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# Induction of meiotic gynogenesis in Atlantic cod (*Gadus morhua* L.) through pressure shock

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ABSTRACT

The Atlantic cod, *Gadus morhua*, is one of the most important species for commercial fisheries and a promising candidate for aquaculture. Precocious sexual maturation of males is one of the major issues compromising large scale production. The potential approaches to this problem include production of all female populations. Consequently, the objective of this study was to develop an effective protocol to induce meiotic gynogenesis in the Atlantic cod by using hydrostatic pressure shock. Our first experiment tested the relevance of gamete quality on achievement of chromosome manipulation and identified the best time interval between fertilization and pressure shock. Our second experiment was designed to determine the optimal pressure value and duration of the pressure shock. Eight combinations of pressure values and durations were tested. Among them, the 34.47 MPa/6 min combination gave the best survival rate (23.6 ± 3.9%), the highest percentage of normal larvae (15.7 ± 3.6%), and the highest percentage of meiotic diploids (88.89%). In both experiments, haploid controls served as an indirect reference for paternal DNA inactivation. Chromosome counting confirmed the restoration of diploidy in gynogenetic fish. The present study optimizes a procedure for the induction of meiotic gynogenesis in the Atlantic cod, thus laying the basis for further applications towards producing monosex and defining the sex determination system.



## 1. Introduction

The Atlantic cod (*Gadus morhua* L.) has historically been, and still is, one of the most important commercial fish species in the Northeast Atlantic (Kurlansky, 1997). However, in recent years, wild-caught Atlantic cod landings have been in severe decline (Christensen et al., 2003; Esmark and Jensen, 2004). As a consequence, the interest in cod aquaculture has increased as a means to meet the market needs with the potential to reach the success of the Atlantic salmon large-scale production (Standal and Utne, 2007). Over the past decades research on cod farming has been undertaken in Norway, Canada, Iceland, UK and USA (Rosenlund and Skretting, 2006). Mariculture systems have been developed, moving from traditional methods towards more intensive techniques including new biotechnologies (Kjesbu et al., 2006).

Precocious sexual maturation, occurring predominantly in males, is one of the major problems in Atlantic cod aquaculture. This may result in reduced growth, flesh quality and cause a potential genetic impact on wild stocks (Taranger et al., 2010). The potential approaches to this problem include induced sterility through triploidy and production of mono-sex stock populations.

Fish species show a wide variety of sex determination systems. Often sex may be influenced by environmental factors, while other times it is strictly relying on genetic/chromosomal mechanisms. Among the latter, the

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most common are the XX/XY (male heterogamety) and ZW/ZZ (female heterogamety) systems. In many species having a male heterogamety system, chromosomal manipulation based on the induction of gynogenesis has resulted in all-female fingerlings production (reviewed in Devlin and Nagahama, 2002). The induction of gynogenesis has been carried out successfully in many freshwater fishes but fewer attempts have been made in marine species (reviewed by Pandian and Koteeswaran, 1998; Felip et al., 2001).

No information on the sex determination system acting in the Atlantic cod is currently available and cytogenetically distinguishable sex chromosomes have never been described for this species (Klinghardt et al., 1995). The induction of gynogenesis in this species, besides being potentially the first step towards the mass-scale production of all-female Atlantic cod populations, could also allow the elucidation of the sex-determination system.

The gynogenesis technique includes: (1) production of gynogenetic haploids through activation of eggs with genetically inactivated spermatozoa, and (2) restoration of diploidy by suppressing the second meiotic division of the egg (meiotic gynogenesis) or by disturbing the first mitotic division of the zygote (mitotic gynogenesis). Several techniques have been used to restore the diploidy of the embryos including thermal shock, pressure shock and, in a few cases, chemical shock. In fish, cold shock is mostly used, possibly due to the fact that no special equipment is required and large volumes of eggs can be treated at once (reviewed in Felip et al., 2001). However the choice of hydrostatic pressure to induce gynogenesis usually results in a higher survival at hatching if compared with other methods (Pandian and Koteeswaran, 1998; Peruzzi and Chatain, 2000). Moreover, the possibility of error in the pressure-shock based procedure is limited, making the achievement of diploidization through this method more reproducible and easily accomplishable when compared with other techniques (Morgan et al., 2006).

An experimental procedure to retain the second polar body by thermal shock was recently published for the Atlantic cod as part of a protocol to induce triploidy (Peruzzi et al., 2007). However, no protocols for retention of the second polar body through hydrostatic pressure shock are currently available for this species.

The objective of the present study was to develop an effective protocol to induce meiotic gynogenesis in the Atlantic cod by optimizing hydrostatic pressure shock parameters.

## 2. Materials and methods

### 2.1. Broodstock management and gamete collection

The work described in this article was carried out in accordance to the EU Directive 86/609/EEC for animal experiments. Sperm and eggs were collected from the wild caught Atlantic cod broodstock (5.0–10 kg) captured in the coastal area of Nordland (Skjerstadfjorden) in Northern Norway and kept at the Bodø University College, Bodø, Norway (67°18′N, 14°32′E). The fish were held in circular tanks (diameter 6 m, water depth 1.2 m, centrally located bottom drain) in flow-through seawater and feed to satiation every second day using dry pelleted feed 17 mm (Skretting Vitalis Repro Cod, Skretting AS, Norway). Water temperature ranged from 6.2 to 7.3 °C, oxygen levels, measured in outlet water, was maintained above 75%, and salinity remained stable around 34.5‰ during the spawning period.

The sperm was obtained by applying slight pressure on the abdomen and individually collected with a syringe. Contact of semen with water or urine was avoided. Based on our previous experience, the milt was diluted 80-fold with Hanks' Balanced Salt Solution (Sigma–Aldrich, H8264) and Modified Turbot Extender (Vermeirssen et al., 2004). The sperm motility after activation with seawater was checked under the microscope (400×) before and after dilution. Leja chambers coated with albumin (Leja products BV, Nieuw-Vennep, The Netherlands) were used for assessing the motility. The ratio sperm:seawater was approximately 1:40. Only sperm showing 80% motility after dilution was used for experiments.

Eggs were stripped by gentle pressure of the belly into glass beakers. Good quality eggs were chosen for experiments after visual evaluation based on morphology of eggs, buoyancy and amount of ovarian fluid. Groups containing mostly white and/or deformed eggs, contaminated with blood or excrement, or samples containing a too large ovarian fluid amount were excluded from the experiments.

### 2.2. Induction of gynogenesis

The induction of gynogenesis was achieved by fertilizing the eggs with UV-inactivated sperm followed by pressure shock.

The optimal UV dose has been determined in a preliminary experiment based on post-treatment spermatozoa motility. Fifteen ml of diluted sperm was poured into a watch glass, kept on crushed ice to a depth of approximately 1 mm, and subjected to UV irradiation (3689 $\mu W/cm^2$) for 60 s while being continuously stirred on a magnetic stirrer. The motility of irradiated sperm was microscopically checked.

Shocks were applied by using a pressure chamber (Aquatic Eco-Systems Inc., Apopka, FL, USA). Various parameters were tested in order to optimize retention of the polar body (summarized in Table 1). A first experiment was designed (1) to investigate the effect of gamete quality on the achievement of the chromosome manipulation, and (2) to identify the best time interval between fertilization and pressure shock. A second experiment was designed to determine the optimal combination of hydrostatic pressure value and duration of the shock.

#### 2.2.1. Experiment 1

Eggs obtained from each of the females were divided into five equal batches (approximately 1000–2000 eggs each). One egg batch was fertilized with 4 ml of diluted cod sperm and served as a diploid control. A second batch was fertilized with 4 ml of UV-irradiated diluted cod sperm and served as haploid control. The other egg batches were fertilized with 4 ml of UV-irradiated diluted cod sperm and pressure shocked to induce gynogenesis. A pressure shock