid by flow cytometry and their sex ratio was 1:1 female to male. The sex ratio of these juveniles supported the assumption of a genetic mechanism of sex determination of female heterogametes (ZW/ZZ) in turbot. This protocol can be used to create homozygous clones of double haploids that are very useful for researches on both the sex determination mechanism and selective breeding of this important farmed fish species.

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1. Introduction

Artificial gynogenesis, a technique of manipulation of the chromosome set, is widely used to ascertain the genetic mechanisms of sex determination in teleost fish. It also can be used to establish homogametic XX male or WW female broodstock (with a sex chromosome system of female “XX/XY” or male homogametic “ZZ/ZW”, respectively) to be used subsequently for the production of all-female juveniles at large-scale for commercial farming (Pandian and Koteeswaran, 1998; Hulata, 2001; Devlin and Nagahama, 2002; Komen and Thorgaard, 2007). Depending on the timing of diploidization of the haploidic embryonic chromosome in eggs fertilized by genetically inactivated sperm, meiotic or mitotic gynogenesis can be induced by blocking respectively the extrusion of the secondary polar body or the first embryonic cleavage with thermal or pressure shocks (Felip et al., 2001). Theoretically, the genetic mechanism of sex determination in teleosts can be ascertained by examining the sex ratio of diploid gynogens, with a female ratio of 100% indicating female homogamety (XX/XY) and 50% indicating male homogamety (ZW/ZZ), respectively. However, in practice, the female ratio of meiotic gynogens in many species of teleost is skewed from the theoretical percentage of 100% (XX/XY) or 50% (ZW/ZZ) because of either environmental effects or the crossover and recombination of sex-related genes between homologous chromosomes during the first meiotic cycle of eggs, or both of these factors (Baroiller et al., 2009; Martínez et al., 2009; Shelton and Mims, 2012; You et al., 2008). Therefore, for the investigation of the genetic mechanism of sex determination, mitotic is preferred to meiotic gynogenesis because it can produce completely homozygous diploids. Thus, it prevents the skewness of sex ratios in diploid gynogens caused by the crossover and recombination of sex-related genes (Devlin and Nagahama, 2002).

The turbot *Scophthalmus maximus* is an important marine fish cultured widely in Europe, Chile and China. Its annual production in China has been maintained at 50–60 thousand metric tons during the last decade, which is about 80% of the world's total output and makes a principal contribution to the production of land-based tank cultured marine finfish in China (Lei et al., 2012). Like other flatfish, such as the Japanese flounder *Paralichthys olivaceus* (Yamamoto, 1999), southern

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flounder *Paralichthys lethostigma* (Luckenbach et al., 2004) and half-smooth tongue sole *Cynoglossus semilaevis* (Chen et al., 2009), female turbot grow much faster than males as early as 8 months after hatching (Imsland et al., 1997). Therefore, all-female turbot populations are preferred for aquaculture because they can greatly shorten the aquaculture cycle and improve production efficiency. However, the technology for the production of all-female turbot juveniles has not been established so far, owing to the uncertainty regarding its sex determination system (Bouza et al., 1994; Cuñado et al., 2001; Cal et al., 2006a; Haffray et al., 2009; Martínez et al., 2009; Viñas et al., 2012). Until now, meiotic gynogenesis has been employed to reveal the genetic mechansim of sex determination of turbot, and both female (XX/XY) and male (ZZ/ZW) homogamety has been rendered (Baynes et al., 2006; Cal et al., 2006a; Piferrer et al., 2004). Mitotic gynogenesis, which could be a better way to solve the puzzle of the sex determination system in turbot because it has been reported that rearing temperature only has a minor effect on the sex differentiation of juveniles (Haffray et al., 2009), has not been reported so far.

The primary objective of this study was to establish a protocol for inducing mitotic gynogenesis in turbot, thereby producing sufficient juveniles of a diploid mitotic gynogen for the analysis of their sex ratio to reveal the genetic mechanism of sex determination and the establishment of either homogametic male (XX) or female (WW) broodstock to produce an all-female population for commercial farming. The ploidy of the mitotic gynogens was also evaluated using NORs (nucleolus organizer regions)-banding and flow cytometric analysis.

2. Materials and methods

2.1. Broodstock maturation and gamete collection

A turbot broodstock of 25 females and 27 males (body weight 1.5–4.0 kg, 5 years old), which originated from artificially reproduced juveniles, was held in two 36,000 l concrete tanks at Tianyuan Aquaculture Co., Ltd (Yantai City, Shandong province, China). To induce their maturation, the broodstock had been maintained under controlled conditions of a photoperiod of 16 h light:8 h dark and a water temperature of 12–14 °C for more than two months prior to the experiment.

When females showed signs of ovulation, with obvious swelling and "softening" of the abdomen, they were checked for ovulation daily by exerting gentle abdominal pressure. Ovulated eggs from each female were collected into a 500 ml glass beaker and their total volumes were recorded individually. Only batches of eggs with a volume more than 100 ml and a quality estimated to be high (McEvoy, 1984; Kjørsvik et al., 2003) were retained at a room temperature of about 14 °C for fertilization later. At the same time, milt from 2–3 running males was collected into a 2.5 ml polypropylene syringe without a needle and kept refrigerated at 4 °C for later use. Sperm motility was checked under light microscopy following activation with sea water.

2.2. UV irradiation of sperm of red sea bream

Cryopreserved sperm of the red sea bream *Pagrus major* were used for the induction of mitotic gynogenesis in turbot in this experiment. Sperm of the red sea bream were cryopreserved in 2-ml cryovials as described by Liu et al. (2006). Before ultraviolet (UV) irradiation, the cryovials were put into a 37 °C water bath for 90–110 s until most of the frozen sperm had thawed. Subsequently, the cryovials were placed at room temperature for about another 60 s to defrost the sperm inside completely. The thawed sperm were diluted 1:20 by volume with Hank's balanced salt solution precooled to 4 °C. Diluted sperm in volumes of 4.5 ml were spread thinly over a glass Petri dish (12 cm diameter) to form a thin layer 0.4 mm in depth and UV irradiated at a dosage of 6480–7200 erg/mm^2 with an ultraviolet crosslinker (SCIENT Z03-II, Ningbo Scientz Biotechnology Co., Ltd.) as described by Meng et al. (2013). The irradiated sperm were refrigerated at 4 °C, and prior to use, their motility was confirmed by checking the sea water-activated sperm using light microscopy.

2.3. Artificial fertilization and egg hatching

Turbot eggs were split into different groups in selected volumes, held in individual 1000 ml dry glass beakers and fertilized with irradiated/non-irradiated diluted sperm of red sea bream or untreated sperm of turbot. The ratio of sperm to eggs by volume was 10:1 for red sea bream sperm and 50:1 for turbot sperm. After being mixed thoroughly with a feather, the gametes were activated by adding seawater at 14.5 °C and twice the volume of the gametes. The moment of activation of the gametes was taken as time zero for the development of the eggs. Sixty seconds later, sea water was added further to a total volume of 900 ml and the fertilized eggs were left undisturbed at 14.5 °C until pressure shock treatment or hatching. Before the treatment, floating eggs were collected and rinsed, and put into individual plastic vials with perforated mesh for the shock treatment. Floating eggs in the control groups (unshocked samples) were also collected, rinsed, and moved to a hatching tank.

After the treatment, fertilized eggs were incubated in net cages which were suspended inside 2500 l FRP (fiberglass reinforced plastics) tanks with flow-through sea water at 14.5 ± 0.5 °C. Dependent on the number of fertilized eggs, small (15 l) net cages or large (100 l) net cages were used.

Survival of developing eggs and viable larvae was recorded at different developmental stages: the fertilization rate at 4 h after fertilization (haf) and the hatching rate at 125 haf were recorded. The abnormality rate (ar) was also recorded at 125 haf as the percentage of abnormal larvae in the total number of hatched larvae.

2.4. Pressure and cold shock treatment

An Electrical Hydrostatic Pressure Chamber (Key-B001, Qingdao Starfish Instrument Co., Ltd, China) was used to inhibit the first division of turbot embryonic cells. The vials holding the eggs for treatment were placed in a 1500 ml stainless steel cylinder filled completely with sea water at 14.5 °C, and the cylinder was sealed with a screw cap. The pressure inside the cylinder was elevated to the required level in less than 5 s and kept stable at that level with an electric air pump. Decompression was instantaneous at the end of the treatments and the treated eggs were moved to the hatchery tank to hatch.

Cold shocks were used to induce meiotic gynogenesis by soaking the vials in a polystyrene box containing ice and water at −2 °C. The temperature was monitored constantly throughout the treatment.

2.5. Optimal parameters of pressure treatment

Three experiments were designed to determine the optima of three key parameters (timing, intensity and duration) of the hydrostatic pressure treatment for the induction of mitotic gynogenesis in turbot. In each experiment, among the three key parameters, one was tested at the selected alternative levels while the other two were maintained individually at a fixed level: alternative timing of 70, 75, 80, 85, 90 and 95 maf (min after fertilization) with a fixed intensity of 70 MPa for 6 min; alternative intensity of 60, 65, 70, 75, 80, and 85 MPa with a fixed timing of 90 maf for 6 min; alternative duration of 2, 4, 6, 8, and 10 min with a fixed intensity of 75 MPa initiated at 90 maf. For each experiment, 70 ml eggs from one female were divided into equal samples and put individually into a 1000 ml beaker; one sample was fertilized with normal turbot sperm to create a counterpart control group, and the other samples were fertilized with UV-irradiated sperm of red sea bream, shocked with hydrostatic pressure, and moved to a small net for hatching as described above. All experiments were repeated up to three times using egg batches derived from different females. Data on the fertilization rate and hatching rate of the eggs, and the abnormality

rate of larvae in each experiment were collected and analyzed to determine the three optima.

2.6. Comparison between mitotic and meiotic gynogenesis

Three repeated experiments were designed to compare mitotic with meiotic gynogenesis. In each experiment, 100 ml of eggs from one female were divided into four equal groups, and each group was held individually in a 1000 ml glass beaker. One group was fertilized with normal turbot sperm to create the group of normal control diploid (NCD). Two groups were fertilized with irradiated or non-irradiated sperm of red sea bream and then shocked with pressure under the optimal conditions determined above (see Section 2.5) to create mitotic gynogenetic diploids (MID) and putative hybrid tetraploids (PHT), respectively. The fourth group was fertilized with irradiated sperm of red sea bream and then temperature shocked in −2 °C sea water for 45 min at 6.5 maf to create meiotic gynogenetic diploids (MED) as described by Meng et al. (2013).

After treatment, the eggs were moved to large nets for hatching. Data on the fertilization rate and hatching rate of eggs, and the abnormality rate of larvae in each experiment were collected with the method described above (see Section 2.3). The ploidy of the newly hatched swimming larvae was determined by counting the number of NORs (nucleolus organizer regions) in the nuclei of 50 cells from each larvae (20 larvae per group) following the method established by Pifferer et al. (2000).

2.7. Production of mitogynogenetic diploid juveniles

Approximately 1100 ml eggs pooled from four females were divided into two groups: one group contained 50 ml eggs that were fertilized with normal turbot sperm to create a control group, and the other group contained 1050 ml eggs that were fertilized with irradiated sperm of red sea bream and shocked with pressure under the optimal condition determined in the above experiment (see Section 2.5) to create the mitotic gynogenesis group. After the fertilization and treatment, eggs of both groups were hatched in large nets using the method described above (see Section 2.3). The hatched larvae were reared using the protocol for turbot developed by Person-Le Ruyet et al. (1991). Juveniles were cultured in indoor tanks with flow-through sea water at a temperature of 18–21 °C and fed on commercial dry feed.

Data on the survival rates of larvae and juveniles in both groups were collected at 1, 40, 60 and 150 days after hatching (dah). The body weights and total lengths of 30 juveniles were measured at 150 dah. After random selection, juveniles were killed with a lethal dose of tricaine methane sulphonate (MS-222, Sigma) at 180 dah. The gonads of 30 gynogens and 80 juveniles from the control group were dissected and sexed by histological examination as described by Cal et al. (2006b). The ploidy of the mitotic gynogens was analyzed individually with a PARTEC cell counter analyzer (CCA-II; PARTEC, Germany) while the control group was used as a diploid standard.

2.8. Statistical analysis of data

Data on the hatching rate of eggs and the abnormality rate of larvae in the experiments in Sections 2.5 and 2.6 were arcsin transformed before analysis with Tukey's honest significant differences test (ANOVA). All differences analyzed were assessed at a significance level of 0.05. SPSS 17.0 software was used for data analysis.

3. Results

3.1. Optimal parameters of pressure treatment

The average fertilization rates in the nine control diploid groups were 84.6% ± 2.7%, 83.6% ± 4.2%, 78.3% ± 4.1%, 77.1% ± 5.0%, 78.1% ± 2.9%, 77.1% ± 5.0%, 82.8% ± 2.6%, 84.4% ± 3.8% and 86.3% ± 1.6% (N = 3 per control group). Although each batch of eggs was from a different female, there were no significant differences by ANOVA analysis ($p > 0.05$) in the fertilization rates among these nine control diploid groups, owing to the adoption of measures for the quality control of eggs and sperm.

With the exception of 70 maf, the other five timings (75, 80, 85,90, and 95 maf) were within the period for the inhibition of the first division of turbot embryonic cells pressure shocked with an intensity of 70 MPa for 6 min (Fig. 1-A). However, the groups treated at 85 and 90 maf had significantly higher hatching rates and lower abnormality rates than the other three groups (75, 80 and 95 maf) ($p < 0.05$), and had no obvious differences between them ($p > 0.05$) in these two rates. Therefore, the optimal timing for the pressure shock was 85–90 maf at a water temperature of 14.5 ± 0.5 °C for fertilization and hatching. The yields of gynogenetic diploid embryos, computed as the percentage of hatched normal larvae over the total number of fertilized embryos, were 1.10% ± 0.32% and 1.19% ± 0.34% when the treatments were initiated at 85 maf and 90 maf, respectively.

When initiated at 90 maf and lasted for 6 min, pressure shocks at all six intensities examined (60, 65, 70, 75, 80, and 85 MPa) could produce viable mitotic gynogens (Fig. 1-B), and both the maximum hatching rate and the minimum abnormality rate were recorded at an intensity of 75 MPa, with the highest yield of normal larvae of 0.90% ± 0.40%. Therefore, 75 MPa was considered to be the optimal intensity for the pressure treatment.

When lasting for 2, 4, 6, 8, and 10 min, respectively, all pressure shocks initiated at 90 maf with intensity of 75 MPa could produce viable mitotic gynogens. The two groups given shocks lasting for 4 and 6 min had significantly higher hatching rates and lower abnormality rates than the three with shocks lasting for 2, 8 and 10 min, respectively (Fig. 1-C, $p < 0.05$). Between two groups with shocks of 4 and 6 min, the difference in hatching rate was significant, but the abnormality rate was not significantly different. Therefore, 6 min was found to be the optimal shock duration, given the higher yield of gynogens, 1.46% ± 0.23% when compared with 1.22% ± 0.45% at 4 min.

3.2. Comparison between mitotic and meiotic gynogenesis

The rates of fertilization, hatching and abnormalities of the four groups (NCD, MEI, MIT and PHT) using eggs from three females are presented in Table 1. There were no significant differences among the four groups in the fertilization rates when analyzed by ANOVA ($p > 0.05$). All the embryos in the three PHT groups died before hatching, therefore hydrostatic pressure shock initiated at 90 maf with 75 MPa for 6 min could not produce viable larvae of a putative hybrid tetraploid of turbot. When compared with the NCD, both the pressure shock in MIT and the cold shock in MEI significantly decreased the hatching rate and increased the abnormality rate, respectively ($p < 0.05$). At the same time, the average hatching rate in MIT (2.26%) was significantly lower than that in MEI (27.97%), and the abnormality rate showed the opposite tendency (46.64% in MIT and 22.89% in MEI, respectively) ($p < 0.05$). The average percentages of normal larvae were 67.8%, 21.6% and 1.2% in NCD, MEI and MIT, respectively, and the differences between each pair were significant ($p < 0.05$).

The newly hatched swimming larvae from the NCD, MEI and MIT were confirmed to be diploid on the basis of the average number of NORs of each larva tested (n = 20 in each group), which ranged from 1.15 to 1.62 (Fig. 2).

3.3. Production of mitogynogenetic diploid juveniles

3.3.1. Survival rate and growth of the diploid mitotic gynogen

The results of the induction of diploid mitogynogenesis on a large-scale with the optimal protocol of pressure shock (see Section 3.2), and its normal control group are presented in Table 2. The fertilization

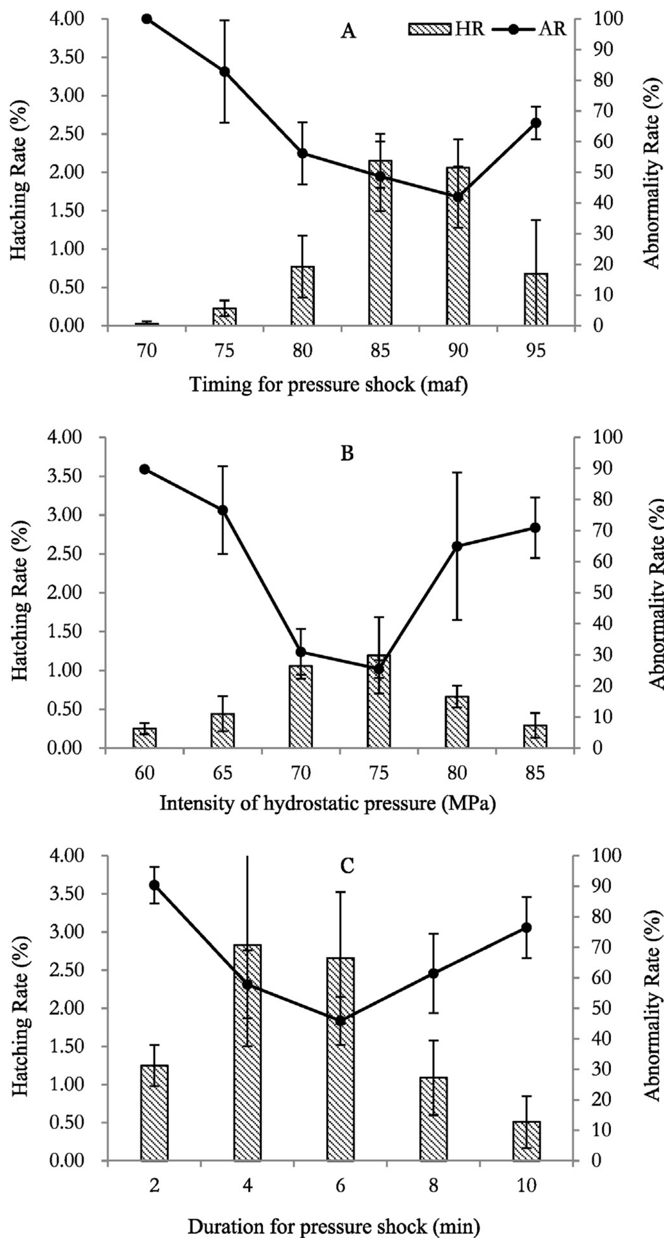

Fig. 1. Effects of timing, intensity and duration of the pressure shock on the hatching rate of eggs and the abnormality rate of larvae of the mitotic gynogens of turbot. Eggs activated with UV-irradiated red sea bream sperm were pressure shocked at: different timings of 70, 75, 80, 85, 90, and 95 maf (min after fertilization) with a fixed intensity of 70 MPa for 6 min (Fig. 1-A); different intensities of 60, 65, 70, 75, 80, and 85 MPa with a fixed timing of 90 maf for 6 min (Fig. 1-B); different durations of 2, 4, 6, 8, and 10 min with a fixed intensity of 75 MPa initiated at 90 maf (Fig. 1-C). Data are presented as means and standard errors of the raw data from three replicated experiments.

rate in the treatment group and the control group was 84.5% and 87.6%, respectively. The hatching rate of the treatment group was much lower than that of the control group, and the abnormality rate was the direct opposite. The survival rate of gynogens in both groups with a period of 1–40 dah (3.3%) and those with 40–60 dah (51.1%) was much lower than the counterpart in the control group (18.8% and 82.1%, respectively), but was similar to that in the control group with a period of 60–150 dah (99.0% in gynogen and 95.4% in control). Besides the lower survival rate of the gynogens before 60 dah, the growth of the gynogens was significantly lower than that of the counterparts in the control group both in total length and in body weight, at 150 dah. The average body weight of the gynogen group at 150 dah (16.2 g) was less than half of that in the control group (33.0 g).

3.3.2. Ploidy and the sex ratio of the mitotic gynogen

At 180 dah, a total of 124 mitotic juveniles survived out of 1050 ml of eggs pooled from four females. The ploidy of all 30 randomly selected 180 dah larvae of the gynogen was identified as diploid with a value of cellular DNA content equal to that of diploid control (Fig. 3). The female ratio was 50% among the 30 juveniles sampled from the mitotic gynogen and 55% in the 80 juveniles sampled from the control group; neither of these ratios deviated from the 1:1 ratio with a χ^2-test.

4. Discussion

In this study, a protocol for the induction of mitotic gynogenesis in turbot utilizing heterologous sperm was developed. A mitotic gynogen of more than 7000 normal larvae was produced by the method established and, among these, a total of 124 juveniles survived at 150 dah. The sex ratio of the mitotic gynogen was 1:1 female to male.

Heterologous sperm has been used to induce gynogenesis in a number of teleost fish, because it is either convenient to obtain or incapable of producing viable diploid hybrids with the receiving species, or both (Morgan et al., 2006; Purdom and Lincoln, 1974; Váradi et al., 1999). Given that turbot are poor producers of milt in terms of both quality and quantity, when compared with most other domesticated marine teleosts (Suquent et al., 1994), heterologous is preferred to homologous sperm for the activation of turbot eggs in gynogenesis. Sperm of the red sea bream was chosen in this study because it comes from a widely farmed species, methods of cryopreservation and genome inactivation for it are well established (Liu et al., 2006), and it has been used successfully to induce gynogenesis in several marine fish (Gorshkov et al., 2002; Peruzzi and Chatain, 2000; Yamakawa et al., 1987; Yamamoto, 1999). Use of cryopreserved sperm can avoid the necessity to synchronize the breeding season of female turbot with male red sea bream and facilitate the standardization of UV irradiation procedures.

Among the three parameters (timing, intensity and duration) of the induction of mitotic gynogenesis with pressure shock treatment to prevent the first division of teleost embryos, the timing is the most critical because it is more species specific than the other two parameters and is usually narrowed down to an instant, or a range of a few minutes (Diter et al., 1993; Francescon et al., 2004; Komen and Thorgaard, 2007). So far, among the four successful procedures for mitotic gynogenesis in marine

Table 1
Fertilization rates, hatching rates, and abnormality rates among the normal control (NCD), meiotic gynogenetic diploid (MEI), mitotic gynogenetic diploid (MIT) and putative hybrid tetraploid (PHT) eggs from three females (F1, F2 and F3, respectively).

Groups	Fertilization rate (%)				Hatching rate (%)				Abnormality rate (%)			
	F1	F2	F3	Ave	F1	F2	F3	Ave	F1	F2	F3	Ave
NCD	90.91	86.19	80.51	85.87	78.84	71.48	64.30	71.54	5.67	5.05	7.08	5.93
MEI	87.20	70.72	60.76	72.89	31.22	26.61	26.08	27.97	21.46	24.08	23.12	22.89
MIT	83.96	67.94	64.86	72.25	4.41	0.78	1.59	2.26	53.06	41.25	45.61	46.64
PHT	88.32	78.46	68.93	78.57	0	0	0	0	–	–	–	–

Note: Ave means the average of equivalent data from the three females.

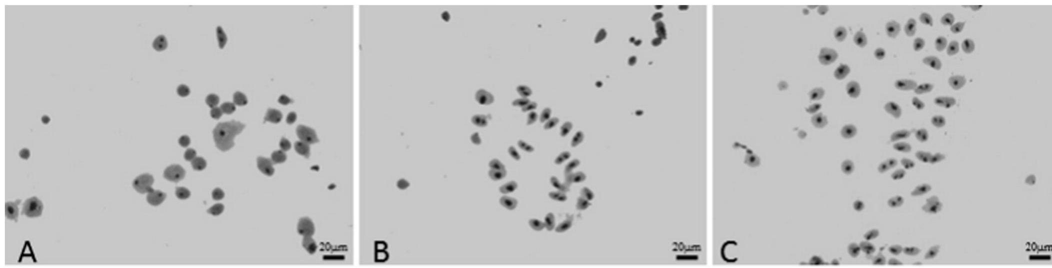

Fig. 2. Ploidy identification of the newly hatched swimming larvae from the normal control diploid (A), meiotic gynogenetic diploid (B) and mitotic gynogenetic diploid (C) by counting the number of NORs in the nuclei of 50 cells from each group of larvae (n = 20 larvae per group).

fish induced by blocking the first embryonic division, each has a sharply fixed timing: 60 maf for Japanese flounder (Tabata and Gorie, 1988), 45 maf for red sea bream (Takigawa et al., 1994), 21.5 maf for tongue sole (Chen et al., 2012), and 85–97 maf for sea bass (Francescon et al., 2004). In this study, the optimal timing for turbot was determined to be 85–90 maf, which is quite similar to that for European sea bass but much later than that for the other three marine fish species. The sharing of similar characteristics and the hatching temperature of eggs may be the reason for the similarity of timing between turbot and sea bass.

According to Francescon et al. (2004), the optimal timing is in accordance with a "time window" of spindle formation to furrow appearance during the first division of the fish embryo. This time window for turbot has been identified as 74–90 maf when eggs are hatched at 15.5 °C (Sun et al., 2005) and will be slightly later if eggs are hatched at a lower temperature of 14.5 °C as in our study. Therefore, the optimal timing of 85–90 maf determined in our study was in accordance with the time window of spindle formation to furrow appearance during the first division of the turbot embryo.

The other two parameters (intensity and duration) are less species specific than the timing. Excepting the confusion caused by the use of multiple units of pressure intensity in relevant studies, such as kg/cm^2 (Hussain et al., 1993) and psi (Goudie et al., 1995; Johnstone and Stet, 1995), the optimal pressure intensity of 75 MPa determined in this study is in agreement with that of 70 MPa determined for most teleost species reviewed by Komen and Thorgaard (2007). With regard to the duration, theoretically it should be related to the speed of embryonic development of the species of fish tested, but for reasons of practicality, an arbitrary shock time of 4–10 min has been applied to most of the reported cases (see review by Komen and Thorgaard, 2007).

All the hybrid embryos between turbot eggs and red sea bream sperm, whether shocked with hydrostatic pressure (in this study) or not (in our previous study, Meng et al., 2013), was dead before hatching. This indicates that these hybrids, either in the form of a diploid or in the form of the putative tetraploid that could possibly be induced by pressure shock, cannot survive past the egg stage. That haploid embryos of turbot cannot survive past the larval stage has also been observed repeatedly in our previous experiments and other related studies (Meng et al., 2013; Piferrer et al., 2004; Xu et al., 2008). The combination of the above observations theoretically excludes the possibility of juveniles, other than gynogenetic diploids, arising from the protocol of mitotic gynogenesis developed in this study: haploid from failure of shocking, hybrid diploid from failure of both UV irradiation and shocking, or hybrid tetraploid from failure of UV irradiation. In addition, juveniles of the mitotic gynogens in this study were affirmed to be diploid by analysis with flow cytometry. Therefore, it is safe to assume that all the surviving juveniles from the mitotic gynogenetic larvae formed in this study were diploid mitotic gynogens.

Compared with the control, both the meiotic and mitotic gynogenesis significantly reduced the hatching rate and increased the abnormality rate in turbot. The decrease in the viability of the gynogens has been attributed to their expected high level of inbreeding, which can increase the opportunity for the expression of lethal recessive alleles in their genome (Arai, 2001; Kavumpurath and Pandian, 1994; Suwa et al., 1994; Taniguchi et al., 1990). Between the two kinds of gynogenesis employed in this study, the yield of normal larvae from mitotic gynogenesis (1.2%) is just 6% of that from meiotic gynogenesis (21.6%). Theoretically, as a completely homozygous diploid, a mitotic gynogen is supposed to have two homozygous alleles in each locus in its genome, and this greatly increases the rate of mortality from lethal recessive alleles. However, for a heterozygous locus holding a recessive lethal allele in the maternal parent, meiotic gynogens could potentially still be heterozygous at this locus owing to the phenomenon of crossing over between the homologous chromosomes during the meiotic division of the ova. This will greatly increase the likelihood of juveniles surviving with lethal recessive alleles in their genome (Francescon et al., 2005; Chen et al., 2009).

The sex ratio of mitotic gynogens in this study was 1:1 female to male, and this was in accordance with the assumption of a genetic sex determination mechanism involving female heterogametic ZW/ZZ in turbot (Baynes et al., 2006; Haffray et al., 2009; Martínez et al., 2009; Taboada et al., 2012; Vale et al., 2014; Viñas et al., 2012). If the rearing temperature truly had a minor effect on the sex differentiation of turbot juveniles in our study, which is very possible because not only has this been reported by Haffray et al. (2009) but also the sex ratio of the counterpart control group was nearly identical to 1:1 in this study, then the genetic mechanism of sex determination of turbot could be ascertained

Table 2
Induction of mitotic gynogenesis in turbot on a large scale and its normal control.

Variable	Normal control	Mitotic gynogenetic diploids
Volume of eggs used (ml)	50	1050
Approximate total number of eggs used	6.0×10^4	12.6×10^5
Fertilization rate of eggs (%)	87.6	84.5
Hatching rate of fertilized eggs at 125 haf (%)	67.8	1.3
Abnormality rate of hatched larvae at 125 haf (%)	8.5	4.0
Survival rate of hatched normal larvae in the period from 1 to 40 dah (%)	18.8	3.3
Survival rate of juveniles in the period from 40 to 60 dah (%)	82.1	51.1
Survival rate of juveniles in the period from 60 dah to 5 months (%)	95.4	99.0
Body weight at 150 dah (g) (n = 20)	33.02 ± 6.23	16.18 ± 11.30
Total length at 150 dah (cm) (n = 20)	12.24 ± 0.77	9.18 ± 2.03
Total number of larvae remaining at 150 dah	4800	124

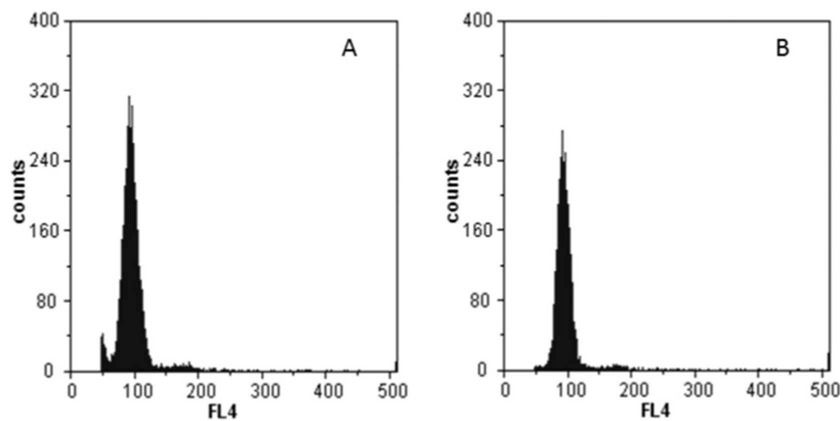

Fig. 3. Ploidy identification of larvae in normal control (A) and mitotic gynogen (B) of turbot by flow cytometry analysis.

to be female heterogametic. The reason for this is that it is almost impossible for a homogametic female teleost (XX/XY) whose sex differentiation is not sensitive to environmental temperature to produce a mitotic gynogen with such a high percentage of males (nearly 50%). However, it will be prudent to test this conclusion by investigating the sex ratios of more families of mitotic gynogens and the effect of temperature on the sex differentiation of juveniles with an experiment designed specifically for this objective.

The protocol for mitotic gynogenesis established in our study will be useful for generating inbred strains of turbot because of the complete homozygosity in diploid individuals. Homozygous clones can also be generated by a second generation of meiotic or mitotic gynogenesis. Maintenance of such clones by normal breeding will be beneficial to both experimental research and commercial applications in turbot aquaculture as demonstrated for other teleosts (Kobayashi et al., 1994; Komen and Thorgaard, 2007; Komen et al., 1991; Bertotto et al., 2005).

In conclusion, a protocol for the induction of mitotic gynogenesis in turbot has been created in this study by applying a hydrostatic pressure shock to eggs activated with UV-irradiated heterologous sperm. More than 100 diploid gynogens with a sex ratio of 1:1 female to male were produced with this protocol. The assumption of a genetic mechanism of sex determination of female heterogametic ZW/ZZ in turbot is supported by the sex ratio of mitotic gynogens in our study and it should be verified by further research on more families of gynogens produced by our protocol. This protocol also establishes the basis for the creation of homozygous clones of double haploids, which will facilitate further genetic research and breeding of this important farmed fish species.

Statement of relevance

Turbot is an important fish species widely cultured in European, Chile and China. The protocol described in this m0061nuscript can be used both for the development of all female production technique and the creation of homozygous clones of turbot, which will improve the growth rate greatly and facilitate its selective breeding.

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