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Gamete quality in triploid Pacific oyster (*Crassostrea gigas*)

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ABSTRACT

Triploidy induction in shellfish aims to obtain faster growth and sterility of reared individuals. Triploid Pacific oysters are most often not totally sterile, but have greatly reduced reproductive capacities compared to those recorded in diploid individuals. The description of gamete output in triploid aquatic animals is most often confined to the assessment of fecundity in females. The present work aims at further describing gamete quality of triploid Pacific oysters and comparing it to values observed in diploid individuals. Diploid and triploid oysters (produced by mating tetraploid males × diploid females) were reared in standard conditions and then transferred to the wild to allow the offspring to attain reproductive maturity. At the end of gametogenesis, the reproductive characteristics of both groups were estimated.

After gonad sampling, gametes could be observed in 92.9% diploid oysters compared with 42.0% in triploids. A higher number of spermatozoa was produced in diploids ($344 \pm 106 \times 10^9$ spermatozoa) compared with triploids ($5 \pm 7 \times 10^9$ spermatozoa). Furthermore, the percentage of motile sperm and sperm swimming speed were higher in diploids compared with triploids. In contrast, a higher intracellular ATP content was found in triploids (99.1 ± 34.0 nmole 10^{-9} sperm) than in diploids (63.6 ± 20.7 nmole 10^{-9} sperm). A higher number of oocytes was collected from diploid oysters ($19.1 \pm 3.8 \times 10^6$ oocytes), than from triploid ones ($0.1 \pm 0.1 \times 10^6$ oocytes). The D-larval yield was 45% higher for crosses (female × male) triploid × diploid, than the control (diploid × diploid). Furthermore, the lowest D-larval yield was measured for triploid × triploid crosses.

Considering the present data, a partial estimation of the reproductive potential of triploid Pacific oysters (triploid × triploid crosses) could be close to 0.06% of that of diploid individuals. However, this estimation is probably over-evaluated because it does not take into account the low sperm production of triploids (1.5% compared to diploids), the unknown frequency of spontaneous spawning in triploids and the low viability of the progeny. The consequences of the low reproductive potential of triploid Pacific oysters on natural populations and on hatchery practices are discussed. In conclusion, the present work confirms that triploidy leads to a limited reproductive potential as estimated by gamete characteristics and embryo developmental success.

Statement of relevance: This study is relevant to aquaculture because triploids are produced by aquaculture and the consequences of their low gamete quality, described in this paper, on the natural environment but also on hatchery practices are discussed.

1. Introduction

The induction of triploidy in shellfish aims to obtain a faster growth and sterility of reared animals, by relocation of energy from reproduction to growth (Piferrer et al., 2009). A superior flesh flavour of triploids has also been reported by consumers due to a firmer texture and a more constant flesh quality throughout the year, resulting from reducted reproductive effort (Nell, 2002). These economically beneficial characteristics have led to triploidy being used in a large range of farmed molluscs including Pacific oyster, in which triploids account for 30% of worldwide production (Dunham, 2011).

Triploids are usually not totally sterile, but their reproductive capacities are much reduced compared with those recorded in diploid individuals. In fish species, triploid females rarely produce eggs and those that are produced are generally unfertilisable (Piferrer et al., 2009). In Atlantic cod (*Gadus morhua*), the hatching success of offspring was halved when sperm collected from triploid males was used to fertilize eggs, compared with results obtained using diploid males (Feindel et al., 2010). In crustacean species, triploid females or males of black tiger shrimp (*Penaeus monodon*) do not produce viable gametes (Sellars et al., 2013). In shellfish, the potential to produce viable scallop

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(*Pactinopecten yessoensis*) offspring from triploid breeders was estimated to be 4×10^{-6}% of diploid ones (Meng et al., 2012).

In oysters, polyploidy was first induced in the American oyster (*Crassostrea virginica*) in the early 1980s, by inhibiting polar body expulsion using cytochalasin B (Stanley et al., 1981). To avoid chemical toxicity, triploids have been more recently produced by crossing tetraploid oysters with diploid ones, leading to all-triploid oyster progenies (Guo et al., 1996).

Regarding their reproductive capacity, gonad development of triploid oysters is reduced: triploids show active gametogenesis during the first stages but their gonad development is then blocked, with most triploids remaining at early stages of gametogenesis (Jouaux et al., 2010; Normand et al., 2008). However, it is possible to collect functional gametes by gonad stripping in certain breeders of both sexes, and larvae can be obtained after controlled crosses of diploids × triploids or triploids × triploids. The fecundity of triploid females is low and was found to vary from 2% to 13% of diploid levels (Gong et al., 2004; Guo and Allen, 1994). Additionally, the reproductive potential of triploid Pacific oysters was estimated to be 0.0008% of diploid ones (Guo and Allen, 1994). No previous studies on reproductive capacities of triploid oysters have described gamete quality of these breeders.

A panel of bio-descriptors can provide a complete picture of gamete quality in aquatic species, including gamete concentration and morphology, sperm motility, intracellular energy content and fertilization capacity (Fauvel et al., 2008). The present work aims to describe gamete quality of triploid Pacific oysters and compare it with that observed in diploid individuals.

2. Materials and methods

2.1. Broodstock conditioning and gamete collection

The oysters used in the present study were produced in 2012 at the Ifremer experimental hatchery in La Tremblade (Charente, France). To produce diploid and triploid oysters, 30 diploid females, 10 diploid males and 15 tetraploid males were selected as genitors. Diploid oysters were three-year-old individuals, bought from local oyster farmers working exclusively with wild-caught spat. These diploid oysters were conditioned to full maturation in an open circulating system at 20–22 °C. Tetraploid oysters used to produce triploid progeny in this work, were two years old and were conditioned to maturation in the same conditions as the diploids, but in a quarantine area equipped with water treatment systems using ozone to prevent dissemination of gametes or larvae. Tetraploid oysters were used as males and sampled from the same tetraploid broodstock used for the mass production of triploids by French commercial hatcheries. This tetraploid broodstock was initially produced in 2006 in the Ifremer experimental hatchery using diploid × diploid crosses according to the method developed by Benabdelmouna and Ledu (2007).

At the end of oyster gametogenesis, gametes were collected from diploid and tetraploid oysters by gonad stripping, then suspended in 1 μm filtered sea water (FSW, 24 °C). Eggs from the 30 diploid females were collected and divided into two pools. The first pool was fertilized using sperm collected from the 10 diploid males to produce diploid larvae, and the second pool was fertilized by sperm from the 15 tetraploid males, to produce triploid larvae. Then, these diploid and triploid larvae were transferred to 150 L tanks filled with FSW and cultured under standard rearing conditions until metamorphosis (24 °C, 100 larvae mL^{-1}, fed with a mixture of microalgae: *Isochrysis galbana*, *Chaetoceros gracili* and *Skeletonema costatum*). DNA ploidy level of each batch was regularly verified by flow cytometry, according to Barranger et al. (2014).

When the spat reached 2 mm, they were transferred to the Ifremer nursery in Bouin (France) for intensive growth using raw sea water enriched with *S. costatum* (June 2012 to October 2013). From the end of 2013, adult diploid and triploid oysters were reared in the field at La Floride (Marennes-Oléron bay, France) bay until the end of July 2014, when they were fully ripe.

2.2. Gamete quality analysis

At the end of the conditioning period, the DNA ploidy level was again individually verified by flow cytometry for all the diploid and triploid oysters. Mature Pacific oysters (diploid n = 20, triploid n = 70) were then transferred to the experimental hatchery in Argenton (Ifremer, Northern Brittany, France). They were maintained in a conditioning environment, according to Song et al. (2009). Oyster whole weight (W) and flesh weight following superficial drying with soft paper (FW) were measured and condition index was calculated: CI = (FW / W) * 100, according to Royer et al. (2008). In order to determine their sex, six samples were pipetted from the gonad of each oyster (covering the whole gonad volume). Diploid and triploid oysters were stripped in seawater (33.7 salinity, 19 °C).

Sperm density was assessed using a Thomas cell after gamete dilution in seawater (ranging from 1:1 to 1:25, depending on individual sperm concentration). The total number of spermatozoa collected from each oyster was calculated (gamete density × seawater volume). To estimate sperm motility, sperm was diluted in an activating solution (AS: seawater, 5 g L^{-1} bovine serum albumin, Tris 20 mM, pH 8.10, caffeine 10 mM, dilution rate: 1:9). Sperm movement was observed at 10 min and 2 h post-activation under a phase contrast microscope (Olympus BX51, ×10 magnification) and recorded (Sony camera, 60 frames s^{-1}, 4 s film duration, 3 × 30 spermatozoa). Then, sperm motility characteristics (percentage of motile sperm and velocity of the average path: VAP) were assessed using a CASA plugin developed for Image J, according to Suquet et al. (2014). To measure intracellular ATP content, 10^7 sperm in 500 μL seawater were transferred into 2 mL cryotubes. ATP was assessed in triplicates by bioluminescence (ATP lite kit, Perkin Elmer).

Oocyte density was estimated by microscopic counts (3 × 50 μL in 10 mL to 2 L, depending on individual oocyte concentration). Total number of oocytes collected from each oyster was calculated (gamete density × seawater volume). Furthermore, morphological characteristics of oocytes (Feret diameter, perimeter, area and circularity ranging from 0 to 1, where a value of 1 indicates a perfect circle) were assessed 30 min after gonad stripping, using Image J (n = 30 oocytes).

Triplicate batches of 25,000 oocytes each were then fertilized according to the following crosses (female × male): diploid × diploid (n = 5), diploid × triploid (4), triploid × diploid (2), and triploid × triploid (3). Oocytes were fertilized using a non-limiting sperm to egg ratio (500). Oocyte lots were incubated in 2 L beakers and the D-larval yield was estimated 48 h post-fertilization: (number of D-larvae / 25,000) * 100.

2.3. Statistical analysis

Data are presented as mean ± standard deviation. Percentages (D-larval yield) were arcsin square root transformed before analysis. Means were compared using Student t tests or ANOVA. For ANOVA and when differences were significant ($P < 0.05$), a Fisher a posteriori test was used for mean comparisons. Because variances were not homogeneous, morphological data on oocytes were compared using a non parametric Mann and Whitney test.

3. Results

The whole weight of triploid oysters (99.3 ± 28.4 g) was significantly higher ($P = 0.014$) than that observed in diploid ones (81.4 ± 24.4 g). Inversely, the condition index was significantly lower ($P = 0.001$) in triploid oysters (13.9 ± 1.6) than in diploid ones (16.2 ± 3.0). After gonad sampling, gametes could be observed in 92.9% diploid oysters compared with 42.0% in triploids. No simultaneous hermaphrodites were observed in either group.