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Induction of triploidy in the turbot (*Scophthalmus maximus*) I. Ploidy determination and the effects of cold shocks ☆

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Abstract

The basis for induction of triploidy in the turbot by applying cold shocks shortly after fertilization (AF) was studied. Since this species exhibits a polymorphism in a number of nucleolar organizing regions (NOR), determination of the ploidy level through NOR analysis was first validated. Results showed that NOR analysis could discriminate well between diploid and triploid individuals whose ploidy level was verified karyotypically ($n = 44$ chromosomes in diploids; $n = 66$ in triploids). In diploids, the mean number of NOR per cell ranged from 1.10 to 1.85, whereas in triploids, it ranged from 1.50 to 2.35. However, histogram distribution of data on mean number of NOR per cell showed that the number of fish in the overlapping region (NOR number between 1.50 and 1.85) was very low. Cold-shocked fish with a NOR value > 1.735 were considered triploids. The error in ploidy assessment using NOR analysis in the turbot was found to be consistently around 3% and always $< 5\%$. In this way, NOR analysis could be safely applied to monitor the effects of cold shocks on triploidy induction. Cold shocks were applied 5 min AF for 5, 10, 20 or 40 min at either 0°C, 2°C, or 4°C. Results showed that the number of triploids

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increased with lower shock temperatures and longer shock duration in the range from 5 to 20 min. In particular, cold shocks of 0°C applied during 20 min consistently resulted in ~90% triploid turbot ($P < 0.001$). Shocks longer than 20 min (40 min) did not increase the number of triploid turbot in contrast to what has been found in other flatfish species. This is probably related to the higher pre-shock temperature at which turbot eggs are incubated. Survival, 1 day after hatching, was ~80% of the untreated controls and not different ($P > 0.05$) from appropriate sham controls, indicating that lower survival is due to the effects of mechanical handling and stress during triploidy induction rather than the triploid condition per se. The highest triploid yield obtained in this study, ~70%, is higher than the triploid yield obtained in other flatfishes.

Keywords: Sex control; Triploidy; Cold shocks; NOR; Turbot; *Scophthalmus maximus*

1. Introduction

Turbot culture is firmly established in Europe with a production of 2966 t in 1995 (FAO, 1997). During intensive farming, females markedly outgrow males. This sex-related difference is among the largest recorded in cultured marine fish (Piferrer et al., 1995). In addition, sexual maturation usually occurs in males and often in females before they reach marketable size. Differential growth implies a higher number of fish sortings to optimize feeding regimes. Sexual maturation reduces somatic growth and increases mortality. Together, this results in an economic loss during the growout phase. There is, therefore, great interest to produce all-female or sterile stocks to avoid these problems.

All-female or sterile fish have been obtained in many species by chromosome set manipulation to induce gynogenesis and triploidy, respectively (Thorgaard, 1983; Benfey, 1989). Triploidy and gynogenesis are both naturally occurring phenomena in fish (Thorgaard, 1983). Fish, in which triploidy has been induced (i.e., they have an extra set of chromosomes in the nucleus of their cells), are not considered genetically-modified organisms in Europe and elsewhere. Recently, chromosome set manipulation has been applied to marine fishes important for European aquaculture including the sea bream, *Sparus aurata* (Gorshkova et al., 1995; Garrido-Ramos et al., 1996) and the sea bass, *Dicentrarchus labrax* (Carrillo et al. 1993; Colombo et al., 1995; Gorshkova et al., 1995; Felip et al., 1997). To the best of our knowledge, no data have been published regarding chromosome set manipulation in turbot despite its importance in European aquaculture (FAO, 1997). This is interesting considering that the pioneering works of Purdom (1972) on chromosome set manipulation were made in flatfish. The induction of triploidy and gynogenesis has been recently reported for some flatfish species: the olive flounder, *Paralychthys olivaceus* (Tabata, 1991; Kim et al., 1993); the common sole, *S. solea* (Howell et al., 1995); and the halibut, *H. hippoglossus* (Brown et al., 1997; Holmefjord and Refstie, 1997). Generally, cold shocks have been used to induce triploidy in these species.

Turbot is known for having lower larval survival than many other cultured marine fish (Devauchelle et al., 1988). Thus, to induce triploidy in the turbot while maintaining the highest possible survival, an approach similar to that employed in the sea bass (Felip

et al., 1997) was used. This approach consisted of testing different cold shocks and examining their bearing on both survival and triploidy. To monitor the effects of cold shocks in a new species, analysis of the nucleolar organizing regions (NORs) is a fast, reliable and inexpensive technique for determining ploidy, even in embryonic cells (Phillips et al., 1989). Thus, for a given species in which diploid fish exhibit 1 or 2 NOR per nucleus, treated fish with up to 3 NOR are considered triploids. The turbot has a maximum of 2 NOR per nucleus (Bouza et al., 1994). However, a recent molecular cytogenetic study has revealed the existence of a polymorphism in the number of NOR, although it affects only a few individuals which exhibit 3 NOR per nucleus (Pardo et al., 1998). This polymorphism could cause difficulties in the application of NOR analysis in ploidy determination in turbot.

Therefore, the objectives of this study were, firstly, to investigate the effects of temperature and duration of cold shocks on survival and triploidy rates in the turbot, and secondly, to validate the use of NOR analysis to check ploidy levels in this species.

2. Materials and methods

2.1. Gamete collection and artificial fertilization

Gametes were obtained during April–June from turbot broodstock reared at the facilities of the Centro Oceanógrafico de Vigo (NW Spain). Sixty days before use, broodstock were switched to a constant photoperiod of 16 h of light:8 h of darkness and to a constant water temperature of 13–14°C to stimulate the onset of maturation. For each trial, eggs from one to two ovulated females and milt from two to four running males were obtained by abdominal massage. Egg quality (egg diameter ~ 1.1 mm; 1 ml of eggs ~ 800 eggs) was assessed according to the criteria of McEvoy (1984). Since turbot are poor milt producers in terms of both quality and quantity compared to other marine teleosts (Suquet et al., 1994), an excess of milt (~ 200 μl of milt for each 10 ml of eggs) was used for artificial fertilization. First, eggs were coated with milt in the proportion described above. Then, each volume of eggs plus milt was mixed with 2 vol of seawater. This triggered sperm motility, and thus, fertilization. This moment was considered time zero. Thirty seconds after fertilization (AF), eggs were gently rinsed with excess seawater for 30 s and then divided into groups of approximately 500 each (~ 100 eggs/ml of water) and kept in clean 20 ml glass vials until use. The seawater used to fertilize and rinse the eggs was at the same temperature as the seawater in which the broodstock were maintained, i.e., 13–14°C. No attempts were made to separate viable and non-viable eggs. Viability was assessed in excess fertilized eggs not needed for the trials by placing them in a graduate cylinder, letting them sit for about 5 min and measuring the proportion that floated. As a precaution, unshocked egg batches with less than 50% survival after 24 h were discarded.

2.2. Cold shock application and experimental design

This study consists of one experiment repeated several times (trial). For each trial, the eggs of one to two females were used. Pooled eggs were divided into 15 groups: three

controls and 12 treated (cold-shocked). Each group was carried out in quadruplicate. Three types of control groups were used. An untreated control group consisted of eggs that were immediately transferred to the incubators (see details below) with no further disturbance AF and rinsing. This group served as a control for gamete quality and artificial fertilization procedures. The other two remaining control groups were sham controls consisting of eggs subjected to the same manipulations to which cold shocked eggs were subjected (pouring into different glass vials and handling), except that they were always maintained at 13–14°C. These controls served to evaluate the effects of possible mechanical disturbances shortly AF. One sham control group was transferred to the incubators and left undisturbed 10 min AF and the other 40 min AF, coinciding with two out of the four durations of the cold shocks (see below).

Triploidy was induced by applying cold shocks shortly AF. For cold shocks, each one of the 12 treated groups of approximately 500 eggs ($\sim$ 5 ml) was poured to another glass vial containing approximately 10 ml of seawater pre-chilled at the desired temperature. These vials were attached to the bottom of a plastic tray filled with a mixture of crushed ice and seawater in such a way that it almost covered the glass vials. Actual water temperature inside the vials fluctuated ± 0.5°C as determined with a precision thermometer graduated in steps of 0.1°C.

Cold shocks started 5 min AF and different combinations of three shock temperatures (0°C, 2°C, or 4°C) and four shock durations (5, 10, 20 or 40 min) were investigated, giving rise to 12 different treated groups.

Control and treated groups were incubated in plexiglass cylinders (15 cm diameter, 3 l capacity) fitted with a bottom mesh (300 μm pore) partially submerged inside a large tub provided with recirculated, filtered, UV-sterilized, and aerated seawater thermoregulated at 13–14°C. Since each one of the three control and each one of the 12 treated groups was carried out in quadruplicate, there were therefore a total of 60 incubators with eggs and larvae in each trial.

2.3. Calculation of survival and ploidy determination

Under the incubation conditions described above, hatching typically took place over 1 day, 5 days AF. The non-fertilized eggs, non-hatched eggs, and larvae were counted and added to obtain the total number of eggs initially used in each replicate of each group. Survival was calculated 1 day after hatching as the number of live larvae with respect to the number of initial eggs and was expressed as a percentage.

Ploidy was determined in larvae collected 1 day after hatching. For this procedure, a sample of larvae from each group in each experiment were kept swimming in a solution of 0.005% colchicine for 6 h. Metaphase spreads were obtained following the technique of Kligerman and Bloom (1977). Slides were stained with silver nitrate as previously described (Howell and Black, 1980). Ploidy was determined by counting the number of NOR in the nucleus of 50 cells from each larvae ($n = 32$ larvae per group) and verified by direct counting the number of chromosomes in five to 10 good metaphase plates in $\sim$ 20% of the examined larvae. In this manner, the mean number of NOR per cell was correlated with its true ploidy level as assessed by karyological analysis for about seven larvae per group in each trial.

2.4. Statistical analysis of data

Only trials in which actual survival 1 day after hatching was > 50% in the untreated controls were used. Thus, the data presented were obtained from three successful, separate trials with eggs from different females. Survival at 1 day after hatching was transformed to percentages and expressed relative to the survival of the untreated control, whose survival was set at 100% (Volckaert et al., 1994; Felip et al., 1997). Percentage data were arcsin transformed before analysis of variance (ANOVA). Data are expressed as mean ± SEM.

3. Results

Karyological analysis showed that 1 day after hatching, all metaphase spreads of larvae from both control and sham control groups had 44 chromosomes (Fig. 1A). In

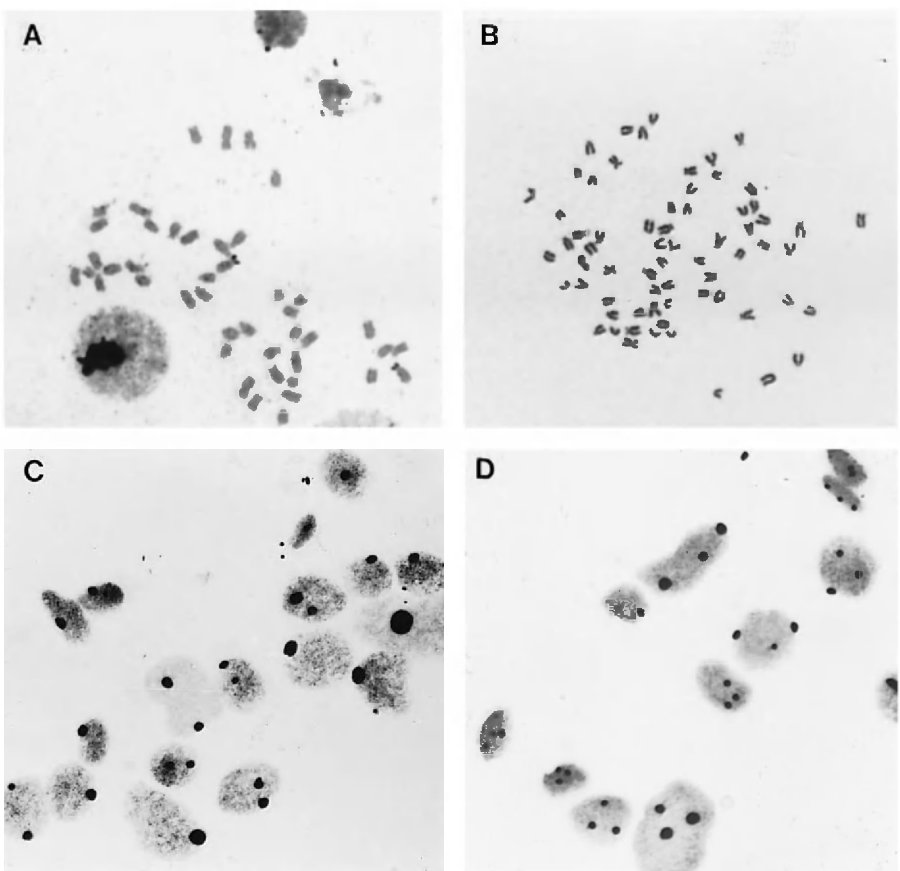

Fig. 1. Ploidy identification in turbot. Typical karyotypes and nuclei of cells obtained from diploid (A and C, respectively; 2n = 44) and triploid (B and D, respectively; 3n = 66) turbot larvae at 1 day after hatching and analysed in this study.

contrast, cold shocked groups had a variable proportion of individuals exhibiting 66 chromosomes (Fig. 1B), depending on which treatment conditions they had been subjected to (see below). These fish were considered triploids. No aneuploid or mosaic individuals were observed.

Examination of Ag-stained slides revealed that the average number of active NOR per nucleus of cells from diploids ranged from 1.10 to 1.85 (Fig. 1C). In nuclei from larvae that were triploids as confirmed by direct counting of the number of chromosomes, this value ranged from 1.50 to 2.35 (Fig. 1D). There was therefore an overlapping range from 1.50 to 1.85. However, when a frequency histogram of mean number of NOR per nucleus of cells from larvae whose ploidy level was verified karyotypically was constructed, a clear differentiation of NOR distributions between ploidies was observed (Fig. 2). Percentiles were calculated from these distributions at the 99% confidence interval. Thus, on average, the mean NOR number per cell that comprised 99% of cells in the diploids (right tail) was 1.84 and the mean NOR number per cell that comprised 1% in the triploids (left tail) was 1.63. The mid point between 1.64 and 1.84 was 1.735 and was used as the threshold average value in the number of NOR (dotted line in Fig. 2).Thus, larvae in the treated groups with an average number of NOR > 1.735 were considered triploids. This value was found to range from 1.70 to 1.77 in initial trials and was used subsequently to discriminate between diploids and triploids. Since the number of diploid larvae that were above 1.735 was very low (see Fig. 2), the error of classifying a treated larvae as being triploid when in fact it could be diploid (i.e., the larvae in the control groups with mean NOR > 1.735; see Fig. 2) was

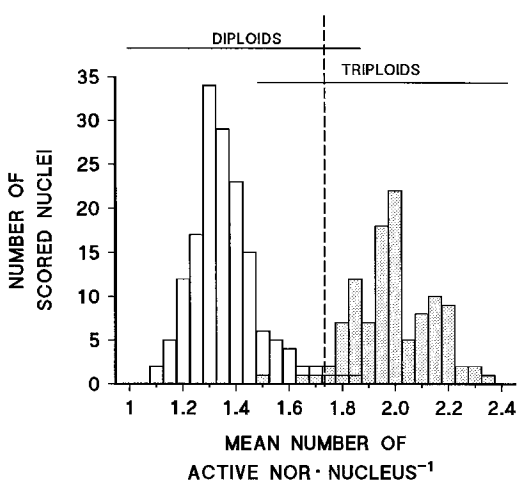

Fig. 2. Frequency histogram of mean number of active NORs per nucleus of cells from individual larvae whose ploidy level was verified karyotypically. Open bars: control diploids; shaded bars: triploids. Note the clear differentiation of both NOR distributions, but also the existence of an overlapping area. The dotted vertical line indicates the threshold average in number of NOR (1.735) used to discriminate between diploid and triploid larvae. About three fish in a 100 would be misclassified. Data from two separate trials. See text for further details.

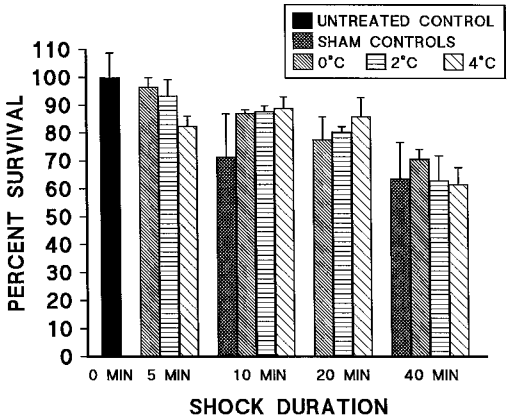

Fig. 3. Relationship between shock temperature and shock duration with survival at 1 day after hatching. Shocks of 0–4°C were applied during 5–40 min, starting 5 min AF. Survival expressed relative to the untreated control, set at 100%. See Section 2 for a detailed explanation of the different types of controls used. Data as mean + SEM of three separate trials, with each treatment in each trial carried out in quadruplicate.

consistently calculated to be $< 5\%$ and averaged $2.79 \pm 0.91\%$, i.e., about three out of 100 fish would be misclassified.

Of the total number of eggs used in each trial, on average $65.0 \pm 9.8\%$ were viable eggs. Mean survival up to 1 day after hatching of these viable eggs was $94.6 \pm 5.4\%$ in the untreated controls. Typically, one or two out of three trials were successful ($> 50\%$ survival 24 h AF in the untreated control). Survival at 1 day after hatching of treated groups relative to that of the untreated control is shown in Fig. 3. Survival decreased with longer shock durations, ranging from 82–97% (5 min shocks) to 61–71% (40 min shocks). Although there was a progressive decline in survival with longer shock durations, ANOVA did not detect significant differences when all groups were com-

Table 1
Analysis of variance of survival at 1 day post hatch in relation to treatment. In each trial, a total of 15 groups were used: one untreated control, two "sham" controls, and 12 cold-shocked groups (see Section 2 and Fig. 3). Analysis performed with the mean of three separate trials

Source of variation	Sum of squares	Degrees of freedom	Mean squares	*F*-ratio	Significance level
A. All 15 groups compared together					
Between groups	2342.59	14	167.32	1.059	0.4284
Within groups	4739.41	30	157.98		
B. "Sham" control for 10 min vs. cold-shocked groups for 10 min					
Between groups	395.96	3	131.98	1.653	0.2531
Within groups	638.78	8	79.84		
C. "Sham" control for 40 min vs. cold-shocked groups for 40 min					
Between groups	532.83	3	177.61	1.075	0.4127
Within groups	1321.56	8	165.19		

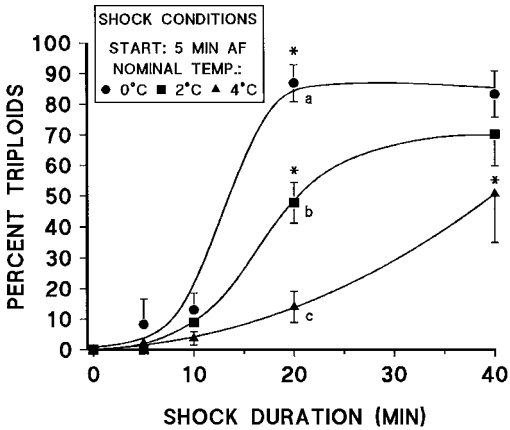

Fig. 4. Relationship between shock temperature and shock duration with the percentage of 3n turbot obtained. Shocks of 0–4°C were applied during 5–40 min, starting 5 min AF. Letters indicate significant differences ($P < 0.001$) between different shock temperatures with a shock duration of 20 min (the differences in the percentage of 3n turbot obtained between different shock temperatures were not significantly different at other shock durations). Within each shock temperature, asterisks indicate significant differences ($P < 0.001$) when compared to previous shock durations. Data as mean ± SEM of quadruplicate determinations per treatment from two separate trials.

pared together ($P > 0.05$; Table 1, A). In addition, survival of cold-shocked groups for 10 and 40 min was not significantly different ($P > 0.05$; Table 1, B and C) from the survival of their appropriate sham controls.

The number of triploid turbot larvae obtained with the different cold shocks assayed is shown in Fig. 4. Percent triploidy increased with both longer shock durations and lower shock temperatures. At 0°C or 2°C and at 4°C, shocks of 20 min or longer and 40 min, respectively, gave a significantly (ANOVA; $P < 0.001$) higher number of triploid turbot when compared to shocks of lesser duration at the same temperature. Maximum differences in triploid production as a function of temperature were evidenced with shocks of 20 min (ANOVA; $P < 0.001$). With these shocks, statistical analysis revealed a clear inverse relationship between shock temperature and number of triploid turbot obtained (Linear regression; $P < 0.001$; $r = -0.94$). Thus, with shocks of 20 min, the percentage of 3n turbot obtained was 78.6 + (−14.8 times the shock temperature used in °C).

4. Discussion

This paper validates the use of NOR analysis for the determination of ploidy levels in the turbot and shows the effects of cold shocks on both triploidy rates and early larval survival in this economically important species. The highest triploidy rate, 87% (survival of 77.5% with respect to the untreated controls), was achieved with a cold shock of 0°C applied for 20 min. This represents a triploid yield of 67.3% and it compares well with

the triploid yield obtained in other species: olive flounder, 24.3% (Kim et al., 1993); halibut, 46–77.9% (Holmefjord and Refstie, 1997).

The chromosome number of control groups was in accordance with the chromosome number previously determined for this species (Bouza et al., 1994). The analysis of the number of nucleoli per nucleus is a straightforward and inexpensive technique to determine the level of ploidy. This technique has been applied when developing methods to induce triploidy in fish (Phillips et al., 1989; Felip et al., 1997), but can only be used when NOR are located in a single chromosome pair which results in the presence of one or two nucleoli per cell in diploids, and one, two, or three nucleoli in triploids. However, multichromosomal distribution of NOR has been observed in several vertebrate species (Arnheim et al., 1982; Volleth, 1987) including fish (Phillips et al., 1989; Castro et al., 1996). This can present difficulties due to the variable expression of available NOR in each cell. The turbot is a marine flatfish with a single NOR located in the short arm of each one of the only submetacentric pair of chromosomes (Bouza et al., 1994). However, a polymorphism in the number of NORs has been recently discovered in this species, although it affects only a small number of individuals (Pardo et al., 1998). The polymorphism manifests itself in that occasionally two different acrocentric chromosomes evidence Ag- and CMA_3-positive signals in their telomeres. Therefore, this fact could introduce some disturbance in evaluating the ploidy level when using the counting of nucleoli per cell in turbot. In the present work, we determined the number of NOR and verified the level of ploidy by karyotyping 20% of the individuals analyzed. The results obtained are in accordance with the previous data of NOR polymorphism in turbot (Pardo et al., 1998). Thus, diploid cells have, in some individuals in the same family, three nucleoli, while triploids exhibit four nucleoli, supporting the existence of additional active NOR. These individuals represent the upper tail of the mean nucleoli per cell distributions in diploids and triploids, respectively. This circumstance, however, only affected a minor fraction of the individuals analyzed, as expected, and diploid and triploid nucleoli number per cell distributions hardly overlapped. Checking the level of ploidy by karyotype analysis showed that the error committed by misclassification of individuals according to its level of ploidy is below 5%. Therefore, despite its NOR polymorphism, NOR analysis can be used to identify ploidy in the turbot.

Both untreated and sham controls were used in this study, the latter groups included to detect the effects of possible mechanical disturbances shortly AF due to handling. It was found that the survival of cold-shocked groups that gave high triploid ratios was not significantly different from that of appropriate sham controls. Thus, decreased survival from fertilization up to 1 day after hatching in the treated groups was probably a consequence of the handling involved in triploid induction, not of the triploidy condition per se. Regarding the actual survival values, here it is worth noting that survival, 1 day after hatching, was near 80% of the untreated controls, which is in accordance with that found in other studies on triploidy induction in marine fish (Felip et al., 1997), even though cultured turbot have typically lower larval survival (Devauchelle et al., 1988).

The timing of the cold shock and the temperature applied are in agreement with those used with other marine fish, i.e., commencement of shock within the first 15 min AF and shock temperature near or at 0°C. Regarding the best shock duration, our data show that shocks longer than 20 min did not increase the number of triploid larvae obtained.

This contrasts with the data obtained in other flatfishes where shock duration typically ranges between 45 min and 3 h (Lincoln, 1981; Tabata, 1991; Kim et al., 1993; Holmefjord and Refstie, 1997) and instead, is more similar to the short shocks (10 min) necessary to induce triploidy in the sea bass (Felip et al., 1997). It could be argued that a peak in the number of triploids obtained could exist at a shock duration between 20 and 40 min which was not tested. However, this is unlikely because for a given shock temperature and timing, once a maximum of triploidy is achieved, longer durations do not usually increase the number of triploids obtained as evidenced also in this study. The observation that shocks of more than 20 min did not increase the number of triploids obtained could be due to the warmer incubation temperatures used in the present study with turbot (and also in the study with sea bass) as compared with the relatively cooler temperatures used with other flatfishes. In fact, Díaz et al. (1993) suggested that the difference between the pre-shock and shock temperatures was more important than the shock temperature itself in obtaining high triploid rates. Thus, since sea bass and turbot eggs are incubated at 12–14°C before the shock in contrast to 6°C in halibut (Holmefjord and Refstie, 1997) or 7°C in plaice (Lincoln, 1981), it is likely that shocks near or at 0°C need to be applied for relatively shorter periods of time when compared to the cold water species referred to above.

This paper provides the basis for triploidy induction in the turbot by cold shocks AF. Although survival after the manipulations was acceptable, no 100% triploidy was achieved. In this regard, the inverse relationship between shock temperature and triploidy induction, i.e., $\%3n = 78.6 + (-14.8 \times °C)$ ($P < 0.001$; $r = -0.94$) derived from these trials predicts that $\sim 100\%$ triploid turbot would be obtained with shocks of 20 min at -1.4°C, starting 5 min AF. This, however, is likely to negatively affect survival and thus other factors also important to ploidy induction, such as the time of commencement of the shock, should be taken into account. Optimization of treatment regimes to achieve 100% triploid turbot while maintaining high survival and the scaling up of these methods to enable the mass production of triploid turbot are currently being investigated.

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