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Heteroploid mosaic tetraploids of *Crassostrea virginica* produce normal triploid larvae and juveniles as revealed by flow cytometry

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ABSTRACT

Advantages associated with sterility in triploids have made them the standard for commercial aquaculture of *Crassostrea virginica* in the Chesapeake Bay. Current hatchery practices for commercial production of triploids employ tetraploids as broodstock, and thus tetraploids are a core asset for oyster culture. Tetraploids undergo reversion, losing entire sets of chromosomes, and become heteroploid mosaics comprised of triploid and tetraploid cells. The possible effects of using mosaic tetraploids for triploid production are a practical concern for commercial oyster culture. We crossed mosaic and non-mosaic tetraploids, males and females, to a reference diploid oyster and compared relative DNA content and phenotypic qualities of triploid progeny. Relative DNA content was measured in somatic and gametic tissue of tetraploid broodstock, as well as in resulting triploid larvae via flow cytometry. Size, abnormality, and survival of larvae were recorded through the rearing process. For six crosses, triploids were raised for 1 year and shell metrics, size, and somatic DNA content were compared. Flow cytometry analysis of broodstock revealed sperm from mosaic tetraploids were consistently di-haploid and virtually indistinguishable from sperm of non-mosaics. For resulting triploid progeny, there were no detectable differences between larvae or juveniles produced from mosaic and non-mosaic tetraploids. Differences existed, however, between triploid larvae produced from di-haploid sperm and those produced from tetraploid eggs: triploids from tetraploid eggs were significantly more abnormal, less viable, and grew slower. Our findings support current commercial practices of using tetraploid males as the principal parent for triploid production and demonstrate no practical consequence from using mosaic tetraploid broodstock for commercial production of triploid *C. virginica*. Further analysis via cytogenetic techniques may provide insight into long term consequences of using mosaic tetraploids, for example, in tetraploid × tetraploid crosses.

1. Introduction

Polyploid induction, more specifically the commercial production of triploids and the creation of tetraploid brood stock to support it, has become an important and successful technique in aquaculture of oysters, including the eastern oyster, *Crassostrea virginica*. In Virginia, about 90% of commercial oysters are triploid (Murray and Hudson, 2013). In the rest of the world, with *Crassostrea gigas*, up to 50% of hatchery oysters are triploid (S. Allen, unpublished). Triploid oysters are valued for their sterility that results from an odd number of chromosome sets (Thorgaard, 1983).

Sterility generates several advantages for oyster culture. Compared to their diploid counterparts, sterile triploids have reduced gonadal development, allowing for higher growth rates and superior market quality during the reproductive season (Allen, 1988, reviews by Beaumont and Fairbrother, 1991; Guo et al., 2009; Nell, 2002; Piferrer et al., 2009). Triploids exhibit higher levels of disease resistance sometimes (e.g., Degrémont et al., 2012; Gagnaire et al., 2006; Hand et al., 1988) and sometimes, not (e.g., Barber and Mann, 1991; Cheney et al., 2000; DeDecker et al., 2011). Sterile stocks also carry less risk of unwanted proliferation and genetic pollution that may threaten biodiversity (Piferrer et al., 2009). Early comparative papers (pre-Guo et al., 1996) between triploids and diploids concerned triploids produced by inhibiting polar body II in newly fertilized eggs, so-called induced triploids. Induced triploids vary considerably from triploids made from more current methods.

With few exceptions, commercial quantities of triploid oysters are currently produced by crossing diploid females with tetraploid males, made possible after a method to generate viable tetraploid oysters was developed (Guo and Allen, 1994). Compared to polar body II inhibition, crossing diploids and tetraploids is better suited for aquaculture because it bypasses the need for an artificial treatment and produces triploids with higher efficiency (Guo et al., 1996). As tetraploid brood stock becomes increasingly important, questions arise about how to domesticate them. As with diploids, selective breeding strategies may be applied to tetraploids. Yet unlike diploids, tetraploids engender new challenges, from improvements in their induction to management of brood stock holdings. For example, since the Guo and Allen method was published, an alternative method to produce tetraploids has been

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published (McCombie et al., 2005b) and a second is presently subject to a US patent (Benabdelmouna and Ledu, 2010). Both alternative processes of making tetraploids provide short cuts for genetic improvement of tetraploid lines, each with its own breeding strategy.

Regardless of how tetraploids are produced in the F_0 generation, the generations thereafter are propagated from tetraploid × tetraploid crosses. At the Aquaculture Genetics and Breeding Technology Center (ABC), we have begun the process of creating tetraploid lines of *C. virginica* by producing families. During this exercise, we frequently observed mosaicism within somatic tissue of individual tetraploids (Allen, 2011).

Mosaicism, or perhaps more correctly, heteroploid mosaicism, was first described in oysters over 10 years ago (Allen et al., 1996), and the best treatment of the subject so far has been by Zhang et al. (2010a). As observed in oysters, heteroploid mosaicism arises from progressive loss of chromosomes from the original polyploid state. The result is an individual comprised of a composite of the original polyploid cells (i.e., either triploid or tetraploid) as well as cells of lower ploidy. Triploid mosaics, for example, contain triploid cells as well as hypotriploid and hyper-, hypo- and euploid diploid cells (Zhang et al., 2010a). An important feature of the Zhang paper is that it showed the correspondence between cytogenetic data (chromosome counts) and flow cytometric data.

The data from mosaics via flow cytometry (FCM) and by cytogenetics are quite different, although supportive (Zhang et al., 2010a). Comparatively, FCM data can be rapidly obtained enabling numerous samples; however, there is little information in FCM data about aneuploidy (e.g., hypo- or hyperploid levels). Rather, what we often identify as evidence for mosaics are discrete peaks both at the original ploidy level (i.e., triploid or tetraploid) as well as at lesser ploidy levels. Little to no signal is observed for intermediate ploidy levels by FCM, presumably because events at these hyper- or hypo- levels do not occur in numbers sufficient to produce a discrete population of cells. That is not to say that FCM cannot be used surgically to evaluate general cytogenetic phenomena. A good example of a more sophisticated use of FCM is Allen et al. (1986), in which reproductive cells from triploid grass carp were analyzed by FCM to determine the likelihood of reproductive sterility.

The loss of chromosomes from tetraploids, which are used exclusively as brood stock, is of major scientific interest as well as serious practical concern (McCombie et al., 2005a; Zhang et al., 2010a). Scientifically, as mechanistically proposed by Zhang et al. (2010a), reversion of this sort provides evidence of a new mode of diploidization. On a practical level, chromosome loss in tetraploids causes two principal concerns. The first is the fate of future generations of tetraploid brood stock if mosaics are used to create them. Will this further exacerbate chromosome loss by encouraging high levels of aneuploidy in tetraploids?

The second and more immediate concern is the fate of the commercial triploid seed produced from mosaic tetraploid brood stock. ABC produces tetraploids for industry hatcheries. Each year tetraploids are distributed for the upcoming spawning season, and when hatcheries are ready to use these tetraploid brood stock, they send sperm to our laboratory for certification. If the sperm are di-haploid, they proceed with the spawning. (Di-haploid is used in this paper to denote sperm from a tetraploid, although technically, the term refers to the doubling of a haploid genome.) Rarely do we certify tetraploid somatic tissue for spawning. What are the ramifications of allowing a commercial hatchery to proceed with producing triploid seed from a mosaic tetraploid?

In the summer of 2012, there was a confluence of observations that led to this study. We began to evaluate the reproductive potential of our tetraploid families that were then two-years old (data in prep.) and, at the same time, received a sample of tetraploid somatic tissue (gill) from a commercial hatchery. Assessment of tetraploid families revealed a surprisingly high proportion of mosaics, while the sample from the commercial hatchery was also from a tetraploid mosaic. This study took advantage of the supply of tetraploids available to us from the ongoing family sampling, male and female tetraploids as either mosaics or non-mosaics. These various types of tetraploids were crossed with standard diploid parents to yield triploids, much as commercial hatcheries would do. We then analyzed the larvae, as well as select crosses after 1 year, for differences between progeny created by mosaics or by non-mosaic tetraploids.

2. Materials and methods

2.1. Experimental population

Tetraploid oysters were used from a set of experimental families held by ABC, designated, for the purpose here, as families HH, GG, and FF. Each family is an F_3 of 4n × 4n crosses of one tetraploid line, but only the previous generation was produced from a pair mating; thus the tetraploids described here are F_2 pair-mated families.

Tetraploid oysters were opened and separated into males and females according to family. For males, a sperm sample was taken with a microcapillary tube and a gill sample was dissected away from one lamella. For females, only gill was sampled. Using FCM (Allen et al., 1996), gill samples were analyzed to indicate somatic ploidy and identify mosaic individuals. Our goal was to obtain approximately equal numbers of tetraploids that were "pure," that is, had no indication of a second ploidy type in the somatic tissue (herein referred to as "non-mosaic"), to be matched with those that had multiple ploidy types in somatic tissue (herein referred to as "mosaics"). Mosaics outnumbered non-mosaics, often considerably (data not shown) among families.

Diploid gametes were obtained from a single pair-mated family derived from other ongoing experiments. Ripe individuals from this family were held in 18 °C seawater during the course of this experiment.

2.2. Crosses

A mosaic and non-mosaic male were crossed with a single diploid female to make pairs of half sib groups. In the reciprocal cross, a mosaic and non-mosaic female were crossed to a single diploid male, also producing two half sib groups. This process was repeated in each of the four experiments (referred to as "spawns" below). One family of diploids was used in all crosses to reduce the variation stemming from the diploid parent among test crosses. All crosses performed in 1 day were deemed a spawn. Crosses are depicted in Table 1.

After confirmation of ploidy in parents, male and female parents were strip spawned. Eggs were aliquoted to comprise a maximum density of 100/ml in the incubation tank containing each cross, and sperm activity was used to estimate the amount of sperm needed for fertilization.

Eggs were examined about 0.5 h post-fertilization to determine fertilization rates. Rates were high, averaging 92% (data not shown).

2.3. Larval rearing

For spawns 1 and 2, larvae were reared in 1 liter. However, larvae cultured this way did not develop properly. Thus all larval data reported here are from spawns 3 and 4. From the first two spawns we report only results from the ploidy analysis of parent tetraploids. Spawns 3 and 4 were reared in 40 liter tanks. Larvae were examined 2 and 4 days post-fertilization for ploidy analysis. On day 2, larvae were isolated on a top (48 μm) and bottom sieve (35 μm). Larvae collected on the 48 μm sieve on day 2 were returned to culture until day 4, at which time they were isolated on larger sieve sizes, 63 μm (top) and 48 μm (bottom). After day 4, all but a subset of the larvae cultures were terminated. For spawn 4 only, larvae from three crosses using sperm from mosaics and three crosses using sperm from non-mosaics, herein referred to as the select crosses, were maintained to setting (≥day 17) following standard protocol. For these select crosses, larvae were sampled on day 8 for ploidy analysis.