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Journal:	<i>Genome</i>
Manuscript ID	gen-2015-0222.R1
Manuscript Type:	Article
Date Submitted by the Author:	08-Feb-2016
Complete List of Authors:	Sousa, Joana; Virginia Institute of Marine Science, Fisheries Allen, Standish; Virginia Institute of Marine Sciences, Baker, Haley; The University of Alabama Matt, Joseph; Virginia Institute of Marine Science, Fisheries
Keyword:	<i>Crassostrea virginica</i> , aneuploidy, triploidy, mitotic instability, chromosomes

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Aneuploid progeny of the American oyster, *Crassostrea virginica*, produced by tetraploid x diploid crosses: another example of chromosome instability in polyploid oysters.

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Abstract

The commercial production of triploids, and the creation of tetraploid broodstock to support it, has become an important technique in aquaculture of the eastern oyster, *Crassostrea virginica*. Tetraploids are produced by cytogenetic manipulation of embryos and have been shown to undergo chromosome loss (to become a mosaic) with unknown consequences for breeding. Our objective was to determine the extent of aneuploidy in triploid progeny produced from both mosaic and non-mosaic tetraploids. Six families of triploids were produced using a single diploid female and crossed with three mosaic and non-mosaic tetraploid male oysters. A second set of crosses was performed with the reciprocals. Chromosome counts of the resultant embryos were tallied at 2-4 cell stage and as 6-hour(h)-old embryos. A significant level of aneuploidy was observed in 6-h-old embryos. For crosses using tetraploid males, aneuploidy ranged from 53 – 77% of observed metaphases, compared to 36% in the diploid control. For crosses using tetraploid females, 51 – 71% of metaphases were aneuploidy versus 53% in the diploid control. We conclude that somatic chromosome loss may be a regular feature of early development in triploids, and perhaps polyploid oysters in general. Other aspects of chromosome loss in polyploid oysters are also discussed.

Key words: *Crassostrea virginica*, aneuploidy, triploidy, mitotic instability, chromosomes

Résumé

La production commerciale d'huîtres triploïdes, ainsi que la création de stocks de géniteurs tétraploïdes pour la soutenir, sont devenues des techniques importantes dans l'aquaculture de l'huître américaine, *Crassostrea virginica*. Les tétraploïdes sont produites grâce à une manipulation cytogénétique des embryons, qui peut provoquer la perte de chromosomes (devenant mosaïque) présentant des conséquences inconnues pour la reproduction. L'objectif de cette étude était de déterminer l'impact de l'aneuploïdie chez les descendants triploïdes produits à partir de tétraploïdes aussi bien mosaïques que non-mosaïques. Six familles de triploïdes ont été produites en utilisant une femelle diploïde croisée avec trois mâles tétraploïdes mosaïques et non-mosaïques. Une deuxième série a été réalisée en utilisant des croisements réciproques. Le nombre de chromosomes ont été comptés sur les embryons au stade « 2-4 cellules » et 6 heures après la fécondation. Après 6 heures, un niveau significatif d'aneuploïdie a été observé. Pour les croisements impliquant les mâles tétraploïdes, dans les métaphases observées, le niveau d'aneuploïdie était compris entre 53 et 57% contre 36% dans le contrôle diploïde. Pour les croisements impliquant les femelles tétraploïdes, le niveau d'aneuploïdie était compris entre 51 et 71% contre 53% dans le contrôle diploïde.

Nous pouvons conclure que la perte de chromosomes somatiques pourrait être une caractéristique normale observée lors du développement précoce chez les triploïdes, et de façon plus générale chez les huîtres polyploïdes. D'autres aspects liés à la perte de chromosomes chez les huîtres polyploïdes sont également abordés au cours de cette étude.

Mots-clés: aneuploïdie, triploïdie, l'instabilité mitotique, chromosomes

Introduction

The eastern oyster, *Crassostrea virginica*, is a highly fecund commercial bivalve and well-adapted to an estuarine existence, being highly tolerant to wide fluctuations of temperature, salinity, suspended sediments, and dissolved oxygen (Kennedy et al. 1996). However, the eastern oyster has declined in many estuaries where it was once abundant due to over-harvesting, habitat degradation, reduced water quality, disease, and interactions among these factors (Kingsley-Smith 2009). In order to overcome the reduction in commercial product, oyster aquaculture has become prominent in the last decade led by genetic improvements, such as selection (Guo 2009, Frank-Lawale et al. 2014) and polyploidy (Dégremont et al. 2012).

Polyploidy is the heritable condition of possessing an additional set (or sets) of chromosomes, being a phenomenon well tolerated in many groups of eukaryotes such as plants, fish, and amphibians (Comai 2005). In bivalves, natural polyploidy is less common but has been observed in two marine species of genera *Lasaea*: *Lasaea australis* (Foighil and Thiriot-Quievreux 1991) and *Lasaea consanguinea* (Thiriot-Quievreux et al. 1988), in the freshwater clams *Sphaerium striatinum* (Lee 1999) and *Sphaerium rhomboideum* (Petkevičiūtė et al. 2007) and in *Corbicula* spp. (Park et al. 2000). As evidenced from the high incidence of polyploidy in some taxa, polyploids can clearly be advantageous. Such advantages can be obtained from inducing polyploidy as well. For example, the commercial production of triploid oysters (Guo et al. 1996) and the creation of tetraploids to serve as progenitors of hatchery-bred triploid spat (Guo and Allen 1994a), has become an essential and successful tool in aquaculture (Nell 2002, Piferrer et al. 2009, Guo et al. 2009).

The first viable tetraploid oysters (*Crassostrea gigas*) were produced by treating fertilized eggs of a triploid with cytochalasin B to block the release of the first polar body to create *de novo* tetraploids (Guo and Allen 1994a). *De novo* tetraploids are genetically unique because they are obtained from a triploid x diploid cross, so consequently the tetraploid genome is made up of three chromosome sets from the mother (triploid) and one from the father (diploid). Moreover, by definition, these types of tetraploids derive from “fertile” triploids (Guo and Allen 1994b, Eudeline et al. 2000). Alternative methods to produce tetraploids exist (McCombie et al. 2005b, Benabdelmouna and Ledu 2015), but for all types, subsequent generations of tetraploids are propagated from tetraploid x tetraploid crosses (Guo and Allen 1997). Virtually, all commercial triploid oysters in the world are now created using the tetraploid x diploid cross. Triploid oysters have several advantages for oyster culture, especially reduced fecundity with consequent higher growth and improved market quality during the reproductive season (Allen 1988).

Although polyploidy can be advantageous, it is often associated with cytological problems. For example, polyploidy increases the occurrence of spindle irregularities, e.g., multiple spindles that can lead to the chaotic segregation of chromatids and the production of aneuploid cells with abnormal numbers of chromosomes (Borel et al. 2002). Across taxa, chromosome loss in polyploids is frequent and occurs to a much greater extent than in diploids (Comai 2005). For bivalves, cytogenetic abnormalities, such as aneuploidy, are common in diploid populations (Thiriot-Quiévreux et al. 1992; Leitão et al. 2001; de Sousa et al. 2011) and this phenomenon is apparent in *de novo* polyploid shellfish as well (Guo and Allen 1994a; Wang et al. 1999; Yang et al. 1999; Yang et al. 2000). For example, induced triploid and tetraploid embryos of the scallop *Chlamys farreri* had up to 53% of aneuploid (hypoploid) cells (Yang et al. 1999; Yang et al. 2000). High levels of aneuploidy also occurred in polyploid Pacific oysters, *C. gigas*, in both

adults (Wang et al. 1999; McCombie et al. 2005a; Zhang et al. 2010a; Zhang et al. 2013) and larvae (Guo and Allen 1994a; Guo and Allen 1997).

For oysters, chromosome loss is not limited to aneuploidy, but also includes the loss of entire sets of chromosomes to become heteroploid mosaics (herein called “mosaics”) through a process called reversion (Allen et al. 1996; Zhang et al. 2010a). It seems that cells undergoing reversion, at least some of them, continuously eliminate chromosomes until a stable euploid state is established (Zhang et al. 2013). The loss of chromosomes from tetraploids is of major scientific interest and of practical concern for commercial oyster culture (Matt and Allen 2014). Previous studies in *C. virginica* revealed that tetraploid mosaics have little impact on triploid production, although this question was not examined at the chromosomal level (Matt and Allen 2014). Until now, flow cytometry (FCM) was our principal research tool for detecting reversion. FCM data can be rapidly obtained enabling high throughput, however, it is difficult to detect small differences in DNA content and, consequently, the data contain little information about aneuploidy.

We initiated this study with the intention of refining the information in Matt and Allen (2014) with chromosome counts. To that end, we established crosses between mosaic and non-mosaic tetraploids with reference diploids. In the course of the investigation, we uncovered a surprisingly and unexpectedly high incidence of aneuploidy in early (6-hour[h]-old) embryos. We then broadened our question by comparing these data to 2-4 cell embryos from the same crosses to try to identify the source of aneuploidy.

Materials and methods

Experimental population

Tetraploid *C. virginica* broodstock were obtained from lines propagated by the Aquaculture Genetics and Breeding Technology Center (ABC). Tetraploid oysters were opened and males and females sorted. From each tetraploid, a 4mm² gill sample was dissected from one lamella and processed for FCM (Allen et al. 1996). Gill cells were stained in DAPI/DMSO (Allen and Bushek 1992) and analyzed on a Partec Cyflow Space flow cytometer. Samples were measured with referencing to a diploid standard for mean relative DNA content as well as variation (CV) in DNA content of cell populations. Gill samples were analyzed for somatic ploidy to obtain tetraploids that had only tetraploid cells apparent (herein called “non-mosaics”) and to obtain tetraploids that had multiple ploidy types in the somatic tissue (“mosaics”). The number of mosaics were more numerous than non-mosaics (data not shown). Diploid gametes were obtained from a single male or female, depending on the test crosses.

Crosses

After confirmation of ploidy in parents, males and females were strip spawned using the technique outlined by Allen and Bushek (1992). In the first set of crosses, three tetraploid *mosaic* males and three tetraploid *non-mosaic* males were crossed with a single diploid female tester creating six half-sib groups. A control cross was made using the diploid female tester and single male diploid. In a second set of reciprocal crosses, three tetraploid *mosaic* females and

three tetraploid *non-mosaic* females were crossed to a single diploid male tester, producing another set of six half sib groups. A control cross was made using the diploid male tester and single female diploid. Thus, each set of reciprocal crosses comprised three mosaic tetraploids and three non-mosaic tetraploids all crossed with the same diploid, and a control.

Cytogenetics

To block the mitosis in metaphase cells, about 300,000 1-h-old (2-4 cell embryos) and 300,000 6-h-old embryos were collected and incubated for 20 minutes in seawater containing 0.005% colchicine. The 1-h-old embryos were fixed directly in Carnoy's solution-freshly prepared absolute ethanol: acetic acid (3:1) (Guo and Allen 1997). The 6-h-old embryos were treated for 10 minutes in 0.9% sodium citrate and then fixed in Carnoy's. The fixed embryos were stored at 4°C until analyzed. Slides for 1-h-old embryos were prepared following the technique of Guo and Allen (1997) and slides for 6-h-old embryos were prepared following the air drying technique of Thiriot-Quéveux and Ayraud (1982). Chromosome counts were made directly by microscope observation (Nikon Eclipse 50i with camera image acquisition incorporated Nikon DS-Fi1) on apparently intact metaphases.

For 2-4 cell embryos, chromosome counts were made on at least 20 embryos. Each count represented the contribution of chromosomes from the sire and the dam. For 6-h-old embryos, at least 30 intact metaphases per cross were counted. A sample size of 30 is the minimal statistical number typically accepted in cytogenetic studies (Leitão et al. 2001). In the case of 6-h-old embryos, chromosome counts represent a random sample of cells from the population of disaggregated embryos and cannot be attributed to any particular embryo. Aneuploidy incidence was estimated as the total number of aneuploid metaphases divided by the total number of

metaphases counted per cross. Average chromosome number from crosses using mosaic or non-mosaic tetraploids was compared with a one-way analysis of variance (ANOVA parametric test) at $\alpha=0.05$.

Remaining embryos were reared in 40 liter tanks for 48h, at which time about 3,000 larvae were examined for ploidy via FCM (Chaiton and Allen 1985). Relative DNA content and CV were measured for the population of cells from each cross.

Results

Tetraploid broodstock

Males

Relative DNA content of gametic cells (di-haploid sperm) from mosaic and non-mosaic males was the same, both with an average relative DNA content of 2.04 (Table 1). Di-haploid sperm of mosaics had 0.52x the relative DNA content of gill tissue of tetraploids; for non-mosaics 0.51x.

For tetraploid cell populations, average relative DNA content for somatic (gill) tissue of non-mosaics was slightly higher than mosaics (3.99 vs. 3.92) (Table 1). Mosaic male oysters had two populations of cells — tetraploid and “triploid.” Average relative DNA content of “triploid” cell populations for mosaic males was 2.98 ($n = 3$) with an average CV of 3.50 ($n = 3$) (Table 1). On average, the ratio of the mean relative DNA content of the “triploid” cell population to the mean relative DNA content of the tetraploid population was 0.76 ($n = 3$), slightly higher than the expected 0.75 (Table 1).

Females

For tetraploid cell populations, average relative DNA content for somatic tissue (gill) of non-mosaics was slightly lower than mosaics (3.95 vs. 4.00) (Table 1). All mosaic females had two cell populations — tetraploid and “triploid”. Average relative DNA content of “triploid” cell populations for mosaic females was 3.10 ($n = 3$) with an average CV of 3.59 ($n = 3$) (Table 1). The ratio of the mean relative DNA content in the “triploid” cell population to the mean relative DNA content of the tetraploid cell population was 0.78 ($n = 3$), also higher than the expected 0.75 ratio and slightly higher than the value in the males. The average percent of tetraploid cells in females was 78.0 ($n=6$) (Table 1).

Larvae from diploid x tetraploid crosses

Day 2 — relative DNA content

FCM analysis of two-day-old larvae confirmed that all crosses produced 100% triploid progeny. For larvae from male tetraploids, average relative DNA content was 2.95 ($n = 6$) and average CV – 5.16 ($n = 6$). For larvae from female tetraploids, average relative DNA content was 2.85 ($n = 5$) and average CV – 5.18 ($n = 5$) (Table 2). Relative DNA content of larvae from males was significantly higher than that of tetraploid females ($p=0.007$). For comparisons of relative DNA content between larvae from non-mosaic versus mosaic parents, there was no difference, either for males ($p=0.95$) or females ($p=0.51$). Similarly, there were no significant differences in CV between crosses made with non-mosaic or mosaic males ($p=0.51$) and non-mosaic and mosaic females ($p=0.92$). The relative DNA content of larvae produced from the

diploid female tester and random diploid male was 1.99 and CV – 5.50. For the larvae from the diploid male tester and random diploid female the relative DNA content was 1.86 and CV – 5.65.

Cytogenetic analysis (6-h-old embryos)

Chromosome counts of 6-h-old triploid embryos from both female and male tetraploids, as well as the diploid control, were analyzed (Figure 1, a-c). Cells from triploid embryos from both non-mosaic and mosaic, males and females displayed a wide variation of chromosome number, ranging from 16 to 38 chromosomes (Figure 2a, b). Despite this wide variation, all triploid crosses had a modal chromosome number of 30 and the diploid – 20 (Table 3). More than half of all metaphase spreads from triploid embryos were aneuploid. For male tetraploids, 64% of the non-mosaic and 63% of the mosaic cells from the progeny were aneuploid. Additionally, 3% and 6% of the cells from non-mosaic and mosaic progeny were diploid, respectively (Table 3). For female tetraploids, 63% for the non-mosaic and 58% for the mosaic cells from the progeny were aneuploid. Five per cent of the cells were diploid ($n=20$) for both non-mosaic and mosaic progeny from female tetraploids (Table 3). There were no significant differences between the proportion of aneuploids from male or female tetraploids ($p=0.63$), and no significant differences in the proportion of aneuploids between progeny of non-mosaic and mosaic females ($p=0.95$), or progeny of non-mosaic and mosaic males ($p=0.72$). Aneuploidy in the diploid controls was also high – 36% and 53%. Of the aneuploid cells, one control cross had only hypoploid cells and the second - 23% hypo- and 13% hyperploid.

Cytogenetic analysis (2-4 cell embryos)

In order to better understand the origin of the high levels of aneuploidy observed in the 6-h-old triploid embryos, we performed a cytogenetic analysis in the 2-4 cell triploid embryos (1-h-old) from the same crosses for both female and male tetraploids and the diploid control. Only the embryos from female tetraploids showed adequate metaphase spreads to perform chromosome counts, perhaps owing to the physical nature of the eggs (Figure 1d). The metaphase spreads of embryos resulting from diploid eggs (for both male tetraploids and the diploid control) presented distended chromatin instead of the condensed chromosomes typical from this phase, making the identification of the individual chromosomes impossible.

Hypoploid cells of $3n$: 20, 26, 28 or 29 were observed in the embryos from both non-mosaic and mosaic females (Figure 2b). Both embryos from non-mosaic and mosaics showed a modal and an average chromosome number of 30. Compared with the 6-h-old triploid embryos, an average of 8% of 2-4 cell embryos from both non-mosaic and mosaic parents were aneuploid (Table 4). There was no significant difference observed between the progeny from mosaic and non-mosaic tetraploid females ($p=0.55$).

Discussion

At the heart of this study was the question about the chromosome stability of tetraploid *C. virginica* parents that have undergone the process of reversion to a state of becoming heteroploid mosaics. Specifically, is the chromosome instability (CIN) of mosaic parents heritable? This question was initially addressed by Matt and Allen (2014) using FCM, and peripherally touched upon in cytogenetic studies of meiotic chromosomes by (Zhang et al. 2010b; Zhang et al. 2014). In a recent paper by Benabdelmouna and Ledu (2015), heritability of CIN was further implicated by observations from tetraploids derived through a completely different method of induction, the

so-called direct method – produced by inhibiting the first polar body in a $2n \times 2n$ cross (also see below). Below, we discuss the observations of mosaicism from the parents in this study, our observations of CIN in the crosses made from these parents, and insights that these observations and other work tell us about the heritability of CIN in mosaic tetraploid oysters.

Non-mosaic and mosaic parents

The tendency for polyploid oysters to lose chromosomes over time has become an important question for commercial production (e.g., Guo and Allen 1994a; Wang et al. 1999; Zhang et al. 2010a; Zhang et al. 2013; Matt and Allen 2014) since it was first reported by Allen et al. (1996) 20 years ago, as well as an interesting and possibly unique mechanism of chromosome loss (Zhang et al. 2010a; Zhang et al. 2013). From the practical side, the short term consequences of chromosome loss may affect commercial production in tetraploid $\times$ diploid hatchery output. The longer term consequences concern how chromosome loss may affect the integrity of tetraploid lines.

For tetraploid male and female parents, relative DNA content of tetraploid cells from non-mosaics and mosaics were the same, confirming the observations in Matt and Allen (2014) and reinforcing the idea that reversion occurs subsequent to the initial tetraploid condition. “Triploid” cell populations, on the other hand, were not uniform. “Triploid” cells in male mosaics were slightly hypo-triploid and “triploid” cells in female mosaics were slightly hyper-triploid (Table 1). It is not difficult to envision why the “triploid,” i.e., the chromosomal condition resulting from CIN, is variable and likely aneuploid, given the mechanism of chromosome loss proposed by Zhang et al. (2010b) and Zhang et al. (2014). The so-called clumping that sheds multiple chromosomes (and likely random numbers of them) over the course

of reversion would be expected to yield imperfect levels of triploid (and diploid, if reversion continued).

For gametic cells, i.e., di-haploid sperm of tetraploids, we found no difference in relative DNA content or in its variance (CV) between the non-mosaics and mosaics by FCM. Similar results were observed by Matt and Allen (2014) between non-mosaic and mosaic individuals of *C. virginica* that underwent severe reversion or between non-mosaic and mosaic individuals of *C. gigas* (McCombie et al. 2005a). That is, we could not detect aberrant meiotic products from tetraploids that had, also, triploid somatic cells present. These results are consonant with cytogenetic evaluations of chromosome pairing in meiosis of both triploid and tetraploid *C. gigas* (Zhang et al. 2010b). In a similar analysis of spermatocytes from tetraploid *C. gigas*, one tetraploid/ triploid mosaic was examined and only 1 of 47 spermatocytes (2%) was a triploid (Zhang et al. 2014). Also in this same Zhang paper, the frequency of aneuploidy spermatocytes was the same between the non-mosaic tetraploids and the one mosaic examined, suggesting that aneuploidy in spermatocytes may be a function of being polyploid, but not necessarily to being a heteroploid mosaic.

Crosses from non-mosaic and mosaic parents

Integrity of the gametes was also confirmed through the crosses that we tested. According to FCM results, all the crosses performed in this study resulted in 100% triploid progeny even when tetraploid mosaics were used as broodstock; no differences in relative DNA content were evident between larvae from non-mosaic and mosaic parents. Similar results were observed in previous studies (Guo et al. 1996; Matt and Allen 2014), confirming the consistency of this method to produce triploid *C. virginica* larvae. There is no evidence that the mechanism giving rise to mosaics affects DNA content of gametes in tetraploid parents. In fact, there was more of

a difference in relative DNA content of triploid progeny between males and females than there was between non-mosaics and mosaics (Table 2). Similarly, there was a noticeable difference in the relative DNA content of diploid progeny produced from the female diploid tester to the diploids produced from the male diploid tester. In contrast, Matt and Allen (2014) found DNA content to be the same in 2-day-old triploid larvae produced from males and females.

In order to better understand the relationship between chromosome loss in tetraploid parents and triploid progeny, chromosome counts of 6-h-old triploid embryos from both female and male tetraploids (non-mosaic and mosaic), as well as a diploid control, were analyzed. One must bear in mind that the aneuploidy we observed was from a population of cells comprised of disaggregated embryos. Therefore, we are observing the pool of aneuploidy cells among many individuals. High levels of aneuploidy, mainly hypotriploidy (58-64%, Table 3), prevailed in the metaphases observed in these 6-h-old triploid embryos. At the same time, there was also a relatively high level of aneuploidy in the two diploid controls (37%, 53%). In adult diploid bivalves, aneuploidy was as high as 26% for *C. gigas* (Leitão et al. 2001) and 19% - 79% in adult European clam, *Ruditapes decussatus* (de Sousa et al. 2011). In triploids of *C. gigas* aneuploidy ranged from 6% - 12% in diploids and up to 56% in triploids (Guo and Allen 1997). Zhang et al. (2010a) observed high levels of aneuploidy in triploid mosaic Pacific oysters (43% for induced and 42% for mated [$4n \times 2n$]) and Zhang et al. (2013) observed about the same level of aneuploidy (46%) in chemically induced (i.e., G_1 tetraploids from $3n \times 2n$ mating) tetraploids. Figure 3 is a graphic representation of data from Table 1 of Zhang et al. (2010a) showing the chromosome distribution in adult triploids. It differs from those that we saw in this study by the relative lack of chromosome counts in the mid-range, from 22-28. The difference in chromosome distribution could be due to the fact that in the present work we analyzed triploid

embryos, versus gill tissues of adult triploids in previous work, suggesting that highly aneuploidy cells are lost over time and that chromosome numbers tend to stabilize, with time, around the euploid state.

The patterns of aneuploidy from non-mosaic parents was the same as that for mosaic ones, suggesting a lack of heritability for CIN, at least the type of CIN that leads to reversion (see Chromosome instability and its consequences). The cytogenetic results from 6-h-old embryos stands in contrast to those obtained from FCM on 2-day-old larvae. At 2-days old, there was little to no sign of high levels of aneuploidy. Indeed, from the many FCM analyses of triploids we have done in our lab, we had never had occasion to suspect aneuploidy in early development: ploidy confirmation of triploid larvae for commercial purposes is always on larvae that are ≥ 2 -days old. We believe this lack of correspondence between aneuploidy at 6-h and 2 days old is due to mortality of embryonic cells with severe aneuploidy, acting as a natural control on the number of aneuploid cells produced by mosaics and non-mosaics alike.

Due to the high level of aneuploidy we observed in the 6-h-old triploid embryos, we decided to further investigate the source of this phenomenon by performing additional chromosome counts in 2-4 cell embryos (around 1-h-old embryos) from the same crosses. Again, non-statistically significant differences were observed for chromosome loss or gain in 2-4 cell triploid embryos produced from mosaic and non-mosaic. However, the levels of aneuploidy were significantly lower in 2-4 cell embryos compared to the 6-h-old triploid embryos, with most of the metaphases presenting 30 chromosomes in the former. Due to the increase of aneuploidy in these first hours of early development, we can assume that the CIN that leads to the high levels of aneuploidy in embryos occurs as a mitotic error during cell division. In polyploids across taxa, aneuploidy, due to aberrant mitosis and errors in chromosomal segregation, is frequent and

occurs to a much greater extent than in diploids (Comai 2005; Storchova and Kuffer 2008). These errors during mitosis leading to abnormal chromosome numbers can culminate in the activation of the apoptotic default pathway and cellular death (Castedo et al. 2004).

In our particular case, the 6h chromosomes counts could only be ascribed to individual cells, not to individual embryos, as with the 2-4 cell data. Therefore, it was impossible to know whether the distribution of chromosome counts represented a high occurrence of aneuploidy in a small percentage of embryos or a lower occurrence of aneuploidy in the majority of embryos. Judging from the tendency for larval cultures of triploids oysters to have similar survival to diploids (Guo et al. 1996; Guo et al. 2009), it would seem that the loss of aneuploid cells may not correspond to the loss of larvae, favoring the view that our cell population at 6h post-fertilization represents a generally low level of aneuploidy among the entire population of embryos, which also assumes low levels of aneuploidy are tolerated.

Chromosome instability and its consequences

We have been acutely interested in the consequences of CIN in tetraploids. It seems, based on this and other work, that there is little evidence for increased levels of aneuploidy in progeny as a consequence of reversion, i.e., mosaic tetraploids seem to produce predominantly euploid gametes (Zhang et al. 2010a, Zhang et al. 2014, Matt and Allen 2014, this study). The production of euploid gametes, or not, in mosaics is likely a completely different issue than the mechanism(s) of chromosome loss in polyploids. That is, gamete production is a meiotic process whereas chromosome loss yielding reversion (mosaics) seems to be a mitotic one.

Zhang et al. (2010a) and Zhang et al. (2013) proposed a mechanism of chromosome loss in triploid *C. gigas* and *Crassostrea ariakensis* as well as in tetraploid *C. gigas* called “chromosome clumping.” They observed that individuals with more chromosome clumps in their cells tended

to have higher percentages of aneuploidy. Furthermore, the clumping was observed in mosaic oysters. Whether clumping is the mechanism of reversion *per se* or the phenotypic manifestation of the mechanism (discussed below) is open to interpretation, but the phenomenon of quantum losses in chromosome number makes sense from all we have observed about mosaics. Supporting evidence for losing chromosomes in “clumps” comes from several observations: with FCM data, there is seldom evidence for intermediate ploidy types between the original and the next level down in adult polyploids (e.g., between tetraploid and triploid); reversion happens relatively quickly and begins at a relatively early age (Ritter and Allen 2015), which could indicate sudden, quantum losses rather than gradual chromosome by chromosome loss; and, once reversion commences, it is progressive over time (Allen unpublished data, Zhou 2002, Erskine 2003). To the latter point, in studies of polyploid yeast, if one chromosome is lost, additional chromosomes are lost at increasingly higher frequencies (Mayer and Aguilere 1990).

Chromosome clumping may only be the phenotype of the actual mechanism for reversion. The causative mechanism may be supernumerary centrosomes causing multipolar spindles yielding chromosome clumps. Centrosomes coordinate important micro-tubule related functions, including chromosome segregation and cytokinesis. Extra copies can result in multipolar spindles and mitotic failures (Nigg 2002). Tetraploidy can result in supernumerary centrosomes and, as studied mostly in mammalian systems, represents an important intermediate on the route to aneuploidy by initiating chromosomal instability (Storchova and Kuffer 2008). Tetraploid induction, as is performed in oysters, interferes with the normal cell cycle development and may give rise to extra copies of centrosomes. Indeed, there are a number of mechanisms posited for centrosome amplification, some associated with cytokinesis failure leading to tetraploidy (Meraldi et al. 2002). It is not hard to imagine a multipolar spindle giving

rise to a “clump” of chromosome that would result in a quantum loss of chromosomes from a polyploid cell, a step on the way to reversion to the next ploidy down. Furthermore, the hypothesis that mosaic formation and/ or aneuploidy is, at least partially, generated by supernumerary centrosomes is widely supported in the literature (Storchova and Kuffer 2008).

It is interesting that besides the observation of chromosome clumping in tetraploid *C. gigas*, Zhang et al. (2013) also observed asynchronous chromatic condensation that they said “could account for losses in later divisions”. Duplication of centromeres occurs only once in each S phase of the cell cycle but increasing the duration of S phase can lead to centrosome amplification. The “later” chromosome losses suggested by Zhang et al. (2013) could be related to delayed mitoses that allow abnormal centrosome duplication.

There is much to confirm about the role of supernumerary centrosomes as a process accounting for mosaicism in polyploid oysters, so for now, it remains a hypothesis. At least it seems consonant with observations of chromosome clumping. Such a mechanism might reasonably be evoked for all types of polyploid shellfish, oysters being the prime example and the only example for which mosaics have been documented so far. Observations of chromosome clumping in mosaics were made on chemically induced triploids and mated ($4n \times 2n$) triploids (Zhang et al. 2010a) and chemically induced tetraploids (Zhang et al. 2013). Other evidence for mosaics have been made through FCM, including chemical triploid *C. gigas* (Allen et al. 1996), chemical triploid *C. ariakensis* (Zhou 2002), mated triploid *C. ariakensis* (Erskine 2003), mated tetraploid *C. virginica* (Matt and Allen 2014) and, now, on direct tetraploids of *C. gigas* (Benabdelmouna and Ledu 2015). It would seem, then, that the general condition of polyploidy provides the substrate for subsequent chromosome loss.

It is curious that there seems such a difference in the incidence of mosaicism in the several types of tetraploids documented by Benabdelmouna and Ledu (2015). They compared three types of tetraploids, which we will name after the authors responsible: GA – those induced from triploid eggs (Guo and Allen 1994a); MC – those produced by fertilizing diploid eggs with tetraploid sperm, followed by second polar body inhibition (McCombie et al. 2005b); and, BL – those created directly by inhibiting the first polar body in a $2n \times 2n$ cross (Guo et al. 1992a; Benabdelmouna and Ledu 2015). In comparison of the three types of tetraploids at two years old, the proportion of mosaics was GA – 45%, MC – 25%, and BL – 7%. These same results were later confirmed in another year class: GA – 50% and BL – 5%. It is remarkable then that there could be such a large difference between tetraploid oysters produced in three different ways, especially when two of those (GA and BL) are produced by a similar cytological manipulation.

The cytological manipulation that gave rise to the BL type oysters was first documented by Guo et al. (1992a) and resulted in tetraploid embryos that did not survive through the larval phase. More than two decades later, Benabdelmouna and Ledu (2015) were able to achieve the zootechnical skill to keep these tetraploids alive. The chromosomal segregations that gave rise to tetraploids when the first polar body of the diploid egg was inhibited were complicated but clearly documented by Guo et al. (1992b). In summary, the segregations that produce the tetraploid embryos were called either tripolar or separated bipolar segregations, either one of which has to be the result of supernumerary centrosomes, by definition. GA tetraploids are also produced by inhibiting the first polar body, but from triploid eggs, not diploid ones. The chromosome segregation that gives rise to tetraploids under those circumstances – also documented by Guo et al. (1992a, albeit in diploid eggs) – was called united bipolar segregation,

and curiously does not involve supernumerary centrosomes. So what could be the explanation for such a vast difference in reversion between GA and BL type oysters?

Benabdelmouna and Ledu (2015) proposed that the “manipulation of meiosis, particularly via the blocking of the expulsion of the first polar body, has a much higher impact on triploid oocytes than on diploid oocytes, due to the greater number of chromosomes that are concerned by the meiotic segregation in triploid oocytes.” They suggested one of these impacts is initial aneuploidy that accompanies the inhibition of polar body I. Another proposed impact was a genetic predisposition transmitted by the triploid female. As to the first “impact,” it should be noted that the cytogenetic divisions giving rise to GA tetraploids are inherently more “normal” (a bipolar segregation) than those giving rise to BL tetraploids (a tripolar segregation or a quadripolar segregation). So it is possible that the level of aneuploidy is the same after either GA or BL tetraploid induction, and, arguably, GA tetraploids might be less aneuploid because of the lack of a multipolar spindle to give rise to tetraploids. To verify any difference in initial levels of aneuploidy between GA and BL, chromosome counts in early embryos will be needed in future studies.

The second “impact,” that of genetic predisposition for reversion, which Benabdelmouna and Ledu (2015) hypothesized would be “directly and fully transmitted by triploid oocytes” is supported by the fact that MC tetraploids (which had $\frac{1}{2}$ of their genetic material inherited from the genetically predisposed tetraploid parent) had intermediate levels of reversion between GA and BL tetraploids. The idea that CIN is heritable is intriguing, although counter to the argument that CIN is an inherent feature of polyploidy in the first place (Comai 2005; Storchova and Kuffer 2008). Whatever this genetic effect is, it seems to be additive (*cf.* Benabdelmouna and Ledu 2015). However, in this study and that of Matt and Allen (2014), we found no evidence of

heritability of CIN between non-mosaic and mosaic parents, as measured through both FCM and chromosome counts in triploid *C. virginica*. It is possible that somatic chromosome loss is of less concern for the long-term chromosomal integrity of 4n x 4n crosses than would be suggested by the levels of reversion observed in tetraploid adults.

Acknowledgements

We would like to thank the entire staff of the Aquaculture Genetics and Breeding Technology Center (ABC) for their technical help, without whose expertise this research would not be possible. Special thanks to Kate Ritter for her assistance with flow cytometry, Shelley Katsuki for her assistance with breeding and rearing the oysters and Eric Guévelou for translating the abstract into French. This work was partly funded through the National Science Foundation's Research Experience for Undergraduates (REU) Program (grant # NSF OCE 1062882). This paper is Contribution No. XXXX of the Virginia Institute of Marine Science, College of William & Mary.

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Tables

Table 1. Parents: Flow cytometric analysis of males and females, mosaics and non-mosaics of *C. virginica* broodstock used for crosses showing relative DNA content (Rel DNA) and coefficient of variation (CV) for cell populations of sperm (2C) and somatic cells: tetraploid (4n) and triploid cells (3n). For mosaics, the ratio of triploid to tetraploid relative DNA content (3n/4n ratio) and percentage of tetraploid cells (% 4n) for each individual is shown. NA – samples not available.

		SPERM		SOMATIC				ANALYSIS	
		2C Rel		3n Rel		4n Rel		3n/4n	
		DNA	CV	DNA	CV	DNA	CV	Ratio	% 4n
MALES	NM 1	NA	NA	--	--	4.03	3.24	--	100
	NM 2	2.03	3.05	--	--	3.96	3.25	--	100
	NM 3	2.05	3.73	--	--	3.99	3.25	--	100
	NM mean	2.04	3.39			3.99	3.25		
	M 1			2.97	3.97	3.95	3.57	0.75	66
	M 2	2.04	3.39	3.06	3.06	3.92	3.24	0.78	42
	M 3	2.04	3.58	2.92	3.46	3.90	3.25	0.75	60
	M Mean	2.04	3.49	2.98	3.50	3.92	3.35	0.76	56.0
	Mean all	2.04	3.44	2.98	3.50	3.96	3.30	0.76	--
FEMALES	SD all	0.01	0.29	0.07	0.46	0.05	0.13	0.02	--
	NM 1	--	--	--	--	4.01	3.64	--	100
	NM 2	--	--	--	--	3.89	3.38	--	100
	MN 3	--	--	--	--	3.95	3.16	--	100
	NM mean					3.95	3.39		
	M 1	--	--	3.11	3.95	4.03	3.41	0.77	50

M 2	--	--	3.10	3.41	3.99	3.35	0.78	62
M 3	--	--	3.09	3.41	3.97	4.21	0.78	56
M mean			3.10	3.59	4.00	3.66	0.78	56.0
<i>Mean all</i>	--	--	<i>3.10</i>	<i>3.59</i>	<i>3.97</i>	<i>3.53</i>	<i>0.78</i>	--
<i>SD all</i>	--	--	<i>0.01</i>	<i>0.31</i>	<i>0.05</i>	<i>0.37</i>	<i>0.01</i>	--

Table 2. Progeny: Flow cytometric analysis of triploid *C. virginica* larvae from day 2 showing relative DNA content (Rel DNA) and coefficient of variation (CV). Larvae were produced by crossing either non-mosaic (NM) or mosaic (M) **tetraploid males** × a diploid female tester or non-mosaic or mosaic **tetraploid females** × diploid male tester. The female tester diploid was crossed to a random diploid male; the male tester diploid was crossed to a random diploid female. NA – samples not available.

		Rel DNA	CV
MALES	NM1	2.97	4.75
	NM2	2.90	5.51
	NM3	2.98	5.07
	M1	2.95	5.21
	M2	2.90	5.51
	M3	3.01	4.89
	Mean	2.95	5.16
	SD	0.04	0.32
	Control	1.99	5.50
FEMALES	NM1	2.84	4.47
	NM2	2.86	4.91
	NM3	2.80	5.45
	M1	2.89	6.01
	M2	NA	NA
	M3	2.85	5.07
	Mean	2.85	5.18

	SD	0.03	0.58
Control		1.86	5.65

Draft

Table 3. Chromosome count data and percentage of spreads among different ploidy classifications for 6-h-old triploid embryos of *C. virginica* produced by crossing non-mosaic (NM) or mosaic (M) **tetraploid males** × a diploid female tester or non-mosaic or mosaic **tetraploid females** × diploid male tester. n = number of metaphase spreads.

			Control	NON-MOSAIC				MOSAIC			
				NM1	NM2	NM3	$\bar{x}$	M1	M2	M3	$\bar{x}$
MALES	NO.	n	30	36	31	30	32	33	31	38	34
		mode	20	30	30	30	30	30	30	30	30
		mean	20	27.1	27.1	27.3	27.2	26.2	27.0	27.7	27.0
	PERCENT	triploid	0	33	23	43	33	24	29	39	31
		diploid	63	6	0	3	3	9	6	3	6
		hypoploid	23	58	71	50	60	67	65	58	63
		hyperploid	13	3	6	3	4	0	0	0	0
FEMALES	NO.	n	36	30	32	30	31	34	30	31	32
		mode	20	30	30	30	30	30	30	30	30
		mean	19	26.3	26.2	28.1	27.0	26.0	27.3	27.6	27.0
	PERCENT	triploid		23	31	40	32	26	37	45	36
		diploid	47	10	6	0	5	3	10	3	5
		hypoploid	53	63	62	50	59	68	53	48	56
		hyperploid	0	3	0	10	4	3	0	3	2

Table 4. Chromosome count data and percentage of spreads among different ploidy classifications for 2-4 cell triploid embryos of *C. virginica* produced by crossing non-mosaic (NM) or mosaic (M) **tetraploid females** × a diploid male tester.

		NON-MOSAIC			MOSAIC			
		NM1	NM2	$\bar{x}$	M1	M2	M3	$\bar{x}$
NO.	n	27	21	24	28	23	24	25
	mode	30	30	30	30	30	30	30
	mean	29.3	29.9	29.6	29.9	29.8	30.0	29.9
PERCENT	triploid	85	95	90	93	87	96	92
	diploid	4	0	2	0	0	0	0
	hypoploid	11	5	8	7	13	4	8
	hyperploid	0	0	0	0	0	0	0

Draft

Figure captions

Figure 1. Examples of metaphase chromosomes from *C. virginica* embryos. (a) Diploid $2n = 20$ from $2n$ control. (b) Hypoploid $3n = 29$ from 6-h-old embryo from $4n$ male non-mosaic x $2n$ female. (c) Eutriploid $3n = 30$ from 6-h-old embryo from $4n$ male mosaic x $2n$ female. (d) Eutriploid $3n = 30$ from 2 cell embryo from $4n$ female mosaic x $2n$ male. Scale bar= $5\mu\text{m}$.

Figure 2a. Male tetraploid parents: Frequency distribution of chromosome number of cells from 6-h-old triploid embryos of *C. virginica* produced by crossing either tetraploid non-mosaic or mosaic **tetraploid males** with a diploid tester female and the diploid control.

Figure 2b. Female tetraploid parents: Frequency distribution of chromosome number of cells from for 6-h-old triploid embryos and for 2-4 cell stage embryos of *C. virginica* produced by crossing either tetraploid non-mosaic mosaic **tetraploid females** with a diploid tester male and the diploid control.

Figure 3. Frequency distribution of chromosome number for adult, triploid, mosaic *C. gigas*, from Table 1 (Zhang et al. 2010a). Tetraploids were G_1 obtained from chemical induction method of Guo and Allen (1994).

Figure 1. Examples of metaphase chromosomes from *C. virginica* embryos. (a) Diploid $2n = 20$ from $2n$ control. (b) Hypoploid $3n = 29$ from 6-h-old embryo from $4n$ male non-mosaic $\times$ $2n$ female. (c) Eutriploid $3n = 30$ from 6-h-old embryo from $4n$ male mosaic $\times$ $2n$ female. (d) Eutriploid $3n = 30$ from 2 cell embryo from $4n$ female mosaic $\times$ $2n$ male. Scale bar= $5\mu\text{m}$.
104x104mm (300 x 300 DPI)

Figure 2a. Male tetraploid parents: Frequency distribution of chromosome number of cells from 6-h-old triploid embryos of *C. virginica* produced by crossing either tetraploid non-mosaic or mosaic tetraploid males with a diploid tester female and the diploid control.
152x221mm (300 x 300 DPI)

Figure 2b. Female tetraploid parents: Frequency distribution of chromosome number of cells from for 6-h-old triploid embryos and for 2-4 cell stage embryos of *C. virginica* produced by crossing either tetraploid non-mosaic mosaic tetraploid females with a diploid tester male and the diploid control.
155x227mm (300 x 300 DPI)

Figure 3. Frequency distribution of chromosome number for adult, triploid, mosaic *C. gigas*, from Table 1 (Zhang et al. 2010a). Tetraploids were G1 obtained from chemical induction method of Guo and Allen (1994).
168x105mm (300 x 300 DPI)