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RESEARCH PAPER

Effects of Triploid Induction to the Early Cleavage Patterns of Black Sea Turbot (*Psetta maxima*) Embryos

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Abstract

Fertilization and embryonic survival rates and cleavage pattern were examined to test the effects of triploid induction in the Black Sea turbot (*Psetta maxima)*. Fertilization rates were similar between cold shock and control groups, but the incidence of 4 different blastomere malformations were higher in cold shock group than that of control group. A positive linear correlation was detected in between normal blastomere morphology and embryo viability in all treatment groups. Except blastomere asymmetry, no other morphological abnormalities found correlated with embryo survival. Blastomere asymmetry showed negative linear correlation with embryo survival only in cold shock group. The results of the study demonstrated that blastomere morphology could be used in assessing egg quality and the negative effects of triploid induction in the Black Sea turbot.

Keywords: Black Sea turbot, *Psetta maxima*, egg quality, blastomere morphology, triploid, cold shock.

Introduction

The Black Sea turbot, *Psetta maxima*, large flat fish species, inhabits in the Black Sea and the Azov Sea basins. Seed production studies for this species have been conducted since 1997 with cooperation of Japan International Cooperation Agency (JICA) and Central Fisheries Research Institute (CFRI) in Trabzon, Turkey.

Large sized triploid fish supply does not only prevent undesired reproduction, but also helps to improvement of survival and growth rates, and meat quality in fish culture because of sterility (Arai, 2001; Felip *et al*., 2001; Mori *et al*., 2006; Segato *et al*., 2007). A reliable protocol for triploidy induction in turbot has been established (Piferrer *et al*., 2000; 2003). Although survival or growth rates do not exhibit any differences between diploid and triploid turbots, triploidy increases the proportion of females, which are the fastest growing sex in this species (Cal *et al*., 2006). Additionally, once fish exceeded 1.7 kg mature, triploid turbot exhibits faster growth than the diploids (Cal *et al*., 2006). Therefore, induction of triploidy presents an asset for large size turbot production.

Triploid turbot can be obtained by cold shock application after fertilization, but this practice causes increased embryonic mass mortality (Piferrer *et al*., 2000; 2003). In addition, induction of triploidy in the turbot creates considerable variation in egg quality and there are no reliable indicators to assess egg quality. Blastomere morphology is widely used for egg quality estimation in many marine fish. Abnormal cell cleavages are thought to be egg defects (McEvoy, 1984; Kjørsvik *et al*., 1990; 2003; Shields *et al*., 1997). There is much supporting evidence, for example, that abnormalities increase the susceptibility of eggs to bacterial contamination (Hansen and Olafsen, 1989; Pavlov and Moksness, 1993) or pollution (Longwell *et al*., 1992). A positive correlation was detected between normal blastomeres morphology and hatching success (McEvoy, 1984; Devauchelle *et al*., 1988; Kjørsvik *et al*., 1990, 2003; Pickova *et al*., 1997). There are few studies about cleavage patterns, which exhibit details of blastomere morphology (Shields *et al*., 1997; Rideout *et al*., 2004; Avery and Brown, 2005). Studies with blastomere morphology as an indicator of triploid egg quality are very rare. For example, Kjørsvik *et al*. (2003) studied blastomere morphology with only normal or abnormal classification in the turbot. The main aim of the present study was to investigate the effect of triploid induction by cold shock on blastomere morphology and its relationship with embryonic mortality in the Black Sea turbot.

Materials and Methods

Broodstock Management, Gamete Collection and Artificial Fertilization

Black Sea turbot broodstock were maintained in a flow-through sea water system with ambient temperatures and natural daylight regime in the Marine Hatchery of the Central Fisheries Research Institute, Trabzon, Turkey. Fish were fed with a diet including whiting with vitamin mix throughout the year except spawning period. Four-year-old females (mean live weight of 3423.1g±962.58g) and males (mean live weight of 2050.7g±165.32g) were transferred into a fiberglass reinforced plastic tank of 1mx2mx0.5m dimensions containing a divider set at the center. During the spawning period temperature was kept at 14°C using a titanium heater. A pellet form of 100µg LHRH-a/kg hormone was implanted into the muscle near the dorsal fin of female turbot using a metal tube.

Eggs from each of five females were stripped into separate clean plastic containers, filled with an adequate amount of sea water (18 ppt and 14°C) in May 2007. Eggs were fertilized in seawater by mixing with stripped milt from two different males for each female. Fertilization was taken as time =0. Thirty seconds after fertilization (AF), eggs from each female were divided into 70 g (900 eggs/g) batches and kept in 500 ml glass beakers until used. Five minutes AF, eggs were rinsed with filtered seawater (Figure 1). The seawater used for fertilization and rinsing the eggs was at the same temperature as the seawater in which the broodstock were maintained. Half an hour AF, the eggs were disinfected for 5 min in 50 ppm PVP iodine and rinsed in filtered seawater. A fraction of the stripped eggs was observed under a microscope and viability was assessed according to criteria of McEvoy (1984). Fertilization rates were calculated by counting the proportion of 8 cell stage eggs at 37.5 degree-hours AF.

Cold Shock Application and Experimental Design

For cold shock administration, eggs were poured into 1000 ml glass beakers containing water pre-chilled to the desired temperature (~ -0.5°C) and placed in a 10 l plastic bucket filled with 7 l of crushed ice and seawater mixture. Then, the bucket was placed into an incubator (Sanyo MIR 153 Sanyo Electric, Osaka, Japan which was set at 2.5°C. This way, water temperature inside the beakers was maintained in between -1 to 0°C as monitored with a precision thermometer (Figure 1).

The experiment consisted of three treatment groups. In the cold shock group, fertilized eggs from each female were exposed to just below 0°C cold water at 6.5 min AF for 20 min as recommended by Piferrer *et al*. (2000). In the control treatment, fertilized eggs were transferred to the incubators with

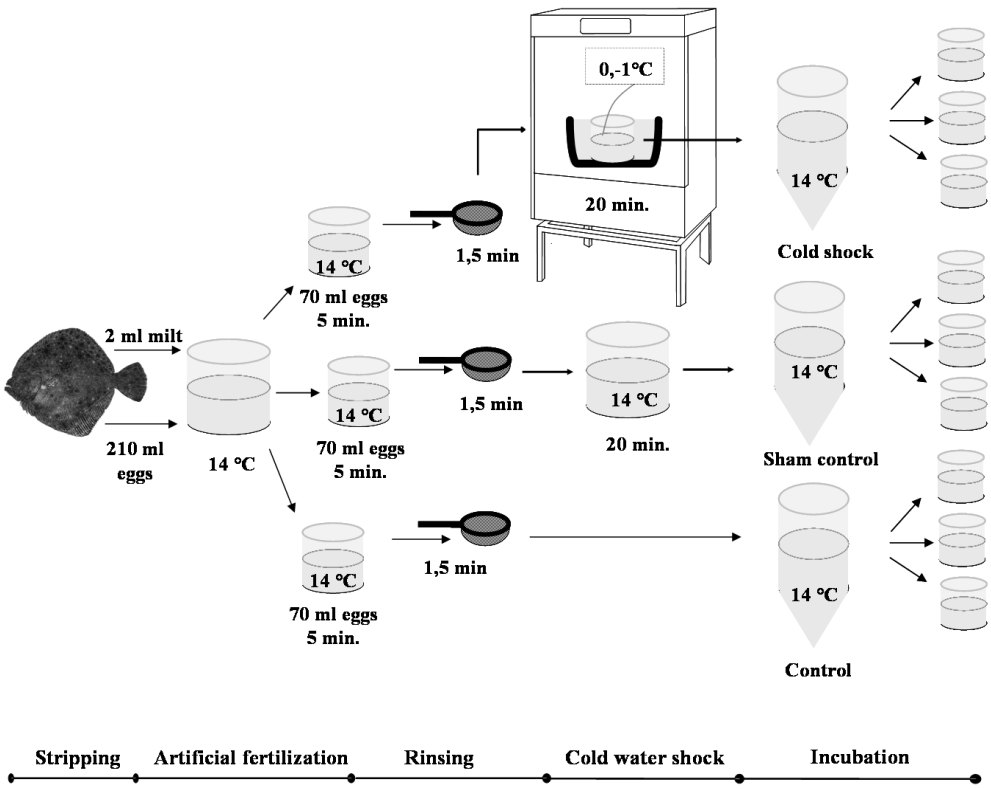

Figure 1. Schematic diagram of the experiment of triploid induction with cold water treatment in the Black Sea turbot. Values shown are means (± SE).

no further disturbance. While eggs in sham control group subjected to the same manipulation with the cold shock group without temperature change to evaluate the possible effects of mechanical disturbance (Figure 1).

Blastomere Morphology Analysis and Incubation

Blastomere morphology was determined on eggs from control and cold shock groups at 42 degree-hours AF, once they reached the 8 cell stage. Eggs were observed at high magnification and low light intensity and pictures were taken for further assessment. Approximately 150 eggs per treatment from each female were assessed by the following criteria, which were adapted from Shields *et al*. (1997):

1. Symmetry: the bilateral symmetry of 8 blastomeres about the axes, normal is symmetrical (Figure 2A) while abnormal is asymmetrical (Figure 2B).
2. Cell size: Uniformity of cell size among all blastomeres, normal means all cells are of equal size (Figure 2A), whereas abnormal indicates unequal blastomere size (Figure 2C).
3. Adhesion: The proximity of adjacent cell membranes, normal means adjacent blastomere cells are in contact (Figure 2A) and abnormal means there are gaps in between the adjacent cells (Figure 2D).
4. Margins: The discreteness of the cell the margin, normal means cell margins are well resolved (Figure 2A) and abnormal is presented in Figure 2E. These blastomere morphology parameters were scored on each individual egg and recorded as either normal or abnormal.

Eggs from the control, sham control and cold shock groups were transferred to 50 l cylindrical tanks (1000 eggs/l) with conical bottom until hatching. For evaluation of embryonic survival, three replicates of approximately 100 floating eggs from each incubation tanks were transferred in to 500 ml glass beakers containing filtered,, UV-sterilized, 18 ppt seawater and 0.5 mg/l penicillin G to reduce risk of bacterial contamination. Beakers were placed into a temperature controlled incubator (Sanyo MIR 153 Sanyo Electric, Osaka, Japan) and temperature was set at 14°C. Dead eggs and larvae were recorded daily in each replicate. Mortality rate was calculated as the ratio of dead eggs and dead larvae to the initial total number of eggs.

Ploidy Assessment

When the experimental fish became 9 months old, 40 diploid and 200 triploid juveniles were bled for ploidy evaluation. Ploidy levels were determined by measuring the long axis of erythrocytes (30 erythrocytes per fish) from dried smears of their blood under a light microscope (Piferrer *et al*., 2000). The long axis of erythrocytes was 11.2±0.13 µm in diploids (with a total length of 13.2±0.20 cm), and it was significantly longer (14.2±0.16 µm, $P<0.001$) in triploids (with a total length of 12.8±0.11cm).

Statistical Analysis of Data

Proportion of blastomere morphology parameter in control and cold shock groups were analyzed by x^2-test. Survival data were tested by Kruskal-Wallis rank test. The level of significance is given as $P< 0.05$. Data on the long axis of erythrocytes and juvenile size

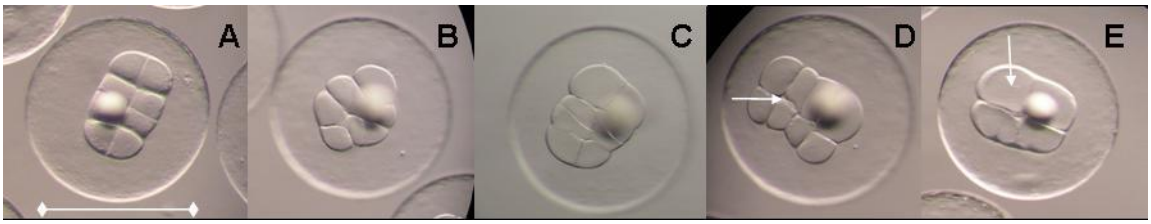

Figure 2. The Black Sea turbot embryos at eight blastomere stage of development. (A) normal blastomere, B-C: types of cleavage abnormalities (B) asymmetric blastomere, (C) unequal blastomere size, (D) the proximity of adjacent cell membranes blastomere, (E) the discreteness of cell margin blastomere. Horizontal bar: 1mm.

Table 1. Mean fertilization rates, major axis diameter of red blood cells in control and cold shock treated Black Sea turbot eggs and the success of triploid induction. Values are shown as means (± SE)' '*Female 4 was not assessed for the success of triploid induction because of low survival

Female No	Fertilization Rates		Cell major axis (µm)		Triploid rates
	Cold shock	Control	Triploid	Diploid	
1	86	87	13.7±0.16	11.2±0.14	91.1
2	77	85	13.9±0.19	11.3±0.13	94.6
3	75	76	14.3±0.14	11.2±0.13	95.9
4	75	79	-	-	-
5	90	92	14.0±0.19	11.0±0.12	92.3

were expressed mean ± SEM. Pearson correlation coefficient was used to evaluate correlation between blastomere morphology parameters and embryo viability (Zar, 1999). All analyses were performed with the program Statistica 7.0 for windows.

Results

The water temperatures inside the beakers before and after shock started are illustrated in Figure 1.

Triploid success varied from 91% to 96% depending on the trials (Table 1).

Fertilization rate did not vary between control and treated groups in any of the females as shown Table 1. No correlation was found between fertilization rate and egg viability ($P<0.01$).

Proportion of eggs with normal blastomere morphology (Figure 2A) were significantly higher in both control groups than that of in cold shock group ($P<0.05$) for all females. However, prevalence of 4 different abnormalities (Figure 2B-E) varied among females. Proportions of eggs carrying any of the 4 abnormal blastomere morphology significantly ($P<0.01$) differed between the control and cold shock groups only for female 1, 3 and 5 (Table 2). No significant correlation was found between blastomere morphology parameters and fertilization rates for all females.

Cracked egg surfaces were observed because of ice crystals formation during the cold shock (<-1°C) inside the beakers due to low salinity (18 ppt) of Black Sea water

Two mass mortality event were recorded in embryos and larvae of all females at 2 DAF (day after fertilization) and 1DAH (day after hatching) (6DAF). No significant differences amongst the treatment groups or females were observed in terms of embryo viability on 1DAF. However, on 2 DAF, survival rates of embryos from female 4 and 5 showed significant differences between cold shock and control groups. On 3 and 4 DAF, survival rates of embryos only from female 2 showed significant variation between sham control and control groups. At hatching (on 5 DAF), significantly different embryo survival rates persisted only in female 5. On 1 DAH (on 6 DAF), significant differences in survival rates were observed between cold shock and control groups in female 1 and 5 ($P<0.05$).

Together with this, eggs in cold shock, control and sham control groups from female 1 had the highest survival rates (72, 78 and 82%) on 1 DAH. Survival rates of eggs on 1 DAH from female 2 and 3 were moderate and ranged in between 55 to 68 % among the treatment groups. Eggs from female 4 in all three treatment groups had the lowest 1 DAH survival rates (< 17%). Survival rates of eggs from female 5 on 1 DAH showed variation among the treatments. Eggs in cold shock groups had the lowest survival rate (32%) while eggs in control groups had very high survival rates (75 and 78%).'

A positive linear correlation ($P<0.05$) was observed between the normal blastomere morphology rates and embryo viability (Table 3) in all treatment groups. Except blastomere asymmetry, no other morphological abnormalities found correlated with embryo survival. Blastomere asymmetry, on the other hand, showed negative linear correlation with embryo survival only in cold shock group. Although they were not significant, the negative correlation coefficient was considerably high in between unequal blastomere size anomaly and embryonic survival in cold shock group. The correlation coefficient in between indiscrete blastomere margin anomaly and embryonic survival was also high both in cold shock and control groups (Table 3).

Table 2. Mean blastomere parameters of Black Sea turbot eggs in cold shock and control groups from the five egg batches used in the experiment calculated as a percentage

(%)	Female 1		Female 2		Female 3		Female 4		Female 5	
	Control	Shock	Control	Shock	Control	Shock	Control	Shock	Control	Shock
Normal	57.6	50.0	50.4	42.3	51.6	38.7	13.2	7.5	14.4	10.1
Asymmetry	7.6	15.0	12.2	11.3	18.3	25.3	13.2	15.0	33.9	25.9
Cell size	10.1	6.0	13.9	16.7	15.1	17.3	15.8	17.7	27.8	23.7
Adhesion	5.7	12.3	11.3	10.7	4.3	13.3	14.0	12.9	7.8	10.1
Margins	19.0	16.2	12.2	19.0	10.8	5.3	43.9	46.9	16.1	30.2

Table 3. Correlation coefficient"r" between blastomere parameteres and survival rate of 1 day old prelarvae in cold shock and control groups and both groups combined

	Shock and Control Combined	Shock	Control
n	10	5	5
Normal	0.749*	0.959*	0.992*
Asymmetry	0.346	-0.999*	0.039
Cell size	-0.046	-0.606	0.012
Adhesion	-0.579	-0.07	-0.737
Margins	-0.855*	-0.875	-0.862

Discussion

A triploid induction protocol similar to Piferrer *et al*. (2000) yielded 96 % triploid populations when applied to Black Sea turbot in this study. Triploid induction conditions could affect embryo development and survival. For example, Piferrer *et al*. (2003) observed that survival rate of turbot embryos at 1DAH was higher in control (71 %) than in treated groups (18 %) and hatching proportion of treated groups was almost 80 % lower than that of the untreated controls in barfin flounder *Verasper moseri* (Mori *et al*., 2004). The effects of triploid induction on embryo survival may depend on species, the magnitude of difference between fertilization temperature and treatment temperature or quality of embryos, etc,. Survival rates of embryos vary among the females for many marine fish species (McEvoy, 1984; Kjørsvik *et al*., 2003; Sahin *et al*., 2008). Such variation, which was also observed in this study, may result from age, feeding, genetic potential or stripping time of females (Kjørsvik *et al*., 1990; Brooks *et al*., 1997). Physical effects other than cold temperature of triploid treatment were investigated with sham control group embryos. There was no significant difference between sham control groups and shock groups in terms of survival at any of the days, although 3 DAF and 4 DAF differences were significant between sham control and control groups in female 2. It was observed that sudden temperature change has more effect on viability of the embryos than handling. At the 2 DAF to 1 DAH significant differences were observed between shock and control groups in females 5 ($P<0.05$). Survival performance of embryos from female 5 could be related to its blastomere morphology patterns.

Blastomere morphology, one of the useful tools for assessing embryo quality can be observed during the early embryogenesis in marine fish. Blastomeres were classified as symmetric (normal) and asymmetric (abnormal) (McEvoy, 1984; Vallin and Nissling, 1998; Kjørsvik *et al*., 2003; Avery and Brown, 2005). However, it may not enough for defining all the problems in embryos. In this study, abnormalities were categorized as asymmetry, cell size uniformity, low adhesion and discreteness of cell margin. Percentage of all types blastomere abnormality varied among females from 5.7% to 43.9% and from 6.0% to 46.9% in control and shock groups, respectively. Similarly, cold shock increased abnormal blastomere level in Atlantic cod, *Gadus morhua* (Rani, 2005).

Results from the present study mainly agree with previous studies that have demonstrated the negative impacts abnormal cleavage patterns on embryonic survival rates both in control and cold shock applied groups (Table 3). However, association of abnormalities such as asymmetry and unequal cell size to embryonic survival showed differences between the control and cold shock groups (Table 3). Rideout *et al*. (2004) also reported that haddock embryo with proportion of asymmetric cells can develop and produce viable larvae. Vallin and Nisling (1998) demonstrated that Baltic cod embryos with various malformations in early development could produce viable offspring. Abnormal eggs are capable of producing viable larvae in yellowtail flounder *Limanda ferruginea* (Avery and Brown, 2005). In contrast with our results, numerous studies used asymmetry as an important indicator of poor egg quality (McEvoy, 1984; Devauchelle *et al*., 1988, Kjørsvik *et al*., 2003). In cold shock groups, there was significant negative correlation between asymmetric cell percentage and embryo viability. Also, there was negative correlation between unequal cell size abnormality percentage and embryo viability (Table 3).

An abnormal blastomere has the potential to have a bigger impact on development than deformity occurring in one or two cell divisions later (Kjørsvik *et al*., 1990). In contrast, Vallin and Nissling (1998) and Avery and Brown (2005) stated that abnormalities at later embryonic stages of development are more severe than those at the earlier stages. We consider that adhesion and indistinct margins reduced hatching rates under the optimum conditions or in the presence of environmental stressors. In contrast, embryos, which have asymmetric or unequal blastomeres, can reach larval stage in optimum conditions. But if they are exposed to negative factors such as a cold shock their hatching rates will be negatively affected. Thus, the relationship between embryo viability and blastomere abnormality should be studied in more detail.

The results presented here validate the use of blastomere morphology patterns as a quick and easy tool for prediction of embryo quality not only on routine hatchery production, but also in biotechnical applications such as triploidy induction via cold shock in the Black Sea Turbot Consequently, the effect of triploid induction related to blastomere morphology patterns and blastomere morphology can be used for egg quality assessment. The effect of triploid induction based on blastomere patterns in larval or juvenile stages of turbot is currently being studied and will be reported elsewhere.

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